CHAPTER 1

General Introduction

Partly adapted from Kipp et al., 2012; Van der Star et al., 2012 and Van der Star et al., book chapter in: “Autoimmunity to Neuronal Proteins in Neurological Disorders”, Nova Science Publishers 2011
Multiple sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS) frequently manifested by episodes of neurological disease (relapses) and recovery (remissions). Worldwide around 2.3 million people are estimated to be affected by MS and the disease is differentially distributed with a higher prevalence in North America and Europe. In the Netherlands 1 in 1000 of the population is affected by the disease (1). The first symptoms of the disease are generally observed in young adults between the age of 20 and 40 and more women are affected than men (2.3:1) (2). MS also occurs in younger adults and children as well as in older people.

Damage to the brain and the spinal cord of persons with MS results in a variety of signs and symptoms, depending on the area(s) affected. Symptoms include visual problems (3), loss of motor and sensory functions (4) and cognitive impairment (5). In many persons with MS the episodes of relapses and remissions lead to accumulation of neurological deficits over years with progression of disability and many patients eventually become wheelchair bound. Besides the variability of symptoms, the severity of the symptoms and duration of the disease differs between individuals with MS. Several forms for the clinical course of MS are defined (4, 6): Relapsing-Remitting MS (RRMS), Secondary Progressive MS (SPMS) and Primary Progressive MS (PPMS, Figure 1). The majority of the people (~80%) have RRMS which translates to the appearance of relapses and remissions (4). However, after years of relapses and remissions, the disease becomes more progressive due to the accumulation of symptoms, and is referred to as SPMS (Fig. 1A). It is estimated that 50-60% of the people with RRMS progress to develop SPMS (6). Approximately 10% of the people with MS have PPMS and experience less recovery from onset of first symptoms (Fig. 1B, gender ratio female to male 1.3:1) (7). Why the majority of the people with RRMS progress to SPMS and why others have PPMS is unknown.

Figure 1. Different forms of MS. A) The majority of the patients (~80%) develop relapsing-remitting MS (RRMS) with relapses as bars above the clinical threshold (dotted line) of clinical disability, and remission below the threshold. After decades, damage in the CNS accumulates with almost no recovery and is called secondary progressive MS (SPMS). B) In approximately 10% of the people with MS damage accumulates until it reaches the threshold of clinical disability and progresses in time without remissions (primary progressive MS, PPMS).
Another form of MS which is less common than the forms described above is primary relapsing MS (PRMS) in which patients show progressive neurological disability with relapses (6). Individuals that experience a single relapsing-remitting episode are classified as clinically isolated syndrome (CIS). A recent study shows that 26% of the persons with CIS develop clinically definite MS after one year (8).

Several other uncommon subtypes of MS have also been described. These include acute and aggressive types such as Marburg’s variant and Balò’s concentric sclerosis (9) or a benign subtype MS, so-called clinically silent MS (10). It is estimated that between 1.7% and 5.6% of the people with MS are children under the age of 18, of which 95% of the cases follow a RRMS course (11, 12). Although several types of MS can be distinguished, the cause of MS has yet to be identified.

**Genetics**

Although the aetiology of MS is unclear, several lines of evidence indicate that genetic variations increase the risk of getting MS. Genome wide association studies have identified polymorphisms in genes coding for cytokines (e.g. Interleukin (IL)2-RA, IL-7R, IL22-RA2), co-stimulatory molecules (e.g. cluster of differentiation (CD)40, CD86) and signal transduction molecules (e.g. signal transducer and activator of transcription 3) (13, 14). The strongest association with the risk of MS is the human leukocyte antigen (HLA)-DRB1*1501 allele, located within the class II region of the major histocompatibility complex (MHC), which increases the risk of developing MS by approximately threefold (15, 16). Furthermore, the concordance rate of MS is 25%-30% in identical twins and the individual risk of getting MS increases 20- to 40- fold when a sibling has MS (17, 18). Mutations in the CYP27B1 gene are also associated with an increased risk of MS (19) and are thought to be due to the immunomodulatory effects of Vitamin D (20-23). Polymorphisms in killer immunoglobulin-like receptors (KIR), which are MHC class I-specific receptors, are associated with MS susceptibility as well. The KIR genes code for stimulating or inhibitory receptors and are expressed by natural killer (NK) cells and subsets of T lymphocytes. The absence of one of the inhibitory receptors, KIR2DL3, is associated with the development of CIS and MS (24, 25).

Nevertheless, the above mentioned genetic mutations do not fully explain the high incidence of MS, suggesting that other factors such as environmental factors might also contribute to the development of MS.

