Differential Regulation of Cytokine Secretion in Multiple Sclerosis

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PhD Thesis

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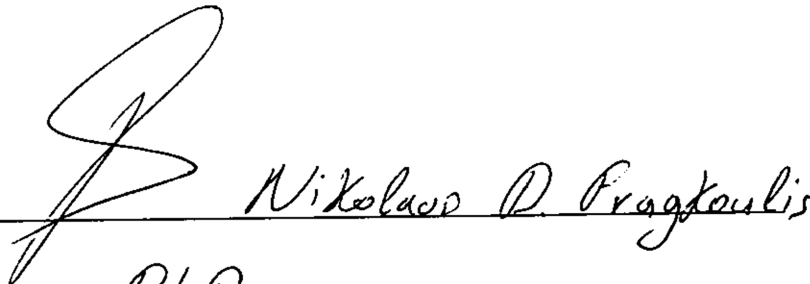

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Abstract

Multiple sclerosis (MS) is the most common autoimmune inflammatory demyelinating disorder of the human nervous system affecting nearly 1 million people worldwide every year. Current treatments for first-line nontoxic therapy of the relapsing-remitting form of the disease are two forms of recombinant IFNβ, 1α and 1β and glatiramer acetate. The pathogenesis of MS is highlighted by an inflammatory response and myelin destruction. Several recent studies have reported alterations in immune variables such as cytokine concentrations, immune-related molecules such as MHC and adhesion molecules and chemokines; which may contribute to an imbalance between Th1 and Th2 T cell-mediated immune response.

The aims of the present study were to characterize the cytokinetic profile of MS patients through investigating the secretion of the main pro- and anti-inflammatory cytokines from peripheral blood mononuclear cells of MS patients compared to controls, and to investigate cytokine receptor expression levels in T and NK cells. In addition, the induction of apoptosis and the involvement of adhesion molecules and metalloproteinases in oligodendrocyte destruction are examined. Finally, the effect of IFNβ treatment on cytokine secretion levels as well as on the aforementioned immunoregulatory molecules was evaluated.

The study population consisted of 60 MS patients (20 untreated, 20 treated with IFNβ-1α and 20 treated with IFNβ-1β) in parallel with 25 controls. Serum/plasma samples and peripheral blood mononuclear cells (PBMCs) were isolated from each participant. Using the enzyme linked immunospot (ELISPOT) assay the numbers of cytokine-secreting PBMCs were measured for the following cytokines: IFN-γ, TNF-α, IL-12, IL-10 and IL-4. The expression levels of the cytokine receptors IL-12Rβ1, IFN-γ, IL-10R, TNFαR were estimated in the surface of lymphocytes using flow cytometry and serum levels of soluble CD95, ICAM-1, VCAM-1, metalloproteinase-9 (MMP-9) and the tissue inhibitor, metalloproteinase-1 (TIMP-1) were evaluated in all samples.

The ELISPOT assay proved a very sensitive and valuable tool for characterisation of the cytokine profile of MS patients. Decreased numbers of PBMCs secreting IL-10 and IL-4, and increased numbers of PBMCs secreting TNF-α, IL-12 and IFN-γ were observed in MS patients compared to healthy controls. Treatment with IFNβ elevated IL-10 and IL-4 levels, and decreased TNF-α, IL-12 and IFN-γ levels in these patients. IFNβ/1α was more efficient in decreasing IL-12 secreting cells and IFNβ/1β in decreasing IFN-γ, and TNF-α secreting cells and elevating IL-4 and IL-10 secreting cells

Increased expression of IL-12Rβ1 and TNF-αR was observed in NK cells and decreased expression of IFN-γR and IL-10R was shown in T cells of MS patients compared to controls. IFNβ therapy reduced IL-12Rβ1 levels in T and NK cells (IFNβ-1α was more effective), and TNF-αR levels in T cells (IFNβ- 1β was more effective), but it increased IFN-γR and IL-10R in T and NK cells of MS patients.

Increased levels of the soluble CD95 apoptosis marker were observed in serum of MS patients compared to controls, which further increased after IFNβ treatment (IFNβ-1β was more efficient).

Increased levels of soluble ICAM-1 and VCAM-1 were observed in serum of MS patients compared to controls, which were further increased after treatment with IFNβ

(IFNβ-1α was more efficient in increasing sICAM-1 and IFNβ-1β in increasing sVCAM-1 levels)
Increased levels of MMP-9, decreased levels of TIMP-1, and increased MMP-9/TIMP-1 ratio was found in serum of MS patients compared to controls, and treatment with IFNβ decreased MMP-9 levels, increased TIMP-1 levels and decreased MMP-9/TIMP-1 levels (IFNβ-1a was more effective).
These results indicate an over expression of Th1 type cytokines in MS accompanied by a concomitant suppression of Th2 type immune response as well as by alterations in the expression of cytokine receptors. A consequent activation of other immunoregulatory elements (sCD95, adhesion molecules, metalloproteinases) was also observed which was partly modified by IFNβ treatment.
This study provides important evidence on the role of cytokines and of other inflammatory molecules in MS pathogenesis and highlights possible mechanisms of action of IFNβ treatment, offering new perspectives for novel, more efficient, diagnostic, and therapeutical approaches for the disease.

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CHAPTER 1: INTRODUCTION

1.1. General introduction

Multiple sclerosis (MS) is the most common inflammatory demyelinating disorder of the human central nervous system (CNS), and the leading cause of non-traumatic neurological disability in young adults in Europe and North America. It affects nearly 1 million people worldwide every year. Neurologic disability generally increases over the course of the disease, which runs over a 10-20 year period (Kumar and Clark 2002). Thus, MS has a major personal and social impact on those afflicted with the illness (Gytten and Maseide 2006).

Unlike type-1 diabetes which becomes clinically evident once the majority of islet cell damage has occurred, the initial presentation in MS occurs when there is reversible clinical disability and minimal nervous system damage (Phadke, 1990; Miller *et al.*, 2005). Thus the opportunity exists to prevent CNS damage and disability by early intervention, followed by more aggressive immunotherapy if the disease continues to be active. The future therapy for MS is to identify the exact mediators of inflammatory damage (like cytokines) investigate their cell signaling pathways and find compounds that can be given early in the course of the disease to allow an aggressive treatment.

The present thesis focuses on the investigation of inflammatory cytokines associated with MS pathogenesis, aiming to identify their mechanisms of actions as well as their regulation in the peripheral tissues of MS patients. Furthermore, a specific cytokine (IFNβ) has been selected as a therapeutic modality for these patients at an early disease stage and its effects in the immunopathology of these patients are clearly presented.

1.2. Multiple sclerosis

Multiple sclerosis has begun to be studied from the beginning of the 19th century, but was established as a particular neurological disease by the famous French neurologist Charcot (1868), who systematized the clinical and pathological features and moreover proposed the first diagnostic tests (Charcot's "three"), by which the disease was separated from other diseases like hysteria and tabes (Compston *et al.,* 1998). Since then, many investigators have studied its etiopathogenesis, which remains enigmatic and indeterminable. It is generally believed, that it is a disease of multifactorial etiology, and not a single disease. That is that what is described today as multiple sclerosis with aspects rare in some, and common, or more common in other patients.

In multiple sclerosis, the white matter of the CNS is more often affected than the gray matter. The peripheral nerves and roots affected only in particular cases (Lublin and Reingold, 1996).

The more typical features of the disease are: firstly, the disseminated development of demyelinated areas of the CNS, a fact which explains the great diversity of its clinical features, and secondly, it is a process of relapses, i.e. it shows characteristic exacerbations and remittances of the clinical features. Another characteristic feature of the disease is the apparent finding at necrotomies of a higher number of demyelinating areas than those shown clinically or in the laboratory (provoked dynamics, imaging methods). It is possible that those clinically silent areas are responsible for some of the inexplicable or unstudied symptoms of the disease, such as observed psychomental disorders, which will be described later (Keegan and Noseworthy, 2002).

1.2.1 Clinical features

Multiple sclerosis as mentioned earlier is characterized by demyelinating lesions in the white matter of the CNS (the gray matter is rarely affected) and can be broadly defined clinically into relapsing/remitting and progressive forms. Although there are mixed patterns and two different classifications of the progressive disease, it is generally the progressive form that leads to disability (Hope *et al.*, 1993). Another distinction used in the past by some authors according to the underlying symptomatology, defines the disease as MS of brain, MS of brain stem, MS of cerebellum, MS of cortex-spinal tract, MS of posterior columns. However, this classification is without practical importance, because in most cases with progressive time there is a mixture and a combination of the symptoms. Further classifications refer to 'disseminated' and 'spinal' forms according to disease characteristics.

Different immunologic mechanisms may be linked to the different stages of the illness, and treatment may also differ depending on the stage of the disease. Most commonly, MS begins as a relapsing-remitting illness, with an average age of onset of 28-30 years. At this stage, the patient experiences discrete neurologic attacks, with full or partial recovery and lack of progression between relapses. The relapse rate tends to decline with time, with an average rate of 1.2-1.8 relapses per person/year at the first year from diagnosis, to 0.8-1.0 in the 5th year and 0.5-0.9 in the 10th year (Rich *et al.*, 2001).

A large percentage (at least half) of patients with the relapsing-remitting form, enter a stage in which the disease becomes progressive (secondary progressive stage). At this

time the clinical course is continuously progressive, with no or only occasional relapses. Another progressive form is termed primary progressive, in which there is continuous progression from the onset. The primary progressive form is different pathologically and perhaps etiologically from the relapsing-remitting and secondary progressive forms. In 10-15% of cases there is minimal accumulation of disability over a 10-15-year period, and the MS is classified as benign (Ramsaransing and de Keyser, 2006). In rare instances MS may be an acute fulminating illness or may present primarily as a cognitive disorder. The course of the disease is usually unpredictable. Sometimes the relapses follow infections, puerperium, trauma, stress, increase of the body temperature. After each relapse the disease leaves additional neurological damage and at the last stages, the patient remains in bed susceptible to respiratory or urinary infections (Hope *et al.*, 1993; Rich *et al.*, 2001).

The clinical features of MS depend mainly on the site of the lesions in the CNS, and although there are no typical symptoms or signs related to the onset or the course of the disease, some regions tend to be more affected than others. These include the optical tract, the brain stem, the cerebellum, and the cortex - spinal tract and the posterior columns (Kumar and Clark, 2002).

Optical disturbances are frequently associated with the disease, and include dazzling of vision, decrease or complete loss of vision (usually unilateral), and disturbances of the optical fields i.e. central scotoma and more rarely homonymous hemianopia. These disturbances are of acute or subacute onset, and the color conception declines earlier and more intensively.

The optic neuritis which is responsible for these signs is thought to be a common feature and a classic form of onset for MS (Cerovski *et al.,* 2005). It involves an inflammation of the optical nerve accompanied by demyelination of the nervous fibres and swelling of tissues around the area. This is demonstrated clinically by the acute establishment of unilateral dazzling of vision and of pain within or behind the bulb caused by the eyes' motion. The vision of the affected eye decreases acutely and importantly, at the optic field appears central or paracentral scotoma, since the fundus of the eye appears normal at the funduscopesis. After few days the pain declines, and after 1-2 weeks vision begins to improve, and return to normal after few weeks (Merle *et al.*, 2005; Mc Donald and Ron, 1999). When the area of demyelination develops at the head of the optical nerve, a swelling of the papilla occurs (mamillitis), which is distinguished from the swelling from posture of the endocranium hypertension, by its small graduation, and from the beginning of decreased vision. Optic neuritis in some people appears independently of MS, but in the majority of cases it's combined with the rest of the symptoms of MS. Since optic neuritis cannot be caused by anything other than MS, the etiology of MS in the patient has always to be investigated extensively (Swanton *et al.* 2006).

Concerning the disturbances of the eye's pupil in MS, the more usual is irregularity of shape, and especially the irregular reaction of the pupil of the affected side in the photomotor reflex, called the Marcus Gunn reaction.

Diplopia may be caused by one single nerve being affected but is often also caused by damage to the median elongated bundle, and in this case is caused by nuclear opthalmoplegia. Binuclear opthalmoplegia is connected, in a great number of patients, to

MS, and especially double binuclear opthalmoplegia is believed to be an almost pathognomonical finding (Cerovski *et al.*, 2005).

Another common feature of the disease especially in women is the development of numbness in the legs, which extends to the buttocks and the perineum, perinatal insensibility and loss of the normal sense of uresis (Hope *et al.*, 1993). These symptoms are retained for long, and because they often are not accompanied by motor disturbances or by important modification of the reflexes, they're underestimated.

Paresthesia symptoms are usual and persistent subjective disturbances, which appear in extensive or restricted areas of the body (i.e. face, hand fingers) and compromise numbness and pricking pains. The Lhermitte's sign also belongs to them and is demonstrated as a feeling of "electric evacuation" to the back or to the ends, during the passive or the active bending of the head of patients. It is attributed to an irritation of the posterior roots and columns of the neck, and is not an exclusive feature of MS. Motor disturbances involve the affection of the pyramidal way and of the cerebellum as well as other further connections. The effect on the pyramidal way is one of the more usual manifestations of the disease. Often onset appears as a development of weakness and motor disturbances, mainly in the form of monoparesis, paraparesis or hemiparesis. These weaknesses can sometimes be sudden and severe whilst others are mild, leading to diagnostic problems. At the late onset of the disease, weakness in most of the cases appears from the beginning, as a slowly establishing spastic paraplegia, which is combined to numbness or disturbances of the proprioceptional sensitivity and sphincter disturbances. These motor disturbances are caused by damage to the cortex-spinal pyramidal way and are expressed by the analogous symptomatology and semeiography.

The cerebellum is also often affected, resulting in dysarthria, walking ataxia, tremor at voluntary motions, asynergy, and nystagmous. Tremors from cerebellar cause may also appear in the head, giving a characteristic retrogression movement. Dysarthria is one of the characteristic features of the disease, expressed as "stumbled speech". As the disease progresses where pseudopromecic paralysis and dyskataposia coexist, the speech is also affected by the damage to the cortex-spinal pathway. Ataxia is a serious and common symptom for the progress of the patient.

Disturbances of uresis during the onset or the progress of the disease, take the form of imperative uresis or urine loss. They are the result of defective or non-existent control of bladder function, and these symptoms must be distinguished from those attributed to urine infection or other local cause before an exact diagnosis of the disease is done.

Sexual disturbances may be present in both sexes (incapability of erection or orgasm, libido loss) and may have an organic cause (Compston *et al.*, 1998).

The possible expressions from the cranial nerves compromise: effect on the V nerve, which is often accompanied by a neuralgic type pain, similar to that of idiopathic neuralgia, usually distinguished by the fact that MS patients are young and idiopathic neuralgia patients are old.

Affection of the VII nerve is usually expressed as a paresis of central type myokymia or hemispasm, and finally vertigo which usually comes from around the IV ventricle, is usually intense, associated with vomiting and appears similar to posterior cranial depression syndrome (Racke *et al.*, 2004).

The variable symptoms of the disease sometimes appear together from the beginning so that the clinical status is integrated at once, whilst in other cases they may arise as distinguished incidents, consisting of one or more symptoms. One can say unreservedly, that MS, at least in the beginning may mimic any other neurological disease.

The named seizure symptoms of MS (pain of the V nerve, dystonia attacks, paresthesias, myokymias or hemispasmus of the face, seizure dysarthria or ataxia), constitute a special group of brief, but especially painful symptoms. The most common of them are the pain of the V nerve and dystonia attacks, which appear like local tetanus and are induced automatically or by a voluntary motion (Nardocci *et al.*, 1995). With regard to epileptic seizures, which according to some authors are classified in this group of symptoms, it should be noted that they are relatively rare, since in MS, white matter of the CNS is mainly affected. The pathogenesis of these seizures is not completely known, but is believed to be associated with the disturbed transmission in the demyelinated fibers (tangent transmission), or with the creation of ectopic irritations in partly demyelinated fibers (Nyquist *et al.*, 2001; Poser and Brinar, 2003).

During the initial stages of the disease, pychomental functions remain normal but during the progress of the disease, changes in emotion and disturbance to the superior intellectual functions (memory, attention, concentration, poverty of ideas) occur leading to intellectual decline (dementia) (Kumar and Clark, 2002; Hope *et al.*, 1993; Feinstein, 2006). The percentage in intellectual decline at the initial stages of the disease is low (2%), but at the end, may be as high as 50% (Leyhe *et al.*, 2005). It is a form of subcortical dementia connected to the degree of affection of the interlobar and to the interruption of the connections of the reticulum system of the brain stem to the cortex, or

to the subcortical areas, or of the connections of the frontal lobe to the posterior cortex areas.

Psychiatric disorders range from unimportant deviations in the patients behavior to the psychosis. Euphoria has been believed, for a long time, to be the most characteristic psychiatric disorder (Euphoria Sclerotica), and its occurrence ranges between 10-18% (Fishman *et al.*, 2004; Papageorgiou *et al.*, 1993). Cheerfulness and excessive optimism shown by the patients often are opposite to their serious physical disability, and laughter usually alternates with crying. Expression of emotional instability has an organic background and is attributed to the deficient control of the pyramidal system at the mimetic muscles (pseudopromecic syndrome).

Depression in MS appears usually as the reactive form, as well as sometimes psychotic (Gold and Irwin, 2006). It is more usual in the brain rather than the spinal form of the disease, and occurs with a frequency of about 18-20%. The incidence of suicide in these patients is high, and is an important reason for the development of a direct and systematic therapy. Bipolar emotional psychosis (depression-mania) is rare and schizophrenic-type symptoms are very rare.

