Blood and CSF biomarkers for investigation of the immunopathogenesis of relapse in Multiple Sclerosis

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Till Petra, Elis & Ella

"The prognosis has hitherto been of the gloomiest….
…After what preceeded, need I detain you long over the
question of treatment? The time has not yet come
when such a subject can be seriously considered…"

JM Charcot

Abstract

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS). MS usually starts with a relapsing-remitting course (RRMS) that later converts into a secondary progressive phase (SPMS). While inflammation is considered predominating in RRMS, neurodegenerative processes are probably more important in SPMS. The pathophysiology of MS includes autoreactive inflammatory cells that invade the CNS, causing demyelination, oligodendrocyte loss, axonal damage, astrocyte activation and subsequently gliotic scars. Over the last decade the importance of neuronal/axonal damage has been re-discovered. Although, most extensive during progressive MS, it is also an early phenomenon and can be noticed already soon after MS onset. It is the main cause of CNS atrophy and irreversible disability in MS. Several studies indicate that MS immunomodulatory therapy reduces this process, at least during RRMS. The most apparent clinical effect of MS therapy is relapse reduction.

The aim of this thesis was to investigate the relationship between inflammation, neuropathological processes and the clinical course of MS in order to identify biomarkers that could be useful for monitoring disease activity and therapy efficacy. The pathophysiological mechanisms behind clinical relapse were explored, including the possible role of T-cell mediated cytotoxicity.
Patients with RRMS or SPMS were included and healthy blood donors served as controls. Serum and cerebrospinal fluid (CSF) were obtained at relapse, remission or progression. A sub-group of RRMS patients with acute relapse were followed-up repeatedly 5 and 15 weeks after relapse onset. Pro- and antiinflammatory cytokines, neurofilament light chain protein (NFL), a marker of axonal damage and glial fibrillary acidic protein (GFAP), an astrogliosis marker were analysed in CSF. T-cell mediated cytotoxicity was investigated by analysing granzyme A and B in serum and CSF and by mRNA gene expression analysis of peripheral T-cells.

NFL in CSF was increased in all MS patients and showed a 10-fold increase during clinical relapse in relation to patients in remission and progression. The levels peaked after five weeks and were sustained for 15 weeks. CSF-levels of GFAP had a strong correlation to the EDSS in MS-patients, with the strongest correlation in the SPMS group. IL-6 in CSF was higher in RRMS patients than SPMS and controls. CCL2 in CSF was lower in RRMS patients with the lowest level at ongoing relapse compared with controls. Granzyme A and B were increased in CSF at acute relapse and increased level of granzyme A were sustained for up to 3 months.

Increased levels of NFL indicate a continuous axonal damage throughout the clinical course of MS with the most extensive damage during acute relapses. Increased levels of GFAP in MS, with a strong correlation to increasing disability, indicate that astrogliosis is more prominent during clinical progression. While demyelination is considered the pathophysiological hallmark of relapse development, our data suggests that axonal damage may be important in this process. Further, the change in the CSF levels of inflammatory markers (IL-6, CCL2) during relapses supports a relationship between inflammation and axonal damage. We also demonstrate that T-cell cytotoxicity within the CNS/CSF compartment might participate in the immunopathogenesis of clinical relapses. Prolonged increase of NFL and granzyme A levels in CSF after a relapse support an ongoing immunological attack even after apparent clinical remission suggesting a dissociation between immunological and clinical remission.

In conclusion, NFL appears to be a marker for relapse and GFAP for clinical progression. Although our data suggests that these markers might be useful in monitoring disease activity and MS therapies, further studies are needed. Intense treatment of relapse and reduction in relapse frequency during immunomodulatory treatments probably decrease axonal damage which should be beneficial for a positive long term outcome

Original articles

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I

MALMESTROM, C., HAGHIGHI, S., ROSENGREN, L., ANDERSEN, O. & LYCKE, J. (2003) Neurofilament light protein and glial fibrillary acidic protein as biological markers in MS. *Neurology,* 61**,** 1720-5.

II

MALMESTROM, C., ANDERSSON, B. A., HAGHIGHI, S. & LYCKE, J. (2006) IL-6 and CCL2 levels in CSF are associated with the clinical course of MS: implications for their possible immunopathogenic roles. *J Neuroimmunol,* 175**,** 176-182.

III

MALMESTRÖM C, LYCKE. J., HAGHIGI S, ANDERSEN O, CARLSSON L, WADENVIK H, OLSSON B (2008) Relapses in Multiple Sclerosis are associated with increased CD8+ T cell mediated cytotoxicity in CSF. Manuscript – Submitted

List of abbreviations

BBB	Blood-brain Barrier
CCL2	C-C Ligand 2
CD-	Cluster Designation
CIS	Clinically Isolated Syndrome
CNS	Central Nervous System
CSF	Cerebrospinal fluid
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated Antigen - 4
EAE	Experimental allergic encephalomyelitis
EDSS	Expanded Disability Status Scale
ELISA	Enzyme linked immunosorbent assay
GA	Glatiramere Acetate
Gd^+	Gadolinium enhancement
GFAP	Glial Fibrillary Acidic protein
HLA	Human Leukocyte Antigen
IgG	Immunoglobulin G
IL-4	Interleukin–4
IL-6	Interleukin–6
Lp	Lumbar puncture
MBP	Myelin Basic Protein
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
MOG	Myelin-Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
MSFC	Multiple Sclerosis Functional Composite
NAWM	Normal appearing white matter
NF	Neurofilament
NFH	Neurofilament – heavy chain
NFL	Neurofilament – light chain
OB	Oligoclonal bands
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PPMS	Primary progressive MS
RRMS	Relapsing Remitting MS
SPMS	Secondary Progressive MS
TCR	T cell receptor
TGF-β	Transforming growth factor β
VLA	Very late antigen

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Introduction

Multiple sclerosis (MS) is considered an organ specific autoimmune disease of the central nervous system (CNS) where autoreactive immune cells target epitopes on myelin, oligodendrocytes or axons. The disease activity comprises of a chronic low grade disseminated inflammation in the CNS (Chard et al., 2002a) and superimposed acute focal inflammation caused by infiltration of inflammatory cells to the CNS (Noseworthy et al., 2000). This results in episodes with rapid deterioration of neurological function followed by a complete or incomplete recovery, the MS relapse. The consequence is a complex injury to nerve- and glial tissue in terms of demyelination of axons, but also damage to axons and oligodendrocytes, astrocyte activation and formation of gliotic scars (Raine, 1997). The dynamic clinical course with relapse followed by remission is unpredictable and reliable methods for measuring disease activity are needed for predicting outcome in individual patients and assessing response to immunomodulating treatment.

Epidemiology of MS

MS is a major cause of disease-burden with around 2.5 million individuals affected worldwide (Ian Douglas, 2006; Rosati, 2001). There is considerable variation of prevalence of MS around the world. Temperate climate zones in both the northern and southern hemisphere are considered high risk areas with a prevalence of MS between 60-200/100.000. In low risk areas, such as Japan, the prevalence is 2-20/100.000 however because of different diagnostic criteria the figures are not entirely comparable (Rosati, 2001). The prevalence in Sweden is estimated to be 100-154 /100.000 (Sundstrom et al., 2001) and incidence to around 5/100000 (Svenningsson et al., 1990) resulting in approximately 12000 affected individuals.

Women are affected approximately twice as often as men; however recent reports have stated an increase in incidence of MS in women (Debouverie et al., 2007). Also an increase in sex-ratio has been reported from Canada where women exceeded men 3.2:1 (Orton et al., 2006). This suggests an increase in MS incidence, a topic however under some debate (Poser and Brinar, 2007) and a recent prevalence survey in the greater Gothenburg area resulted in a prevalence of 94/100000 (C Ahlgren, pers comm.) and could thus not confirm an increase in MS incidence.

Aetiology of MS

The aetiology of MS is so far unknown; however several lines of evidence support environmental as well as genetic factors. The latitude dependent prevalence and migration data (Elian et al., 1990) have focussed the search on external factors towards environmental factors such as sunlight (Freedman et al., 2000), vitamin D intake (Munger et al., 2004), and cigarette smoking (Hernan et al., 2001)**.**
Several infections have been suggested however none have been convincingly associated with the disease (Giovannoni et al., 2006). During recent years candidate infections have been herpes virus -1 (Bergstrom et al., 1989), human herpes virus 6 (Swanborg et al., 2003), Chlamydia *pneumoniae* (Munger et al., 2003), and Epstein Barr virus (EBV) (Bray et al., 1983; Sumaya et al., 1985). EBV is a herpes virus capable of establishing a chronic infection in B-lymphocytes and has been reported to

affect the risk of MS (Thacker et al., 2006). This meta-analysis showed an increased risk of developing MS among individuals who contract the primary EBV-infection in the form of mononucleosis compared with having the infection during infancy with a relative risk (RR) of 2.3 and further having had mononucleosis a RR of 38 compared with those who have never had an EBV-infection. Furthermore EBV has recently been found in B-lymphocytes in ectopic lymphoid tissue in the meninges of post mortem MS brains (Serafini et al., 2007).

The genetic influence in MS does not follow classical Mendelian laws of inheritance. However the genetic influence is estimated to increase the risk of MS from 0.1 % in western population to approximately 3 % in first degree relatives; siblings and dizygotic twins, and to about 30 % in monozygotic twins of affected patients (Willer et al., 2003). Although several investigations with whole genome scans have been performed for associations with MS (Sawcer et al., 2005) the reported candidate genes are regulating immune-responses and particularly within the HLA II-gene complex (Olerup and Hillert, 1991). The first the non-HLA genes recently found were polymorphisms in the receptor of interleukin 7, with an estimated aetiological fraction of 12%, although not coinciding with the HLA positive cases (Gregory et al., 2007; Lundmark et al., 2007) and interleukin 2-receptor α polymorphism (Hafler et al., 2007).This supports the concept that MS is a complex non Mendelian hereditary disease with low penetration (Dyment et al., 2004).

Clinical Course of MS

The first relapse noted is designated the clinically isolated syndrome (CIS) (Figure 1) as dissemination in space and time is required for a diagnosis of MS (McDonald et al., 2001). A majority of the patients experience a relapse onset, relapsing remitting MS (RRMS) and about 10 to 15 % start with an insidious progressive course from onset, termed primary progressive MS, (PPMS). After several years with RRMS a majority transforms to secondary progressive MS (SPMS). In SPMS common symptoms are a slowly increasing spastic paraparesis and/or cerebellar ataxia with or without superimposed relapses. The rate of conversion to SPMS has been calculated to 2-3% per year (Vukusic and Confavreux, 2003) and median time from CIS to conversion to SPMS is about 16 years (Runmarker and Andersen, 1993).

Clinical characteristics of MS relapse

MS onset generally occurs between 10 and 60 years of age, and characteristically in early adulthood between 20 and 40 years of age where about 2/3 of the patients have their first symptoms. The clinical presentation of MS is frequently with a clinical relapse being defined as new or reappearing neurological symptoms (Poser, 1995) lasting more than 24 hours, and consistent with affection of the white matter of the CNS and in absence of fever (Poser et al., 1983). A decline in neurological function may occur in a step-wise manner as a consequence of incomplete recovery after a relapse or as a gradual unalterable deterioration in absence of relapses in progressive forms of MS. Incomplete remission after relapses is common. In a follow-up two months after relapse onset 42% of patients had an increase of 0.5 points or more and 28 % had an increase of 1.0 or more on the Expanded disability status scale (EDSS)

(Kurtzke, 1983; Lublin et al., 2003). Residual symptoms after first relapse and high initial relapse frequency are predictive factors for a more severe outcome (Eriksson et al., 2003).

Clinical Courses
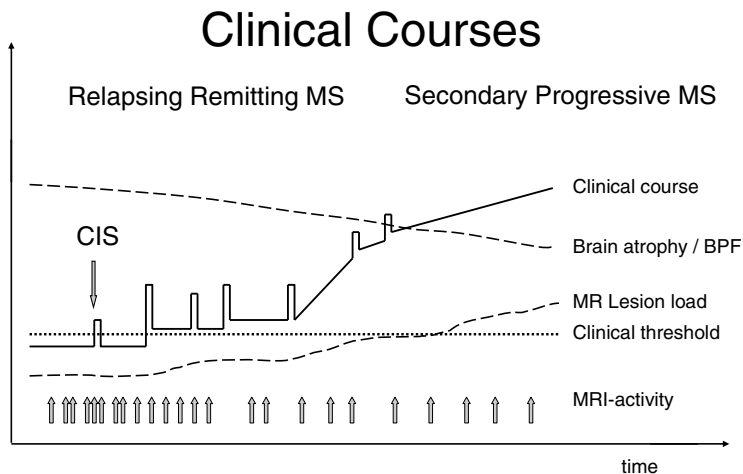
Figure 1. *CIS; Clinically isolated syndrome: an initial demyelinating event suggestive for onset of MS, BPF; Brain parenchymal fraction. A MRI based brain atrophy measurement.*

Diagnostic criteria in MS

The diagnosis of MS still depends on dissemination in space and time of white matter lesions in the CNS. The present set of criteria, the McDonald-criteria (McDonald et al., 2001) had its predecessor in the Poser-criteria (Poser et al., 1983) and MacAlpine criteria (1971) which in turn replaced the Schumacher-criteria (Schumacher et al., 1965). This evolution reflects the development of more reliable techniques to detect demyelinating lesions of the CNS. There is no single test or finding with sufficient sensitivity and specificity to be considered pathognomonic of MS, therefore combinations of findings in neurological- ; magnetic resonance imaging (MRI); and cerebrospinal fluid (CSF) examinations weighed together builds up the diagnosis. The concept of dissemination in space and time, i.e. neurological symptoms occurring at different points in time and affecting different parts of the CNS and hence not attributable to a single lesion has been a cornerstone since the Schumacher-criteria. The McDonald-criteria emphasise the role of MRI and repeated MRI examinations showing newly developed lesions can replace clinical relapses in estimating

dissemination in time and hence facilitating an early diagnosis in suspected MS-cases. The criteria were further revised in 2005, to enhance the role of spinal cord lesions and the criteria for PPMS (Polman et al., 2005).