**Environmental factors**

Although MS occurs worldwide, geographical studies show that the prevalence of MS is not equally distributed (26). In general, the incidence of MS is low in tropical areas, higher further away from the equator but again low in the most northern regions. Migration early in life from a high-risk area to a low-risk area decreases the risk of getting MS (4, 20). Variations in Vitamin D levels are proposed as one of the contributors to the unequal distribution of MS prevalence. This is supported by differences in MS prevalence in Norway between fishing villages and farming villages inland, which is probably due to the high Vitamin D in the diet (16, 26). Contrary to the protective effect of Vitamin D is cigarette smoking, which is a risk factor for MS development (27-30), although a protective effect of nicotine is also proposed (31, 32). Other environmental factors, such as exposure to infections, might influence the risk of MS as well. Electron microscopical and virological studies support a role for infections in MS by revealing the presence of viruses such as measles (33), human Herpesvirus 6 (HHV-6) (34, 35) and Epstein-Barr Virus (EBV) (36) in the
CNS and cerebrospinal fluid (CSF) of people with MS. Furthermore, antibodies to EBV nuclear antigens are present in the serum of people with MS (37, 38), as well as B cell follicles containing EBV-infected B cells in post-mortem MS brain tissue (36). However, these results are contradictory and the contribution of the EBV to the development of MS is still a matter of debate (39-43). A viral aetiology of MS is also supported by the discovery of structural similarities between viral antigens and myelin proteins in people with MS (44-46). In addition, several viruses e.g. measles and John Cunningham (JC) virus cause demyelination in humans (47, 48), but the exact impact of viruses in the development of MS is unclear.

Pathology
The major pathological characteristics of MS are inflammation, as reflected by the presence of innate immune activation and the presence of adaptive immune responses i.e. T and B cells, demyelination, axonal damage and sclerotic plaque formation. It is proposed that lesion development starts with the formation of pre-active lesions (49). These are small clusters of activated microglia which are HLA-DR+ (Fig. 2A), resembling an activated cell type, and stressed oligodendrocytes, but without apparent myelin damage (Fig. 2B) (49, 50). At that stage, the blood-brain barrier (BBB) is still intact (49, 51). Pre-active lesions might develop into active lesions characterised by accumulation of activated and phagocytic HLA-DR+ cells, oligodendrocyte loss, demyelination and axonal damage. At this stage, cells from the periphery including macrophages, dendritic cells and lymphocytes infiltrate the CNS, the BBB is damaged and lesions contain immunoglobulin (Ig) and complement deposits (49, 52-55). In active lesions, microglia and macrophages show a round morphology, contain intracellular (myelin and axonal) debris (Fig. 2C and D), and are also present in the perivascular space together with other leukocytes (52). HLA-DR+ cells containing debris are also present in cervical lymph nodes, a site for antigen presentation, lymphocyte activation or lymphocyte tolerance (56, 57). Active lesions gradually progress into chronic active lesions which are characterised by a rim of phagocytic HLA-DR+ cells (Fig. 2E, G) and a demyelinated hypocellular centre (Fig. 2F, H). Finally, due to an unknown trigger, phagocytosis of myelin and axonal proteins stops, leaving a rim of HLA-DR+ cells (Fig. 2I and J) and a demyelinated centre (Fig. 2K) with reactive astrocytes (Fig. 2L) forming a sclerotic plaque. Contrary to plaque formation, regeneration of damaged axons and remyelination occurs as well (58, 59), although it is believed that these restored areas function less as before the damage.
Figure 2. Pathology of MS and schematic representation of lesion development. A) A small cluster of HLA-DR cells form pre-active lesions. B) No damage to myelin in pre-active lesions as shown by immunostaining of proteolipid protein (PLP). An active lesion with HLA-DR cells containing debris (C, arrowheads) or Oil-Red-O particles resembling uptake of neutral lipids (D, arrowheads). E-H) Chronic active lesion, circled by dotted line, is characterised by a rim of HLA-DR cells (E, G) and a demyelinated hypocellular centre as stained for PLP (F, H). Asterisks in G and H indicate the location of the lesion. I-L) Inactive lesion leaving a rim of ramified HLA-DR cells (I, J), a demyelinated centre as shown by PLP staining (K) and a centre of reactive astrocytes (L, asterisk, glial fibrillary acidic protein staining). The dotted line in L indicates the lesion. Cell nuclei are stained blue. M) Schematic and simplified representation of lesion development in MS.
Axonal damage and neurodegeneration in MS
Although the extent of axonal injury is variable in lesions of people with MS, it is clearly present in all demyelinated lesions and normal appearing white matter (Fig. 3A-C) (53, 60-64). In chronic inactive lesions axonal density is reduced up to 70% (65, 66). Studies from post-mortem spinal cord tissue from people with MS reveal that axonal damage correlates with irreversible neurological disability (65) indicating that arresting progressive degeneration would be a crucial step in controlling disability. With the improvement of magnetic resonance imaging (MRI) and histochemical staining techniques, it has become clear that demyelination and axonal degeneration is not limited to the white matter (WM) but also occurs in the cortical regions (67). Since 1999 a classification system for grey matter (GM) lesions is used (67, 68). Leukocortical lesions (Type I lesions) are localised both in WM and GM (Fig. 3D). Intracortical lesions (Type II lesions) are localised in the cortex, thus subcortical WM and the surface of the cortex are not involved (Fig. 3E). Sub-pial cortical lesions (Type III lesions) only include superficial layers of the cortex (Fig. 3F) and finally transcortical lesions (Type IV lesions) affect the subcortical regions but not the WM (Fig. 3G) (68). In GM lesions, infiltration of macrophages and lymphocytes is lower compared to WM lesions, so low to be virtually absent (63, 69). This is also reflected at the border between the GM and WM part of leukocortical lesions, where sometimes a clear rim of inflammatory cells in the WM is seen, whereas the GM is devoid of these cells (63).