Finally, hysteric symptoms may coexist in MS patients usually as an excess or exaggeration of existing symptoms e.g. the patient's fingers become numb and he/she maintains that the whole hand is numb. It has, however, to be emphasized that after an increase in the patients' body temperature (hot bath, fever) some temporary symptoms may appear which are not hysteric (Uhthoff phenomenon, Guthrie and Nelson, 1995).

Prognosis is very difficult to accurately predict. However, the majority of patients follow the usual path, described earlier, and only a few have unimportant symptoms lifelong, or a very serious disability from the beginning. Proportional to progress and to the severity of development, the following forms of the disease may be distinguished: a) Benign: with mild and rare relapses, complete or almost complete restoration and little or no disability, 20% of the patients. b) Relapsing-remitting form: with more often relapses at the start, imperfect restoration, long remitting periods but always with some degree of disability, 25% of the patients. c) Chronic relapsing-remitting form: with less remitts and continously increasing disability. It concerns 40% of patients, and finally d) Chronic progressive form: with unclear onset, stable and progressive worsening of the symptoms, 15% of patients.

Today, life expectancy of the patient is significantly increased when compared to the past, because of better care. Death usually comes from co-existing non-controlled infections of the respiratory or of the urinary system, or from infection of wounds from bedlying, and rarely directly from the disease (Compston *et al.,* 1998).

Certain factors have been associated with a better prognosis. Most studies show that female gender, early age of onset (Tremlett and Devonshire, 2006), onset of a relapsing-remitting course as opposed to a primary progressive course, complete recovery from the first attack, low frequency of attacks in the early course, and a long period of time to moderate disability is associated with a better outcome. In addition, the type of sign or symptom at the onset may have some predictive value, as patients with optic neuritis or sensory symptoms at onset have a better prognosis (Rich *et al.,* 2001).

1.2.2 Diagnosis and laboratory findings

Although there is no pathognomonic test for the disease, diagnosis is generally based on clinical presentation, examination of cerebrospinal fluid (CSF), performance of electrophysiological methods, and magnetic resonance imaging (MRI) examinations (Freedman *et al.*, 2005; Hope *et al.*,1993; Kumar and Clark, 2002).

The preference for demyelinating areas to develop in the brain's white matter around the ventricles and area around the spinal tube makes CSF composition an important source of information for the underlying disease. Usually five parameters are measured in CSF samples; the number of the white cells is usually increased in CSF (more than 40 cells per mm^3 in about 30% of the patients). This increase is taken as an index of the clinical activity of the disease, but the substantial importance of the test remains unclear, since it has not been elucidated which exact subgroup of leucocytes is increased in CSF during the relapse and for what reason (Rich *et al.*, 2001).

A mild increase in total protein concentration of CSF is found in about 40% of the patients. However, a large increase in protein concentration (much more than 100 mgr/ %) although not excluded, is not usual, and may create doubts about the appropriate diagnosis.

IgG is often increased and consists of more than 25% of the total protein of CSF in 60-80% of the patients. This percentage of IgG is much higher than the normal upper limit (12-16%). Smaller increases are observed in the first years and in the milder types of the disease, whereas higher values are present in more serious and chronic cases. Clinically,

it is believed that the estimation of the γ-globulin index and not total percentage is more useful.

This index is estimated from the ratio:

$$\frac{\gamma\text{-globulin of CSF} \times \text{serum albumin}}{\gamma\text{-globulin of serum}} \times \text{CSF albumin} \quad \text{[normal values less than 0.7]}$$

An increase in the γ-globulin index, indicates an increased composition of IgG globulin in CNS, which can also occur in other inflammatory diseases of the CNS, in addition to MS.

During the electrophoresis of normal CSF in agar, homogenous staining of IgG is observed. In most MS patients, a heterogeneous staining is observed which appears in the form of several zones, clearly separated, known as oligoclonal bands. These zones may appear even when the total γ-globulin in CSF is not increased, and occurs in many patients with recent onset of the disease and mild symptoms (Rich *et al.,* 2001). This is a most important point since it enforces the diagnosis of a clinical uncertain form of the disease. The oligoclonal bands of IgG are found in about 87% of patients with the progressive form of the disease and serious disability and in 73% of the patients with mild symptoms. In practice this indicates that most MS patients have IgG oligoclonal bands in the CSF.

These quantitative and qualitative alterations to IgG globulin are not unique and specific to MS, since they are observed in many other diseases, such as acute and chronic diseases of the CNS and peripheral neuropathies, where the blood-brain barrier (BBB) is disrupted (Yenari *et al.,* 2006; Sellner and Leib, 2006).

The estimation of myelin basic protein (MBP) and of its antibodies has been used by some investigators for the definition of identity and of clinical activity of the disease (Rauer *et al.,* 2006). However, the false positive results found in other diseases have reduced initial optimism, so that this test has now ceased to be considered as specific for MS.

Electrophysiological tests include provoked potentials, electroencephalography, and electronystagmography.

The registration of provoked responses from the brain cortex by the use of optical, hearing, and somatosensory irritations has proved valuable in the discovery of clinical asymptomatic focuses (Persson and Sachs, 1978). Optical provoked potentials are a great help in clinically uncertain cases because they often reveal subclinical affects of the optical tract (21-83% depending on the form of the disease) (Sisto *et al.,* 2005). Provoked potentials of hearing give less, but important, information for subclinical damage, especially for the brain stem whereas somatosensory provoked dynamics provide evidence for the existence of damage to the spinal cord (Lublin and Reingold, 1996).

Electroencephalographic abnormalities are found in about 35% of patients with MS, especially in the acute phase of the disease but are rarer in the remitting phase. They are mainly of slow wave type and are believed to express a non- specific brain reaction to the pathologic process of the acute phase of the disease (Lublin and Reingold, 1996).

Electronystagmography contributes to the better study of binuclear opthalmoplegia, of the divisioned nystagmous and for the investigation of vestibular function, peripheral or central, which is often affected in MS. It has been confirmed that proper electronystagmography testing contributes to the safer diagnosis of patients with only

spinal symptoms of the disease, as effectively as provoked dynamics (Lublin and Reingold, 1996; Papageorgiou *et al.*, 1993).

The most valuable diagnostic aid for MS is magnetic resonance imaging (MRI), which is a very sensitive method for the identification of multiple white matter lesions in more than 90% of patients (Mills *et al.*, 2006; Racke *et al.*, 2001; Stone *et al.*, 1995). A characteristic distribution and morphology of plaques in T2-weighted MRI are associated with MS (Meier & Guttmann, 2006). MS plaques are found in the white matter in a periventricular distribution, the posterior poles of the lateral ventricles and the area of the centrum semiovale being more frequently involved (Vaneckova, 2001; Papageorgiou *et al.*, 1993). The most common appearance is of homogeneously hyperintense lesions whereas less commonly, rings or cystic lesions may occur. Non specific white matter abnormalities may be confused with MS, particularly in patients older than 50 years with encephalopathies or other CNS disorders. An alternative method is electronic counting brain tomography (CT); but this has a major disadvantage in being unable to identify small affected regions (Rich *et al.*, 2001).

Diagnosis of MS continues to remain mainly clinical and it does not usually reveal specific difficulties (Table 1). The disease has a characteristic clinical picture, process and symptoms of the CNS, from anatomically unconnected areas, which cannot be attributed to other diseases.

Table 1-1. Clinical forms of multiple sclerosis and their diagnostic criteria according to Rose (Papageorgiou *et al.*,1993):

Diagnosis of MS	***Criteria***
I. Clinically certain form of MS	a) Relapsing-remitting progress and at least two affects with time spaced at least one month between each other. b) Slow or graduated progress taking more than 6 months. c) Proved neurological signs attributed to more than one affected area of the white matter of the CNS. d) Onset of the symptoms usually begins in patients of age 10-50 years. e) No better explanation of the neurological symptoms.
II. Possible form of MS	a) Relapsing-remitting history of symptoms and clinical picture with only one neurological sign usual for MS. b) Proved presence of symptoms with signs of multifocus disease of white matter. c) No better explanation for the neurological symptoms.
III. Probable form of MS	a) Relapsing-remitting history of symptoms without proved signs. b) Objective neurological signs insufficient to determine more than one affected area of white matter of the CNS. c) No better explanation for the neurological symptoms.

Conditions that may be confused with MS include acute disseminated encephalomyelitis, Lyme disease, Friedrich's ataxia, sarcoidosis, Behcet's syndrome, HIV or HTLV-I myelopathy, Arnold-Chiari dysplasia, spinal-cerebellar degenerations, angiomas and especially gliomas of the brain stem with a relapsing/remitting progression and CNS vasculitis (Hope *et al.*, 1993; Kumar and Clark, 2002; Rich *et al.*, 2001).

1.2.3 Epidemiology and genetics

The epidemiological characteristics of the disease are very interesting. MS has not a uniform geographical distribution through the world and the geographical latitude is believed to be a major distribution index for the disease. Some other external factors used to interpret this peculiar distribution (climatology conditions, diet, way of living, sunshine, geographical longitude) have not proved useful (Hogancamp *et al.*, 1997). Disease frequency is considered 'high', when, at a given time period, more than 30 patients per 100,000 people are affected, 'mild' when 5-25 patients are found, and 'low' when less than 5 patients are observed (Compston *et al.*, 1998).

Near the equator, MS practically does not exist, but further away, the frequency increases significantly and in some countries of the North such as the Shetland Islands and Orkney (north of Scotland); it considerably exceeds 150 per 100,000 people (Delasnerie-Lauprertre and Alperovitch, 1991; Poskanzer *et al.*, 1980). Countries with a high frequency of the disease in the Northern hemisphere are: Northern Europe, Northern states of USA and Southern Canada. In the Southern hemisphere, New Zealand and South Australia have a high incidence (Kurtzke, 1975). Southern Europe, Southern states

of USA and the larger part of Australia have a medium frequency, and Asia and Africa, generally independent of geographical latitude, are areas of low frequency, with the unique exception of regions of South Africa inhabited by whites (Prange *et al.*, 1986; Weinshenker *et al.*, 1989; Andersen *et al.*, 1993).

MS is approximately twice as common in females as in males and prevalence and incidence appears to increase with distance from the equator. It affects all races, except very rare cases such as the Bantu race of Africa, where no indication of MS has been found (Papageorgiou *et al.*, 1993). It is not incidental that all those areas with increased or medium frequency of the disease are inhabited by Caucasians, and, moreover, that between veterans of the 2nd World War, and between several minorities in the USA (Japanese, Chinese) there exists an unequal distribution and tolerance to the disease, even in those areas with increased risk of the disease (Weinshenker *et al.*, 1989).

The disease affects 1-1.8 people per 1000, resulting in a prevalence of 250,000 – 350,000 cases in the United States. MS is unusual before adolescence, and then steadily rises in incidence from teens to 35 years of age, gradually declining thereafter (Filippi *et al.*, 2001; Smith *et al.*, 1999).

Epidemiological studies support both genetic and environmental components of susceptibility. Reports of clusters, small epidemics, geographical variation in prevalence and alteration of MS susceptibility after migration supports an environmental factor or factors (Poser, 2006). The higher risk of MS in Europeans and in relatives of patients, especially in monozygotic versus dizygotic twins, adoption studies and the existence of MS-resistant ethnic groups supports a genetic predisposition (Hansen *et al.*, 2005). In MS patients of European descent, a strong association occurs for HLA-DR2, the extended

haplotype of HLA-DRB1*1501/DQA1*0102/DQB1*0602 (Holmes *et al.*, 2005). Also several chromosomal regions have been identified as having a mild to moderate influence on genetic risk for MS, among them the major histocompatibility region on chromosome 6. The results support a multifactorial etiology, including environmental factors and multiple genes of a moderate effect (de Jong *et al.*, 2002; Zipp *et al.*, 1995; Oksenberg, 2001).

There is no evidence for a single or unique environmental agent in MS although correlation has been found between upper respiratory tract viral infections and exacerbations of relapsing-remitting MS (Sibley *et al.*, 1985; Andersen *et al.*, 1993). After a rational relationship between frequency of disease and geographical latitude, was proposed important information for disease risk came from studies of emigrants from South Africa, and, later Israel (Limburg, 1950). Data have shown that the crucial age for getting the disease was about 15 years of age (Papageorgiou *et al.*, 1993). Conclusively, the contribution of several external factors are implicated: 1) the peculiar worldwide distribution of the disease, 2) the modification of the disease risk by emigration before 15 years of age and, 3) the existence of clusters and the appearence of the disease in the form of epidemics. Internal factors are indicated by reports of: 1) the complete absence of the disease in some African races (i.e. Bantu) and its relative rareness in others (i.e. Asians), 2) the increased consequence of the disease between members of patients' families, with respect to the closeness of the relationship, 3) the studies of pairs of twins, which show that the disease risk of the other member of the pair is 50% for monoozygotic twins and 17% for dyoozygotic ones and 4) studies of the histocompatibility system (HLA antigens) (Compston *et al.*, 1998; Hogancamp *et al.*, 1997; Lublin and Reingold, 1996).

1.2.4 Pathology

The external surface of the brain and the spinal cord appear to be in general, normal. Macroscopic pathological findings consist sometimes of atrophy of the brain helixes, expansion of the ventricles, gray formations in the white matter, especially around ventricles which represent demyelination regions, spinal cord atrophy or intumescences with demyelination regions clearer those in the brain's, and atrophy of the optic nerve (Dutta and Trapp 2006; Papageorgiou *et al.*, 1993). It is evident that the most characteristic pathological findings of the disease are the demyelination regions (sclerosis plaques) which are spread in different parts of the white matter of the CNS and appear as gray or red-gray spots, of various shape and magnitude. They are often found around the ventricle network, in the semi oviform center, the brainstem, the cerebellum, and the spinal cord. The number of plaques depends on time, number of impulses and gravity of the disease, since their position determines the pattern and the severity of the clinical symptoms. In postmortem material the earlier plaques appear softer and red-colored, and the older ones gray-colored and hard. The distribution of sclerosis plaques is not uniform, nor determinate. Plaque sclerosis arises from the hypertrophy of astrocytes and the creation of projections, whilst deficient hematosis may cause necrosis and cystic degeneration into the plaque (Chesik *et al.*, 2006; Frohman *et al.*, 2006; Papageorgiou *et al.*, 1993). Axons remain always in good condition, and rarely display swelling and vacuum degeneration. The whole process of gliosis produces a characteristic sclerosis in the earlier plaques, and gives a name to the disease (Hope *et al.*, 1993; Kumar and Clark, 2002; Luccinetti *et al.*, 2000).

The main histo-pathological appearance of MS lesions includes perivascular infiltration of monocytes, destruction of the myelin sheath (demyelination), axonal damage, loss of oligodendrocytes, astrocytic hypertrophy and increased expression of MHC Class I and II proteins (Butinx *et al.*, 2002; Ayers *et al.*, 2004) . The inflammatory infiltration in the MS lesion is composed of T lymphocytes, small numbers of B cells and plasma cells, and activated microglia and macrophages (Benevista, 1997; Raine, 1991) which interact with the myelin sheath and are actively engaged in the demyelinating process (Lassmann, 2004, Pouly and Antel, 1999). In addition, there is a local production or expression of immune-related molecules such as MHC molecules, adhesion molecules, cytokines and chemokines (Rich *et al.*, 2001). In brain biopsies the myelin sheath appears thin, fragile, and swollen caused by the continuing destruction by the macrophages (Papageorgiou *et al.*, 1993).

Neuropathology of the MS lesion is heterogeneous. There is a selective destruction of the myelin sheath and relative sparing of axons. However, axonal destruction does occur from the beginning of MS, is more pronounced in progressive MS patients and correlates with the permanent neurological deficit. In the early stages of the disease there is also partial remyelination after the inflammation subsides.

Trends of remyelination are observed, but usually the remyelinated axons are associated with the dense tissues of the developing glia, they are abnormal in shape and diameter, and may cause secondary consequences in clinical symptomatology of the disease whithout the creation of new foci. Generally little is known of the effect of remyelination in MS. It is frequently impossible to determine if remyelination is in developement or in a

phase of restoration. It is possible that myelin loss is not the only cause of creation of the clinical symptoms of the disease, since other factors have been identified that –at least *in vivo*- inhibit synaptic transmission, but this area remains under investigation (Wegner *et al.*, 2006).

Lassmann and colleagues have classified MS into different neuropathological categories: (1) demyelination with no or minor oligodendrocyte loss; (2) demyelination with concomitant destruction of oligodendrocytes; (3) primary demyelination with secondary loss of oligodendrocytes in inactive plaque areas; (4) primary oligodendrocyte destruction with secondary demyelination. These categories correspond to different mechanisms of demyelination (Lassmann, 1998).

1.2.5 Immunopathogenesis

The pathogenesis of the disease remains largely unknown. The most commonly believed theory involves an infection during childhood (e.g. viral) that may trigger exacerbations or influence the immune system and lead, through a series of events (B-cell activation, destruction of the oligodendrocyte's membrane, modification of the blood-brain barrier, production of antibodies against the basic myelin protein), to the appearance of the disease later in life (Lovvet-Racke and Racke, 2006; Pannitsch, 1994; Poser, 1993; Sibley *et al.*,1985; Prat & Antel,2005).