Immunomodulating treatment of MS

Since the 1990s immunomodulating treatment has become available for patients with RRMS. Interferon-β (IFN-β) is a polypeptide normally produced in human fibroblasts with antiviral and anti-proliferative effects. IFN-β reduces antigen presentation, T-cell proliferation and shifts the cytokine pattern from a proinflammatory T_H-1 pattern to a more anti-inflammatory T_H-2 pattern, (see text below). IFN-β1a for intramuscular (im) or subcutaneous (sc) administration and IFN-β1b for sc administration have in large pivotal trials shown partial effect with a 30 % reduction in the relapse rate (Ebers et al., 1998; Jacobs et al., 1996; Polman et al., 2006; Sibley et al., 1993), 60 – 80% reduction of Gd^+ enhancing T1 lesions seen with MRI (Li and Paty, 1999) and a moderate effect on disability progression (Kappos et al., 2006b). Glatiramere acetate (GA) is a randomly assembled polymere built up of four aminoacids mimicking the composition of myelin basic protein (MBP). It is one of the main components of the myelin sheath and suggested to be one of the targets of the autoimmune attack in MS (Schmidt, 1999). GA is administered sc daily and has similar efficacy in relapse reduction as IFN-β however it has shown no convincing effect on disease progression (Johnson et al., 1995). It is suggested that GA induces a shifts in the T cells population from proinflammatory T_H1 cells to regulatory T_H2 cells that suppress the inflammatory response. It is further suggested that due to its resemblance to MBP, GA also act as a decoy, diverting an autoimmune response against myelin.

Mitoxantrone, a cytotoxic agent acts antiproliferative on B-cells and T-cells, reduces antigen presentation and reduces synthesis of proinflammatory cytokines such as interleukin 2 (IL-2), tumor necrosis factor alfa (TNFα) and interferon gamma (IFNγ) (Baker et al., 1992; Watson et al., 1991). In MS mitoxantrone has shown effect in reducing relapse rate by 67 % and reducing disease progression by 64 % (Hartung et al., 2002).

Natalizumab is a humanized monoclonal antibody targeting VLA-4 (very late antigen-4), an adhesion molecule expressed predominantly on T-cells but also on B-cells and monocytes. VLA-4 is a bottleneck molecule for migration of immune-cells over the Blood-brain Barrier (BBB) into the CNS. Natalizumab have shown a reduction of relapse rate by 68 %, reduction of new or enlarging T2 lesions on MRI by 83% and Gd^+ lesions on MRI by 92% (Polman et al., 2006).

Immunology of MS

About 5% of the western population is affected by autoimmune disease where the immune system mistakenly attacks the body's own tissues leading to tissue destruction (Davidson and Diamond, 2001). A definition of autoimmunity is "Witebskys postulate about autoimmunity", revised 1993 (Rose and Bona, 1993). It states (i) The presence of autoantibodies or autoreactive T-cells, (ii) Binding of auto antibodies to target structures, (iii) The disease should be reproduced by active immunization by autoantigen, passive transmission of autoantibodies or autoreactive T-cells and (iv) Response to immunomodulation or immunosuppression.

MS fulfill several of the requisites and further shares clinical and immunological similarities with experimental allergic encephalomyelitis (EAE) an animal model of MS (Steinman, 2001b). In EAE preparations of myelin proteins, usually myelin-basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) in combination with adjuvant evokes an autoimmune reaction with demyelination of the CNS (Rivers, 1935). Depending on the inducing antigen, animal strain, and type of immunisation, the clinical course varies from acute monophasic to chronic relapsing remitting, the latter clinically mimicking RRMS. EAE is considered to be primarily a T-cell mediated disorder, and $CD4^+$ antigen-specific T_H-1 cells have a crucial although not exclusive role in the development of EAE. It can be transmitted to naïve animals from affected animals predominantly by autoreactive $CD4^+$ T-helper cells, designated adoptive transfer EAE (Hemmer et al., 2002).

Immunopathgenesis of MS

The current hypothesis of MS pathogenesis, although not yet proven, comprises an initial autoimmune inflammatory phase, subsequently developing into a phase of gradual neurodegeneration (Steinman, 2001a). Present theories of MS pathophysiology are largely developed from EAE where formation of CNS lesions involves a number of immunological mechanisms in which $CD4^+$ T-cells play a central role. In the prevailing hypothesis of MS autoimmunity, reviewed by (McFarland and Martin, 2007) $CD4^+$ T-cells become autoreactive in peripheral lymph nodes when encountering antigens e.g. myelin fragments presented by antigen presenting cells, (APC). Attracted by chemokines, such as C-C motif ligand 2 (CCL2) and CCL5 (also known as RANTES) they are directed to the site of inflammation in the CNS. They cross the BBB by interaction between adhesion molecule VLA-4 and its endothelial ligand vascular cell adhesion molecule-1 (VCAM-1) among other up-regulated adhesion molecules. They become reactivated within the CNS by APC i.e. microglia, macrophages or astrocytes, again presenting autoantigens for the $CD4^+$T-cells.

T-cells proliferate to either a proinflammatory phenotype, $CD4^+$ type 1 helper T cells (T_H-1) synthesizing proinflammatory cytokines (e.g., interleukin-12, IL-12 and interferon- γ, IFN-γ) inducing immune-mediated injury to myelin and oligodendrocytes (Figure 3) or an antiinflammatory phenotype T_H-2 with release of anti-inflammatory cytokines (interleukin-1, IL-1, interleukine-4, IL-4, interleukine-

10, IL-10) (Noseworthy et al., 2000). Candidate autoantigens are fragments from MOG, MBP, or other myelin derived proteins, reviewed by (Schmidt, 1999).

Several mechanisms are proposed as contributors to the resulting damage of the myelin sheets, axons and oligodendrocytes. (i) Complement dependent antibody-mediated damage, as complement deposit is demonstrated in MS-lesions (Lucchinetti et al., 2000), (ii) Digestion of surface myelin by macrophages after targeting myelin-antibodies (Trapp et al., 1998), (iii) Injury mediated by release of toxic mediator such as cytokines and proteases (Schaecher et al., 2002), (iv) Reactive oxygen and nitrogen species (Bitsch et al., 2000b; Rejdak et al., 2007), (v) Direct injury by CD8^{+} cytotoxic T-cells by reslease of granzymes among other molecules (Neumann et al., 2002). The result is tissue damage ranging in a spectrum from axons lacking myelin-sheets and loss of saltatory conduction properties, where remyelination and reconstitution of axonal function can still occur, to irreversible axonal transection (Trapp et al., 1998). Proposed mechanisms for the immune attack and putative biomarkers in MS are shown in Figure 3.

White matter lesions
Areas of focal lesions, disseminated across the white matter of the CNS are the most prominent findings in MS brains. Most frequent sites for lesion formation are the optic nerves, periventricular regions, subcortical, brainstem and the spinal cord.
MS-lesions of the white matter are traditionally divided into three major categories based on distribution and density of inflammatory cells, and on demyelination. They are active or acute-, chronic active- and chronic inactive or silent lesions (Cannella and Raine, 1995). The active lesion shows a high density of evenly distributed small lymphocytes and indistinct margins, demyelination and axonal damage. The chronic active lesion has sharper edges less cellular density in the centre, deposition of immunoglobulin and remyelination. The chronic inactive lesion is regarded as the common endpoint in the process and features sharp edges, few infiltrating leukocytes, reduced number of demyelinated axons and astroglial scars (Frohman et al., 2006).
Heterogeneity between MS-lesion is suggested in a well cited paper by Luccinetti and co-workers (Lucchinetti et al., 2000). They reported four different immuno-pathological patterns of MS lesions. In pattern I demyelination was associated with T-cells and macrophages, remyelination and survival of oligodendrocytes. Pattern II had similar properties but additional deposition of immunoglobulin and complement suggesting a component of humoral immune response. Pattern III showed active lesions with a diffuse loss of oligodendrocytes due to apoptosis and pattern IV displayed a primary oligodendrogliopathy suggesting a neurodegenerative cause rather than an inflammatory. Apart from a correlation between pattern IV and PPMS they seemed not to correlate with any specific clinical course. The lesions appeared to be homogenous in individual patients suggesting different underlying pathophysiological mechanisms operating in different patients. That these findings are caused by a constitutional heterogeneity between affected individuals is a matter of discussion and other groups have found lesions consistent with several of the above described patterns in the same patient (Barnett and Prineas, 2004; Gay et al., 1997).

Gray matter lesions

Demyelinating lesions in the cerebral cortex are a feature that is gaining increasing attention. Previous studies, correlating MRI data with histopathological examinations indicate that MRI underestimates the prevalence of cortical lesions and that small cortical lesions are common in MS (Kidd et al., 1999). Recent MR-spectroscopy (MRS) studies have demonstrated grey matter metabolic changes suggestive of both demyelination and axonal damage (Chard et al., 2002a).

Morphological studies generally separate cortical lesions into three different types; the leukocortical lesion, stretching over the border between white and grey matter small perivascular entirely intracortical lesions and subpial lesions affecting several, and sometimes all cortical layers (Bo et al., 2003a). Pathologically the lesions are characterized by demyelination, activated microglia and apoptotic death of neurons. The number of infiltrating T-cells and macrophages are significantly fewer than in white matter lesions (Peterson et al., 2001). Subpial lesions appear to be associated with meningeal inflammation and to have a predilection to basal areas of the brain such as the cingulate gyrus, the temporal- and insular cortex (Bo et al., 2003b). Although cortical lesions appear early in RRMS they are a more general feature of PPMS and SPMS, where they can be extensive, with up to 70 % of the cortex affected, suggesting it is a predominantly late phenomenon in MS pathology (Kutzelnigg et al., 2005).

Neurodegeneration in MS

Besides the inflammatory activity in the CNS there are signs of an ongoing degenerative process in MS that appears to start early in the disease. Patients who have been diagnosed less than two years and with a mean EDSS of 1.0 have a significant cerebral atrophy, compared with healthy controls (Chard et al., 2002b). Pathological alterations in normal appearing white matter (NAWM), with low concentrations of N-acetylaspartic acid (NAA), a marker for axonal damage shown by MRS in MS patients with low scores on EDSS (range 2-3) suggests axonal damage apart from the inflammatory areas detectable on conventional MRI (De Stefano et al., 2002). The atrophy of the CNS is most pronounced in the progressive phase of MS, and correlates with the rate of decline in neurological function (Losseff et al., 1996a). The pathology in the chronic inactive lesions consists of areas devoid of myelin and demarcated from adjacent parenchyma, an intense fibrillary astrogliosis, with bundles of GFAP-fibres, interlaced with macrophages and myelin debris, making up the glial scar tissue (Raine, 1997). Furthermore oligodendrocytes are depleted in the lesions, and oligodendrocyte death has been associated with apoptosis (Lucchinetti et al., 2000), however oligodendrocytes have been observed to proliferate in adjacent unaffected tissue (Raine, 1997).

Axonal damage

Even though axonal injury in MS lesions was described in the initial works of the 19th century (Charcot, 1868) it has been deemphasized up until the 1990s since the main pathological events appeared to be related to myelin destruction and the label

"axonal sparing" has long been tagged to the disease. During a relapse the neurological symptoms have mainly been considered to be caused by axonal conduction blocks secondary to demyelination. The disability accumulated during the progressive phase has been seen as a result of later permanent axonal damage. However acute axonal injury can be detected by accumulation of amyloid precursor protein (APP) in MS lesions early in the disease (Kuhlmann et al., 2002) in terms of swelling where axons have been transected and axonal transportation has been interrupted (Ferguson et al., 1997). An association between the numbers of CD8^{+} T-cells and the extent of axonal damage suggests the involvement of T-cell mediated cytotoxicity in axonal injury (Bitsch et al., 2000b). It is uncertain whether the axonal damage is caused by a direct attack on axons or if it is a phenomenon secondary to demyelination. It has been shown that cytotoxic T-cells (CTL) can co-localize to naked axons in MS lesions and that cytotoxic granules polarize towards the axon indicating release of effector molecules such as granzymes and perforins (Neumann et al., 2002). It appears that axonal damage is not restricted to areas with inflammation. Histopathologically, axonal loss can also occur in NAWM (Bjartmar et al., 2001).
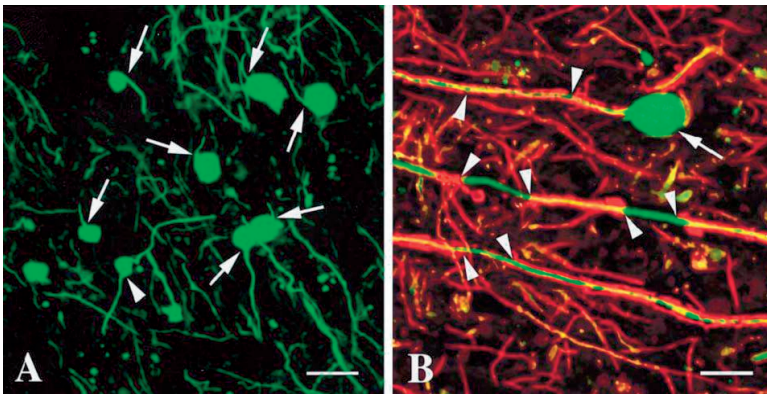
Figure 2. *Confocal Microscopical Images of axonal changes in MS lesions. Nonphosphorylated neurofilaments are green in both panels. Red indicates myelin in Panel B.* *Panel A shows the centres of active lesions. Panel B shows the edges of active lesions. Panel A shows "stacked images" of terminal axonal ovoids with single axonal connections (arrows), an axonal ovoid with dual axonal connections (arrowhead), and many normal-appearing axons. Panel B shows three large, nonphosphorylated-neurofilament–positive axons undergoing active demyelination (arrowheads). One axon ends in a large terminal ovoid (arrow). The scale bar in Panel A represents 64 μm; in Panels B, 45 μm.*

Axonal damage visualized by staining with an antibody against non-phosphorylated neurofilament showed ovoid swellings in the ends of transected axons (Figure 2), and activated macrophages containing myelin debris adjacent to the swelling (Trapp et al., 1998). Cortical lesions also exhibit transected axons and dendrites and neurons undergoing apoptosis, especially in chronic cortical lesions (Peterson et al., 2001). Thus axonal loss is substantial in white as well as in grey matter, and in all stages of MS.