In summary, improvements of techniques to detect axonal damage have contributed to the knowledge about neurodegeneration in MS.

Figure 3. Axonal damage and grey matter lesions in MS. A) A neuron with a thick axon extending from the cell body, stained with an antibody to neurofilament light. B) Axonal bulbs, identified with an antibody directed to amyloid precursor protein (APP), representing possible damage (arrowheads) in the rim of a chronic active MS lesion. C) Abnormal structure along the axon (arrowhead) indicating possible damage (antibody to APP). D-G) Schematic representation of grey matter MS lesion in the brain. D) Leukocortical lesions affect both the cortical GM and the WM. E) Intracortical lesions are localised in the cortex. F) Sub-pial lesions include only the superficial layers of the cortex. G) Transcortical lesions affect the subcortical regions but not the WM. L, lesion.
Neurofilaments
The cytoskeleton of neurons and axons consists of actin microfilaments, microtubules and intermediate filaments (70). Neurofilaments, classified as intermediate filaments, are the most important proteins in the axonal cytoskeleton (71) that determine the axonal calibre and contribute to transport of proteins along the axons (72). Neurofilaments consist of the subunits neurofilament heavy (NF-H), medium (NF-M) and light (NF-L) (Fig. 4) and have their nomenclature related to their molecular weight. Regarding the structure of the subunits, all neurofilaments consist of a head (N-terminal), rod and tail (C-terminal) domain. While the head and rod domains are highly conserved between the subunits, the tail domain is variable (Fig. 4).

Post-translational modifications e.g. phosphorylation and glycosylation of the neurofilament subunits, are involved in neurofilament assembly (71). Since NF-H is the most phosphorylated of the three subunits, monoclonal antibodies recognising non-phosphorylated or phosphorylated NF-H are used to detect changes in NF-H phosphorylation status in axons (71, 73). Especially in damaged brain areas dephosphorylated neurofilaments are detected (71, 73), whereas in a healthy environment the majority of neurofilament subunits are phosphorylated (71, 74). On the contrary, increased immunoreactivity to phosphorylated NF-H is also seen in brain tissues of people with Alzheimer’s disease (75, 76) and in active MS brain lesions (74).

Mutations in the gene encoding NF-H are reported to cause aggregation of neurofilaments in a subset of people with amyotrophic lateral sclerosis (ALS) (77), the most common form of motor neuron disease in adults and a rapidly progressive disorder resulting in muscle weakness and muscle atrophy (78). In Charcot-Marie-tooth disease, an inheritable neuropathy, several mutations in the NF-L gene have been linked to aggregation (79-82).

This thesis focuses on one form of neurofilaments, namely NF-L, since antibodies to NF-L are present in the CSF and serum of people with MS (discussed below) and correlate with cerebral atrophy in people with MS (83). Antibodies to NF-L are suggested as biomarkers for disease progression in MS (84-88). In addition, NF-L is the most abundant of the neurofilament subunits (89) and immunisation of Biozzi antibody high (ABH) mice with the NF-L protein results in spasticity and paralysis, which are symptoms observed in people with MS (90, 91).
**Therapies**