Several lines of evidence suggest that an immune mediated mechanism plays a role in MS (Bar-Or *et al.*, 1999; Barnett and Sutton, 2006; Compston, 2004; Grigoriadis *et al.*, 2006; Hemmer *et al.*, 2002; Hemmer *et al.*, 2006; Martin *et al.*, 1992; Reder *et al.*, 1985;

Traugott, 1990). Laboratory findings show a considerable decrease in the number of $CD8^+$ suppressor T-lymphocytes in the blood and CSF of MS patients, and an increase in the relative and absolute number of $CD4^+$ T-helper lymphocytes, as well as a correlation of increased antibody titer against several viruses and against the myelin basic membrane (MBP), with disease progression (Haase and Faustmann, 2004; Soderstrom *et al.*, 1993). Furthermore, the putative immunological events that lead to the pathology of MS are based on animal experiments in experimental autoimmune encephalomyelitis (EAE) and observations in human MS. Autoreactive T cells specific for myelin and other antigens of the CNS pre-exist in the normal immune system of both animals and human. These clones have escaped deletion by thymic negative selection and are normally controlled by one of the modes of peripheral tolerance, such as the maintenance of immunological ignorance, clonal inactivation or active suppression by regulatory cells. In MS these autoreactive clones undergo activation outside the CNS (Sun, 1991; Ratts *et al.*, 2006). There are several possibilities for such activation, one being molecular mimicry of a viral or bacterial antigen and cross-stimulation of autoantigen-specific T cells. Upon activation, autoreactive T cells express chemokine receptors and adhesion molecules that enable them to migrate into the brain tissue. Upon entering the brain, the autoantigen-specific T cells encounter autoantigen through its presentation by MHC Class II molecules expressed by microglia and macrophages. Cytokines such as interferon-γ, lymphotoxin and TNF-α produced by the entering CD4 T cells (Th1-type cells), as well as by activated local antigen-presenting cells, induce an up-regulation of MHC Class II expression. Once the inflammatory reaction begins, there is an increased production of inflammatory mediators, leading to a change in the BBB and to additional inflammatory

cells entering the brain (Kermode *et al.*, 1990). These mediators also activate astrocytes, endothelium and pericytes, resulting in an increased expression of MHC Class II molecules and, therefore, perpetuation of the autoimmune response (Brosnan and Raine, 1996).

Different autoantigens, other than myelin basic protein are encephalitogenic in animal models; these include proteolipid protein, myelin oligodendrocyte glycoprotein, S100b, alpha-B crystalline, transaldolase oligodendrocyte myelin glycoprotein and 2'3'-cyclic nucleotide 3'-phosphodiestarase (Kuchroo *et al.*, 2002; von Budingen *et al.*, 2001; Schmidt, 1999). Different autoantigens and different epitopes of a particular autoantigen may be targeted by the autoimmune response during different stages of the disease (Rich *et al.*, 2001; Sun *et al.*, 1991).

The humoral immune system also participates in pathogenesis, as there is an overproduction of IgG in the CSF and antibodies are detected in the lesions. B cells are usually found in MS plaques and their number relative to T cells, increases as the lesion becomes chronic (Qin and Duguette, 2003). There is evidence that after binding to the myelin surface, demyelinating autoantibodies activate complement and attract macrophage/microglia. Glial cells presumably also contribute to myelin damage, as reactive astrocytes in the MS plaques contain NADH diaphorase activity which reflects inducible nitric oxide synthase (iNOS). High expression of iNOS has also been detected in macrophages in the region of active demyelination in MS (Acar *et al.*, 2003; Merill *et al.*, 1993; Bo *et al.*, 1994; Sarchielli *et al.*, 1997).

Oligodendrocyte destruction in MS lesions may be either due to direct lysis mediated by T-cytotoxic lymphocytes or through an indirect mechanism in which activated T cells

induce toxic cytokine production (e.g. nitric oxide, TNF) by accessory cells. T cell produced cytokines may also be involved in the damage to oligodendrocytes (Brosnan *et al.*,1988; Ruddle *et al.*,1990; Traugott and Lebon, 1988). Studies have demonstrated that cytotoxicity mediated by cytotoxic T cells may be secondary to the secretion of electron-dense cytoplasmic granules (perforin and serine esterase granzyme B) or a non-secretory pathway based on the interaction of Fas ligand with Fas (CD95) on target cells and the induction of apoptosis (Dowling *et al.*, 1997).

Recent data indicate that the apoptotic process, mediated by the CD95/Fas cell surface receptor, is impaired in activated lymphocytes of patients with relapsing remitting multiple sclerosis (Bilinska *et al.*, 2002; Giusiani *et al.*, 1998; Ichikawa *et al.*, 1996; Macchi *et al.*, 1999; Pender, 1998; Sharief and Semra, 2001; Zip *et al.*, 1999; Gomes *et al.* 2003). CD95/CD95L interaction results in activation-induced apoptosis, thereby regulating clonal expansion of T cells outside the thymus (Bilinska *et al.*, 2001; Dhein *et al.*, 1995; Gold *et al.*, 1997; Hong *et al.*, 2004; Papoff *et al.*, 1996; Zurak, 1997).

More specifically there is data that shows that, being homozygotic for the G allele of CD95 5'(-670)A-->G SNP and for the C allele of CD95 E7(74)C-- >T SNP increased suspectibility in MS exclusively in women (Hong *et al.*, 2004). There was a marked upregulation of Fas/APO1 (CD95) receptor expression in MS tissue (Dowling *et al.*, 1996) and soluble CD95 is elevated in MS patients with active disease (Sakai *et al.*, 1999). Thus MS sera contains biologically active inhibitors of T cell apoptosis that may allow for prolonged abnormal immune responses (Zipp *et al.*, 1998), and that patients with detectable gadolinium-enhancing lesions had lower expression of CD95 (Gomes *et al.*, 2003).

On the other hand, there is also data which shows that measurement of soluble CD95 is not suitable for monitoring MS disease courses (Bansil *et al.*, 1997; Heesen *et al.*, 2000), and there was no difference in CD95 expression when MS patients and healthy individuals were grouped and compared according to HLA-DR status (Zipp *et al.*, 1998). Matrix metalloproteinases (MMPs) may be another important factor for MS pathogenesis (Khrestchatisky *et al.*, 2003). MMPs are a family of at least 14 zinc-dependent extracellular matrix remodeling proteases important in normal development, angiogenesis, wound repair, and degradation of protein components of the extracellular matrix (Nagase and Woessner, 1999; Oh *et al.*, 1999). In addition, MMPs and related enzymes can also process a number of cell surface cytokines, receptors, and other soluble proteins (Goetzl *et al.*, 1996; Opdenakker *et al.*, 2001). In particular it has been shown that the the release of the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α) from its membrane-bound precursor is an MMP-dependent process (Gearing *et al.*, 1995). MMPs are expressed by inflammatory cells associated with CNS lesions in animal models of MS and in tissues from patients with the disease (Clements *et al.*, 1997; Cuzner *et al.*, 1996; Dubois *et al.*, 1999; Gijbels *et al.*, 1992; Hartung *et al.*, 2000; Maeda and Sobel, 1996; Mair *et al.*, 2002; Yong *et al.*, 2001). They are synthesized in zymogens and, under physiological conditions, are selectively regulated by endogenous inhibitors (Brew *et al.*, 2000; De Clerck, 1989). MMP expression therefore contributes to tissue destruction and inflammation in MS (Minagar and Alexander, 2003; Kieseier *et al.* ,1999). Drugs which inhibit MMP activity are effective in animal models of MS and may prove useful therapies in the clinic (Bever and Rosenberg, 1999; Borkakoti, 2004; Brundula *et al.*, 2002; Chandler *et al.*, 1997; Docherty, 1992; Gijbels *et al.*, 1994;

Hewson *et al.,* 1995; Liedtke *et al.,* 1998; Opdenakker *et al.,* 2003; Rosenberg, 2001; Nelissen *et al.* ,2003).

More specifically, MMPs attack the basal lamina macromolecules which line the blood vessels, opening the blood-brain barrier (BBB), and contribute to the remodeling of the blood vessels that causes hyalinosis and gliosis. They also attack myelin (Lukes *et al.,* 1999; Gijbels *et al.,* 1993; Leppert *et al.,* 1995). During the acute inflammatory phase of MS, they are involved in the damage caused to the blood vessels and may be important in the disruption of the myelin sheath and axons (Kurzepa *et al.,* 2005; Mun-Bryke and Rosenberg, 1998; Newman *et al.,* 2001; Ries and Petrides, 1995; Rosenberg, 2002). Normally under tight regulation, excessive proteolytic activity is detected in the blood and cerebrospinal fluid of patients with acute MS associated with a decrease in tissue inhibitors of MMPs (TIMPS) activity (Wjtowicz-Praya *et al.,* 1997). Because they are induced in immunological and non-immunological forms of demyelination, they act as a final common pathway to exert a "bystander" effect (Rosenberg, 2002; Kurzepa, 2005). Intrathecal synthesis of MMPs and especially of MMP-9 has been suggested (Liuzzi *et al.,* 2002).

Some studies investigating a correlation between MMPs and TIMPs in MS have indicated that: serum levels of MMP-9 are higher in MS patients than controls, circulatory MMP-3 levels are correlated with disease activity in relapsing-remitting MS (higher levels during the relapsing phase), there are significantly lower MMP-9 serum levels in primary progressive multiple sclerosis patients compared to relapsing-remitting ones, MMP-12 is expressed on phagocytic macrophages in active multiple sclerosis lesions, and significantly higher TIMP-1 and TIMP-2 levels occur in MS patients than in

healthy controls (Anthony *et al.*, 1997; Correale *et al.*, 2003; Ichiyama *et al.*, 2006; Kanesaka *et al.*, 2006; Kieseier *et al.*, 1998; Kouwenhoven *et al.*, 2002.; Leppert *et al.*, 1998; Lee *et al.*, 1999; Sastre-Carriga *et al.*, 2004; Vos *et al.*, 2003; Waubant *et al.*, 1999).

1.2.6 Therapy

Since MS is confirmed by diagnostic methods, we have to predict, if possible, the process of the disease and estimate possibilities of therapeutical intervention. Although there is no, as yet, etiological therapy for MS, nor any certain way to inhibit the disease processes, or reduce the chronic symptomatology.

Generally the main therapies for MS use drugs that are effective in the treatment of other inflammatory or autoimmune diseases, and comprise of therapies which: (1) prevent the ongoing destruction of the nervous system tissue by inflammatory cells, (2) are a symptomatic treatment to allow the damaged nervous system to function at a higher level, and (3) are a treatment designed to repair damaged CNS myelin (Trebst and Stangel, 2006). If the destructive inflammatory process in the CNS can be arrested at an early stage it may prevent the accumulation of neurological disability.

During the phase of acute symptoms of the disease corticosteroids (usually prednizone or methylprednizolone) or corticotropine (ACTH) are administered at several doses. These drugs are given intramuscularly, intravenously, or *per os*. Corticosteroids act against swelling, are immunosuppressive and accelerate the improvement of the clinical symptoms of the disease (Leussink *et al.*, 2001; Gelati *et al.*, 2002; Petelin *et al.*, 2004).

However, their continued and inconsiderate use is absolutely prohibited, because of serious undesirable actions.

To restrict the frequency and seriousness of the relapses in MS, several imunnosupressive factors are used in the hope they have a prophylactic action. None of them have proved particularly effective as indicated by long time preventive studies and MRI use. For instance, azathioprine and cyclosporine failed to prevent relapses of the disease to any statistically significant degree, and the use of cyclophosphamide, intended to stabilize the progressively worsening disease, did not give the expected results (Rudge, 1998; Brochet, 1998). The same is true for the combined use of immunosupressive drugs and plasmaferesis, although there are contradictory data.

The use of immunosupressive drugs (azathioprine, cyclosporine, cyclophosphamide, methotrexate), was based on the conviction that MS is the result of superimmune reaction. Studies (Laplaud and Confavreux, 2006; Becker *et al.,* 1995) have shown that there is superimmune stimulation, and by the use of these substances, the frequency of relapses is decreased at least in some patients, and the quantity of IgG in CSF is reduced, although only temporarily. These results were accepted with reservation, because they were not statistically superior to other therapies, and, moreover, the uncontrolled use of these drugs is potentially harmful and dangerous.

The use of immune stimulating factors (Levamisol, Transfer factor), according to the theory that the disease is a condition of immune deficiency, failed to offer encouraging results. Similar failure was acquired with the use of a number of other therapies, (e.g. body radiation, plasmaferesis, and hyperbaric oxygen).

Consequently, the use of corticoids or ACTH, still remains for clinicians, the only solution for a therapy for the disease, since they do shorten the duration of the symptoms, and have relatively less side effects (Wenning *et al.*, 1994; Glass-Marmor *et al.*, 2006). However, these drugs have no action upon the already damaged myelin, and do not modify the pathogenetical causes of MS.

The use of azathioprine is intended to reduce relapses and recommended to retard the coming disability, although it also may suppress bone marrow function, so that it predisposes to infection (Putzki *et al.*, 2006). Its use therefore must be done with hematological and liver tests, especially for the first time (general blood examination, platelets, and serum transaminases). It is believed that it helps at least at some of the patients when administrated in time and correctly.

Current treatments for first-line nontoxic therapy of the relapsing-remitting disease are two forms of recombinant IFNβ, 1α and 1β and glatiramer acetate (copaxone) (Amason, 1996; Biegler *et al.*, 2006; Chezzi, 2005; Coppola *et al.*, 2006; Edan, 2001; Fellay *et al.*, 2001; Galetta *et al.*, 2002; Greek National Drugs Organization, 2000; Hughes, 1994; IFN-β Multiple Sclerosis Study Group, 1995; Kappos *et al.*, 2006; Khan *et al.*, 2001; Marckmann *et al.*, 2004: Rio *et al.*, 2005; Yong *et al.*,1998; Wolinsky, 2004). IFN-α and IFN-γ were also tested for MS treatment because of their iostatic and immunomodulatory properties (Biron, 2001), but IFN-α failed to give the expected results and the use of IFN-γ aggravated the patients (Balkwill, 1989; Panitch *et al.*, 1987). IFNβ is the most widely used drug and has been shown in clinical trials to decrease the frequency of relapses, favorably affect MRI lesions and, in some instances, slow disease progression (Calabresi *et al.*, 1997; Paty and Li, 1993). Its mechanism of action is not clearly understood but it

may affect the trafficking of cells into the nervous system (Lou *et al.*, 1999; Hartrich *et al.*, 2003; Rudick *et al.*, 1993). Other possible mechanisms of action of IFNβ are: lowering IFN-γ secretion and inhibiting responses to IFN-γ, increasing IL-10 secretion, and increasing the apoptosis rates of immune cells (Ahn *et al.*, 2004; Boz *et al.*, 2006; Gniadek *et al.*, 2003; Rep *et al.*, 1999; Yushenco *et al.*, 2003; Billiau *et al.*,2004; Hua *et al.*, 2002; Lu *et al.* 1995; Revel *et al.* 1995; Weyenbergh *et al.*, 1998; Weinstock-Guttman *et al.*, 2003).

IFNβ-1α (Avonex) is a glycosylated recombinant mammalian-cell product with an amino acid sequence identical to that of natural interferon. IFNβ-1β (Betaferon) is a nonglycosylated recombinant bacterial cell product in which serine is substituted for cysteine at position 17. IFNβ-1α is given as a weekly intramuscular injection whereas IFNβ-1β is given as alternate-day subcutaneous injections (Khan *et al.*, 1996). The choice between the two preparations depends on the patient and the individual physician. IFNβ-1α is most commonly prescribed as it requires only weekly injections and appears to be better tolerated (Alam *et al.*, 1997; Liu *et al.*, 2001; Rich *et al.*, 2001; Runkel *et al.*, 1998; The Multiple Sclerosis Collaborative Research Group (MSCRG), 1996).

Glatiramer acetate, a mixture of random synthetic peptides (copolymer) synthesized initially as a compound to mimic myelin basic protein, has been studied for a considerable time as an autoantigen in MS. It appears to act as an MBP analog that induces Th2 and Th3 regulatory cells (Aharoni *et al.*, 1997; Dhib-Jabut *et al.*, 2002; Yong *et al.*, 2002; Tennakoon *et al.*, 2006). Glatiramer acetate is given as a daily injection and has been shown to reduce the relapse rate in MS patients (Heesen *et al.*, 1998; Karandikar *et al.*, 2002; Karandikar and Racke, 2005; Neuhaus *et al.*, 2001; Ratts *et al.*, 2006).

The choice of whether to start a relapsing-remitting patient on IFNβ or glatiramer acetate has not been resolved. Patients with depression, those very early in their course or those who cannot tolerate IFNβ are often preferentially treated with glatiramer acetate (Rich *et al.*, 2001).

The general weakness of the patients and their ataxia are symptoms resistant to any therapy. The spasticity of hands and feet may also improve with the use of several drugs (such as Diazepam, Baclofen, Tizanidine, Dantrolen), but their choice and dose should be decided with great care. Painful hand tremor and action tremor of hands correspond, sometimes effectively, to treatment with clonazepame, propranolole, or isoniazide (together with vitamine B6) (Charles *et al.,* 1999). Otherwise the pains MS patients have differ in origin and distribution (nerve pains, musculosceletals), and is the reason why they must be confronted in various ways (salicylics, carvamazepine, amitryptiline, physiotherapy) (Solaro, 2006). However, care is needed in the choice of drug, because some of these non steroid analgesics, such as indomethacin, actually aggravate neurological symptoms.

Successful treatment for the functional disturbances of the urine cysts of the patients is of special importance, because it prevents lithiasis and dangerous infections of the uriniparous system. The form of treatment is not always unique (Papageorgiou *et al.,* 1993).

It has been reported that the taking of vitamins, linoleic acid or the restriction of the animal fat from the patients' diet may act favorably (van Meeteren et al., 2005). But no diet has been judged to be the most effective (Schwarz and Leweling, 2005).

Finally, the proper training of the patients to fully use their remaining abilities, with assistance, is extremely useful since it contributes to the preservation of any remaining mobility, and thus prevents premature bed lying , which can cause serious complications (muscle contractures, atrophies, crusts from lying down, infections). The correct physiotherapy is always beneficial with probably the best possible exercise being swimming in warm water (Peterson, 2001; Papageorgiou *et al.,* 1993).