Astrogliosis

Astrocytes have several functions in the CNS. They have an anatomically strategic position; being in close contact with the peripheral circulation, as their end-feet processes closely surround blood vessels of the CNS as a part of the BBB and also cover the nodes of ranvieres along the axons (Williams et al., 2007). They are the major source of glial fibrillay acidic protein (GFAP) in the body. They react within hours to any kind of injury or stress to the CNS such as trauma, infections and inflammation (Pekny and Nilsson, 2005). Further they have phagocytic capabilities and appear to play an important role in removing debris after apoptosis (Herndon, 2002). The response termed astrogliosis comprises proliferation, hypertrophy of the astrocytic soma and synthesis of GFAP-containing fibrils. The final gliotic scar is a complex structure of astrocytes, microglia, macrophages and other cells as well as extracellular matrix and it prevents axonal and neurite regeneration (Hatten et al., 1991).

Key cell populations in MS immune response

Macrophages and microglia

Macrophages are bone marrow derived immune cells which phagocytose cellular debris and pathogens, and stimulate lymphocytes and other immune cells to respond to pathogens. Microglia are the stationary variant of macrophages and a resident APC in the CNS. They make up 10–20% of all glial cells in the CNS and populate the CNS during foetal development (Benveniste, 1997). Microglia continuously survey the CNS for infectious agents, damaged CNS cells and other foreign antigens. They then migrate to the injured site engulfing dead cells and debris (Gehrmann et al., 1995). In MS, microglia are activated, express high levels of MHC class II and function as APCs and further express a broad range of cytokines, such as IL-1, TNF-α, IL-6 and IL-12, most of which have proinflammatory properties and are involved in inflammation and demyelination within the CNS (Ambrosini and Aloisi, 2004).

Astrocytes

Astrocytes are immunologically active and have the ability to synthesize both chemokines (Van Der Voorn et al., 1999) and cytokines (Farina et al., 2007).TNF- α mRNA expression is positively correlated with active demyelinating activity and oligodendrocyte apoptosis in MS brain (Bitsch et al., 2000a). They are further a part of the innate immune response and are a major source of complement in the CNS

(Gasque et al., 2000). Astrocytes have been shown in vitro to act as inducible APC, although their role in MS is debatable (Soos et al., 1998).

B-cells

The involvement of the humoral branch of the immune system in MS is well recognized and production of myelin-specific antibodies which activate the innate immune system is an important step in MS pathogenesis reviewed by (Antel and Bar-Or, 2006). Elevated IgG-index in a majority of MS-patients and persisting oligoclonal bands (OB) in CSF indicate intrathecal B-cell clones synthesizing immunoglobulin (Tibbling et al., 1977). B-cells do not cross the intact BBB, but can, together with antibodies and complement pass in inflammatory activated areas. Depletion of B-cells in MS with a monoclonal antibody directed against CD20, has resulted in a reduction in Gd^+ lesions on MRI and reduced relapse frequency (Bar-Or A, 2007).

T-cells (CD3+)

T cells have a central role in cell-mediated immunity. They can be distinguished from other lymphocytes by the expression of the T cell receptor (TCR, CD3) on the surface. Several subsets of T cells are active in the immune response in MS.

T_H-1/T_H-2 (CD4+)

T-helper cells (T_H cells) are the hub of the adaptive immune response. T-cells recognize the target antigen by the TCR which is co-expressed with either CD4 on T_H-cells or CD8 on CTL. CD4 is crucial for ligand formation between TCR and MHC class II antigens and CD8 anchors the TCR with MHC class I-antigen. Naïve $CD4^+$ helper T-cells, proliferate to either of two major T-cell-pools, the T_H-1 regarded as proinflammatory or the T_H-2 regarded as anti-inflammatory in autoimmune disease (Figure 3) (Neumann et al., 2002). The T_H-1 response has also been shown to promote cell-mediated cytotoxicity by promoting proliferation of CD8+ T-cells via dendritic cells (Smith et al., 2004).

The T_H-1/T_H-2 hypothesis has been extended in recent years as other specific subpopulations of T-cells have been identified (Steinman, 2007).

Regulatory T-cells (CD25+)

Regulatory T-cells (T_{REG}) exert an active control over naturally occurring autoreactive T-cells. They proliferate under the influence of transforming growth factor beta (TGF-β) and have a high expression of the IL-2 receptor, ($CD25^{high}$) (O'Garra and Vieira, 2004). T_{REG} has been reported as having impaired function in MS (Viglietta et al., 2004) and reduced ability to suppress proliferation of T-cells from MS patients (Kumar et al., 2006).

γδ T-cells

γδ T-cells are a subpopulation of T-cells, usually $CD4^-CD8^-$, and as a consequence are not restricted to MHC presentation of antigen and therefore are considered a part of the innate immune system, (Sospedra and Martin, 2005). They express γδ subunits

of the TCR (CD3), constitute 1-5% of peripheral blood mononuclear cells (PBMC) and have both host defence and regulatory functions. They have effector capacity similar to CTL and a cytokine pattern with closer resemblance to T_H-1 than T_H-2 (Carding and Egan, 2002).

T_H-17 cells

T_H-17 cells are a specific subpopulation of CD4^{+} T-cells producing interleukin -17 (IL-17). IL-17 is a potent proinflammatory cytokine that can induce IL-6 in human fibroblasts and endothelial cells (Kolls and Linden, 2004) and further induce expression of CCL2 in rat intestinal epithelial cells in vitro (Awane et al., 1999). T_H-17 cells are generated from naïve T-cells under the influence of IL-6 in combination with transforming growth factor β (TGF-β). T_H-17 cells are highly encephalitogenic in EAE (Langrish et al., 2005) and have been shown to have the ability to cross the BBB in an in vitro model (Kebir et al., 2007). Blocking IL-6 signalling pathways results in a reduction of T_H-17 cells (Kimura et al., 2007). They express granzyme B, have the ability to kill neurons and are found in human MS lesions (Kebir et al., 2007).

Cytotoxic T-cells (CD8+)

The role of CD8^{+} CTL in MS has not been studied to the same extent as CD4^{+} cells. However EAE was recently transferred by CD8^{+} T-cells, (Huseby et al., 2001). There are divergent results in therapy trials in CD4^{+} induced EAE and MS, e.g. anti- TNF-α agents cure CD4^{+} EAE (Steinman, 1999) whereas it worsens MS (Lenercept_Group, 1999). Several results suggest that CD8^{+} CTLs may play a role in the pathogenesis of MS (Jacobsen et al., 2002; Zang et al., 2004). CTL bind to MHC class-I molecules on the target cells (Figure 3) which are expressed on all nucleated cells in the organism and immunostaining indicates that MHC class I is upregulated on axons and neurons within active MS lesions (Hoftberger et al., 2004). Expanded CD8^{+} T-cell clones have been identified in the CNS, CSF and blood of patients with MS, and some of these clones persist for five years or more (Skulina et al., 2004). CD8^{+} T-cells dominate the inflammatory infiltrate of MS lesions (Babbe et al., 2000; Gay et al., 1997) and correlate with the amount of acute axonal damage in MS (Bitsch et al., 2000b). In a clinical trial, a monoclonal antibody against CD4, cM-T412, failed to affect the course of the disease (van Oosten et al., 1997) whereas treatment with the anti-CD52 monoclonal antibody Alemtuzumab, which induced a long term depletion of CD4^{+}and CD8^{+} T-cells resulted in fewer relapses (Coles et al., 2006).

CD8^{+} T-cells are capable of damaging oligodendrocytes, axons and neurons in autoimmune demyelinating CNS diseases (Giuliani et al., 2003; Jurewicz et al., 1998; Medana et al., 2000) and may also exhibit regulatory properties (Koh et al., 1992). Direct CD8^{+} T-cell cytotoxicity is triggered by cell contact between the CTL and the target cell. The cytotoxic activity of CTL is mediated by two major pathways. Either a granule mediated pathway with release of perforine and granzymes or by the receptor-coupled pathway by Fas-Fas-ligand complex (Kagi et al., 1994b) (Barry and Bleackley, 2002).

Granule mediated cytotoxicity

Several different proteins in the cytotoxic granule of CTL participate in the killing of the target cell. Perforin, which upon exocytosis polymerizes to form a pore in the target cell membrane, allows intracellular cytotoxic proteins such as granzymes to enter the cell. Granzymes are exogenous serine proteinases with highly specific sites of cleavage. There are several different granzymes identified and the most abundant are granzyme A and B. They are stored and released from cytoplasmic granules of CTLs and natural killer cells (NK-cells) (Barry and Bleackley, 2002). The granzymes enter the target cell via perforin pores or via a receptor mediated pathway (Froelich et al., 1996). In the cytoplasm granzyme B can activate different pathways. One is a caspase-dependent pathway that leads to apoptosis others attack mitochondria and give rise to necrosis of the target cell (Lieberman, 2003). Granzyme A initiates a caspase-independent pathway, affecting DNA and preventing cellular repair, thereby forcing the cell into apoptosis (Beresford et al., 2001).

Receptor-mediated cytotoxicity

Fas (CD95), is a surface receptor expressed on several cell types, It is a member of the tumor necrosis factor receptor (TNFR) superfamily and is important for inducing apoptosis in target cells (Rouvier et al., 1993). The receptor Fas-ligand, (FasL; CD 95L; CD178), is expressed on activated T cells and NK-cells, and is inducible in other cell types. Fas and FasL are reviwed by (Nagata and Golstein, 1995) and the putative role in MS by (Zipp et al., 1998).

Possible biomarkers in different pathophysiological steps in MS

CCL2
GFAP
IL-6
NFL
Granzymes
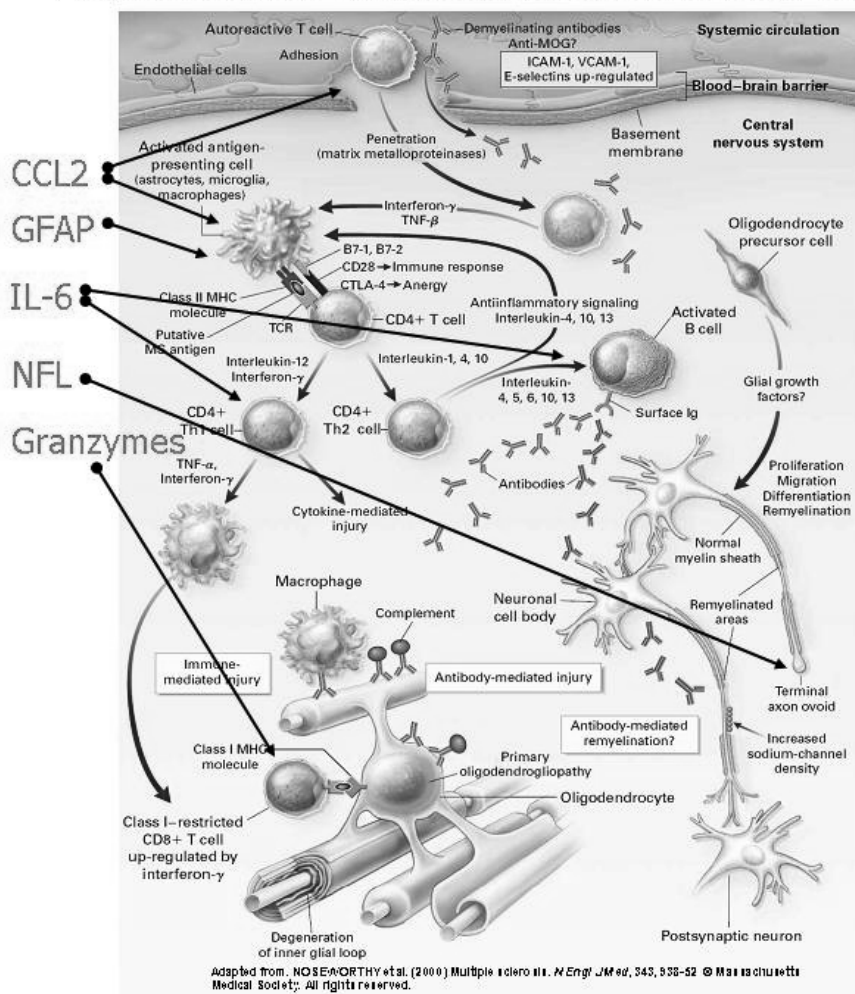
Figure 3.

Infiltration from system circulation:

    CCL2 - CC-motif ligand 2; expressed in astrocytes in inflammatory lesions

Pro-inflammatory activity

    IL-6 – Interleukin-6; pro-inflammatory cytokine

T-Cell Cytotoxic activity

    Granzyme A / B ; CD8+ cell activity

Axonal damage

    NFL – Neurofilament light protein; Cytoskeletal protein specific for axons, mainly large caliber axons

Astrocyte reactivity

    GFAP– Glia fibrillary acidic protein; Marker for astrocyte activation and astrogliosis.

Measurements of disease activity in MS

The golden standard for evaluating MS disease activity in clinical trials and in clinical routine is the measurement of the annualized relapse frequency and scoring progression of neurological deficit over time using clinical scales (Whitaker et al., 1995). However, an average relapse rate of about 0.5-1 relapses/year and a slow progression rate require large patient groups and follow up time in clinical trials (Polman et al., 2006).

MRI is the major paraclinical method for assessing disease activity and is estimated to have a five to ten fold higher sensitivity to detect sub-clinical disease activity than clinical observation (Thompson et al., 1992). However, the correlation between MRI measurements and neurological disability is weak, (rho=0.18) regarding new or enlarging T2 lesions (Filippi et al., 1995), or there is no correlation at all between total lesion load and EDSS (Thompson et al., 1990). This is sometimes referred to as the clinico- radiological paradox (Barkhof, 2002) and emphasizes the need for more sensitive disease activity markers in MS.

Clinical Scales

Expanded Disability Status Scale, EDSS
The dominating clinical scoring scale for MS-associated deficit is the EDSS. It is based on neurological examination/evaluation of seven functional systems (FS), and ranges from 0 to 10 in half steps. In steps up to 3.5 the different FS contribute equally to EDSS, whereas from 4.0 to 6.5 walking ability scoring overrules. In the upper part of the scale (EDSS>6.5) the degree of autonomy in ambulation and daily activities defines the score. This leads to a non linear development of scoring as disease proceeds. EDSS is an ordinal scale allowing only non-parametrical statistics. In EDSS an inter-rater agreement, ranging from 0.3 to 0.5, as expressed by kappa index, has been reported and in particular the mental and sensory FS scored low (Amato et al., 1988). Furthermore perfect inter-rater agreement for EDSS was only achieved if agreement was defined as a difference ≤ 1.5 EDSS points. (Goodkin et al., 1992). Similar results and the conclusion that at least two units of change on the EDSS are needed for a reliable indication of clinical change were reported by (Noseworthy et al., 1990). This is important as clinical trials still use an increase of at least 1.0 on the EDSS score as endpoint for sustained progression (Kappos et al., 2007).