First-line therapies of RRMS and SPMS with relapses include the disease-modifying treatments with interferon-β1a, interferon-β1b or glatiramer acetate (GA) (92). Patients treated with interferon-β1a show a ~30% decrease in relapses, fewer exacerbations and less gadolinium enhancing lesions on MRI than controls (93, 94). Also treatment with GA reduces the relapse rate with ~29% in patients with RRMS (95). Following the development of these disease-modifying therapies, drug development has progressed into target-specific therapies using monoclonal antibodies targeting specific structures on cells. The first officially approved humanised monoclonal antibody for RRMS is natalizumab, a monoclonal antibody that targets a receptor on leukocytes, Very Late Antigen 4 (VLA-4) (96). Blocking of VLA-4 obstructs leukocytes, including lymphocytes, to enter the CNS. Natalizumab decreases the annualised rate of relapses by 68%, reduces the mean number of hyperintense lesions by 83% and reduces progression of clinical disability compared to placebo (97). Unfortunately, patients positive for the JC virus treated with natalizumab have an increased risk of progressive multifocal leukoencephalopathy due to immunodeficiency (48, 98). Other therapies have been developed that inhibit lymphocyte trafficking, such as fingolimod, (99-101) or deplete lymphocytes, like alemtuzumab (102). Moreover, B lymphocyte depletion by a monoclonal antibody to CD20, rituximab, is currently used in trials for MS, and is already used for treatment of non-Hodgkin lymphoma and rheumatoid arthritis (103). Some people with MS benefit from plasma exchange, although temporarily (104-106). Keegan and colleagues propose the removal of potentially pathogenic antibodies as favourable in persons with complement and antibody deposits in the CNS (105).

Unfortunately, therapies such as GA, fail to have an effect on disease progression in people with PPMS (107). In another study, Coles and colleagues show a decrease in neuroinflammation in people with SPMS treated with alemtuzumab but ongoing accumulation of disability (102). Possible explanations might be that in people with progressive MS without relapses, inflammation is less involved or that in people with SPMS accumulation of axonal damage continues as a consequence of the active inflammatory episodes during the RRMS stage (4, 102).

In conclusion, the increase in therapies for RRMS so far has not been beneficial for people with progressive MS with accumulating neuronal damage. Therefore, unravelling the neuropathological mechanisms in MS, partly facilitated by experimental models, will contribute to development of new therapeutic agents.

**Experimental models of MS**

Although the primary cause of MS is unknown, the widely accepted view is that aberrant (auto)immune responses, possibly arising following infection(s), are responsible for the destructive inflammatory demyelination and neurodegeneration in the CNS. This notion, and the limited access of human brain tissue (early) in the course of MS, has led to the development of autoimmune, viral and toxin-induced demyelination animal models as well as the development of human CNS cell cultures and organotypic brain slice cultures in an attempt to understand events in MS. The autoimmune model, known as experimental autoimmune encephalomyelitis (EAE), and the viral models have shaped ideas of how environmental factors may trigger inflammation, demyelination and neurodegeneration in the CNS. Understandably, these models have also strongly influenced the development of therapies targeting the inflammatory aspect of MS.
Experimental autoimmune encephalomyelitis

The animal model EAE, characterised by inflammation, myelin damage and neurodegeneration induced following immunisation with brain antigens, strongly supports MS as an autoimmune disease (90, 91, 108, 109). Despite differences in the course and pathology between MS and EAE, EAE is still the most intensively used experimental model of MS. In addition, EAE studies have provided important contributions to our understanding of neuro-immune interactions within the CNS.

Immunisation of susceptible animals with CNS tissues and adjuvant elicits either a monophasic neurological episode of paralysis, from which the animals recover and are refractory to re-induction of disease, or chronic paralysis from which the animals do not recover (Table 1). While the use of adjuvants, such as complete Freund’s adjuvant (CFA), boosts the immune response (CD4+ T cell-mediated), injection of pertussis toxin (PT) from *Bordetella pertussis* is thought to make the BBB more permeable as well as to induce non-specific activation of T cells. EAE can also be induced following adoptive transfer of activated lymph node cells, or specific T cell lines and clones derived from myelin-immunised animals to naive recipients (110). While initial studies use CD4+ T cells to induce EAE, the finding that CD8+ T cells can also induce EAE (111) is important since CD8+ T cells dominate inflammatory MS lesions (112-114). Depleting CD4+ T cells or therapies inhibiting MHC class II interactions block the induction phase and severely reduce clinical relapses in EAE (115, 116).
Whatever the induction regimen, the initial phase of clinical disease is usually termed the acute phase (Fig. 5A), and correlates with the presence of mononuclear cell infiltrates in the CNS. The scoring system ranges from 0 representing control or no clinical disease to 4 representing severe clinical paralysis commonly observed by flaccid paralysis of the hind limbs. For ethical reasons score 4 is generally the most severe score although in some reports score 5 is used to represent a moribund state or death from EAE. In species and strains where the animals recover, this recovery period is referred to as remission (Fig. 5A). If animals do not recover the disease is referred to as chronic EAE (Fig. 5B). Several animals, particularly strain 13 guinea pigs, Dark Agouti rats, SJL and ABH mice exhibit recurrent phases of neurological deficit (Fig. 5C). It is still unclear why some EAE animals develop relapses while others exhibit a chronic neurological disease. In the relapse phase myelin damage