MS patients must be encouraged to organize their life in the best possible way, and not to give up their interests and special capabilities (Ennis *et al.,* 2006; Sharief, 2004). MS is a peculiar and complicated disease, with obvious indications and symptoms during the patient's life, and as such, it demands careful and "human" therapy (Paty *et al.,* 1999).

1.3. The involvement of cytokines in MS pathophysiology

1.3.1 General aspects

Cytokines are a heterogeneous group of molecular soluble proteins or glycoproteins (Mwt<80 kDa) that participate in both non-specific and immunologically dependent inflammatory reactions, in cell growth, differentiation, cell development and in repair processes vital to host defence. Cytokines are generally produced in small quantities in response to local stimuli, such as the presence of antigens or endotoxins, or the transduction of signals provided by other cytokines, during the active phase of adaptive and innate immune response, which affect induction and regulation (Germenis, 2000). Their secretion is fast, short term and self restricted (Germenis, 2000). In general cytokines are not stored intracellulary as preformed molecules (Germenis, 2000). Their

composition is a result of new, temporary genetic transcription, which is achieved through new and short living RNA's. They are secreted and act on a variety of cells (pleotropism). They include the ones secreted by lymphocytes (called lymphokines), macrophages (monokines) and chemotactic inflammatory cells (chemokines). More than 100 structurally and genetically different cytokines have previously been recognized (Germenis, 2000).

Cytokine action is not specific and does not depend on the antigen. In addition, they can exert multiple actions at the same cellular targets. Their action often affects the composition and the biological action of other cytokines, resulting in a sequence of events in which the next cytokine acts as a mediator to the action of a previous one (Germenis, 2000). The result of this cooperative action can be competitive, or accumulative, stimulation or inhibitory or possibly to enforce partial actions, or creation of a new different action.

Cytokines are divided, based on their function, into four different groups: a) Mediators of innate immunity, secreted mainly by mononuclear phagocytes under the action of infectious agents (IFN-α, IFN-β, TNF-α, IL-1, IL-6, chemokines); b) Regulatory factors for the activation, multiplication and differentiation of lymphocytes, produced from T-cells upon antigen stimulation (IL-2, IL-4, TGF-β); c) Regulatory factors for the immune response, by activating non-specific active cells (IFN-γ, TNF-β, IL-10, IL-5, IL-12); d) Stimulatory factors of leukocyte proliferation, produced by stimulated lymphocytes, and other cells, known as growth factors (GM-CSF, M-CSF, G-CSF, IL-3, IL-7) (Roitt *et al.*, 1998; Germenis, 2000). The main types of cytokines are shown in Table 2.

Cytokines act on target cells expressing the appropriate cytokine receptor in an autocrine or paracrine way. Cytokine receptors are transmembrane proteins with an extracellular binding domain to the appropriate cytokine and an intracellular part involved in transmission of the signal to the cell nucleus (Germenis, 2000; Roitt *et al.*, 1998).

Table 1-2. Individual cytokine classification based on main cytokine types (Roitt *et al.*, 1998)

Cytokine Types	***Individual cytokines***
Lymphokines	MAF (macrophage activating factor), MMIF (macrophage migration inhibition factor), MCF (macrophage chemotactic factor), LMIF (leukocyte migration inhibition factor), HRFs (histamine releasing factors), TF (transfer factor)
Interleukins	IL-1, IL-2, ...-IL15
Tumour necrosis factors	TNF-α (cachectin), TNF-β (lymphotoxin
Interferons	IFN-α, IFN-β, IFN-γ, IFN-ω
Colony stimulating factors	G-CSF (granulocyte colony stimulating factor), GM-CSF (granulocyte-macrophage CSF), M-CSF (macrophage CSF), multi-CSF (IL-3)
Polypetide growth factors	aFGF (acidic fibroblast growth factor), bFGF (basic fibroblast growth factor), EGF (epidermal growth factor), NGF (nerve growth factor), PDGF (platelet-derived growth factor), VEGF (vascular endothelial growth factor)
Transforming growth factors	TGF-α, TGF-β
α-Chemokines	IL-8, NAP-2 (neutrophil-activating protein 2), PF-4 (platelet factor 4), βTG (β-thromboglobulin)
β-Chemokines	MCP-1 9monocyte chemoattractant protein 1), MCP-3, MIP-3, MIP-1α, MIP-1β (macrophage inflammatory protein 1β) RANTES (Regulated upon Activation Normal T Expressed and presumably Secreted chemokine)
Stress proteins	HSPs (heat shochk proteins), GRPs (glucose-regulated proteins), ubiquitin, superoxide dismutase (Mn)

Cytokine receptors are divided into five main groups depending on the motifs or the homologous amino acid sequences of their molecules, as shown in Figure 1-1.

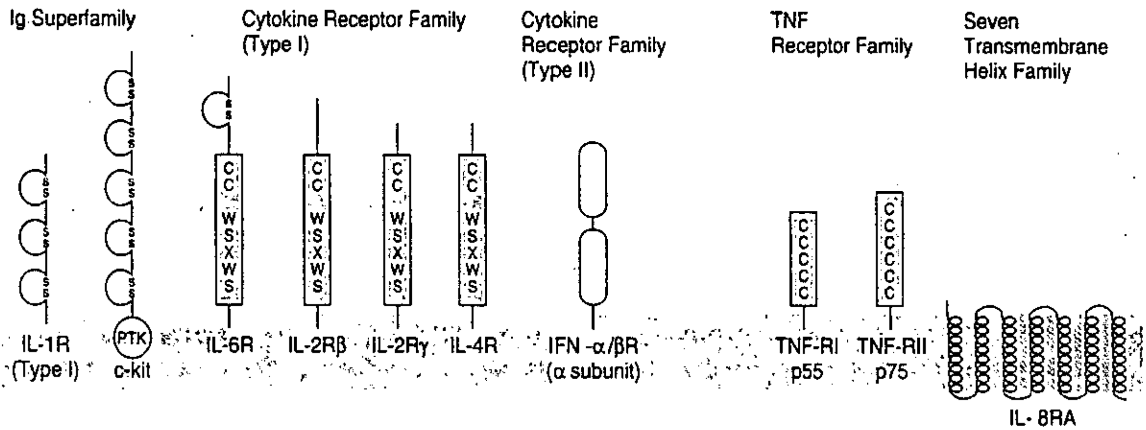

Figure 1-1. Cytokine receptor families
Cytokine receptors share various structural motifs, allowing them to be categorized into families (from Abbas *et al.*, 1997).

The receptors of immunoglobulins' superfamily, like type I and II IL-1Rs, have at their extracellular part, a number of domains with homology to those of immunologlobulins.

The type I cytokine receptor has at its extracellular part, close to the transmembrane one, a conserved motif consisting of tryptophane-serine-X-tryptophane-serine (WSXWS), and bind cytokines which contain a four α-helix structure such as IL-2, IL-3, IL-4, IL-5,IL-6, IL-7, GM-CSF and G-CSF (Hecht *et al.*, 2006).

The type II class of cytokine receptor consists of the type I and type II receptor for interferon.

The type III class of cytokine receptor is composed of the two receptors for TNF, which have the homologous areas p55 and p75, which are also homologous to the Fas protein, to CD40, and to the receptor for nerve growth factor (Ryffel & Myhatsch, 1993; Germenis, 2000).

The chemokine receptors have a transmembrane structure of seven α-helices, common to all receptors, which bind hetero-trimeric GTP-bound proteins, such as β-adrenergic receptors and rhodopsin (Germenis, 2000).

Most cytokine receptors consist of two or more transmembrane polypeptidic chains which act as a complex.

IL-12 actions (promotion of Th1 responses) are mediated through a high affinity receptor, composed of two subunits, designated b1 and b2. Of these two subunits, b2 is more restricted and its distribution and regulation of its expression serves as a central mechanism by which IL-12 responsiveness is controlled (Airoldi *et al.*, 2005). Expression of IL-12R subunits is upregulated in both microglia and splenic macrophages upon stimulation with LPS or IFN-γ. In experimental allergic encephalomyelitis, maximum levels of IL-12 p40 and IL-12p35 mRNA are detected in the CNS at the peak of disease, and expression of IL-12 p35 mRNA is more sustained than that of IL-12 p40 (Li *et al.*,2003). In MS, a higher percentage of IL-12R b1 and IL-12R b2 positive T cells occurred in cerebrospinal fluid (CSF) compared to blood (Ozenci *et al.*, 2001.) The functional importance of high IL-12R β2 in MS has been underlined by the finding that that IL-12 stimulated IFN-γ production and proliferation of PHA-activated T cells correlated with levels of IL-12R β2 expression (Durali *et al.*, 2003). According to other studies, patients with a relapsing-remitting and secondary progressive form of the disease

displayed increased levels of IL-12p40 mRNA in blood white cells compared to controls, since patients with the primary progessive form of the disease showed decreased IL-12R β1 mRNA (Gately *et al.*, 1998; Li *et al.*, 2003; Ozenci *et al.*, 2001; van Boxel-Desaire *et al.*, 2001). These findings raise the possibility that the pathogenetic mechanism of primary progressive multiple sclerosis may be different from the relapsing-remitting's and secondary progressive's, and dosen't use the IL-12R β1 pathway for Th1 immune activation.

IL-10 receptor's levels were markedly increased in the spinal cord of a rat model of chronic relapsing EAE, and strong immunoreactivity for IL-10R was detected in the macrophages of both parenchymal and perivascular areas and in reactive astrocytes in active and chronic lesions in postmortem human brain tissue obtained from MS patients (Hulshof *et al.*, 2002; Ledebeer *et al.*, 2003).

Functionally, cytokines have been classified as either pro-inflammatory (Th1 type, stimulatory) or anti-inflammatory (Th2-type, inhibitory), depending on the final balance of their effects on the immune system (Figure 1-2). In order to maintain homeostasis, a dynamic balance must be struck between pro- and anti-inflammatory cytokines (Miosec, 1995). Inadequate concentrations of pro-inflammatory cytokines may result in excess inflammation, which can be harmful to the host, as in the case of autoimmune disease. However, an excess of anti-inflammatory cytokine concentrations may disrupt the clearance of microbial pathogens. The cytokine network reflects a homeostatic balance, with mutual cross-inhibitory effects exerted by pro- and anti-inflammatory cytokines on cytokine production (Fiorentino *et al.*, 1989; Miosec, 1995; Roitt *et al.*1998).

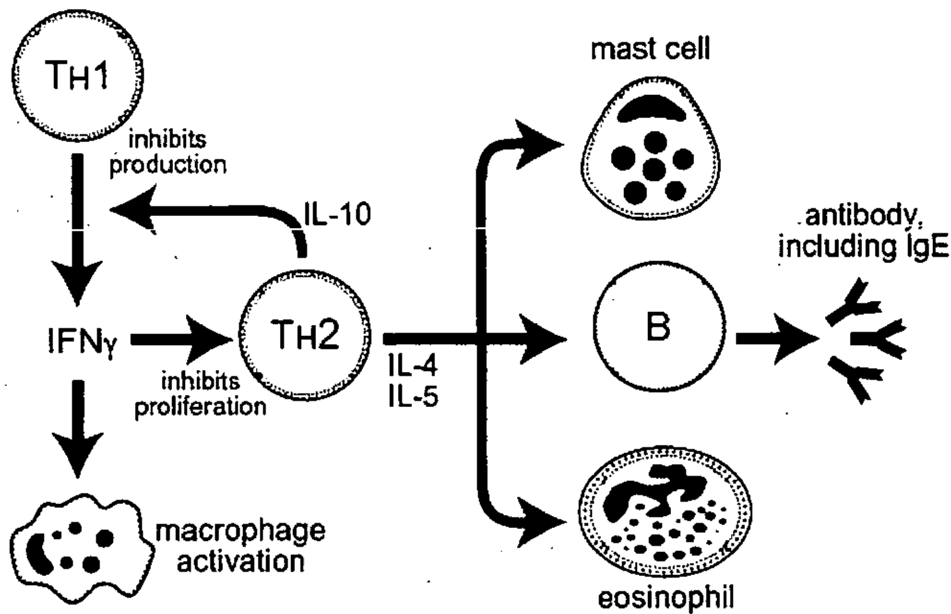

Figure 1-2. Representation of the Th1/Th2 hypothesis
Th1 cells secrete the cytokine IFN-γ and activate inflammatory pathways mainly via macrophage activation. Th2 cells secrete cytokines IL-4 and IL-5 that upregulate antibody formation via B cells, mast cells, eosinophils, and other pathways. Th1 and Th2 cells can cross-inhibit each other (Adapted from Roitt *et al.*, 1998).

In at least two cases, IL-1 and TNF-a, the cytokines, except their soluble form, have been recognized as membrane proteins, since many of the cytokine receptors have also been detected in soluble forms (sRs) (Hecht *et al.* 2006). It is very possible that these molecules play a role in the regulation of cytokine production and/or their action in several cellular populations (Rose-John *et al.*, 2006; Germenis, 2000). Although in many cases sRs result from proteolytic reactions, which lead to the extractment of the extracellular part of the corresponding membrane molecule, in many others it is observed that different lengths of mRNA entail the production of soluble or membrane receptors. This suggests that cells have some factors that regulate receptor gene transcription, which

are mobilized by the action of external messages (probably other cytokines), in order to produce soluble and membrane molecules at a certain ratio (Classen-Linke, *et al.*, 2004). Many sRs are competitively bound to the membrane molecules of cytokines, resulting in the exclusion of the latter's action. However, they may also be bound to cytokines, and in this way protect them from proteolysis in the serum. In this case, they can act as transporters and permit the distant (endocrinal) action of these cytokines (Germenis, 2000). It is possible, therefore, that the inhibition of local cytokine action is a biological role of sRs, whilst they enforce cytokine action at distant areas, where they arrive in very small quantities. Such biological activity as this has been clearly recognized for the complex IL6/IL-6R at the increased sceletal sensitivity at PTH in estrogen-deficient women (Masiukiewisz *et al.*, 2002).

This probably is not the general situation, since the connection of other sRs to the corresponding cytokines entails the inhibition of their action (McDermott, 2001). This is the case for IL-2RII, where the a-chain is detached from the membrane IL-2R, as well as of IL-4R, and of the receptor that binds to TNF proteins I and II (TNF binding proteins I and II, TNF BPI, TNF BPII), (Germenis, 2000). Another characteristic example is calmodulin, found in the urine of pregnant women and which binds to IL-1 in an inhibitory way (Germenis, 2000). Furthermore, A2- microglobulin of serum is bound, between several other proteins, to IL-1, IL-2, PDGF and FGF, resulting either in the enforcement, or in the inhibition of action (Germenis, 2000).

Finally, inhibition of cytokine action occurs after the connection of their receptor to several other substances. Such a substance is a glucopeptide, reported to be a secreted competitor of IL-1R (secreted IL-1 receptor antagonist, sIL-1ra) produced by monocytes,

neutrophils, macrophages and fibroblasts, and which appears to act as an inhibitory factor of the inflammatory reaction (Learn *et al.*, 2001).

1.3.2 Cytokines and central nervous system

Cytokines are involved not only in the immune response, but in a variety of physiological and pathological processes, including events in the peripheral and the central nervous system. They are, therefore, both immunoregulators and neuromodulators (Hadjilambreva *et al.*, 2005). In fact, cytokine production is under tonic control of the peripheral and central nervous system, and cytokine balance may also be modulated by the action of neurotransmitters (Kin and Sanders, 2006). Neuroimmune interactions are therefore bi-directional. Cytokines and other products of immune cells can modulate the action, differentiation and survival of neuronal cells, while neurotransmitters and neuropeptides released from neurons play a pivotal role in influencing the immune response (Szelenyi, 2001).

Cytokine might also exert effects in the CNS both directly and indirectly. A direct action implies that the cytokines themselves are present in the brain, in and/or around the various neuronal cells, while secondary effects are the result of cytokine action on other targets in the indirect pathway (Han and Suk, 2005).

The various cytokines directly affecting the CNS have two possible origins: a) they may originate from peripheral immune organs and cross the blood-brain-barrier. Stimulation of the peripheral immune system may signal the brain in both a local and systemic manner (Planas *et al.*, 2006). Cytokines can reach the CNS directly by crossing at leaky

areas the BBB through the circumventricular organs even in healthy, basal conditions; b) cytokines may be produced by neuronal cells within the CNS. Most of the cytokines and their receptors have been demonstrated and/or postulated in various cell types of the CNS in both healthy and disease states (Rasley *et al.*, 2006; Choi *et al.*, 2005; Hofman *et al.*, 1989). It has been proposed that cytokines, produced by a cascade of neurons and glial cells within the brain, may participate in the complex autonomic, neuroendocrine, metabolic, and behavioral responses to infection, inflammation, ischemia and other brain injuries (Szelenyi, 2001).

1.3.3 Cytokine receptors and Central Nervous System

Recent studies support the presence of cytokine and chemokine receptors in the CNS (Ambrosini et Aloisi, 2004; Dzenko *et al.*, 2005; Limaota *et al.*, 2003; Shideman *et al.*, 2006; Valles *et al.*, 2006). More specifically human microglia were found to express mRNA transcripts for most cytokine receptors (namely IL-1-I, IL-1-II, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-13 and IL-15) (Friedmann, 2001; Lee *et al.*, 2002). A large number of chemokine receptors have also been found in animal and human studies to be expressed in neurons, astrocytes, microglia and oligodendrocytes, either constitutively, or induced by inflammatory mediators (Bajetto *et al.*, 2002; Banisadr *et al.*, 2002; Coughlan *et al.*, 2000; Gosselin *et al.*, 2005; Liu *et al.*, 2005; Nguyen and Stangel, 2001; Sorensen *et al.*, 2002). Astrocytes express IFN-γ receptors (Hindiger *et al.*, 2005). Previous studies show that IL-15 receptor alpha-1 isoforms are expressed in selected structures of the human fetal brain with greater expression in the hippocampus and cerebellum, than the

cortex and thalamus (Kurowska *et al.*, 2002), and TROY- a member of the tumor necrosis factor superfamily- is strongly expressed in the mouse ventricular and subventricular zone, which contains neuronal and glial precursors during embryogenesis (Hisaoka *et al.*, 2003).