Multiple Sclerosis Functional Composite, MSFC
Multiple sclerosis functional composite (MSFC) score was developed by a taskforce in 1999 (Cutter et al., 1999). It was initiated to overcome the weaknesses of EDSS by using quantitative tests and normalizing them towards a control population. It resulted in a composite of three tests for (i) ambulation (Timed 25 foot walk, T25W), (ii) arm function, (Nine hole peg test, 9HPT) and (iii) cognitive function, (Paced auditory

serial addition test, 3 sec, PASAT3). The result from each test is converted to a z-score by normalizing it to a control population. The z-score is the number of SD-units a patient's score is above or below the average score. The three z-scores are then added to MSFC. MSFC has shown an inter-rater agreement of 0.95 (Cohen et al., 2000).

MR measurements of disease activity

The most commonly used sequences in MRI examinations in MS are (i) T1 hypo-intense lesions (black holes), (ii) Gadolinium contrast enhancement on T1 (Gd$^+$) and (iii) T2 lesions. A black hole on T1-weighted images reflects a more destructive type of lesion in which axonal damage is an important feature (van Walderveen et al., 1998). T1 lesion load shows a stronger correlation with clinical disability of MS patients as measured by EDSS than T2 lesion load (Rovaris et al., 1999). Gd$^+$ lesions reflect the acute inflammatory step in lesion development with BBB breakdown (Bruck et al., 1997) and lasts generally for up to five weeks (Meier et al., 2007). T2-weighted images have high sensitivity to alteration in brain tissue composition, and thus low pathological specificity. Increased T2 signal can reflect a range of pathological changes from acute inflammation to profound tissue loss and consequently there is a weak relationship between T2 lesion-load and disability.

New hyperintense lesions on T2-weighted sequence and occurrence of Gd$^+$ on T1-weighted sequence, distributed in a typical manner, (dissemination in space) and with confirmation of newly developed lesions (dissemination in time) constitutes the basis for the current diagnostic MRI-criteria (Barkhof et al., 1997; Tintore et al., 2000). The amount of T2 lesions on MRI in patients with a first demyelinated event is predictive for the risk of conversion to MS (Brex et al., 1999) and the later development of disability according to EDSS (Brex et al., 2002). Furthermore atrophy of the spinal cord is correlated to EDSS evolution (Losseff et al., 1996b).

MRS measures certain proton-containing metabolites in defined voxels of the brain. N-acetylaspartate (NAA) is an amino acid almost exclusively limited to neurons and a reduction of NAA on MRS is regarded to be a marker of axonal damage, as confirmed in autopsy studies (Bjartmar et al., 2000). Decreased levels of NAA have been found in putative MS-lesion (Narayanan et al., 1997), and in both NAWM and the cortex of MS patients early in the disease (Chard et al., 2002a).

Biomarkers: General considerations

A suggested definition of a **Biomarker** is "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological response to a therapeutic intervention" (De Gruttola et al., 2001). This is in relation to a **Clinical endpoint**, "A characteristic or variable that reflects how a patient feels or functions or how long a patient survives", and **Surrogate endpoint** a biomarker intended as a substitute for a clinical endpoint. In order to function as a surrogate endpoint it is suggested that a biomarker fulfills two requisites. First it needs to have strong and significant correlation to a clinical endpoint and secondly that it captures any relationship between a treatment and the "true" clinical endpoint. The second requisite is very restrictive as both positive effects and unanticipated adverse consequences on the clinical endpoint should be counted for (Prentice, 1989).

Biomarkers can be classified as process-specific based on the pathophysiological process they are involved in and a proposed process-specific classification for MS follows the general steps of MS pathophysiology namely markers for changes in the immune system, BBB disruption, demyelination, oxidative stress and excitotoxicity, axonal/neuronal damage, gliosis and demyelination, where immune system markers are divided into several subgroups (Bielekova and Martin, 2004).

Blood and CSF are the most widely used biological fluids but others such as urine (Giovannoni and Thompson, 1998) and tears (Devos et al., 2001) have been discussed and tested. Blood has a weakness in mirroring CNS processes as results are influenced by BBB-integrity. CSF has probably the highest specificity as no biological barrier needs to be passed but it is not suitable for repeated routine follow up. The most recent steps in biomarker research include development of high throughput unbiased techniques such as microarray platforms for gene expression profiling and proteomics (Balboni et al., 2006).

In studies of MS disease activity, both short term markers suitable for detection of changes during relapse such as immune system markers and markers reflecting more permanent long term changes are needed. Markers for inflammatory activation are expected to change during relapses but are also influenced by non MS factors such as infections. Biomarkers from structural proteins such as neurofilament (NF) and GFAP have high specificity for CNS damage. At this point they are primarily analysed in CSF although development of assays for neurofilament heavy chain, (NFH) has been described in blood (Shaw et al., 2005).

Inflammatory biomarkers in MS

Cytokines and chemokines

Cytokines are small proteins (~25 kDa), mediators of intercellular communications and are involved in a wide variety of biological activities. They are typically produced in response to an antigen and ongoing balance between proinflammatory and antiinflammatory cytokines is required to maintain homeostasis. There is a degree of redundancy with overlapping functions between cytokines both via cytokine

production in several cell types and receptor molecule-expressions shared between different cell types (O'Shea et al., 2002). Chemotactic cytokines or chemokines are a group of small polypeptide mediators (8-14 kDa), attracting various types of leukocytes to sites of infection or inflammation. They are divided into two major subfamilies on the basis of the arrangement of the two conserved N-terminal cysteine residues, CXC and CC, depending on whether there is a non conserved amino acid in between, CXC or if they are adjacent, CC which is crucial for tertiary structure and function (Zlotnik and Yoshie, 2000).

Tumor necrosis factor α (TNF- α)

Tumor necrosis factor α (TNF-α) is a proinflammatory cytokine. It is produced mainly in activated macrophages and mononuclear cells. It activates several cell types including macrophages and endothelial cells and induces production of several cytokines including IL-1 and IL-6 (O'Shea et al., 2002). TNF-α is expressed in MS brain lesions (Bitsch et al., 2000a; Hofman et al., 1989) and at high levels in chronic active MS lesions (Cannella and Raine, 1995). Levels of TNF-α in serum and CSF have been found to correlate with disease activity in MS (Maimone et al., 1991) and with severity and progression of MS (Sharief and Hentges, 1991). Despite positive results in EAE, therapy with TNF-α blockers in MS patients have lead to an unexpected increased disease activity (Lenercept_Group, 1999).

Interferon-γ (IFN- γ)

Interferon- γ is the prototypic T_H-1 cytokine. It is produced by activated T-cells and natural killer cells (NK-cells) functioning as activator of macrophages and increases the expression of MHC class I and II in several cell types (Murphy and Reiner, 2002). INF- γ mRNA has together with IL-6 and TNF- α been found in MS-lesions (Woodroofe and Cuzner, 1993). IFN- γ inducible protein (IP-10), (chemokine receptor CXCR3) is elevated in serum and CSF in MS patients in active phase, remission and in SPMS but not in PPMS compared with other non inflammatory neurological conditions (Scarpini et al., 2002). In an open clinical trail IFN- γ increased the exacerbation rate in MS patients (Panitch et al., 1987).

Interleukin 6 (IL-6)

IL-6 is a cytokine considered to have multiple properties (Hirano, 1998). One important role is induction of growth and proliferation of B-cells into immunoglobulin-secreting cells. Further IL-6 acts as an acute-phase reactant suggesting proinflammatory properties (Hirano et al., 1990). In-vitro studies provide evidence for IL-6 involvement in neural differentiation, protection and survival (Hirota et al., 1996). IL-6 is an essential factor for induction of EAE as IL-6 deficient mice are resistant to the disease (Okuda et al., 1999; Samoilova et al., 1998). High levels of IL-6 and other cytokines are found in association with active MS lesions (Cannella and Raine, 1995; Woodroofe and Cuzner, 1993). IL-6 is secreted by

activated immune cells and additional sources within the CNS are activated microglia, astroglia, neurons and endothelial cells. (Frei et al., 1989; Schonrock et al., 2000). Although serum concentrations of IL-6 soluble receptors have been associated with disease severity (Padberg et al., 1999; Stelmasiak et al., 2001) no role has previously been described for IL-6 as a biomarker for disease activity in MS.

Interleukin 4 (IL-4)

IL-4 is a cytokine mainly produced in T_H-2 pattern $CD4^+$ T-cells. It induces proliferation of naïve T-cells into $CD4^+$ T-cells and stimulates proliferation of B-cells (Seder et al., 1992). IL-4 is considered to induce immune deviation in autoimmune disease as $IL-4^+$ T_H-2 cells reduce EAE (Racke et al., 1994). In MS, immune reactivity for IL-4 has been demonstrated in section from several types of lesion suggesting immunomodulatory mechanisms (Cannella and Raine, 1995).

Chemokine (C-C motif) ligand 2, CCL2

CCL2 is a proinflammatory chemokine and a key mediator of early inflammation. It exerts its effect on perivascular transmigration and accumulation of monocytes, NK-cells and T-cells (Carr et al., 1994) to sites of tissue damage (Cai et al., 1996). Astrocytes together with mononuclear cells in both active and chronic MS lesions have been found to be immunoreactive for CCL2 and also to CCL8 and CCL7 (McManus et al., 1998). In white matter adjacent to MS lesions hypertrophic astrocytes alone were positive for CCL2 (Van Der Voorn et al., 1999). Several other chemokines have been shown to be increased in CSF after acute MS relapses and the levels of CCL2 are decreased in the same patients (Franciotta et al., 2001; Mahad and Ransohoff, 2003; Sindern et al., 2001; Sorensen et al., 2004; Sorensen et al., 2001; Sorensen et al., 1999). Data also suggests that CCL2 plays a role in the degeneration of neurons and for astrogliosis (Kalehua et al., 2004; Muessel et al., 2000; Wyss-Coray et al., 2003).

Markers for Cytotoxicity

Granzymes and Perforin

During the cytotoxic attack not all granzymes released from the CTL granule enter the target cell but leak to adjacent body fluids. Granzymes other than A and B found in the granules of CTLs are granzyme H, and granzyme M. These are homologous duplications of granzyme B and A respectively but expressed in less quantities. (Lieberman, 2003). In vitro experiments have shown that $\gamma\delta$ T-cells from blood and CSF can mediate cytotoxicity against oligodendrocytes via the perforin pathway, without the need for APC suggesting a pathogenic role for granzymes (Zeine et al., 1998). Studies of CTLs deficient in perforin showed that all aspects of granular killing was impaired while the FAS-mediated killing was normal (Kagi et al., 1994a). Furthermore, increased expression of perforin in peripheral blood

lymphocytes of MS patients during relapses has been reported (Rubesa et al., 1997). Granzyme B is further expressed in T$_H$-17 cells (Kebir et al., 2007). To our knowledge Granzyme A and B levels in serum or CSF have not previously been specifically studied as biomarkers in MS.

Structural biomarkers

Neurofilament protein

Neurofilament is the major protein of the axonal cytoskeleton and is a member of the intermediary filament family with a diameter of 8-10 nm. It is expressed in large myelinated axons where its function is to maintain axonal calibre and participate in axonal transport (Lee and Cleveland, 1996). Neurofilament is assembled from three subunits, neurofilament light (NFL), molecular weight (MW) 68 kDa, neurofilament medium (NFM), MW 150 kDa and neurofilament heavy, (NFH), MW 200 kDa. The three subunits share a conserved head-domain in the N-terminal end of the molecule, followed by an intermediate rod-domain on which a variable C-terminal chain is linked (Lee and Cleveland, 1996). The light subunit (NFL) is an essential unit of the neurofilament core on which the other subunits copolymerize. The neurofilament subunits display varying amounts of phosphorylation. NFH is the most heavily phosphorylated proteins in the human brain whereas NFL is less phosphorylated, a factor influencing development and maintaining axonal calibre and hence conduction velocity of the axon (Lee and Cleveland, 1996). The degree of phosphorylation also affects the antigenecity of the molecule where NFL has a lower antigenecity than NFH (Karlsson et al., 1989). Several epitopes have been used in immunization, and the head-domain, common to NFL, NFM, and NFH has been suggested as the cause of cross-reactivity in immunoassays (Petzold et al., 2003). Monoclonal antibodies against the tail are suggested to be more specific to either subunit (Petzold et al., 2003). Measurement of the light chain of NFL as a marker of axonal damage in CSF has shown increased levels in neurodegenerative diseases(Holmberg et al., 1998), acute CNS infections(Studahl et al., 2000), cerebral vasculitis (Nylen et al., 2002) and in autoimmune CNS diseases, including MS (Lycke et al., 1998; Rosengren et al., 1995).

Glial fibrillary acidic protein

GFAP is expressed only in nervous tissue and is the major intermediate filament of the cytoskeleton of astrocytes. GFAP was originally isolated from MS-lesion with severe astrogliosis (Eng et al., 2000).It has been considered as a marker for astrocyte disintegration in acute injury or trauma to the CNS and for astrocyte activation during inflammatory conditions. Increased levels in CSF have been demonstrated in dementia (Wallin et al., 1996), in normal pressure hydrocephalus (Tullberg et al., 1998) and in MS patients with RRMS (Rosengren et al., 1995). Serum levels of GFAP are elevated in patients with severe head injury (Nylen et al., 2006) and related to outcome in subarachnoid haemorrhage (Nylen et al., 2007).

Tau

Tau is a microtubule associated protein with a molecule weight of 55-74 kDa. It is involved in assembly and stabilisation of axonal microtubuli. Elevated CSF levels of tau have been found in alzheimers disease, amyotrophic lateral sclerosis and stroke (Andreasen et al., 1999; Blennow and Hampel, 2003). In MS, CSF levels of tau have been found elevated compared with non inflammatory controls (Bartosik-Psujek and Archelos, 2004) and differed in active v.s nonactive phase (Sussmuth et al., 2001). The concentration of tau protein in the CSF probably reflects the intensity of neuronal degeneration in chronic neurodegenerative disorders (Blennow and Hampel, 2003).