### Table 1. Spectrum of EAE

<table>
<thead>
<tr>
<th>EAE</th>
<th>Animal</th>
<th>Clinical Pathology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperacute</td>
<td>Rats</td>
<td>Hyperacute EAE</td>
<td>(117)</td>
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<tr>
<td></td>
<td>Adoptive transfer of lymphocytes after SCH immunisation</td>
<td>one day after transfer and PT</td>
<td></td>
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<td></td>
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<td>Deposits of fibrin and neutrophils</td>
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<tr>
<td>Acute</td>
<td>Biozzi ABH mice</td>
<td>Acute monophasic disease</td>
<td>(118, 119)</td>
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<tr>
<td></td>
<td>Immunisation with MAG or MBP in CFA + PT</td>
<td></td>
<td></td>
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<tr>
<td>Acute</td>
<td>Lewis rats</td>
<td>Acute monophasic disease</td>
<td>(120)</td>
</tr>
<tr>
<td></td>
<td>Immunisation with MBP in CFA</td>
<td>Minimal demyelination</td>
<td></td>
</tr>
<tr>
<td>Clinical optic neuritis</td>
<td>Rhesus monkeys Oligodendrocyte specific protein in CFA</td>
<td>Optic neuritis</td>
<td>(121)</td>
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<tr>
<td></td>
<td></td>
<td>Demyelination and inflammation in the optic nerve</td>
<td></td>
</tr>
<tr>
<td>Chronic</td>
<td>MOG 35-55 in C57BL/6 or Biozzi ABH mice in CFA + PT</td>
<td>Chronic disease no or very infrequent recovery</td>
<td>(109)</td>
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<tr>
<td></td>
<td></td>
<td>Extensive neuronal loss associated with inflammation in the spinal cord</td>
<td></td>
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<tr>
<td>Chronic</td>
<td>rMOG in CFA</td>
<td>Chronic demyelinating</td>
<td>(122, 123)</td>
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<td></td>
<td>Native MOG in myelin in CFA</td>
<td>Demyelination in CNS</td>
<td></td>
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<tr>
<td>Chronic relapsing</td>
<td>Biozzi mice Immunisation with rMOG, MOG 8–21 PLP, PLP 56–70 or SCH in CFA/CFA + PT</td>
<td>Chronic relapsing</td>
<td>(118, 124-126)</td>
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<td></td>
<td></td>
<td>Minimal demyelination in acute EAE and more extensive in relapses. Axonal damage and neurological deficit increases with time and number of relapse</td>
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<tr>
<td>Chronic relapsing</td>
<td>Dark Agouti rats Immunisation with rMOG, SCH in IFA</td>
<td>Chronic relapsing</td>
<td>(120, 127)</td>
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<tr>
<td></td>
<td></td>
<td>Mainly inflammatory with varying degrees of myelin damage</td>
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<tr>
<td>Chronic relapsing</td>
<td>Guinea pig Hartley and Strain 13</td>
<td>Acute and chronic relapsing</td>
<td>(128, 129)</td>
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<td>Inflammation and extensive demyelination with remyelination in relapse EAE</td>
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<tr>
<td>Secondary progressive</td>
<td>ABH mice Immunisation with SCH in CFA</td>
<td>Secondary progressive EAE</td>
<td>(130, 131)</td>
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<td></td>
<td>Marked gliosis, demyelination, remyelination and axonal and neuronal loss</td>
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<tr>
<td>Spontaneous</td>
<td>Humanised double transgenic mice TCR for MBP and HLA-DR15</td>
<td>Monophasic with severe paralysis</td>
<td>(132)</td>
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<td></td>
<td></td>
<td>Inflammation in CNS, limited demyelination</td>
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<tr>
<td>Spontaneous</td>
<td>Humanised double transgenic mice MOG TCR and HLA-DR15</td>
<td>Monophasic</td>
<td>(133)</td>
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<td>Inflammation and demyelination in spinal cord and optic nerve</td>
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<tr>
<td>Spastic paresis</td>
<td>ABH mice Immunisation with NF-L in CFA + PT</td>
<td>Spastic paresis and paralysis</td>
<td>(90, 91)</td>
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<td>Axonal loss, grey matter damage and myelin damage</td>
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SCH, spinal cord homogenate; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; IFA, incomplete Freund’s adjuvant; TCR, T cell receptor.
and axonal loss are more prominent than in the acute phase (125, 129, 134). An advantage of chronic relapsing EAE is the development of a secondary progressive disease. The pathology of ABH mice immunised with spinal cord homogenate shows extensive demyelination, axonal and neuronal loss, and marked gliosis, all features of MS (130, 135). Omission of pertussis toxin decreases the clinical scores (Fig. 5D, black line) and less demyelination is observed (Fig. 5E). Injection of anti-myelin antibodies, such as anti-myelin oligodendrocyte glycoprotein (MOG) antibodies in MOG-immunised ABH mice (136), exacerbates EAE (Fig. 5D, dotted line) and results in extensive demyelination (Fig. 5F).