Cytokine and chemokine receptors are responsible for a number of roles in the physiology and pathology of the CNS (Yang *et al.* 2002). Evidence supports the suggestion that chemokines are involved in many neuropathological processes in which an inflammatory state persists, as well as in brain tumor progression and metastasis (Cartier *et al.*, 2005; Dogan and Karpus, 2004; Mennicken *et al.*, 1999; Sorensen *et al.*, 2002; Krumbholtz *et al.* 2006). Moreover, there is evidence for a crucial role for CNS chemokines similar to those which regulate the immune system, such as proliferation and developmental patterning (Bonavia *et al.*, 2003; Peters *et al.*, 2000; Rezaie *et al.*, 2002; Tissir *et al.*, 2004), but, in addition, peculiar to the CNS, such as regulation of neural transmission, plasticity and survival (Bajetto *et al.*, 2002; Cho *et al.*, 2002; Davis *et al.*, 2003; Dziembowska *et al.*, 2005; Ma *et al.*, 2002; Ragozzino, 2002).
Indicatively, signaling through the chemokine, CXCR2 inhibited oligodendrocyte precursor migration during spinal cord development (Filipovic *et al.*, 2003; Tsai *et al.*, 2002), and the CXCR4 affects cell-cycle proteins in neurons (Khan *et al.*, 2003), a fact which raises the possibility that chemokines may contribute to neuronal survival by repressing the activity of E2-F apoptotic genes and maintaining neurons in a highly differentiated state (Khan *et al.*, 2003).

In pathological and other special conditions (aging, behavioral models) of the CNS, there are several data supporting the involvement of cytokines and chemokine receptors. Specifically they have been observed to reduce neuropathetic pain responses in mice lacking the chemokine receptor CCR2 (Abbadie *et al.*, 2003; Lindia *et al.*, 2005).

In addition, during status epilepticus, IL-1-I has been induced in both hippocampal neurons and astrocytes localized in limbic and extralimbic areas (Rizzi *et al.*, 2003). Neuronal IL-1-I expression in the hippocampus outlasted the duration of spontaneous electroencephalographic seizures, and was not observed in degenerating neurons. Astrocytic expression occurred transiently, between 6 and 18 hours after the induction of status epilepticus and was invariably found in regions of neuronal damage (Ravizza and Vezzani, 2006). Furthermore, up-regulated IFN-γ receptors have been identified in aged spinal cord motor neurons (Eldstrem *et al.*, 2004) and induction of neuronal apoptosis by the Amyloid-beta protein has been observed through the TNF 1 receptor in Alzheimer's disease (Li *et al.*, 2004). Deficiency of TNF receptor 1 causes a decrease in the inflammatory response and tissue damage following brain injury, suggesting an active role for this receptor in this process (Quintana *et al.*, 2005).

In MS, there is some evidence for the implication of cytokine and chemokine receptors in the pathogenesis of the disease, (De Groot *et al.*, 1999; Glabinsk and Ransohoff, 2001; Misu *et al.*, 2003; Muller *et al.*, 2004; Selleberg *et al.*, 2000; Sorensen *et al.*, 1999; Trebst and Ransohoff, 2001; Zhang *et al.*, 2000; Omari *et al.*, 2006), including expression of the chemokine receptor CXCR2 in normal and proliferating oligodendrocytes in active MS lesions. This further suggests a novel mechanism for the recruitment of oligodendrocytes in the area of damage (Omari *et al.*, 2006), and indicates a pivotal role for CCR2 and

CXRC3 in leukocyte recruitment into the CNS in multiple sclerosis (Krumbholtz *et al.,* 2006; Mahad and Ransohoff, 2003; Sellebjerg and Sorensen, 2003; Sorensen, 2004). Finally, genetic studies have identified IL-4 receptor polymorphisms indicating a role for this receptor in MS susceptibility (Suppiah *et al.,* 2005).

1.3.4 Cytokines in MS immunology

There is evidence that cytokines are possibly implicated in three sites during the course of the disease: a) peripheral immune activation; b) entry of activated immune cells into the CNS, and 3) damage to the oligodendrocyte myelin unit (Cannella and Raine, 1995; Hollifield *et al.,* 2003; Matusevicious *et al.,* 1996; Navikas and Link, 1996; Prat *et al.,* 2002; Steinman, 1996).

A proposed model for the involvement of cytokines in the pathogenesis of multiple sclerosis is shown in Figure 3.

The CNS is separated from the general blood circulation by tight-junctioned endothelial cells that form the blood brain barrier (BBB). In the normal CNS, immune cells are sparse, in contrast to what is observed in MS. Thus it might be assumed that immune cells, including T cells, found in the MS brain are of blood origin and that before entering the CNS, they have passed the BBB. Only activated T cells are able to cross the BBB. This happens because adhesion molecules are overexpresed on activated T lymphocytes and therefore their entry into the CNS through blood-brain-barrier is facilitated (Nouza and Krejcova 1997; Shibagaki *et al.,* 1999). Therefore, an initial activation process is necessary for the subsequent trafficking of T cells into the CNS. The antigens that trigger

the immune response in MS are still unknown but, presumably, CNS-derived myelin antigens are presented to T cells in the periphery (Link *et al.,* 1994; Ruiz-Vazquez and de Castro, 2003; Sun J-B *et al.,* 1991).

Co-stimulatory molecules, together with the signal generated by formation of the trimolecular complex, are necessary for the activation of T cells. Recognition of the MHC-peptide complex by the T cell receptor (TcR) induces expression of CD40L on T cells. Activation of antigen presenting cells (APC) by T cells, through the simultaneous engagement of MHC by TcR and of CD40 by CD40L, leads to upregulation of the B7 family of molecules on APCs. Subsequently, other molecules present on APCs bind to molecules on T cells and this interaction is responsible for their activation.

APCs and T cells secrete several cytokines that may orchestrate immune responses. Among these cytokines, IL-12, IFN-γ and TNF-α induce Th1-type immune response while IL-4 and IL-10 induce Th2-type responses (Martino *et al.,* 1998; Druet *et al.,* 1995). In MS, it has been suggested that skewing the balance between Th1 and Th2 responses in favor of the latter may be beneficial (Charlton and Lafferty, 1995; Hintzen and Polman, 1997).

The transmigration of leukocytes into the CNS is a multistep process that occurs in an ordered sequential fashion. First, there is a co-ordinated expression of vascular (selectins, ICAM-1, VCAM-1) and leukocyte (LFA-1, VLA-4) adhesion molecules which facilitate adhesive interactions between leukocytes and endothelial cells. The expression of the selectins, ICAM-1 and VCAM-1 on endothelial cells is upregulated by the proinflammatory cytokines IFN-γ and TNF-α (Shrikant *et al.,* 1994). Thus, upregulated TNF-α in MS may play a role in increasing the expression of adhesion molecules, which

in turn results in the docking of activated leukocytes to vessel walls. The adhesion of molecules to the extracellular substance and to each other mediates a series of biological events. The specificity of the adhesion, which is demanded for each one of these cases, is ensured by the contribution of specific adhesion molecules (Elovaara *et al.,* 2000). These molecules are expressed at the cell membrane under the action of specific stimulation.

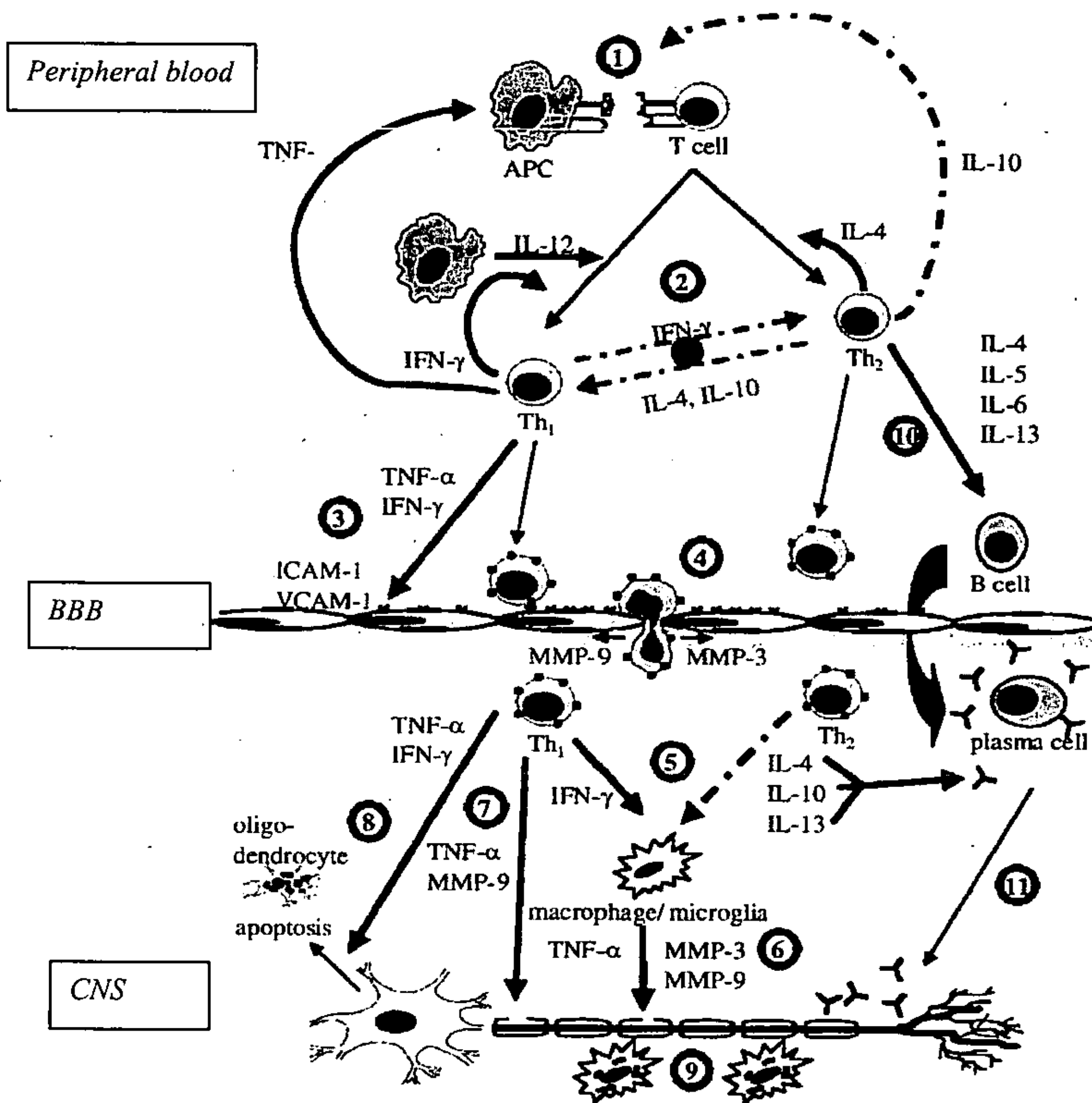

Figure1-3. Proposed model for the involvement of cytokines in the pathogenesis of MS Regulation of APC-T cell interaction by cytokines (1); mutual bidirectional cross-inhibitory effects of pro- and anti-inflammatory cytokines (2); induction of adhesion molecules by TNF-α and IFN-γ (3); entry of T cells into the CNS through disruption of the BBB by MMP-3 and MMP-9 (4); induction of macrophage/ microglia by IFN-γ (5); myelin damage by TNF-α, MMP-3 and MMP-9 (6-7); induction of apoptosis of oligodendrocytes by TNF-α and IFN-γ (8); macrophage with engulfed myelin peptides (9); induction of B cells by anti-inflammatory cytokines (10); myelin damage by antibodies directed against myelin components (11). (Diagram adapted from Ozenci *et al.*, 2002)

The biological importance of cellular adhesion is not so much the multiplicity of functions that contribute but more its dynamic nature. Adhesion molecules not only ensure a very strong intracellular connection but also transmit intracellular messages which promote multiplication, differentiation and activation of the cells (Davey *et al.*, 2005). At present, adhesion molecules are classified into four classes: selectins, intergrins, molecules that belong to the immunoglobulins' superfamily, and CD44. Some representative adhesion molecules are ICAM-1, ICAM-2, ICAM-3 and VCAM-1 which are connected to the intergrins, and mainly serve the adhesion of cells to the endothelium. As mentioned earlier, these molecules appear to have an important role in the pathogenesis of MS. Several studies concerning the connection of the adhesion molecules to multiple sclerosis have shown that the VCAM-1/VLA-4 adhesion pathway is expressed at higher levels in chronic MS lesions while ICAM-1/LFA-1 is found in lesions, of all ages (Cannella and Raine, 1995), serum ICAM-1 levels are elevated in MS patients compared to patients with other neurological diseases and healthy controls, CSF VCAM-1 is elevated over control and disease control samples, levels of serum VCAM-1 are significantly elevated in Primary Progressive Multiple Sclerosis (PPMS) compared with Relapsing Remitting Multiple sclerosis (RRMS) in remission, while ICAM-1 was significantly elevated in PPMS compared to all others MS groups. (Bilinska *et al.*, 1999; Dore-Duffy *et al.*, 1995; Droogan *et al.*, 1996; Elovaara *et al.*, 2000; Mc Donell *et al.*, 1998; Mc Donnel *et al.*, 1999; Rieckman *et al.*,1994; Rieckmann *et al.*, 1998; Sellebjerg and Sorensen, 2003).

Following adhesion, the next step involves chemoattraction of immune cells into the CNS mediated through a chemokine gradient, with chemokine concentrations augmented in

the CNS relative to blood (Stuve *et al.,* 1997). Activated cells, capable of expressing chemokine receptors, migrate along this chemokine gradient. Immune cells, including activated T cells cross the BBB by passing either through or between endothelial cells (Tsukada *et al.,* 1993). They then traverse the basement membrane composed of proteins of the extracellular matrix. Metaloproteinases (MMP) secreted by immune cells such as MMP-3 and MMP-9 may disrupt the basement membrane allowing cells to enter the CNS (Leppert *et al.,* 2001; Lindberg *et al.,* 2001).

Damage to the oligodendrocyte myelin unit is thought to be a critical event in MS pathogenesis, involving the synergistic actions of various cells and molecules (Boccaccio and Steinman, 1996). Astrocytes and microglia present in the CNS are capable of secreting a number of cytokines and MHC Class II molecules and therefore may interact with blood-derived immune T cells leading to oligodendrocyte damage directly or indirectly (Aloisi *et al.,* 1999). The secretion of soluble mediators from T cells, such as IFN-γ and TNF-α has been proposed to exert toxic effects on oligodendrocytes *in vitro* (Ozenci, *et al.,* 2002). Furthermore, the same cytokines, along with IL-1b, are potent activators of microglia and macrophages which may further damage myelin (Takeuchi *et al.,* 2006).

Another mechanism of demyelination in MS is related to apoptosis of oligodendrocytes. Activated T cells may play a role in this process through IFN-γ secretion. Addition of IFN-γ to cultured oligodendrocytes renders them susceptible to Fas ligand-mediated apoptosis by inducing Fas expression on their cell surface (Racke *et al.,* 1994; Sharief *et al.,* 2001).

The humoral response might also be important in MS pathogenesis. Augmented B cell response and autoantibodies to multiple myelin components are present in MS CSF (Zabaleta *et al.*, 2002). B cell proliferation, differentiation and antibody production are augmented by cytokines, including IL-10. The process by which damage occurs to the oligodendrocyte myelin unit may be complex and involve all of the above mechanisms (Hemmer *et al.*, 2004).

1.3.5 Previous studies on main cytokines and MS

A number of studies have been performed on the secretion of cytokines during various stages of MS with conflicting results (Crucian *et al.*, 1996; Crucian *et al.*, 1995; Frei *et al.*, 1991; Inoges *et al.*, 1999; Link, 1998; Link *et al.*, 1994; Rieckmann *et al.*, 1994; Rieckmann *et al.*, 1995; Sharief and Hentges, 1991; Hermans *et al.*, 1997; Navikas *et al.*, 1996). Elevated, normal and decreased levels of almost all cytokines have been reported (Ozenci *et al.*, 2002). Experimental animal models of MS have also been proposed in order to study different treatment protocols but outcome data is far from clear. Such contradictory results with regard to cytokine levels in MS most probably reflect methological dilemmas in addition to the complex biology of cytokines (Scrijcver *et al.*, 2004).

Three main cytokines, IFN-γ, IL-10, and TNF-α are believed to play an important role in the active phase of the immune response underlying MS (Karni *et al.*, 2006; Tran *et al.*, 2006) and they constitute a major part of this thesis, however, several others such as IL-12, and IL-4 are also of great interest.

1.3.5.1 IFN-γ

IFN-γ is the only proinflammatory cytokine so far shown to have any clear influence on the course of MS (Dai *et al.,* 2001; Vartarian *et al.,* 1995). It is a homodimeric glycoprotein of 21-24 KDa molecular weight and is the central regulator of non-specific inflammation (Germenis, 2000). IFN-γ is produced from Th1 $CD4^+$ cells, from the majority of $CD8^+$ T-lymphocytes and a small number of NK cells (Germenis, 2.000). Therefore, IFN-γ production from NK cells represents a T-cell independent pathway for macrophage activation, involved in stimulation of the cytokine network after bacterial exposure of the organism. IFN-γ has potent proinflammatory properties, including the capacity to induce the production of other proinflammatory cytokines, such as IL-12, and the expression of MHC Class II molecules on monocyte/macrophages (Aloisi *et al.,*1999; Segal *et al.,* 2002; Hugh *et al.,* 1995; Kudinov *et al.,* 2003). IFN-γ-mediated signaling leads to increased intracellular Ca^{2+} levels, therefore lowering the excitability threshold of T cells (Billiau, 1996; Young and Hardy, 1995).