Metabolic biomarkers

S100B

S100B is a calcium binding protein involved in various intracellular processes such as cell cycle regulation and cell growth. It is highly abundant in astrocytes and further expressed in other tissues such as melanocytes, skeletal muscle cells and adipocytes. It has an extracellular function, contributing to cell signalling between astrocytes and neurons (Zimmer et al., 1995). In the CNS S100B has been investigated as a marker of astrocyte activity and is an established marker for traumatic brain injury (Ingebrigtsen et al., 2000; Raabe et al., 1999). S100B CSF levels as a marker for CNS inflammation has been reported to show an increasing trend from PPMS via SPMS to RRMS (Petzold et al., 2002). A recent report showed S100B levels in CSF to be higher in RRMS than in controls but they did not correlate to EDSS or disease duration of MS (Rejdak et al., 2007). In plasma however no difference was found between RRMS, SPMS and PPMS (Koch et al., 2007).

Neuron specific enolase (NSE)

Enolases are metabolic proteins involved in glycolysis. Neuron specific enolase (NSE) is an isoform specific to neurons and other neuroendocrine cells. It is localized in neuronal cytoplasm and axons and to a lesser extent expressed in erythrocytes and platelets (Marangos and Schmechel, 1987). Increased levels of NSE were detected in CSF of patients with cerebral ischemia and correlated to infarction volume on computer tomography, but not to clinical outcome (Cunningham et al., 1991). In MS plasma levels of NSE were decreased in patients with PPMS and SPMS patients with a significant progression, and correlates inversely to EDSS (Koch et al., 2007). NSE in CSF might be a feasible biomarker for disease activity in MS grey matter lesions. However, previous studies on NSE have shown similar CSF levels in MS as in other neurological diseases and healthy controls (Royds et al., 1983), and equal levels in RRMS and SPMS (Jongen et al., 1998).

Aims of the study

The general aims of this study were to investigate the immunopathogenic processes of MS at clinical MS relapse and at different clinical phases of MS and to identify biomarkers for inflammatory activity, structural damage and T-cell mediated cytotoxicity in MS. The specific aims were to:

- Investigate the CSF levels of NFL, GFAP, NSE and S100B as neuropathological biomarkers during different clinical phases of MS with emphasis on their dynamics during clinical relapse. (Paper I).

- Investigate serum and CSF levels of TNF- α, IFN- γ, IL-6 and C-C motif ligand 2, (CCL2) as proinflammatory- markers - and IL-4 as anti-inflammatory marker during clinical MS relapse and their association to different clinical phases of MS. (Paper II).

- Investigate the involvement of T-cell mediated cytotoxicity during acute relapse discerning the gene expression of cytotoxicity in peripheral CD3^{+} T-cells and by determining serum and CSF levels of granzyme A and B. (paper III).

Subjects and methods

Main MS population and controls (Paper I, II & III)

Sixty-six patients with MS according to (McDonald et al., 2001) were consecutively recruited at the Department of Neurology, Sahlgrenska University Hospital, Gothenburg to participate in the studies of this thesis, (Paper I, II & III). Forty-one patients had RRMS, 23 were included at the time of an acute relapse (RRMS-rel), 18 patients were in clinical remission (RRMS-rem), and 25 patients had secondary progressive MS (SPMS). None of the patients had had clinical relapses within the last three months before inclusion but three SPMS-patients had experienced a relapse within the last year. In paper II the main MS population was reduced by 2 RRMS-rel and 1 RRMS-rem and in paper III the main population was reduced to 19 RRMS-rel, 8 RRMS-rem and 19 SPMS. However, when each MS group was compared with its reduced group in paper II and III there were no statistical differences between them in terms of age, albumin ratio, mean IgG-index or EDSS.

Fifty healthy blood donors served as controls. None of them had a history of neurological symptoms that would indicate an acute or chronic neurological disease and all had a normal neurological examination. Forty-four control samples were analysed in papers II and forty in paper III. In the consecutive part of paper III 10 age and sex matched samples from the controlgroup was used. There was no statistical difference in terms of age, albumin ratio or mean IgG-index between the groups.

The studies were approved by the Ethics Committee of the University of Gothenburg. Clinical characteristics of the patients and healthy controls are shown in table 1.

All patients were examined and neurological deficits were scored according to EDSS (Kurtzke, 1983).The clinical syndrome of the relapses were classified topically as optic neuritis, hemispheric, brainstem or myelopathy and as monofocal or polyfocal. One RRMS-rel patient and one RRMS-rem patient had ongoing interferon-beta treatment at the time of their inclusion. CSF samples were obtained by lumbar puncture (Lp). The first 12 ml of CSF were mixed and centrifuged, then frozen in 0.5 ml aliquots and stored at -80° C.

Table 1. Characteristics of patients and healthy control subjects, paper I-III

Group		RRMS-relapse	RRMS-remission	SPMS		Healthy Controls	
N		23	18	25		50	
Female/Male	(n)	12/11	13/5	16/9		15/35	
Age	[mean (range) ; y]	32.4 (17-52)	37.8 (21-58)	47.7	(32-57)	35.4	(21-59)
MS duration	[mean (range) ; y]	7.9 (0.5-25)	14.1 (5-27)	20.4	(4-37)	N/A	
EDSS score	[median (range)]	2.5 (1.5-6.5)	3 (1-4)	6.5	(2-8)	N/A	
Subjects with OB	(n)	22	17	24		2	
Albumin ratio	[mean (S.D.)]	5.01 (3.31)	5.19 (2.22)	6.10	(2.27)	5.09	(2.16)
IgG index	[mean (S.D.)]	1.03 (0.51)	1.08 (0.68)	0.95	(0.60)	0.46	(0.04)

RRMS = relapsing remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; EDSS = Expanded Disability Status Scale; OB = Oligoclonal bands; S.D. = standard deviation; N/A = not applicable.

Subgroup of MS patients with extended follow-up of acute relapses (Paper I, II & III)

A subgroup of 13 patients from the RRMS-rel group, were followed with re-examinations and two additional blood and CSF samplings. The group consisted of six female and seven male patients, 25-45 years of age, (mean 32.7 years), with MS duration of 0.5–25 years (mean 8.6 years) and EDSS 1.5-6.5 (median 3). The time between relapse onset and the first Lp was 16 days (median, range 5-34 days), and a second Lp were performed at 5 weeks (median 34 days, range 26-53 days). Two patients did not proceed after the second Lp and the remaining 11 patients underwent a third Lp 15 weeks after relapse onset (median 106 days, range 88 -142 days). CSF from the first Lp was absecent in one patient in the consecutive group in papers II and III, and from three patients from the last Lp in paper II which meant three complete consecutive CSF samples were analysed in 11, 9 and 10 out of 13 patients in the CSF follow up group in papers I, II and III respectively.

Clinical characteristics and EDSS scoring of the consecutive patients are shown in table 2.

MS and control subjects for investigation of gene expression and analysis of T-cell cytotoxicity during acute relapse (Paper III)

A separate group of 10 patients, 6 males and 4 females, with RRMS according to (McDonald et al., 2001) were included in the study during acute relapse. They had a mean age of 38 years, mean disease duration of 9.5 years (range 0.5-37 years), median EDSS of 3.5, and mean time from relapse onset to obtaining the first blood samples of 14 days. Six were on disease modifying therapy (interferon beta) and seven received methylprednisolone intravenously after the initial blood sampling. Ten gender and age matched healthy individuals were recruited as controls. Clinical characteristics and EDSS scoring are shown in table 3.

Table 2. Clinical characteristics of subpopulation of relapse patients consecutively followed with CSF sampling x 3, paper I-III

Pat	Male/ Female	Age (years)	MS Duration (years)	EDSS -1	EDSS -2	EDSS -3	Treatment	Sampling after onset (days)	IVMP
1	M	25	10	2.5	2	2		24	N
2	M	30	10	3	2	2		6	N
3	F	27	5	2	1	2	IFN-β1b*	29	Y
4	M	25	2	1.5	1.5	N/A	IFN-β1a[1] *	7	N
5	F	26	15	3	1	1		11	N
6	M	40	25	4	4	4.5		16	Y
7	F	42	7	4.5	4.5	4.5		5	Y
8	M	38	3	6.5	6.5	2		10	N
9	F	25	7	2	1	N/A		8	N
10	M	43	7	2.5	2	2		12	Y
11	F	45	12	3	2.5	2	IFN-β1a[2]	18	Y
12	M	28	0.5	2	2	0	IFN-β1a[1]*	16	N
13	F	28	0.5	3.5	3	3		34	Y

*EDSS: Expanded disability status scale; IFN-β1a: Interferon beta1a; ¹: 30 µg i.m. once weekly; ²: 22 µg s.c. 3 times weekly; IFN-β1b: Interferon beta1b, 250 µg s.c e.o.d.; * initiated after sampling one; IVMP: Methylprednisolone 1g iv for 3 days; N/A = not applicable.*

Table 3. Clinical characteristics of MS patients included in the DNA microarray analysis, paper III.

Pat	Male/ Female	Age (years)	MS Duration (years)	EDSS -1	EDSS -2	Treatment	Sampling after onset (days)	IVMP
1	F	64	37	6	6	IFN-β1a[1]	12	Y*
2	M	38	9	4	3.5	IFN-β1a[2]	17	N
3	M	26	0.5	4.5	3.5	IFN-β1a[2]*	25	Y*
4	M	48	6	3.5	3.5	IFN-β1a[1]*	4	Y*
5	M	34	11	2	1	-	10	N
6	F	31	7	2	0	-	18	N
7	M	36	8	3	0	-	30	Y*
8	F	36	7	2.5	2.5	IFN-β1a[1]	9	Y*
9	M	24	1.5	6	3.5	[IFN-β1a[2]/Mitox*	5	Y*
10	F	44	8	3.5	3.5	-	9	Y*

*EDSS: Expanded disability status scale; IFN-β1a: Interferon beta1a; ¹: 30 µg i.m. once weekly; ²: 44 µg s.c. 3 times weekly; Mitox: mitoxantrone; * initiated after sampling one; IVMP: Methylprednisolone 1g iv for 3 days.*

Immunoassays of inflammatory biomarkers

Assay of IFN-γ, TNF-α, IL-4, IL-6, and CCL2
Flow-cytometry multiplex immunoassay - Luminex

Serum and CSF samples were analysed for IFN-γ, TNF-α, IL-4, IL-6, and CCL2 with Bio-Plex human cytokine assays (Bio-Rad Laboratories AB, Sundbyberg, Sweden). The system can separate up to 100 different colour tagged sets of beads marked with different capturing antibodies. The method is suitable for multiple analyses on small sample volumes. Beads coated with capture antibodies (5000 beads per cytokine) were incubated with premixed standards or sample supernatants (50 μL) in 96-well filter plates and incubated at room temperature for 30 minutes. After incubation and washing, the beads were re-suspended in 125 μL of Bio-Plex assay buffer and read by the Bio-Plex assay reader (Bio-Rad). Data were analysed with the Bio-Plex ManagerTM software version 2.0 (Bio-Rad) with 4PL and 5PL curve fits. The intra-assay variability for cytokines and CCL2 is 1-2% and their inter-assay variability is 4-8%, according to the manufacturer. For samples below the detection level, the detection level was chosen for statistical calculations.

Immunoassays of structural biomarkers

Neurofilament light protein (NFL) assay

CSF concentrations of NFL were analyzed according to an in-house developed sandwich ELISA (Rosengren et al., 1996). Hen anti-NFL IgG was adsorbed to microtitre plates, after which CSF samples or reference NFL were incubated for 2 h at room temperature. Rabbit polyclonal anti-NFL IgG was used as the secondary antibody that was detected using peroxidase-conjugated donkey anti-rabbit IgG. The standard curve ranged from 125 to16000 ng/L. The sensitivity of the assay was 125 ng/L. Samples with levels below the detection limit were assigned this value. The cross reactivity with NFH has previously been estimated to about 15 % (Rosengren et al., 1996). Storage of samples of CSF up to three days at +4 degrees Celsius was found not to influence degradation of NFL neither did repeated freezing and thawing of samples affect NFL concentrations (Van Geel et al., 2005).

Glial fibrillary acidic protein (GFAP) assay

GFAP was measured with a previously described ELISA procedure (Rosengren et al., 1994). In brief: capturing antibody (hen anti-GFAP IgG) was adsorbed to microtitre plates. CSF samples or reference GFAP were added and incubated for 2 h at room temperature. Rabbit anti-GFAP IgG was used as a secondary antibody and incubated for 1 h at room temperature. Secondary antibody was detected using peroxidase-conjugated donkey anti-rabbit IgG. The standard curve ranged from 32-16000 ng/L. The sensitivity of the GFAP assay was 16 ng/L.

Immunoassays of metabolic biomarkers

S100B and NSE assay

S-100B protein and NSE concentrations were measured by commercially available luminescense immunoassays (Sangtec Medical, Bromma Sweden) and the sensitivity was 0.02 µg/L and 1.0 µg/l respectively. Normal reference values for CSF were for S100B <5µg/L and NSE <10.5µg/L.

Assay for T-cell mediated cytotoxicity

Granzyme A/B assay

Analysis of granzyme A and B in plasma and CSF was performed using a commercially available ELISA (Sanquin, Amsterdam, The Netherlands), according to the manufacturers instructions. Briefly, the 96-well plates were coated with the primary antibody overnight. On the following day, standards, controls and plasma were added in duplicates to the wells and incubated at room temperature (RT) for 1h. Thereafter the plates were washed and the secondary biotinylated antibody was added. After 1h of incubation at RT, the plates were once again washed, and streptavidin conjugated horse radish peroxidase was added. The plates were 30 min at RT before the addition of 3, 5, 3', 5' tetramethylbenzidine (TMB), which served as substrate. The reaction was stopped after 30 min with H_2SO_4. The absorbance was recorded at 450 nm in a microplate reader (Milenia kinetic analyzer, Diagnostic Products Corp., Los Angeles, CA.). The results are expressed in units /mL.

Gene-expression analysis

Affymetrix[TM] gene chip contains 25-mer oligonucleotides, synthesized onto glass slides using photochemical methods. The oligonucleotides are organized in pairs with perfect match (PM) and mismatch (MM) adjacent. The HG-U133 plus 2.0 arrays contains over 38,500 well-characterized human genes and are represented by over 47000 transcripts.