Besides the use of myelin antigens, immunisation with neuronal antigens can be used to induce autoimmune mediated neuronal damage.

**Figure 5. Disease courses in EAE.** Immunisation of animals with myelin antigens gives rise to a spectrum of EAE in which the clinical scores represent the neurological deficit observed. 0 Healthy, 1 limp tail, 2 impaired righting reflex, 3 partial hind-limp paralysis, 4 complete hind-limp paralysis. 

**A)** Acute phase in which animals recover (remission) yet do not develop further episodes of disease. In some cases animals do not recover from the acute phase and the disease is referred to as chronic EAE (B). In those models where animals recover, the remission phase is followed by relapse phases (C). In this case the animals develop subsequent relapses in which neurological deficit accumulate and eventually do not return to baseline (0) when the animals enter the secondary progressive EAE phase. 

**D)** Clinical course of acute EAE in ABH mice in which pertussis toxin has been omitted (black line, note the reduced clinical score compared to A) do not show extensive demyelination as shown by luxol fast blue (LFB) staining (E). To augment myelin damage injection of antibodies to myelin oligodendrocyte glycoprotein at the onset of EAE (day 10) exacerbates clinical disease (D, dotted line) concomitantly with myelin loss (F, arrow points to remnants of myelin, LFB staining).

**Autoimmune mediated neuronal damage**

EAE models induced by immunisation with myelin proteins or peptides show preferential white matter pathology with some (reversible) neuronal damage (137,
Extensive axonal damage is also observed in the secondary-progressive EAE model in ABH mice immunised with spinal cord homogenate (130). Since in MS both GM and WM are affected (62, 67), animal models reflecting GM and WM pathology are more useful to investigate the mechanisms behind this aspect of the disease. Immunising animals with neuronal and axonal proteins or peptides induces more severe axonal pathology, depending on the animal, the strain and the antigen. Axonal pathology is often seen in the spinal cord and includes cellular infiltrates and Ig and complement deposits (90, 91). Immunisation of Biozzi mice with NF-L leads to paralysis and spasticity (91), which are clinical features of MS. In this model of axonal damage, Igs are observed in axons in the spinal cord of mice with disease but not in mice that do not exhibit overt clinical signs (91). This observation suggests that antibodies to the intracellular protein NF-L are able to reach their target and possibly contribute to the development of axonal damage.

In MOG-deficient mice, transfer of T cells with a MOG-specific T cell receptor (TCR) results in demyelination and GM pathology caused by an autoimmune response to one of the neurofilament subunits, NF-M (139). The authors identify shared epitope sequences between MOG$^{35-55}$ and NF-M$^{18-80}$, responsible for recognition by the TCR. These findings indicate that myelin-specific T cells could also induce neuronal damage via cross-reactivity with neuronal antigens (139).

Furthermore, similar to antibodies to myelin, antibodies to neuronal antigens are demonstrated to cause damage in animals. For example, an antibody directed to neurofascin (NF) induces axonal pathology and exacerbation of EAE in Dark Agouti rats, but does not enhance inflammation or demyelination (140). In this study the authors use a monoclonal antibody directed to both NF-155 (myelin-related) and NF-186 (located at the nodes of Ranvier) isoforms in vitro, but could only find binding of the antibody to NF-186 in vivo, indicating NF-186 as the primary target (140). More recently, the pathogenicity of monoclonal antibodies to NF-155/186 and NF-186 alone as well as purified human Igs from MS sera was shown in myelinating spinal cord co-cultures, revealing that in some people with MS pathogenic antibodies contribute to pathology (141).

To conclude, depending on the question of interest, different antigens can be used for immunisation of animals to model certain aspects of MS pathology.