There are conflicting data on IFN-γ levels in MS. Elevated numbers of IFN-γ mRNA-expressing blood mononuclear cells and increased serum IFN-γ levels compared to controls have been reported (Karni, *et al.,* 2002). Alternatively, other studies have found no difference between MS patients and controls with regard to either the percentage of IFN-γ expressing cells in the blood detected by flow cytometry or blood levels of IFN-γ mRNA (Link *et al.,* 1994; Noronha *et al.,* 1993; Olsson *et al.,* 1990).

1.3.5.2 IL-10

IL-10 (also known as cytokine synthesis inhibitory factor) is the most important anti-inflammatory cytokine described to date (Asadullah *et al.*, 1998; Germenis, 2000; Kim *et al.*, 1992; Moore *et al.*, 1993). It is produced from Th2 T-helper cells and by some activated macrophages and has inhibitory effects on potentially dangerous proinflammatory cytokines, including TNF-α, IL-2, IFN-γ, IL-12, and some chemokines (de Waal-Malefyt *et al.*, 1991; Fiorentino *et al.*, 1989; Fiorentino *et al.*, 1991; Germenis, 2000; Mossman and Moore, 1991; Pahan *et al.*, 2000; van der Veen *et al.*, 1993; Vieira *et al.*, 1991;). The inhibitory effects of IL-10 on cytokine production are mainly mediated via suppression of expression of MHC Class II molecules and of adhesion and costimulatory molecules in monocytes/macrophages and dendritic cells (de Waal-Malefyt *et al.*, 1991). A potential therapeutic role for IL-10 in autoimmune diseases, such as RA, type 1 diabetes mellitus and psoriasis has been proposed (Asadullah *et al.*, 1998; D'Andrea *et al.*, 1993; Howard and O'Garra, 1992; Kennedy *et al.*, 1992; Mekala *et al.*, 2005; Mossman, 1994; Pennline *et al.*, 1994; Rott *et al.*, 1994; Oswald *et al.*, 1992; Porrini,*et al.*,1998).

A number of studies have evaluated IL-10 in MS, with partly contradictory results (Brod *et al.*, 1997). Elevated numbers of IL-10 mRNA-expressing blood mononuclear cells, compared to controls, have been reported in patients with MS (Rep *et al.*, 1999; Navikas *et al.* 1995). However, decreased IL-10 has also been observed in MS: serum IL-10 levels and numbers of IL-10 secreting blood mononuclear cells were reported to be lower in MS

patients than in controls (Ozenci *et al.*, 1999; Rep *et al.*, 1996; Xiao *et al.*, 1998; Zdanov *et al.*, 2004).

1.3.5.3 Tumor necrosis factor-α (TNF-α)

TNF-α is a potent proinflammatory cytokine of 17kDa molecular weight (Germenis, 2000. The main cellular source of TNF-α are the LPS-activated macrophages, T cells and NK cells (Flohe *et al.*, 2000; Germenis, 2000). TNF-α can be found in soluble and membrane forms and its function depends upon the quantity of its production (Germenis, 2000). Its effects on cells range from the promotion of proliferation to apoptosis. At small concentrations TNF-α acts as an autocrine or paracrine regulator of leukocyte and endothelial cells functions (Robinson *et al.*, 2002; Shrikant *et al.*, 1994). TNF-α up-regulates the expression of adhesion molecules on the endothelium, may activate polymorphonuclear cells to destroy microbes and induces CD80 and MHC Class II expression important in T cell activation (Germenis, 2000; Roitt *et al.*, 1998). TNF-α stimulates production of IL-12, which in turn induces IFN-γ production (Ozenci *et al.*, 2000). Because of its potent pro-inflammatory properties, it has been hypothesized that TNF-α is detrimental in autoimmune disease (Shankar and Handa, 2004; Hofman *et al.* 1989). This has been demonstrated in RA and anti- TNF-α treatment has become part of the standard therapeutic arsenal for this disease (Strokus *et al.*, 1998; Trinchieri, 1995).

A number of studies have reported elevation of TNF-α in patients with MS compared to controls (Navikas *et al.*, 1996). Numbers of TNF-α mRNA-expressing blood cells, concentrations and levels of TNF-α-secreting blood mononuclear cells were higher in MS

patients than in controls. Elevated levels of TNF-α-secreting cells may be important for the induction of pro-inflammatory immune responses in MS (Dowling *et al.*, 1997).

1.3.5.4 IL-12

IL-12 (IL-12p70) is a heterodimeric cytokine consisting of two covalently bound subunits, named p35 and p40 on the basis of their approximate molecular weights. IL-12 promotes the development of Th1-type immune responses and is a powerful inducer of IFN-γ production by T cells and natural killer cells (Adorini, 1999; Aste-Amezaga *et al.*, 1994; Gately *et al.*, 1998; Seder *et al.*, 1993; Sieling *et al.*, 1994; Trinchieri, 2003). IL-12 also induces cell-mediated cytotoxicity and exerts co-mitogenic effects in T cells (Rogge *et al.*, 1997).

IL-12 is thought to play a central role in the pathogenesis of a group of organ-specific autoimmune diseases that may include MS, uveitis, type I diabetes mellitus and rheumatoid arthritis (RA) (Kang and Kim, 2006). IL-12 acts through its receptor, which is composed of two subunits, IL-12Rβ1 and IL-12Rβ2. The interaction of IL-12 with IL-12R represents a pivotal crossroad in the development of Th1-type immune responses (Rogge *et al.*, 1999). IL-12Rβ2 is thought to be a central molecule in these responses (Gillespie *et al.*, 2000).

It has been shown *in vitro* that IL-12Rβ2 is a marker for Th1 cells and that it determines IL-12 responsiveness (Matusevicious *et al.*, 1998; van Boxel-Dezaire, 1999). It has also recently been demonstrated that pulmonary T cells from patients with sarcoidosis, but not from patients with asthma, exhibit enhanced IL-12Rβ2 expression, suggesting the

importance of IL-12Rβ2 as a marker for Th1-type autoimmune diseases (Fassbender *et al.*, 1998; Ozenci *et al.*, 2001)

In MS, conflicting results regarding IL-12 p40 levels have been reported (Balashov *et al.*, 1997; Leonard *et al.*, 1995; Rohowsky-Kochan *et al.*, 1999; Fassbender *et al.*,1998). Increases, compared to controls, both in the number of IL-12 p40 mRNA-expressing blood MNC and of IL-12 p40 mRNA levels in blood have been described (Heesen *et al.* 1999). In contrast, levels of IL-12 p40 were found to be similar in CSF of both MS patients and controls (Drulovic *et al.*, 1997).

1.3.5.5 IL-4

Interleukin 4 (IL-4) is a pleiotropic Th2-derived immune cytokine, which is predominantly produced by activated T lymphocytes, mast cells and basophils (Germenis, 2000). IL-4 has been shown to have various activities in many different cell types, such as T cells, B cells, monocytes, endothelial cells, and fibroblasts. These activities include inhibition of cell proliferation, regulation of adhesion molecules, and induction of signal transduction through the JAK/STAT pathway (Duvey *et al.*, 2006; Luscinskas *et al.*, 1994; Roitt *et al.*, 1998; Rahaman *et al.*, 2002).

To induce biological activities, IL-4 must bind to its specific receptor, which is generally present on the plasma membrane of target cells. IL-4 initiates transmembrane signaling by activating two types of transmembrane receptors. The type I receptor comprises the IL-4Rα subunit, which binds to IL-4, and the γc chain that is shared by IL-2, IL-7, IL-9, and IL-15. The type II receptor comprises the IL-4Rα chain and the IL-13Rα1 subunit, a low affinity receptor for IL-13 that constitutively associates with Jak2 or Tyk2.

In MS, conflicting results exist regarding IL-4 secretion, several studies indicate a downregulation of this anti-inflammatory cytokine during relapses (Bartosik-Psujek and Stelmasiak, 2005; Franciotta *et al.*, 2000) whereas others indicate that IL-4 levels in CSF was significantly higher during the stable stage as well as during relapse when compared to controls (Bienvenu *et al.*, 2000).

1.3.6 Cytokine methodology

Different methods focus on different stages of cytokine production and secretion (Bienvenu *et al.*, 1998; Nguyen *et al*, 1999). Cytokine protein levels in extracellular fluids may be measured by enzyme-linked immunoassay (ELISA). However, cytokines are very rarely found in an unbound state but are nearly always bound to soluble cytokine receptors, anti-cytokine antibodies or binding proteins. Since cytokines typically have short half-lives, i.e. are rapidly taken up and degrade, results obtained by ELISA are frequently negative and/or difficult to interpret. Furthermore, unlike hormones, cytokines mostly exert their effects at short distances from the site of production. Therefore, the overall serum concentration of cytokine may not reflect its rate of production and/or secretion. Detection of cytokines at the levels of the individual cell, i.e. the cytokine-producing/secreting cell may be more relevant for an understanding of the involvement of an individual cytokine in the immune process (Stott, 2000; Sedwick *et al.*, 1983).

Cytokine mRNA levels can be examined by Northern blot, semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) or *in situ* hybridization. These methods are sensitive enough to detect cytokine mRNA in the absence of stimulant. A

drawback with these methods is that results based on determinations of mRNA levels do not necessarily reflect levels of secreted cytokines (Froncillo *et al.*, 1996; Hein *et al.*, 2001).

Immunostaining allows phenotyping and, to some extent, detects the amount of cytokine expressed in the cell. However, the sensitivity of this method in cytokine detection is low, and the method generally requires *in vitro* stimulation, which may yield results which are unrepresentative of the levels of cytokines *in vivo* (Ozenci *et al.*, 2002).

ELISPOT assays, originally described for the enumeration of antibody-secreting cells and then modified for the detection of cells secreting cytokines, offer the possibility of determining the number of cells releasing a specific translated protein. The assays allow real-time cytokine detection by identifying the cells actively secreting cytokines at a given time point and are sensitive enough to detect cytokine-secreting cells in the absence of stimulant. Instead of measuring mRNA or the accumulation of cytokine in the cytoplasm of pharmacologically treated cells, ELISPOT assays detect immunologically relevant secretion of the cytokine, downstream of any post-transcriptional or post-translational regulation. Recent developments in ELISPOT assays, including double-colored ELISPOT assays which allow detection of two different cytokine-secreting cells in the same well, and automatic readers which minimize the risk of subjectivity in reading plates, have brought new advantages to the use of ELISPOT assays in cytokine research (Czerkinsky *et al.*, 1988; Duddy *et al.*, 1999; Jansson *et al.*, 2003; McCutcheon *et al.*, 1997; Masse and Gray, 2002).

However, even ELISPOT assays have some drawbacks including difficulties in establishing the assay for new cytokines due to the scarce availability of suitable antibody

pairs, and an ability to determine the amount of cytokine secreted by the individual cell, i.e., current ELISPOT assays are semi-quantitative.

Therefore, it is clear that production of cytokines is regulated at several different levels, and it is difficult to design a single method ideal for cytokine measurements in general. Furthermore, the method of choice in cytokine research is totally dependent on the question that is being asked.

1.4. Working Hypothesis

Taking the previous literature into account, a working hypothesis for the development of MS may be suggested. The pathogenesis of multiple sclerosis is highlighted by an inflammatory response and myelin destruction. The inflammatory infiltrate in MS lesion is composed of T lymphocytes, a small number of B lymphocytes, activated microglia and macrophages which interact with myelin sheaths and are actively engaged in the demyelinating process. There is a local production or expression of immune-related molecules such as MHC, adhesion molecules, pro- and anti-inflammatory cytokines and chemokines which contribute to an imbalance between Th1 and Th2 T cell-mediated immune response.

There are two predominant hypotheses to explain the immune-mediated mechanisms of myelin destruction in MS lesions: either direct T-cytotoxic lymphocyte-mediated lysis of oligodendrocytes (through their products such as lymphotoxin and perforin or through activation of the CD95-signalling pathway) or an indirect mechanism in which activated T cells induce toxic cytokine production (e.g. nitric oxide or TNF-α) by accessory T cells. T-cell produced cytokines may be involved in the direct damage to oligodendrocytes. If

the destructive inflammatory process in the CNS could be arrested at an early stage it would prevent the accumulation of neurological disability.

Therefore, by investigating cytokine secretion patterns as well as the other immune-regulatory molecules in MS patients and by controlling their effects through administration of IFNβ products to these patients, it would be possible to diminish disease progression, reduce relapses and improve quality of life. Investigation of the mechanism of action of the two IFNβ products will be able to suggest a more efficient therapeutic modality than current treatments.

1.5 Aims of the Study

The aims of the present study were: a) to characterize the cytokinetic profile of MS patients by investigating the secretion of the main pro- and anti-inflammatory cytokines of peripheral blood mononuclear cells using ELISPOT methodology, b) to investigate: the expression levels of cytokine receptors in the three major T cell populations (CD4, CD8, NK cells), and to study the induction of apoptosis by measurement of CD95 levels in the serum of MS patients, and the involvement of adhesion molecules, VCAM-1 and ICAM-1 in lymphocyte trafficking. Also c) to measure the involvement of metalloproteinases in oligodendrocyte destruction, d) to study the effect of IFNβ treatment and to elucidate the mechanism of action of the different forms of IFNβ treatment (IFNβ-1α/1β) on cytokine secretion, percentages of peripheral blood mononuclear cells expressing cytokine receptors, expression of adhesion molecules, metalloproteinases and apoptotic markers (CD95). e) Finally it was important to evaluate the effectiveness of the ELISPOT assay in MS disease monitoring.

CHAPTER 2. MATERIALS AND METHODS

2.1 Clinical methods

2.1.1 Description of the study population

The study involved 60 patients diagnosed with multiple sclerosis and 25 age-matched healthy controls without any neurological or autoimmune disease randomly admitted to the Henry Dynant Hospital in Athens. Informed consent was obtained from all patients and the study was approved by the University of Athens Medical School Ethics Committee.

All MS patients were diagnosed with the relapsing-remitting form (RRMS) of the disease based on laboratory, electrodynamic and imaging control (MRI) examinations. At the time of blood sampling the mean age of the patient group was 34±3.2 years (ranging from 29-39 years). All enrolled patients had at least two relapses in the last 2 years, and they were not treated with any immunomodulating drug before participating in this study.

Based on whether or not receiving IFNβ therapy, the patients group was separated into two main subgroups, twenty patients who were not receiving IFNβ therapy and forty patients who were receiving IFNβ treatment for six months. The group that was receiving IFNβ treatment was further divided into two subgroups: twenty patients were injected intramuscularly once a week with 30 μg of Avonex (IFNβ-1α) and twenty patients were injected subcutaneously every other day with 250 μg of Betaferon (IFNβ-1β) for 6 months. For the patients who were receiving IFNβ, the mean time from the onset of the disease was 3.2±1.2 years, whereas for patients who were not receiving therapy the mean time from the onset of the disease was 3 years.

The control group consisted of age- and sex-matched individuals: twenty five patients (mean age 38.8±6.9 years, range 34–43) with headache who underwent lumbar puncture for exclusion of subarachnoidal hemorrhage or patients who underwent spinal anesthesia (for hernia repairs or leg, gynecological or urological operations).

Peripheral blood was sampled from each MS patient without and after 6 months therapy, during a clinically inactive and at least 1-month steroid-free period. Samples in month 6 were obtained 66–72 h after the most recent treatment of IFNβ. Serum samples of the controls were collected in a similar time frame. Aliquots were centrifuged and the serum immediately frozen at -80°C.

2.1.2 Evaluation of personal history (Description of the questionnaire)

All participants, after clinical diagnosis, laboratory diagnostic examination and MRI results confirmed their inclusion in one of the two main groups, were asked to complete a questionnaire which was filed on a statistical package for further analysis. For accurate completion of the questionnaire the help of relatives and caregivers was used when necessary. It contained (1) demographic data: sex, age, marital status, occupation and educational level, (2) personal history: history of physical illness, history of viral infections, vaccinations, autoimmune and inflammatory disorders (e.g. allergies), and medication, (3) family history of autoimmune disorders, and (4) life-style information (see Appendix I).

2.1.3 Blood Sampling

Blood samples were collected in Heparin-containing tubes and were immediately transferred to the Department of Biological Chemistry in The University of Athens Medical School for separation of lymphocytes using the Ficoll procedure (see below). Serum/plasma samples were also collected from each patient.

2.1.4 Peripheral blood mononuclear cells (PBMC) isolation using the Ficoll-Hypaque density gradient centrifugation

Peripheral blood (15ml) collected in heparin-containing tubes was layered on top of Ficoll-Hypaque solution (10ml) (Amerhsam). The mixture was centrifugated at 1500 rpm for 20 min, at room temperature, and the distinct cell layers obtained. Erythrocytes and granulocytes sedimented at the bottom of the tube and PBMCs were isolated in the interface. By using aseptic techniques, PBMCs were collected in separate tubes, washed, counted and resuspended in solution containing fetal calf serum (FCS) (80%) and DMSO (1.5ml) (20%). Cell viability was determined by trypan blue exclusion.

Cryovials containing the lymphocytes ($2x10^6$cells/ml) were stored in liquid nitrogen until analysis.