RNA extraction

The isolation of T-cells has previously been described (Olsson et al., 2003). In brief, PBMCs were separated from heparinized blood by density gradient centrifugation. After removal of $CD14^+$ cells by magnetic microbeads, T-cells were positively selected using $CD3^+$ magnetic microbeads, according to the manufacturer's recommendations (MACS, Miltenyi Biotec, Surrey, UK).
RNA was isolated from the $CD3^+$ T-cell preparations using the Chomczynski method, (Chomczynski and Sacchi, 1987) followed by RNeasy clean up (Qiagen, Hilden, Germany). The RNA concentration was measured spectrophotometrically and the A260/A280 ratio was 1.8–2.0. The quality of the RNA was verified by agarose gel electrophoresis before reverse transcription into cDNA. cRNA was prepared and hybridized as recommended in the Affymetrix Gene Chip Expression Analysis

manual. Briefly, biotin-labelled target cRNA was prepared by in vitro transcription (Enzo Diagnostics, Farmingdale, NY) and hybridized to Human Genome HG-U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA). Arrays were scanned with a confocal laser scanner (GeneChip Scanner 3000, enabled for High-Resolution Scanning, Affymetrix).

Microarray data analysis

To allow cross-comparisons between different samples, the mean target signal on each microarray was scaled to an average intensity of 100. We compared the expression profiles between each MS patient and its individually matched control, at acute relapse and at 3 months into clinical remission. Comparisons of expression profiles were also done between acute relapse and at 3 months into clinical remission. The difference was analyzed with a call parameter in the gene chip operating software (GCOS). A difference of the call score of over 6, of a maximum of 10, was set as significant.

rt-PCR analysis for confirmation of gene expression in peripheral T-cells

Real-time RT-PCR reagents for analysis of granzyme B, Perforin, Fas ligand and CTLA-4 (Assays-on-Demand, TaqMan Reverse Transcriptase reagents, and TaqMan Universal PCR Master Mix) were aquired from Applied Biosystems (Foster City, CA) and used according to the manufacturer's protocol. cDNA was synthesized from 500 ng of total RNA in a total reaction volume of 50 µL. cDNA corresponding to 10 ng of RNA per reaction was used for real-time PCR amplification. Specific products were amplified and detected with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using default cycle parameters. A standard curve was plotted for each primer-probe set with a serial dilution of pooled T-cell cDNA. All standards and samples were analyzed in triplicate.

Flow cytometry analysis of peripheral T-cells

PBMCs were incubated with anti-CD3-Peridine Chlorophyll Protein (PerCP), anti-CD4-Fluorescein Isothiocyanate (FITC), anti-CD8-Allophycocyanin-Cy7 (APC-Cy7) and anti-CD16-Phycoerythrin-Cy7 (PE-Cy7). Isotype matched control antibodies were used. The samples were analyzed with a FACSCanto (Becton Dickinson, Mountain View, CA) and data were analyzed using the FACSDiva software (Becton Dickinson). First a gate for lymphocytes was set followed by a gate for CD3+ and CD16- identifying T-cells. Helper T-cells were identified by the expression of CD4 and cytotoxic T-cells by the expression of CD8. All samples were analyzed in triplicates

Statistics

Statistical inference analyses were performed using a SPSS 10.1 software package using nonparametric tests: Kruskal-Wallis test and Mann-Whitney U test for unpaired data, Wilcoxon signed rank test for paired data, Spearman´s rank correlation test and partial correlation test were used for calculating correlation coefficients. T-test was used for analyzing parametric differences of albumin ratios and IgG-indices between groups. To calculate the discriminatory level of NFL the value 124 ng/L was applied for patients with CSF NFL levels below the detection limit of the assay. This level was also applied to determine the specificity and sensitivity of the NFL assay in identifying relapses. Partial correlation coefficients were determined in order to control the reciprocal dependence between different variables.

Differences in expression of genes between the three groups were analyzed using a paired sample T-test. The procedure complies with Minimum Information about a Microarray Experiment (MIAME) (Brazma et al., 2001).

Results

Paper I
Increased CSF NFL levels in MS were associated with clinical relapses

The level of NFL in CSF was elevated in all stages of MS. The level peaked during the clinical relapse (mean 1727 ng/L, SD 1711, range <125-6217 ng/L) yielding a 10-fold increase compared with NFL levels obtained from patients in remission or progression (Figure 4). Regardless of disease course MS patients had significantly higher levels of NFL in CSF than healthy controls. There was no difference between patients in remission and those in secondary progression. Applying a discriminatory level of 674 ng/L, that is, 2 SD above the mean CSF NFL level of RRMS-rem patients resulted in a specificity of 94% and a sensitivity of 70% to identify a relapse.
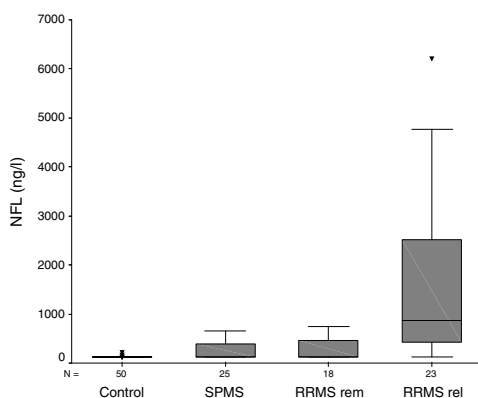

Figure 4. *Boxplot indicating the concentration of NFL in CSF of patients at different stages of MS and in healthy controls. RRMS rel; at relapse; RRMS-rem; in remission; SPMS secondary progressive MS. N = number of subjects*

The temporal change of the CSF NFL levels after relapse onset was further analyzed on an individual basis in 13 RRMS patients (Figure 5). They were examined in close relation with relapse onset and re-examined twice during a median follow-up time of 15 weeks. A large inter-individual range of NFL levels was revealed. The highest level occurred at the second Lp at a median time of 34 days after relapse onset. However the median EDSS score of the patients with repeated LP was 3, 2 and 2 respectively (Table 2) where the decrease between first and second scoring was significant (p=0.01). Thus there was a temporal dissociation between the peak of the clinical symptoms and that of the levels of NFL in CSF. With two exceptions, NFL values were lower at the last Lp and significantly lower when CSF samples were obtained 60 days or later from relapse onset (p=0.03).
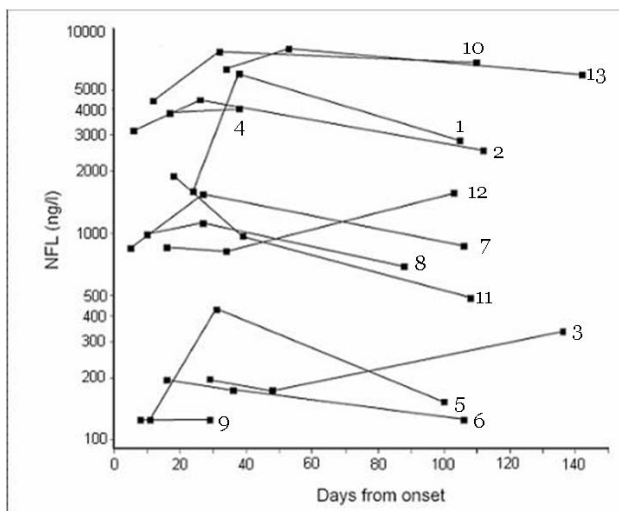
Figure 5

Figure 5. *Logarithmic line plot indicating concentrations of NFL in CSF in individual relapsing remitting MS patients measured after onset of acute relapses. Squares indicate the concentration at each lumbar puncture. Individual patients see Table 2.*

Increased CSF GFAP levels were associated with MS progression

MS patients had higher levels of GFAP in CSF compared with controls, (611 ng/L, SD 308 vs. 435 ng/L SD 160 p<0.001). This difference was most pronounced in the SPMS group and also significant in the RRMS-rel group but not in the RRMS-rem group (Table 4).

Table 4. Levels of GFAP in CSF from different MS patient groups and controls

Group	GFAP (range) ng/l	S.D.	p-value vs control
RRMS-relapse	575 (144-1169)	269	0,024
RRMS-remission	576 (214-2049)	405	0,160
SPMS	671 (332-1287)	262	<0,001
Controls	435 (209-870)	160	

GFAP levels in CSF correlated with the EDSS-score in MS-patients (rho=0.473, p<0.001). The correlation was most pronounced in SPMS patients (rho=0.732, p<0.001) and also found in patients in remission, RRMS-rem (rho=0.627 p<0.005) but not in patients with ongoing relapse, RRMS-rel. There was a weak correlation with age (rho=0.28, p=0.02) but not with disease duration or to gender. In control subjects the CSF GFAP levels also correlated with age (rho=0.37, p=0.009). Partial correlation controlling for age, still showed a correlation between GFAP and EDSS in the combined RRMS-rem/SPMS group (rho=0.48, p<0.001) and for SPMS (rho=0.74, p<0.001).

The CSF levels of S100B and NSE did not differ between MS-patients and control subjects and no association of there CNS-derivied proteins to demographic or clinical data was noted.
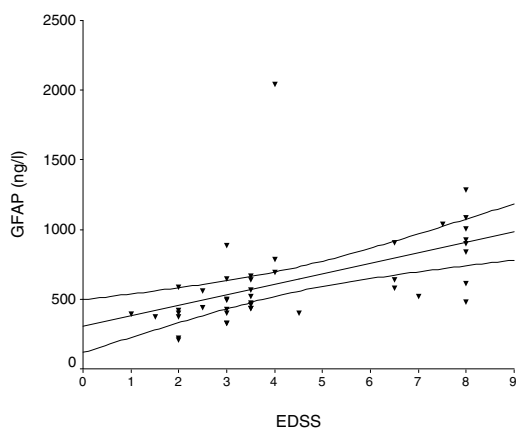

Figure 6. *Scattergram of the concentrations of GFAP in CSF against neurological deficit, scored with EDSS from relapsing remitting patients in remission and secondary progressive patients.(r=0.521, p<0.001). Lines indicate means and 95% CI*

Paper II

CSF CCL2 levels decreased during clinical relapses

The chemokine CCL2 had a significantly lower level in CSF in MS patients than in controls (p<0.001). The levels were lower in RRMS than SPMS. The lowest levels were noted in the group with acute relapses which had significantly lower levels than SPMS (p=0.008) (Figure 7). Patients with SPMS did not differ from healthy controls. In the prospectively followed group of RRMS patients, the lowest level was found at the time for the first LP, with a median time of 16 days from relapse onset. CCL2 levels in CSF increased at sampling 5 weeks and 15 weeks after onset, not reaching statistical significance (p=0.065). No differences were found in serum concentrations of CCL2 between MS-patients and controls.

CSF IL-6 levels were increased in RRMS with clinical relapses

The level of interleukin 6 (IL-6) in CSF was significantly higher in patients with RRMS, with the highest levels noted during acute relapses compared with both SPMS (p=0.028) and controls (p=0.020). In the prospectively followed group, a peak of the CSF IL-6 level occurred 5 weeks after relapse onset (mean 16.95 pg/ml); however, this increase was not significantly higher than levels recorded at onset or 15 weeks later. In serum only 5% of the samples exceeded the detection limit of 5 pg/ml.

Serum and CSF levels of TNF- α, INF-γ and IL-4

Serum analysis of TNF- α**,** INF**-**γ and IL-4 showed that less than 10 % of the samples exceeded the detection limit of the assay. In CSF these cytokines yielded no or only low levels above the detection limit of the assay, and hence no further evaluation was possible.
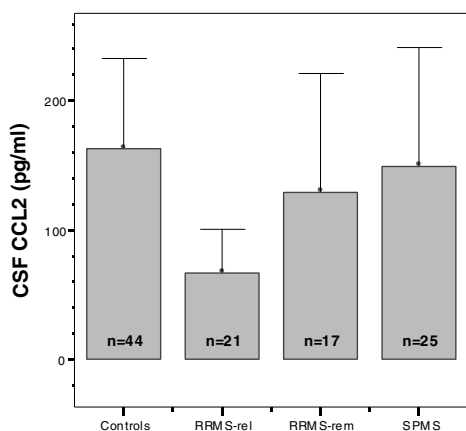
Figure 7. *Bar graph indicating CSF levels of CCL2 of relapsing –remitting multiple sclerosis during relapse (RRMS-rel), remission (RRMS-rem), secondary progressive MS (SPMS) and in healthy controls. Decreased levels of CCL2 were found in both relapsing-remitting MS groups (p<0.001 vs. controls). Bars show mean; error bars ± 1 S.D*

Correlations between CSF-levels of CCL2 and structural biomarkers, albumin ratio and CSF IgG-indices.

Cytokine and CCL2 levels in CSF were compared with levels of NFL and GFAP reported in paper I. There was a negative correlation between the level of NFL and CCL2 in MS patients, (rho=-0.364, p=0.003). However this correlation was not independent and partial correlation controlling for albumin ratio reduced the correlation to a non-significant level (rho=-0.24, p=0.060).

CSF levels of cytokines did not correlate with age, disease duration EDSS IgG-index or albumin ratio. An inverse correlation between CSF CCL2 and IgG index was found in MS-patients (rho=-0.583, p<0.001). Partial correlation revealed that this association was independent to and only slightly influenced by other factors such as EDSS, duration of disease or albumin-ratio.

Paper III

Peripheral T-cell mRNA expression of genes involved in T-cell mediated cytotoxicity was decreased in RRMS patients

The expression of genes involved in T-cell-mediated cytotoxicity (Lieberman, 2003) was lower in T-cells from patients both at relapse and 3 months into clinical remission compared with controls and there were no difference in the expression between RRMS at relapse and three months into clinical remission (Figure 8).