Proposed mechanisms of axonal damage in MS
As mentioned above, axonal pathology is present early in MS. Although the pathological mechanisms of axonal injury is unknown, two models have been proposed, based on a viral model in mice (142). These are the so-called ‘inside-out’ and ‘outside-in’ demyelination and neurodegeneration paradigms (Fig. 6) (142). In the inside-out model, pathological mechanisms lead to neurodegeneration, axonal damage, and dying back of axons, leaving so-called empty myelin sheaths that eventually degrade. In contrast, in the outside-in model damage to the myelin sheath leaves the naked axons vulnerable that, without the trophic support of myelin, degenerate as a secondary event.
Studies demonstrating an association between axonal damage and inflammation are in line with both models (64, 143, 144). Axonal damage (145, 146) and intracellular axonal changes, e.g. changes in post-translational modifications of neurofilaments (147) in the normal appearing white matter might be indications of an inside-out mechanism. Axonal injury might also be caused by autoimmunity to neuronal proteins (90, 148). In particular autoantibodies to neuronal antigens have been clearly demonstrated to be involved in axonal pathology in other diseases. For example, in paraneoplastic neurological syndromes (PNS), patients with tumours located outside the CNS experience neurological disability (149). In these disorders, tumour cells express neuronal antigens on their surface leading to an immune response causing encephalitis that is often associated with onconeural antibodies and T cells (149-151). Among the target antigens are surface proteins, such as the N-methyl-D-aspartate receptor (152) and gamma-aminobutyric acid-B receptor (153) or intracellular antigens including nuclear proteins such as Hu (154) or synaptic proteins like amphiphysin (155). In people with neuromyelitis optica, antibodies to aquaporin-4, which is expressed on astrocytes, lead to demyelination possibly through complement-dependent cytotoxicity (156-160). In Guillain-Barré syndrome, antibodies to gangliosides, including anti-GM1 and anti-GQ1, cause neurotoxicity through complement-mediated calcium influx resulting in paralysis (161). The realisation that MS may also be classified as a neurodegenerative disorder in which autoantibodies to neuronal antigens might play a role, may not only help to uncover the pathogenic role for such autoimmune responses but may also lead to novel therapeutic strategies. The question now is whether there is any evidence for the involvement of autoimmunity to neuronal antigens in MS.

Autoimmunity to neuronal/axonal antigens in MS
Evidence for a role of autoimmunity against neuronal antigens in MS is supported by the finding of axon-reactive T and B lymphocytes and antibodies in the serum and CSF of people with MS. Autoreactive T cells to the neuron specific proteins enolase, arrestin (162) and NF-L (163) are present in the serum of people with MS. However, T cell responses to NF-L are also present in healthy controls, indicating that these cells are part of the normal immune repertoire (163). This suggests that other factors may be involved in the development of autoimmunity to NF-L besides...
only T cell (re)activation (163). The presence of antibodies in the serum and CSF of people with MS might be one of the possible contributors of autoimmunity to NF-L in MS. Igs are detected in the CSF, referred to as oligoclonal bands (OCBs) and are one of the diagnostic criteria for MS (164, 165). Furthermore, B cells and Igs have also been detected in MS brain lesions (36, 52, 166). Antibodies to neuronal antigens including NF-L have been demonstrated in the serum and CSF of people with MS by several groups (167-169). Levels of antibodies to NF-L are increased in the serum and CSF of people with progressive disease (167, 168) and correlate with clinical disability (168) and cerebral atrophy (83). Nevertheless, it is unknown how Igs in the CSF arise and whether they are part of the disease mechanism in MS.

Mechanisms of axonal damage in MS

Several studies indicate a strong correlation between axonal damage and the degree of inflammation in the CNS of people with MS (59-61, 170). The release of toxic factors is one of the mechanisms by which activated inflammatory cells may contribute to axonal injury. For example, the expression of inducible nitric oxide (NO) synthase is increased in acute MS lesions (171, 172), which could lead to neurotoxic levels of NO. Subsequently, NO may inhibit mitochondrial function causing reduced adenosine triphosphate production (173). This is supported by mitochondrial dysfunction in MS lesions (174) which contributes to axonal damage and neuronal loss (175). Also an imbalanced glutamate homeostasis, reflected by an increased glutamate production, might contribute to axonal injury (176, 177). Furthermore, recognition of neuronal antigens and thus T cell activation leading to neurological damage requires the expression of key molecules for antigen presentation, such as MHC and co-stimulatory molecules. Although it has become clear that CD8+ T cells outnumber CD4+ T cells in MS lesions (178), little is known whether, or how, T cells kill neurons in the CNS of people with MS. In the healthy brain, neurons express negligible levels of MHC class I (179). However during inflammation neurons and axons increase the expression of MHC class I molecules (179, 180). In MS lesions axons positive for MHC class I are detected (181). Fissolo et al. purified MHC class I and MHC class II molecules from brain autopsy samples from people with MS and analysed the eluted peptides from these molecules using mass spectrometry. Interestingly, the NF-L, NF-M and α-synuclein peptides are found in this analysis (182). This suggests that T cells may become (re)activated through HLA class I and class II molecules and attack neuronal components resulting in neurodegeneration. In vitro, axons up regulate MHC class I after interferon-gamma and tetrodotoxin treatment, resulting in transection of neurites by cytotoxic T cells (183).