2.1.5 Freezing and thawing procedures for human PBMCs

For freezing, PBMCs were suspended at a concentration of $2x10^7$/ml in 2ml of freezing medium A (60% FCS, 40% RPMI) at room temperature. An equal volume of freezing medium B (20% DMSO, 80% FCS), also at room temperature, was added dropwise, while gently mixing by shaking the tube. The resulting cell suspension (4ml) was pipetted

in aliquots (1.5ml) into cryovials (1.8ml) (Greiner Labortechnik, Frickenhausen, Germany). The tubes were placed into a pre-chilled (4°C) Nalgene Cryogenic Controlled-Rate Freezing Container (Fisher Scientific, Hanover Park, IL) that was placed into a -80 °C freezer. After 24 h, the samples were transferred to a liquid nitrogen tank for storage until required for assay (Kreher *et al.*, 2003).

For thawing, cryotubes were placed in a water bath (37 °C) and, as soon as the samples were completely thawed, they were pipetted into a 15ml tube containing a 2-fold amount of complete RPMI medium (93% RPMI- 1640, 5% heat-inactivated AB serum, 1% L-glutamine, 1% Penicillin–Streptomycin) at room temperature. The cells were washed twice at room temperature. Cell recovery and viability were determined by acridine orange and ethidium bromide (Becton Dickenson, San Jose, CA) staining (Kreher *et al.*, 2003).

2.2 Characterisation of the biochemical profile of MS patients

2.2.1 Estimation of triglycerides levels in plasma samples

The method was based on the enzymatic hydrolysis of serum or plasma triglycerides to glycerol and free fatty acids (FFA) by lipoprotein lipase (LPL) (Figure 4). Glycerol was phosphorylated by adenosine triphosphate (ATP) in the presence of glycerokinase (GK) to form glycerol-3-phosphate (G-3-P) and adenosine diphosphate (ADP). G-3-P was oxidized by glycerophosphate oxidase (GPO) to form dihydrooxyacetone phosphate (DHAP) and hydrogen peroxide.

A red chromogen was produced by the peroxidase (POD) catalyzed coupling of 4-aminoantipyrine (4-AA) and phenol with hydrogen peroxide (H_2O_2), proportional to the concentration of triglyceride in the sample.

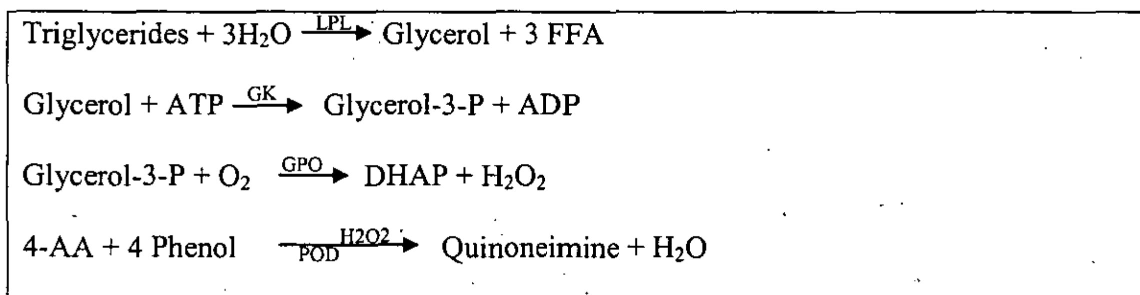

Figure 2-1. Enzymatic hydrolysis of serum or plasma triglyceride to glycerol and free fatty acids (FFA) by lipoprotein lipase (LPL)

Procedure

An aliquot of 1 ml of monoreagent was mixed with either sample or standard (10μl) (see Appendix II). The tube was allowed to stand for 15 min at room temperature (16-25 ^{0}C). The absorbance (A) of the samples and the standard was read at 500 nm against the reagent blank (1 ml of monoreagent only).

The triglyceride concentration was estimated using the following formula:

$(A_{sample}/ A_{standard}) \times C_{standard}$ = mg/dl triglycerides.

Samples with concentrations higher than 600 mg/dl were diluted 1:2 with saline and assayed again and the results multiplied by 2.

The reference values according to the manufacturer's instructions (Linear chemicals, Spain) were: normal < 150 mg/dL, borderline (150-199 mg/dL), high (200-499 mg/dL) and very high ≥ 500 mg/dL.

The precision of the method for 10 replicates of each level are shown in the table below:

Table 2-1. Precision of triglycerides method

mg/dl	Within-run			Between-run		
Mean	220	368	512	220	372	490
SD	1.8	2.6	2.4	1.9	2.8	3.7
CV%	0.81	0.7	0.7	0.47	0.27	0.76
N	10	10	10	10	10	10

In order for the results to be expressed as SI units, a converting factor (mg/dL x 0.0113 = mmol l^{-1}) was used.

2.2.2 Estimation of glucose concentration in plasma samples

The method was based on the oxidation of glucose, present in the sample to gluconic acid in the presence of glucose oxidase (Figure 2-2). Liberated hydrogen peroxide was detected by a chromogenic oxygen acceptor, phenol-ampyrone and the red quinine formed was proportional to the amount of glucose present in the sample.

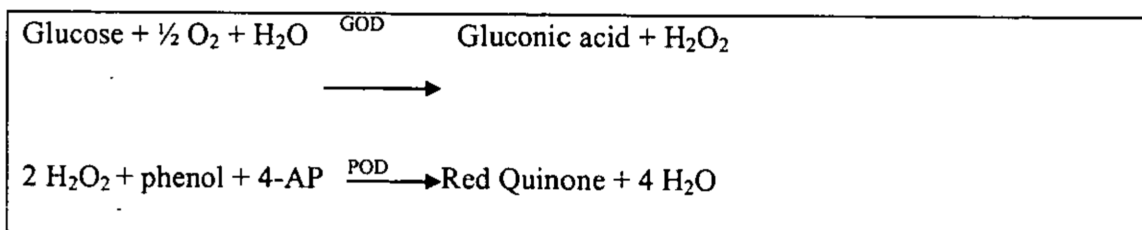

Figure 2-2. Glucose oxidation reactions

Procedure

An aliquot of 1 ml of glucose monoreagent was mixed with either sample or standard (10μl) and incubated for 10 min at 37 ^{0}C (or 20 min at 15-25 ^{0}C) (see Appendix II). The absorbance of the unknown and the standard was measured against a blank reagent at 510 nm (500-530 nm).

Estimation of glucose concentration in the sample was performed using the following formula:

$(A_{sample}/ A_{standard})$ x $C_{standard}$ = mg/dl glucose

The linear range was up to 500 mg/dL and normal values, according to the manufacturers instructions (Linear chemicals, Spain) were 76-110 mg/dL. In order for the results to be expressed as SI units, the converting factor was: mg/dL x 0.0555 = mmol/L.

2.2.3 Estimation of HDL-Cholesterol in plasma samples

The direct method for quantifying cholesterol in high density lipoproteins (HDL) was a homogeneous enzymatic test without the need for any off-line pre-treatment or centrifugation steps.

The procedure comprised of two steps. In the first step, cholesterol in lipoproteins other than HDL in the test sample were decomposed by the simultaneous action of cholesterol esterase and cholesterol oxidase at pH 7.0, giving, as end-products, cholestenone and hydrogen peroxide, the latter being decomposed to water and oxygen by catalase.

In the second step, a surfactant, which specifically acts on HDL, was added to the reaction product of the first step being the remaining cholesterol quantified by a Trinder's type reaction in which the aniline derivative, HDAOS [N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline], and 4-aminoantipyrine (4-AA) as a coupling reagent were condensed by H_2O_2 in the presence of peroxidase (POD) to form a red quinoneimine dye proportional to the concentration of HDL-cholesterol in the sample.

Procedure

A volume of 300 μl of HDL-C enzyme reagent was mixed of sample (4μl) (see Appendix II). The mixture was incubated for 5 min at 37 ^{0}C. Then, POD/4-AA (100μl) reagent was added and mixed. After 30 seconds, the absorbance of the sample (A_{1s}) and the calibrator (A_{1c}) was read at 37 ^{0}C at 600 nm against the reagent blank and after 3 minutes a second absorbance reading was taken at 37 ^{0}C of the sample (A_{2s}) and the calibrator (A_{2c}).

The reagent blank was a mixture of enzyme reagent (300μl) and H_2O (4μl) and the calibrator was a mixture of enzyme reagent (300μl) and LDL/HDL calibrator reagent (4μL) (under the same procedure as the sample).

Estimation of the concentration of HDL-C was performed using the following formula:

$(A_{2s} - A_{1s}) / (A_{2c} - A_{1c}) \times C_{calibrator}$ = mg/dl HDL-cholesterol

The clinical values of HDL-cholesterol used to classify risk groups (based on Warnick & Wood, 1995) are shown below:

Table 2-2. Clinical values of HDL-cholesterol used to classify risk groups

Cholesterol from lipoproteins of high density		RISK
Men	> 55 mg/dL	Low
	35-55 mg/dL	Moderate
	< 40 mg/dL	High
Women	> 65 mg/dL	Low
	45-65 mg/dL	Moderate
	< 45 mg/dL	High

The characteristics of the analytical performance according to manufacturers instructions (Linear Chemicals, Spain) were (a) precision, the coefficient of variation of the within-run test (N=10) was commonly found under 3%; (b) sensitivity, using a 1:75:25 sample reagents at 600 nm, cholesterol (50 mgr) produced a net absorbance between 0.050/0.090 and (c) correlation, this test (y) was compared with a commercially available method (x) and the results were the following: $r= 0.997$, $y=0.987x + 1.4$

2.2.4 Estimation of total cholesterol in plasma samples

The method for total cholesterol measurement in plasma involved the use of three enzymes: cholesterol esterase (CE), cholesterol oxidase (CO) and peroxidase (POD). In the presence of the former, a mixture of ADPS (N-ethyl-N-propyl-m-anisidine) and 4-

aminoantipyrine (4-AA) was condensed by hydrogen peroxide to form a quinoneimine dye proportional to the concentration of cholesterol in the sample (Figure 2-3).

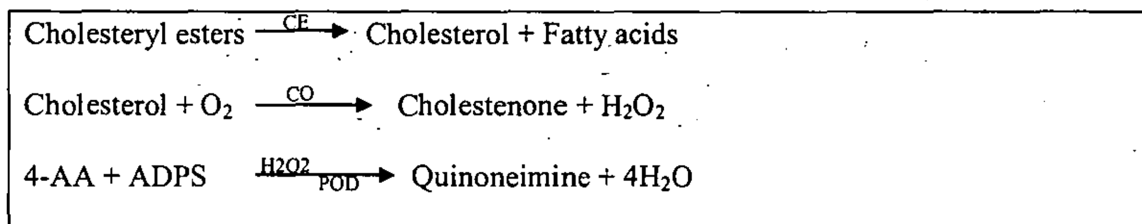

Figure 2-3. Reactions for cholesterol measurement in plasma

Procedure

Sample (10μl) was mixed with monoreagent (1.0 ml) and the mixture (see Appendix II) incubated for either 10 min at room temperature or 5 min at 37 ^{0}C. The absorbance (A) of either the sample or the standard was read at 550 nm against the reagent blank.

The reagent blank contained monoreagent (1.0 ml) and the standard solution contained monoreagent (1 ml) and cholesterol standard (10 μl).

Estimation of total cholesterol was performed using the following formula:

$(A_{sample}/ A_{standard}) \times C_{standard}$ = mg/dl total cholesterol

Samples with concentrations higher than 600 mg/dl were diluted 1:2 with saline and assayed again and the results were multiplied by 2.

Updated clinical values of total cholesterol were used to classify risk groups as shown below:

Table 2-3. Clinical values of total cholesterol used to classify risk groups

Total Cholesterol concentration	*Risk Classification*
< 200 mg/dl	Desirable
200-239 mg/dL	Borderline high
> 240 mg/dL	High

The linearity of the method was up to 600 mg/dL.

According to the manufacturer's instructions (Linear Chemicals, Spain), the precision of the method for 20 replicates of each level is shown in the table below:

Table 2-4. Precision of the cholesterol method

mg/dl	Within-run			Between-run		
Mean	143	162	267	143	162	267
SD	2.4	2.1	1.7	2.9	3.1	4.3
CV%	1.7	1.29	0.64	2.02	1.91	1.61
N	20	20	20	10	10	10

Sensitivity: Using a 1:100 sample/reagent dilution at 550 nm, 10 mg cholesterol produced a net absorbance of approximately 0.030.

2.3 Study I: Evaluation of cytokine secretion levels in MS patients and controls

2.3.1 Detection and enumeration of cytokine-secreting human PBMC by ELISPOT methodology

Human cytokine ELISPOT assays were performed as described by Helms et al., 2000. Briefly, cell suspensions were prepared at different densities, ranging from 5 x 10^5 to 2 x 10^6 cells/ml. Each cell suspension (100 μl/well) was added into ELISPOT plate microwells which contained nitrocellulose membranes in the bottom pre-coated with the desired anti-cytokine capture antibody. Cell suspensions were incubated at 37°C in a 5% CO_2 humidified incubator for 24hrs. The next day, cell suspensions were aspirated, the wells were washed 2x with deionized (DI) water and allowed to soak for 3 – 5 min during each wash step. They were further washed three times with well prepared washing buffer (200μl/ml PBS with 0.1% Tween-20). The Detection Antibody Solution (towards the cytokine in question) was added (100 μl /well), and the plate incubated for 2 hr at room temperature. The wells were again washed three times with washing buffer (200 μl/well), allowed to soak 1 – 2 min during each wash step and Streptavidin-HRP solution (100 μl) added to each well. The plate was then incubated for 1 hr at room temperature and after washing wells four times with washing buffer (200μl/ml); they were further washed twice with PBS (200 μl/ml). AEC Substrate Solution (100 μl) was added to each well and spot development monitored between 5 – 60 min. Substrate reaction was stopped by washing wells with DI water and the plate air-dried at room temperature for 2 hr to overnight, until it was completely dry.

2.3.2 Image analysis of ELISPOT data

An ImmunoSpot Image Analyzer (Aid, Germany), specifically designed for the ELISPOT assay was used. Digitized images were analyzed for the presence of areas in which color density exceeds background by an amount set on the basis of the comparison of experimental wells (containing T cells, APC and antigen) and control wells (containing T cells and APC only). After background and noise subtraction, custom software was used to analyze spot morphology for circularity and density distribution to identify and separate touching and overlapping spots (Karulin *et al.*, 2000). Objects that met these criteria were recognized as spots and counted. The measurement of spot-size distribution was also a built-in function of the software; it was based on the array of spot sizes in a given well sorted according to distinct size categories.

2.3.3 Optimization of ELISPOT methodology

Optimization of the ELISPOT assay for sensitivity (among different ELISPOT company kits) and minimization of background signal was performed before evaluation of the experimental samples. The Becton Dickinson ELISPOT plates were selected for their higher sensitivity and the camera settings were adjusted based on the company's specific requirements by an experienced technician of the manufacturing company (Aid, Germany).

2.3.4 ELISPOT data statistical analysis

ELISPOT frequencies were compared between groups using a t-test calculated by SigmaStat (version 7.0, SPSS, Chicago, IL). Statistical significance was set at $p<0.05$. The coefficient of variation (CV) to assess the relative variability of intra-assay variations was calculated according to the formula CV= (Standard deviation of spot counts from triplicate wells/Mean spot counts in the same triplicate wells) x100.

2.4 Study II: Percentage of peripheral lymphocytes expressing cytokine receptors in MS patients and controls

2.4.1 Cell cultures for studying of percentages of peripheral lymphocytes expressing IFNγR, IL-10R, TNF-αR and IL-12R.

PBMCs ($2x10^6$/ml) were resuspended in Dulbecco's modified Eagle medium supplemented with penicillin (50 U/ml), streptomycin (50μg/ml), L-glutamine (2 Mm), MEM non-essential amino acid solution (1%) and fetal calf serum (10%) (all from Gibco), and plated in plates (12-well) (Nunc, Roskilde, Denmark) at $5X10^6$/well. Cells were cultured for 72 h (37°C, 5% CO_2) with or without PHA (2μg/ml), collected and used for flow cytometric determination of IFN-γR, IL-10R, TNF-αR and IL-12Rβ1 subunits.

2.4.2 Flow-cytometric detection of PBMC expressing IFN-γR, IL-10R, TNF-αR and IL-12R in MS patients

For flow cytometric analysis, untreated or phytohemagglutinin (PHA)-activated blood PBMC were washed with PBS, counted and resuspended at $5X10^6$/ml in PBS containing BSA (1%). Cell suspensions (100 μl per test) were used. Cells were incubated with unlabelled anti- IFN-γR, anti-IL-10R, anti-TNF-αR and anti-IL-12Rβ1 mAbs (Becton-Dickinson Mountain View, CA), or irrelevant IgG2a (final concentration 1 μg/ml for all) for 15 min at RT, washed and incubated with biotinylated mouse anti-rat $F(ab')_2$ fragments (1:500, 15 min, RT). After another wash, cells were incubated with PE–streptavidin (1:100, 15 min, RT). During the same step, cells were co-stained with PerCP-anti-CD3/FITC-anti-CD4, PerCP-anti-CD3/FITC-anti-CD8, or FITC-anti-CD56, to detect CD4 T cells, CD8 T cells or NK cells, respectively (Becton-Dickinson Mountain View, CA). After a final wash, cells were resuspended in PBS (200 μl) and analysed by FACScan flow cytometer using CellQuest software (both from Becton-Dickinson). A total of events (20.000 per test) were routinely acquired. CD4 T cells, CD8 T cells and NK cells were gated electronically, and expression of IFN-γR, IL-10R, TNF-αR and IL-12R subunits were analysed separately for these cell populations.