The microarray-results were confirmed by real-time PCR analysis of granzyme B, perforin and FasL. In contrast, the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a factor that inhibits cell-mediated cytotoxicity and initiating an anergy response was increased in patients at both time points compared with controls, Figure 8.
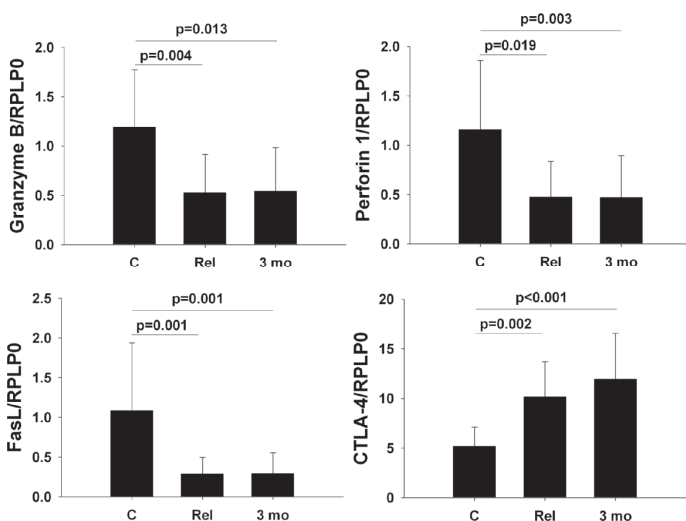

Figure 8. *Real-time PCR analysis of peripheral T-cells confirms reduced expression of granzyme B, perforin 1, FasL and increased CTLA-4 in patients with RRMS (n=9) compared with controls (n=10). Data are mean±SEM*

Gene expression profile in peripheral T-Cells

To investigate whether the expression profiles reflected disease activity, hierarchical clustering was performed on the gene expression profiles of peripheral T-cells from RRMS patients (at relapse and 3 months into clinical remission) and controls. The expression profiles were divided into two separate nodes: (1) all samples from patients, irrespective of time point and (2) all samples from controls. The peripheral T-cell gene expression profiles from patients at relapse were similar to those 3 months into clinical remission (Figure 9). This similarity was confirmed using the Affymetrix change call algorithm, which showed only an increase in 5 genes and a

decrease in 6 genes in the profiles from patients at relapse compared with 3 months into clinical remission. In addition, a paired T-test analysis corrected with Bonferroni did not reveal any differentially expressed genes between relapse and 3 months into clinical remission.
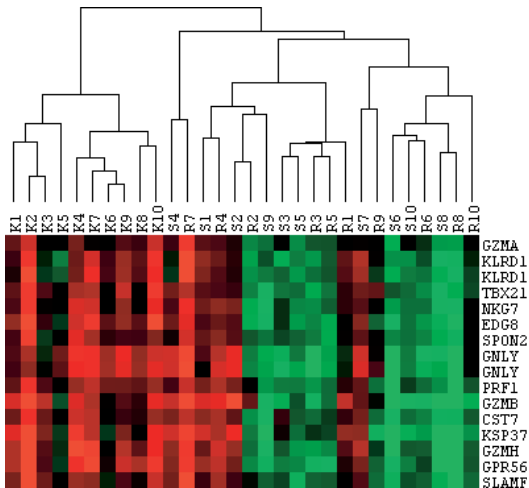

Figure 9. *Hierarchical clustering of gene expression profiles of peripheral T-cells from patients with RRMS (at relapse and 3 months after relapse) and controls. The hierarchical clustering divides the expression profiles into two separate groups: one containing all RRMS patients irrespective of time point and the other containing all controls. The X-axis shows the individual expression profiles divided by nodes and the Y-axis displays a cluster of genes, some of which are involved in T-cell-mediated cytotoxicity. Low expression in green, high in red. Expression profiles from RRMS at relapse (S), RRMS 3 months after relapse (R) and controls (K).*

Effect of immunomodulating treatment and T-cell composition on expression of genes involved in T-cell mediated cytotoxicity

A comparison between untreated patients (n=6 and 4 at relapse and 3 months into clinical remission, respectively) and patients who received treatment (n=4 and 6 at relapse and 3 months into clinical remission, respectively) showed that there was no differences in expression of genes involved in T-cell mediated cytotoxicity at any time point (data not shown). Furthermore, analysis of T-cell subsets with flow cytometry in 7 MS patients and 9 controls showed no significant difference in the percentage of $CD3^+/CD8^+$ cells (Relapse onset=30.1±3.1, 3 months into clinical remission=28.6±3.8, Control=32.6±2.8) or $CD3^+/CD4^+$ cells (Relapse onset=57.8±5.1, 3 months into clinical remission=53.3±8.1, Control=47.9±3.4) between MS patients and controls.

Unchanged plasma levels but increased CSF levels of granzymes during clinical relapse in RRMS patients

Granzyme A levels in the CSF were significantly elevated in RRMS patients during acute relapse compared with controls, (p=0.013) though no significant difference was observed between RRMS in remission, SPMS and controls (Figure 10A).

At all three time points CSF the levels of granzyme A were at least 3-fold higher in patients compared with controls (Figure 10B). The CSF levels of granzyme B were also significantly higher at relapse, and there was a trend towards increased levels at 1 and 3 months into clinical remission compared with controls, (Figure 10C).

Analysis of granzyme A and B levels in plasma showed no difference between prospectively followed RRMS patients, at acute relapse, 1 and 3 months into clinical remission, and controls, Figure 10D-E.
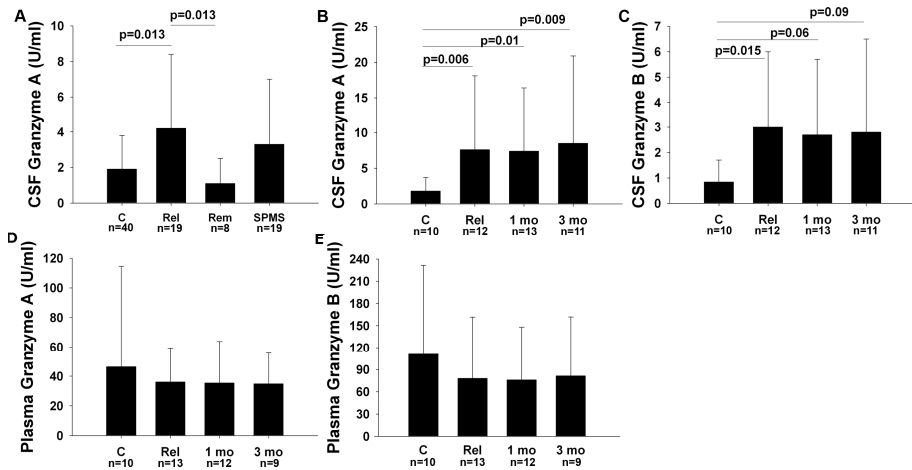
Figure 10. *CSF and plasma levels of granzymes in patients with multiple sclerosis and controls. (A) CSF levels of granzyme A in patients with RRMS during acute relapse (Rel), RRMS 1 year into remission (Rem), SPMS and controls (C). (B) CSF granzyme A, (C) CSF granzyme B, (D) plasma granzyme A and (E) plasma granzyme B levels in prospectively followed RRMS patients and controls. Rel: relapse onset; 1 mo: 1 month after relapse onset; 3 mo: 3 months after relapse onset. Data are mean ± SD*

Discussion

General considerations of biomarkers in MS

Over the last decade it has become evident that MS is a disease with more or less continuous inflammatory activity within the CNS, giving rise to tissue damage that subsequently result in brain and spinal cord atrophy and irreversible neurological disability. Most of these immunopathogenic processes are insidious, at least in the early phase of the disease, and not apparent neither for the patient nor for the clinician. Only a minority of new MS lesions will give rise to relapses (Thompson et al., 1990) and the neurological examination may be unaffected during clinically stable periods. Although there are clinical characteristics that have a predictive value, e.g. age at disease onset, early relapse frequency, and features of the onset relapse (Eriksson et al., 2003; Runmarker et al., 1994), they are not reliable enough for counselling of individual patients. MRI has become a valuable method in the early diagnosis of MS (McDonald et al., 2001) and also as an objective surrogate method in clinical trials for evaluating new MS therapies (Ebers et al., 1998; Johnson et al., 1995; Polman et al., 2006; Sibley et al., 1993). However, there are only weak to moderate correlations between the frequency of lesion formation, lesion volumes and disability development (Filippi et al., 1995). Although, atrophy seems to be a more reliable MRI marker for disability (Fisher et al., 2002; Losseff et al., 1996b), the method is not yet available for the daily clinical setting. Moreover, ordinary MRI examinations do not reveal immunopathogenic process involved in lesion formation.

Biomarkers for MS relapse

NFL as a biomarker for relapse

The results in this thesis indicate that clinical symptoms during MS relapse are not confined to demyelination but also associated with axonal damage. The temporal correlation between high release of NFL to CSF and the appearance of relapses suggests a pathophysiological connection between them. The fact that repeated MRI examinations of the brain reveal 10-fold more frequent active MS lesions than the number of clinical exacerbations (Thompson et al., 1990) has previously been considered indicating that a relatively low number of lesions are located in eloquent areas and that some of the clinically active lesions are located in the spinal cord. However, our results indicate that axonal damage is an important factor for the appearance of relapses.

Essentially all high levels of NFL in CSF were detected during relapse and only moderate or low release of NFL was observed in the CSF during the clinically more stable stages of the disease. NFL levels above 674 ng/L had a specificity of 94 % and a sensitivity of 70% in detecting a relapses. This might be useful in the evaluation of new or aggravating symptoms in order to determine if it is a relapse or due to an infection, i.e. pseudorelapse. The NFL level could also be a useful complement to Gd^+ T1-scans in assessing severity of relapse and when to revise therapeutic strategy for individual patients. However such an evaluation should take in consederation the delay of the NFL peak level of approximately 5 weeks after relapse onset. Thus this

indicates an extended axonal damage that is ongoing also during the clinical remission.

Furthermore, the leakage of NFL to CSF is not an inert phenomenon and the role of NFL as a target for immune attacks has also attracted attention. Elevated IgG anti-NFL antibodies have been reported in the CSF from MS patients (Eikelenboom et al., 2003; Silber et al., 2002). These findings were further supported by immunocytochemistry observations indicating that CSF and serum from MS patients seem to bind to axonal cytoskeletal structures and that CSF oligoclonal IgG bands are formed in conjunction with NFL (Silber et al., 2002). The possible immuno pathogenic role of NFL was also demonstrated by correlations between intrathecal NFL antibody levels and MRI measurements of lesion load and cerebral atrophy (Eikelenboom et al., 2003).

Although axonal damage is extensive in chronic MS lesions, recent studies based on immunohistochemical methods report axonal damage also in acute lesions (Ferguson et al., 1997; Trapp et al., 1998). MR investigations present evidence that axonal damage is an early event in MS (De Stefano et al., 2002; Kuhlmann et al., 2002) and that it might even be present already at the clinical onset of the disease (Filippi et al., 2003). The most striking clinical effect of immunomodulating treatment in MS is reduction of the relapse rate. Immunomodulating treatment of CIS has showed a delay of a definite MS-diagnosis, (Jacobs et al., 2000; Kappos et al., 2006a) and in definite MS-cases it seems to reduce the rate of axonal loss (Rudick et al., 1999). Although the results in this thesis support an ongoing neurodegeneration with increased NFL levels during all stages of MS, the notion that the highest levels are associated with relapses, indicate that immunomodulating treatment might also reduce the rate of axonal degeneration. This could have an impact on the risk for progression and the development of irreversible disability.

Cytokines/chemokines as biomarkers for relapses

CCL2 as a biomarker for relapses

The involvement of pro- and antiinflammatory cytokines/chemokines has been extensively investigated in MS and EAE. There are several studies of their possible role in the immunopathogenesis of MS and their association with different phases of MS disease. In this thesis we found support for an involvement of CCL2 and IL-6 in the development of relapses. While other chemokines are increased in CSF after acute MS relapses, the levels of CCL2 is decreased (Franciotta et al., 2001; Sindern et al., 2001). We found a similar pattern where levels of CCL2 were decreased at relapse onset. We showed that CSF CCL2 levels thereafter slowly returned and 15 weeks after the relapse onset the level had returned to about half of that recorded for clinically stable RRMS patients. A correlation between elapsed time since reported exacerbation and increasing levels of CCL2 in CSF has been reported previously (Mahad et al., 2002) but a repeated CSF sampling one week after intervention with methylprednisolone revealed no changes of the chemokine levels (Franciotta et al., 2001) and changes of the CCL2 levels were more likely to be confined to variations of the clinical activity than to a therapeutic effect (Iarlori et al., 2002). We found no correlation between levels of CCL2 and the CSF cell count, disease duration or EDSS

score confirming the independence of CCL2 from these factors (Scarpini et al., 2002). Although cross-interacting with other receptors in-vitro (Zlotnik and Yoshie, 2000), in-vivo studies of the CCL2-CCR2 complex suggests it to be non-redundant and is thus more attractive as a putative biomarker (Mahad and Ransohoff, 2003).The mechanism for the observed decrease of CSF CCL2 in clinically active MS is not fully understood and different mechanisms have been proposed (i) consumption of CCL2 by its receptor (ii) a decreased expression in the astrocytes due to a refractory state and (iii) translocation of CCL2 to the luminal side in foot-processes (Mahad and Ransohoff, 2003). Our results do not resolve which of these explanations is more likely, however, increased expression of CCL2, detected in astrocytes in acute MS lesions (Van Der Voorn et al., 1999) might suggest a consumption of the CCL2-CCR2 complex as inflammatory cells are recruited. These cells subsequently participate in several immunopathological processes of the acute MS lesion and the temporal changes with a decrease of CCL2 levels at the relapse onset and a peak of the NFL level five weeks thereafter, (Paper I), support the suggested time-course of immunopathological events during acute relapses, including axonal damage and the release of NFL.

IL-6 as a biomarker for relapses

CSF-levels of IL-6 were increased in RRMS compared with SPMS and controls. The IL-6 level peaked 5 weeks after relapse onset. Results from previous MS-studies on IL-6 have varied compared with controls; IL-6 concentrations from MS patients have either been unchanged in serum and CSF (Padberg et al., 1999; Vladic et al., 2002), increased in plasma (Frei et al., 1991) or in CSF (Maimone et al., 1991) or in both serum and CSF (Navikas et al., 1996; Stelmasiak et al., 2001) but the level has not been associated with clinical activity or course of the disease. That we could associate CSF IL-6 levels with disease course and previous studies have may be due to differences in the selection of patients and controls. The varying IL-6 results in MS probably reflect its multiple functions (Akira et al., 1993) and sources (Frei et al., 1989).