More evidence for the impact of T cells on neurons comes from research on the interaction of proteolipid protein (PLP)-specific T cells in brain slice cultures. Activated PLP-specific T cells induce calcium changes in neurons leading to neuronal damage (184). Likewise myelin basic protein (MBP)-specific T cells are more effective in activating microglia that have axon-damaging ability as observed in organotypic CNS cultures (185), suggesting that CNS-specific T cells augment CNS damage.

As mentioned above, autoantibodies are present in the CSF and serum of people with MS. Mathey et al. report the presence of antibodies to NF-155/186, a neuronal protein expressed at the nodes of Ranvier, in the serum of people with MS (140). Antibodies to contactin-2, also located at the nodes of Ranvier, are also identified in the CSF and serum of people with MS (186). In this latter study, Derfuss and
colleagues use a proteomic approach to reveal that this axonal protein is recognised by both autoantibodies and T helper 1 cells (Th1) and Th17 cells. Antibodies directed to NF-L (187-189), NF-M (190), NF-H (191), tau (192) and tubulin (193) are also detected in the serum and/or CSF of people with MS. Among all subgroups in MS, people with progressive disease have elevated levels of antibodies to NF-L in serum or CSF (167, 168). In one study, people with PPMS have higher levels of antibodies to NF-L in the serum, but not in the CSF, than people with SPMS (167). In another study, people with PPMS or SPMS have higher levels of antibodies to NF-L in CSF than people with RRMS (168). These data indicate that anti-neuronal immune responses may be important in a subset of people with MS. The level of antibodies to NF-L in people with MS, especially in the relapsing-remitting subgroup, correlates with MRI measures for axonal loss (83). However, in that study no adjustments are made for possible confounding factors, such as age which is known to influence both NF-L antibody levels and cerebral atrophy (167, 194).

Concluding, the contribution of antibodies (to neuronal antigens) to the disease mechanism in MS is still unclear. Understanding the mechanisms by which autoimmunity to neuronal antigens contribute to MS pathology is key to the development of effective therapies to prevent progression of disease and irreversible damage.

Outline of the thesis

Hypothesis
We hypothesise that autoimmunity to NF-L contributes to axonal damage in MS.

Aim
The research has several aims:

1. Investigate how autoimmunity to neuronal antigens in people with MS might arise.
2. Use EAE as a model to investigate the mechanism(s) by which NF-L-specific T cells cause axonal damage.
3. Determine whether antibodies to NF-L are biomarkers of disease or whether they contribute to axonal damage in people with MS.

Neurodegeneration is a pathological hallmark of MS contributing to irreversible neurological disability. In actively demyelinating lesions, myelin is phagocytosed by microglia and macrophages, while the fate of degenerating or damaged axons is unclear. In chapter 2 we investigated phagocytosis and degradation of neuronal debris by microglia/macrophages. In MS, grey matter pathology is characterised by less pronounced microglia activation and lymphocyte infiltration compared to white matter lesions. Such differences are highlighted by leukocortical lesions that extend across white and grey matter offering an opportunity to examine differences in grey and white matter microglia activation in one lesion. In chapter 3 we examined grey and white matter parts of leukocortical lesions with respect to microglia activation, axonal damage and phagocytosis of axonal debris. In vitro studies were performed with white and grey matter-derived microglia from mice to compare these cells from both regions in functional assays. To further characterise the immune response to NF-L we used a proposed animal model of MS, EAE (chapter 4). In this chapter we focussed on T- and B-cell responses to NF-L peptides and the pathogenicity of immunodominant epitopes in the NF-L amino acid sequence. As mentioned above,
antibodies to NF-L are suggested as biomarkers. Therefore we evaluated the NF-L antibody levels in sera of people with MS with clinical variants and investigated whether these antibody levels change during treatment with natalizumab (chapter 5). Besides that antibodies to NF-L are suggested as surrogate markers, it is at present unknown whether antibodies to NF-L are pathogenic. In chapter 6 we investigated whether monoclonal antibodies to NF-L and purified human Igs of people with MS with high levels of NF-L antibodies were pathogenic in vitro and in vivo.

Finally, we conclude with chapter 7 where we discuss our aims and the results of this thesis and put the results in perspective of the recent literature and suggest further research goals.

References


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Chapter 1