Although it has been suggested that IL-6 promote survival, migration and proliferation of oligodendrocyte precursor cells into MBP-positive cells (Louis et al., 1993) its proinflammatory properties have come into focus with the recent findings of T_H-17 cells as an emerging cell type in autoimmunity (Kimura et al., 2007). The T_H-17 cell hypothesis (Steinman, 2007) supports the role of IL-6 as an important cytokine in MS inflammation. IL-6 together with TGF-β promotes proliferations of encephalithogenic T_H-17-cells (Langrish et al., 2005) that expresses granzyme B and have been shown to be able to kill neurons. They have further been found in human MS lesions (Kebir et al., 2007).

Thus, our results indicate that MS lesions causing relapse are frequently associated with axonal damage and that IL-6 and CCL2 are involved in the immunopathogenesis of MS. Their activity is predominantly associated to RRMS and relapses. However, the specific mechanisms that are involved need further investigations and it is important to incorporate MRI in future studies as it is currently the golden standard for evaluating subclinical disease activity.

The role of T- cell cytotoxicity in MS pathogenesis

Over the last years the role of CD8^{+} T-cells has drawn increasing attention and it has been speculated that T-cell mediated cytotoxicity is involved in both demyelination and neuroaxonal damage in MS (Friese and Fugger, 2005).

Granzymes as biomarker for relapse

CSF levels of granzymes A and B were higher in RRMS patients at relapse compared with patients in remission and controls, and elevated granzyme A levels in CSF at relapse were also found in patients that were prospectively followed after onset. There were no differences in serum levels of granzymes between patients and controls. This discrepancy indicates that the increased T-cell mediated cytotoxicity was confined to the CSF/CNS compartment in MS.

RRMS patients, approximately one year into clinical remission, had similar levels of granzyme A in the CSF as the controls. The difference in granzyme A levels between relapse and 1 year into remission was not due to a difference in disease duration between the two since there was no difference in granzyme A levels between the quartiles with the longest and shortest disease duration (data not shown). This may indicate that T-cell mediated cytotoxicity is increased for several months into clinical remission, but eventually normalizes during long periods of clinical stability. In paper I, we reported high levels of NFL, in the CSF during acute relapse in MS that remained elevated during the 15 weeks of follow-up, indicating a prolonged destructive activity after clinical remission. It is striking that the levels of granzymes and NFL show a parallel increase in the CSF at relapse and into clinical remission and suggest that T-cell mediated cytotoxicity may be involved in axonal damage and demyelination. Granzyme A and B are produced both in CD8^{+} T-cells and natural killer cells. However, since there are few natural killer cells compared with T-cells in the CSF of MS patients, the majority of granzyme molecules in these patients are thought to originate from CD8^{+} CTLs (Merelli et al., 1991). That CTL is involved is further supported by the fact that oligodendrocytes and neurons express MHC class I but not class II (Neumann et al., 2002) and hence can only be recognized by CD8^{+} CTLs and not by CD4^{+} T-cells (Jurewicz et al., 1998; Medana et al., 2000).

The mRNA expression analysis of peripheral T-cells of genes involved in T-cell-mediated cytotoxicity was significantly decreased in RRMS patients at both time points compared with controls (Figure 9) and these findings were confirmed by real-time PCR (Figure 8). In a previous investigation, IFN-β was shown to have a regulatory effect on CD8^{+} T-cell function (Jensen et al., 2006). However, in the present study, the mRNA expressions of genes involved in T-cell mediated cytotoxicity were not affected by immunomodulatory therapy (IFN-β or mitoxantrone). Furthermore, the reduced expression was not caused by an altered T-cell composition in MS patients as the ratio of CD3^{+}/CD8^{+} and CD3^{+}/CD4^{+} T-cells were unchanged compared with controls. However, we did not find lower levels of either granzyme A or B in plasma suggesting that T-cell mediated cytotoxicity is not reduced on a protein level in peripheral blood.

The reduced expression of cytotoxic genes in peripheral cells has to our knowledge not been reported previously. In contrast, granzyme B (also known as CTLA-1)

expression was up-regulated in PBMC in RRMS-patients (Mandel et al., 2004). This difference may be due to different sampling techniques where only $CD3^+$ cells were studied, and different clinical stages. However, T-cells in peripheral blood of MS patients have an increased resistance to apoptosis via a reduced expression of caspase-3. Caspase-3 is an intracellular protein with a bottleneck function in the granzyme induced apoptosis pathway (Jones J, 2007). Apoptotic elimination of naturally occurring autoreactive T-cells is a major mechanism in maintaining haemostasis and dysfunctional peripheral CTL might contribute to an autoimmune reaction (Sharief and Semra, 2001).

No conclusions can be drawn from the present experiments regarding the cause of the low gene-expression of peripheral T-cells but hypothetically it is an active down-regulation of cytotoxicity which might be might be an active down regulation of peripheral cytotoxicity. The increased expression of CTLA-4, an inhibitory signal in the immune-response, might support this. Another explanation of the low expression of cytotoxic genes could be that MS-patients have a primary deficiency in the ability to mount a CTL-response in the peripheral circulation against autoreactive T-cells, and that this induces a skew in the normal homeostasis and opening for an attack of the autoreactive cells on the CNS. A reduced caspase-3 expression in T-cells and monocytes of MS patients found in the Campath-study could be in line with this hypothesis (Jones J, 2007). To speculate further an early event in MS pathogenesis could be an inherited inability of the immune system to control and remove autoreactive T-cells. If so, an appropriate treatment strategy would be to enhance the immune response against autoreactive cells rather than to suppress it.

The converse regulation of granzyme A and B between peripheral blood and CSF indicate that T-cells from MS patients are divided into two distinct populations, one in CSF and one in peripheral blood which are regulated separately.

In conclusion, our findings indicate that $CD8^+$ T-cell-mediated cytotoxicity is specifically increased in the CSF/CNS compartment and may contribute to relapses and axonal damage in RRMS patients. Furthermore, the activity continues several months into remission allowing cytotoxic damage of the CNS for extended periods. The involvement of $CD8^+$ T-cells has been described in other autoimmune diseases and experimental models, e.g. type 1 diabetes, aplastic anemia and ankylosing spondylitis, (Hermann et al., 1993; Risitano et al., 2004; Santamaria, 2003). Furthermore, our co-authors have previously demonstrated that the autoimmune haematological disease idiopathic thrombocytopenic purpura (ITP) is a heterogenous disease where platelets are destroyed by both $CD8^+$ T-cell-mediated cytotoxicity and auto-antibodies (Olsson et al., 2003). In analogy, $CD8^+$ T-cells are most likely to be one among several cell types contributing to the pathogenesis of MS.

GFAP as a biomarker for relapse and progression
Earlier reports have demonstrated increased concentrations of CSF GFAP in RRMS patients with an increase during a 2-year observation period (Rosengren et al., 1995). In paper I we report increased levels of GFAP in CSF in all patient groups except in patients in remission. This is in contrast to other reports where GFAP in CSF is

elevated in patients with more severe disability (Albrechtsen et al., 1985) or active disease (Noppe et al., 1986). There are methodological differences and differences in selection of control subjects that could probably explain these discrepancies. Petzold and colleagues report that RRMS patients in remission had higher levels of GFAP in CSF compared with other subtypes and that more severely disabled patients, defined as EDSS >6.5, had higher levels than patients with mild or moderate disability (Petzold et al., 2002).

The role of astrocyte activation under inflammatory conditions has been proposed as preventive of further tissue damage, and EAE has been shown to be more severe in GFAP knockout mice, than in wild-type control mice (Liedtke et al., 1998) This might mirror two separate causes of elevated GFAP in MS (i) an increase, secondary to astrocyte activation in the inflammatory phase, where the response is appropriate for limiting damage to the tissue and (ii) increased expression of GFAP as a part of the late degenerative phase where astrocytes has a role as scavengers and are involved in the formation of gliotic scars. Our results support this dual explanation as GFAP was significantly elevated during relapse in contrast to controls and correlated strongly to EDSS in SPMS but not in patients in remission. The findings supports that astrogliosis is a major contributor to secondary progression and suggests that GFAP is a putative biomarker for disease-progression in MS.

Concluding remarks and future perspectives

The pathophysiological process in MS is complex and a number of mechanisms contribute to the disease activity. This suggests that no single biomarker can cover the whole spectrum from inflammation, over demyelination and axonal damage to repair. From our studies we conclude that NFL appears to be a marker for relapse and GFAP for clinical progression. Although our data suggests that these markers might be useful in monitoring disease activity and MS therapies, further studies are needed to validate these findings.

The golden standard for measurements of disease activity in MS today is MRI and future studies on proposed biomarkers needs to incorporate MRI examninations for criterion crossvalidation. To accomplish this we have initiated prospective studies for evaluating the effect of clinically silent MS lesion formation on the level of inflammatory- and CNS parenchymal biomarkers. We have also initiated an investigation on the treatment effect of immunomodulating and immunosuppressive therapies on their levels.

Studies of the immunological mechanisms in MS must take into account the immense complexity of the immune response and the large degree of redundancy. Experiences from the divergent results in EAE compared to MS emphasises though the necessity of studies in MS. However, the increased use of immunomodulating therapies leads to difficulties in sampling immunologically unbiased patients, and this must be taken in consideration when designing future studies. The ultimate cause of the axonal damage is at large unknown and the growing evidence of $CD8^+$ cytotoxicity in MS warrant further exploration. Our results suggest that in future studies on $CD8^+$ T-cell involvement in MS cells from CSF should be analysed. This requires more sensitive methods for extraction and amplification of mRNA as the low cell-count in CSF limits the amount of available mRNA. Furthermore needs the so far predominantly preclinical results on the role of T_H-17 cells to be expored in MS.

Summary and conclusion

Axonal damage is of importance for development of clinical relapses suggesting that treatment of relapses and reduction in relapse frequency during immunomodulatory therapies is probably beneficial for a positive long term outcome

T-cell mediated cytotoxicity as measured by levels of Granzyme A and B in the CSF is of pathogenic importance in MS and is confined to the CNS-compartment.

Prolonged increase of NFL and granzyme A in CSF after a relapse indicate an ongoing immunological attack even after apparent clinical remission suggesting dissociation between the immunopathological activity and clinical remission.

Development of long term diability is associated with astrocyte activation, gliotic scar formation and correlates with increased levels of GFAP in CSF.

Svensk sammanfattning

Multipel skleros (MS) är en av de vanligaste orsakerna till neurologiska handikapp hos unga vuxna i västvärlden. I Sverige lever omkring 12000 personer med MS varav två tredjedelar är kvinnor. MS betraktas som en autoimmun sjukdom i centrala nervsystemet (CNS). Förloppet utgörs av episoder av försämring (skov) med efterföljande återhämtning (remission) och som i en stor andel av fallen övergår in fas med progressiv sjukdomsutveckling, sekundärt progressiv MS (SPMS). Ett skov är en episod med uppblossande inflammatorisk aktivitet där celler från immunförsvaret felaktigt invaderar CNS och orsakar skada på myelinskida och nervfibrer (axon); aktivering av stödjeceller (astrocyter) med påföljande ärrbildning och atrofi. Under den mer utdragna progressiva fasen tycks inflammationen vara mindre betydelsefull och neurodegeneration vara den dominerande orsaken till skada. Det senaste årtiondet har betydelsen av axonala skador rönt ett allt större intresse. Trots att axonala skador är mest uttalade under den progressiva fasen har studier påvisat förekomst tidigt i sjukdomsförloppet, även i samband med debut av MS. Den axonala skadan är den huvudsakliga orsaken till bestående handikapp vid MS. Flera studier talar för att behandling med immunomodulerande preparat kan bromsa denna utveckling. Nedsatt skovaktivitet är den tydligaste kliniska effekten av MS behandling.

Syftet med avhandlingens studier var att undersöka sambandet mellan inflammation, neuropatologiska processer och kliniska förlopp vid MS, för att identifiera användbara biomarkörer för monitorering av sjukdomsförlopp och behandling.
I blod och cerebrospinalvätskan (CSV) undersöktes nivåerna av cytoiner, inflammatoriska mediatorer och enbart i CSV undersöktes neurofilament (NFL), en biomarkör för axonskada, och gliafibrillärt surt protein (GFAP), en markör för astrocytaktivering. Vi undersökte också betydelsen av T-cells medierad cytotoxicitet bakom uppkomsten av MS-skov. MS-patienter undersöktes i samband med skov varav en undergrupp följdes upp under 3 månader. Vidare undersöktes patienter under andra sjukdoms stadier samt friska kontrollpersoner.

NFL var förhöjt under alla stadier av MS och visade en 10-faldig förhöjning under kliniska skov jämfört med patienter i remmission och progression. Ökade GFAP nivåer i CSV visade en stark korrelation till ökande neurologisk påverkan. Starkast var sambandet hos patienter med progressivt förlopp. Cytokinen IL-6 i CSV var högst hos patienter med akuta skov. Kemokinen CCL2 hade en signifikant lägre nivå i CSV hos MS patienter med skovvist förlopp och den lägsta nivån förelåg under akuta skov. Det fanns vidare en negativ korrelation mellan nivån av NFL och CCL2 hos MS patienter. Förhöjd CSV nivå av granzyme A, en biomarkör för T-cells medierad cytotoxicitet förelåg vid skov och kvarstod upp till tre månader. T-celler i blod visade ingen signifikant skillnad i mRNA uttryck av cytotoxiska gener mellan akuta skov och remission. Däremot sågs ett signifikant lägre uttryck av dessa gener jämfört med kontroller.

Sammanfattningsvis förefaller NFL vara en markör för skov och GFAP en markör för klinisk progression. Förändringarna i CSV av de inflammatoriska markörerna IL-6 och CCL2 ger stöd för ett samband mellan inflammation och axonal skada vid skov.

T-cells medierad cytotoxicitet har patologisk betydelse vid MS och är avgränsad till CNS. Kvarstående förhöjning av NFL och granzyme A i CSV indikerar en fortgående immunologisk attack även under pågående klinisk återhämtning. Våra resultat talar för att dessa biomarköre kan vara användbara vid monitorering av sjukdomsaktivitet och MS behandling men ytterligare studier behövs för att bekräfta detta. Intensiv behandling av skov samt reduktion av skovfrekvens med immunmodulerande behandling minskar troligtvis den axonala skadan vilket torde fördröja och minska risken för utveckling av svårare neurologiskt funktionsbortfall.

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Hofstadter's Law:

It always takes longer than you expect, even when you take into account Hofstadter's Law.

Gödel, Escher, Bach:
an Eternal Golden Braid