2.5 Study III: Estimation of the apoptotic marker CD95 (Fas) expression

2.5.1 Quantitative determination of soluble CD95 in human plasma samples

The sCD95 kit (Diaclone Research, France) is a solid phase sandwich enzyme-linked-immunosorbent assay, using a monoclonal antibody specific for sCD95 coated onto the

wells of a microtiter plate. Samples, including standards of known sCD95 concentrations and unknowns, were pipetted into these wells. During the first incubation, the sCD95 antigen and a biotinylated monoclonal antibody specific for sCD95 were simultaneously incubated. After washing, the enzyme (streptavidin-peroxydase) was added. After incubation and washing to remove the entire unbound enzyme, a substrate solution, acting on the bound enzyme, was added to induce a coloured reaction product. The intensity of this coloured product was directly proportional to the concentration of sCD95 present in the samples.

Procedure

An aliquot of sCD95 standard (100 μL) (ranging from 93.75 to 3000 pg/ml), plasma sample or control sample was added to the sCD95 microwell plate followed by diluted biotinylated anti-sCD95 antibody (50 μl). The plate was covered and incubated for 1 hour at room temperature (18 ^{0}C – 25^{0}C). At the end of the incubation time, the plate was washed three times with washing buffer and of streptavidin-HRP solution (100 μl) distributed to all wells, including blank wells. The plate was again covered and incubated for 30 min at room temperature.

After a further three washes, ready to use tetramethylbenzidine (TMB) substrate solution (100 μL) was added to all wells, including the blanks. The plate was incubated in the dark for 12-15 min at room temperature. Direct exposure to light was avoided by wrapping the plate in aluminum foil.

The enzyme-substrate reaction was stopped by quickly pipetting H_2SO_4 1M (100 μL) stop reagent into each well, including the blank wells, to completely and uniformly

inactivate the enzyme. Results were read immediately after the addition of the H_2SO_4 stop reagent.

The absorbance of each well was read on a Chemwell spectrophotometer (Awareness, USA) using 450 nm as the primary wavelength and optionally at the 620 nm as the reference wavelength.

Data analysis was performed by the generation of a linear standard curve by plotting the average absorbance on the vertical axis versus the corresponding sCD95 standard concentration on the horizontal axis. The amount of sCD95 in each sample was determined by extrapolating OD values to sCD95 concentrations using the standard curve. In terms of assay sensitivity, the minimum detectable dose of sCD95 was less than 47 pg/ml (based on manufacturer's instructions).

Concerning the precision of the assay according to manufacturer's instructions, for intra-assay the upper values were 1719 ± 106, CV 6.1% and the lower values were 703 ± 39, CV 5.5%. For inter-assay variation, the upper values were 1752 ± 145, CV 8.2% and the lower values were 856 ± 68, CV 7.9%.

2.6 Study IV: Estimation of expression of the adhesion molecules VCAM-1 and ICAM-1

2.6.1 Quantitative determination of soluble Vascular Cellular Adhesion Molecule-1 (sVCAM-1) in human plasma samples

The VCAM-1 kit (Diaclone Research, France) is a solid phase sandwich enzyme-linked-immunosorbent assay, using a monoclonal antibody specific for VCAM-1 pre-coated onto wells of microtiter strips. Samples, including standards of known sVCAM-1

concentrations, controls and unknowns were pipetted into these wells. During the first incubation, the sVCAM-1 antigen and a biotinylated monoclonal antibody specific for VCAM-1 were simultaneously incubated. After washing, the enzyme (streptavidin-peroxidase) was added. After incubation and washing to remove the unbound enzyme, a substrate solution, which acted on the bound enzyme, was added to induce a colored reaction product. The intensity of this colored product was directly proportional to the concentration of sVCAM-1 present in the samples.

Procedure

An aliquot of sVCAM-1 standard (ranging from 50 to 1.56 ng/ml), plasma sample or control sample (100 μl) was added into the VCAM-1 microwell plate followed by the diluted biotinylated anti-sVCAM-1 antibody (50μl). The plate was covered and incubated for 1 hour at room temperature (18 ^{0}C – 25^{0}C). At the end of the incubation time, the plate was washed three times with washing buffer and streptavidin-HRP solution (100μl) distributed to all wells, including blank wells. The plate was again covered and incubated for a further 30 min at room temperature.

After a further three washes, ready to use tetramethylbenzidine (TMB) substrate solution (100 μl) was added to all wells, including the blanks. The plate was incubated in the dark for 12-15 min at room temperature. Direct exposure to light was avoided by wrapping the plate in aluminum foil.

The enzyme-substrate reaction was stopped by quickly pipetting H_2SO_4 1M (100μl) stop reagent into each well, including the blank wells, to completely and uniformly inactivate the enzyme. Results were read immediately after the addition of the H_2SO_4 stop reagent.

The absorbance of each well was read on a Chemwell spectrophotometer (Awareness, USA) using 450 nm as the primary wavelength and optionally at the 620 nm as the reference wavelength.

Data analysis was performed by the generation of a linear standard curve by plotting the average absorbance on the vertical axis versus the corresponding sVCAM-1 standard concentration on the horizontal axis. The amount of sVCAM-1 in each sample was determined by extrapolating OD values to sVCAM-1 concentrations using the standard curve.

The average concentration of sVCAM-1 in normal serum samples (according to manufacturer; Diaclone Research, France) was 626 ± 340 ng/mL as detected in 80 normal human serum samples. The minimum detectable dose of sVCAM-1 was less than 0.6 ng/mL (based on manufacturer's instructions). For intra-assay variation, the upper values were 48.79 ± 0.221, CV 0.45% and the lower values were 5.81 ± 0.132, CV 2.27%.

2.6.2 Quantitative determination of soluble Inter Cellular Adhesion Molecule-1 (sICAM-1) in human plasma samples

The ICAM-1 kit was a solid phase sandwich enzyme-linked-immunosorbent assay, using a monoclonal antibody specific for ICAM-1 pre-coated onto wells of microtiter strips. Samples, including standards of known sICAM-1 concentrations, controls and unknowns were pipetted into these wells. During the first incubation, the sICAM-1 antigen and a biotinylated monoclonal antibody specific for ICAM-1 were simultaneously incubated. After washing, the enzyme (streptavidin-peroxidase) was added. After incubation and washing to remove the unbound enzyme, a substrate solution, which acted on the bound

enzyme, was added to induce a colored reaction product. The intensity of this colored product was directly proportional to the concentration of sICAM-1 present in samples.

Procedure

A volume of sICAM-1 standard (ranging from 8 to 0.25 ng/ml), plasma sample or control sample (100μl) was added to the ICAM-1 microwell plate followed by the diluted biotinylated anti-sICAM-1 antibody (50 μL). The plate was covered and incubated for 1 hour at room temperature (18 ^{0}C – 25^{0}C). At the end of the incubation time, the plate was washed three times with washing buffer and streptavidin-HRP solution (100 μl) distributed to all wells, including the blanks. The plate was again covered and incubated for 30 min at room temperature.

After a further three washes, an aliquot of ready to use TMB substrate solution (100 μl) was added to all wells, including the blanks. The plate was incubated in the dark for 12-15 min at room temperature and direct exposure to light avoided by wrapping the plate in aluminum foil.

The enzyme-substrate reaction was stopped by quickly pipetting H_2SO_4 stop reagent (100 μl) into each well, including the blank wells, to completely and uniformly inactivate the enzyme. The results were read immediately after the addition of the H_2SO_4 stop reagent.

The absorbance of each well was read on a Chemwell spectrophotometer (Awareness, USA) using 450 nm as the primary wavelength and optionally at the 620 nm as the reference wavelength.

Data analysis was performed by the generation of a linear standard curve by plotting the average absorbance on the vertical axis versus the corresponding sICAM-1 standard concentration on the horizontal axis. The amount of sICAM-1 in each sample was

determined by extrapolating OD values to sICAM-1 concentrations using the standard curve.

The average concentration of sICAM-1 in normal serum samples (according to manufacturer, Diaclone Research, France) was 571 ± 168 ng/mL as detected in 77 normal human serum samples. The minimum detectable dose of sICAM-1 was less than 0.1 ng/mL. For intra-assay variation, the upper values were 7.82 ± 0.081, CV 1.03% and the lower values were 1.17 ± 0.033, CV 2.82%. For inter-assay variation, the upper values were 8.05 ± 0.317, CV 3.93% and the lower values were 1.03 ± 0.084, CV 8.15%.

2.7 Study V: Estimation of metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) levels in serum samples

2.7.1 Quantitative determination of metalloproteinase-9 (MMP-9) in human plasma samples

The metalloproteinase assay employed the quantitative sandwich enzyme immunoassay technique where a monoclonal antibody specific for MMP-9 has been pre-coated onto a microplate. Standards and samples were pipetted into the wells, and MMP-9 was bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for MMP-9 was added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of total MMP-9 (pro and/or active) bound in the initial step. Color development was stopped and the intensity of the color measured.

Procedure

Assay diluent (100 µl) (R&D systems, Minneapolis, MA) was added to each well, followed by Standard, Control, or sample (100 µl) per well. The plate was covered with adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit).

Wells were aspirated and washed, repeating the process three times for a total of four washes. Washing was performed by filling each well with Wash Buffer (400 µl) using a squirt bottle. Complete removal of liquid at each step was essential to good performance. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

MMP-9 conjugate (200 µl) was added of to each well, the plate was once again covered and incubated for 1 hour at room temperature on the shaker.

Wells were aspirated and washed, repeating the process three times for a total of four washes. Substrate Solution (200 µl) were added to each well and the plate was incubated for 30 minutes at room temperature on the benchtop, protected from light.

Stop Solution (50 µl) was further added to each well and the color in the wells changed from blue to yellow.

The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm with reference wavelength at 540 nm.

According to the manufacturer's instructions (R&D systems, Minneapolis, MA), the precision of the method for three samples of known concentration tested twenty times on one plate to assess the intra-assay precision or in forty separate assays to assess the inter-assay precision are shown in the table below:

Table 2-5. Precision of the MMP-9 assay

	Intra-assay			Inter-assay		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/ml)	0.833	2.04	11.0	0.972	2.95	122
SD	0.017	0.099	0.316	0.077	0.184	0.845
CV%	2	1.9	2.9	7.9	7.8	6.9

Sensitivity: The minimum detectable dose of MMP-9 was typically less than 0.156 ng/mL.

2.7.2 Quantitative determination of Tissue Inhibitor of Metalloproteinase 1 (TIMP-1) concentrations in human plasma samples

The TIMP-1 assay employed the quantitative sandwich enzyme immunoassay technique where a monoclonal antibody specific for TIMP-1 has been pre-coated onto a microplate. Standards and samples were pipetted into the wells, and TIMP-1 was bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked

polyclonal antibody specific for TIMP-1 was added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of total TIMP-1 bound in the initial step. The color development was stopped and the intensity of the color was measured.

Procedure

Assay diluent (100 μl) (R&D systems, Minneapolis, MA) was added to each well, followed by of Standard, Control, or sample (100 μl) per well. The plate was covered with adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit).

Wells were aspirated and washed, repeating the process three times for a total of four washes. Washing was performed by filling each well with Wash Buffer (400 μl) using a squirt bottle. Complete removal of liquid at each step was essential to good performance. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

TIMP-1 conjugate (200 μl) was added to each well, the plate once again covered and incubated for 1 hour at room temperature on the shaker.

Wells were aspirated and washed, repeating the process three times for a total of four washes. Substrate Solution (200 μl) was added to each well and the plate was incubated for 30 minutes at room temperature on the benchtop, protected from light.

Stop Solution (50 μl) was further added to each well and the color in the wells changed from blue to yellow.

The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm with reference wavelength at 540 nm.

According to the manufacturer's instructions (R&D systems, Minneapolis, MA), the precision of the method for three samples of known concentration tested twenty times on one plate to assess the intra-assay precision or in forty separate assays to assess the inter-assay precision are shown in the table below:

Table 2-6. Precision of the TIMP-1 assay

	Intra-assay			Inter-assay		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/ml)	1.23	3.45	6.09	1.26	3.45	6.38
SD	0.054	0.116	0.181	0.086	0.197	0.467
CV%	4.4	3.4	3.0	6.8	5.7	7.3

Sensitivity: Sixty-eight assays were evaluated and the minimum detectable dose (MDD) of TIMP-1 ranged from 0.004 - 0.064 ng/mL. The mean MDD was 0.011 ng/mL.

2.8 Statistical analysis

The results were statistically processed with the use of a SPSS programme for Windows and the Pearson chi-square test used to compare categorical variables. The values of the laboratory findings were initially checked for possible outliers with the use of boxplots. Since many statistical tests assume the data were normally distributed, individual variables were tested for normality totally and within groups. In order to determine

whether the distributions of the quantitative variables matched, with the normal distribution, histograms were used to display the distribution of values for the variables and probability plots (Normal Q-Q plots). Additionally the Kolmogorov-Smirnov test (p<0.05) confirmed that the individual variables differed significantly from a normal distribution. Due to the lack of normality in the distribution of values of most of the studied parameters, the selected statistical methods for the process were non-parametrical. A Kruskal-Wallis test was used for the comparison of values between the patient groups. Data are presented as mean ± standard deviation (SD) and all results were tested at a statistical level of significance of $p < 0.05$.

CHAPTER 3. RESULTS

3.1 Demographic data

Table 3-1. Principal socio-demographic characteristics of subjects (n=85)

	MS w/o treatment	MS with IFNβ-1α	MS with IFNβ-1β	Controls
Number	20	20	20	25
Age (years) (mean±SD)	32±1.3	36±3.1	34±3.2	38.8±6.9
BMI (Kgr/m^2)	24.2±2.1	22±3.4	23.5±3.6	25.3±3.8
Gender				
Male	5	10	7	10 (40%)
Female	15	10	13	15 (60%)
Marital status				
Married	42	38	40	13 (52%)
Single	18	22	20	12 (48%)
Education				
0-6 years	6	2	4	1 (4%)
6-12 years	25	29	27	6 (24%)
> 12 years	27	31	29	18(72%)
Employment				
Currently employed	52	48	50	18 (72%)
Unemployed	8	12	10	7 (28%)

The principal demographic characteristics of the two groups are shown in Table 3-1. Of the sixty MS patients, seventy one comma six per cent (forty three) were women and twenty eight comma three per cent (seventeen) were men. During statistical analysis, the MS group was assessed as a whole without taking into consideration the different types of IFNβ treatment or not. The mean age (±SD) of the MS and control group was 34 ± 3.2 and 38.8±6.9 years respectively. There was no statistically significant difference for the age of individuals in the two groups (p>0.05). The mean BMI varied significantly

(P<0.01) between the two groups; the MS patients' mean score was lower than that of the controls.

Female gender was dominant in all groups with a percentage of 71.6% for MS patients and 60% for the controls. The majority of the individuals were married. Forty five per cent and twenty four per cent of the MS and control groups respectively had basic education (up to high school). However, the majority of the subjects in both groups (48.3% for MS and 72% for controls) were well-educated (beyond high school).

A total of 83.3% of the MS population had paid employment at the time of study as well as 72% of the controls.

Table 3-2. Main lifestyle features of the subjects (n=85)

	MS patients	Controls
Cigarette smoking		
Non-smokers	23 (38.3%)	19 (76%)
Smokers	37 (61.6%)	6 (24%)
Alcohol		
> 1 drink daily	8 (13.3%)	5 (20%)
2-3 drinks weekly	20 (33.3%)	5 (20%)
< 2 drinks weekly	32 (58.3%)	15 (60%)
Leisure physical activity		
No activity	45 (75%)	3 (12%)
Less than 1 hour per week	10 (16.6%)	2 (8%)
Between 2-3 hours per week	5 (8.3%)	8 (32%)
3 hours or more per week	0 (0%)	12 (48%)

Table 10 shows some characteristics of the life-style of the subjects. The study focused on alcohol consumption, cigarette smoking and leisure physical activity of the subjects, because the participants shared a large number of every-day habits. They followed a similar diet (Mediterranean diet, with the use of olive oil and fresh vegetables); lived in

the same geographical region and their occupations were mostly in the private sectors (e.g. bankers, accountants). However, smokers were outnumbered by non-smokers in the MS population (61.6%) but not in controls (24% were smokers). The majority of MS (58.3%) and control subjects (60%) consumed low quantities of alcohol (<2 drinks weekly). Regarding physical activity in their leisure, an increased percentage of MS patients (75%) were inactive at all whereas only 8.3% exercised between 2-3 hours per week. Among the control subjects, 32% exercised for between 2-3 hours per week and 48% more than 3 hours per week.

3.2 Clinical data

The clinical characteristics of MS patients are summarized in Table 3-3.

Table 3-3. Clinical characteristics of MS patients

	MS patients
No of subjects	60
Sex (F/M)	43/17
Mean age (years)	34±3.2
Age of onset (year)	27.3±8.8
Age at inclusion (year)	32.2±9.8
Mean disease duration (years)	3±1.5
No. of relapses in the last 2 years	2±1.3
Mean treatment duration (month)	6
Mean baseline EDSS score	2.5±1.2

Degree of disability	
Low	10 (16.6%)
Moderate	43 (71.6%)
High	7 (11.6%)

(EDSS=expanded disability status scale)

It is important to note the predominance of female gender among the patients indicating a possible predictive factor of the disease. Furthermore, the patients were relatively young with a mean age of 34±3.2 years. Mean disease duration was 4±1.5 years for MS patients, with 2±1.3 relapses in the last two years. Mean EDSS score of the patients was 2.5±1.2 and the degree of disability was moderate in most cases (seventy one comma six per cent).

Upon admittance to the Neurologic Clinic and following diagnosis of relapsing-remitting form of MS, all patients had to undergo a routine analysis of biochemical parameters as summarized in Table 3-4.

Table 3-4. Baseline laboratory data analysis of MS patients

	Mean ±SD	*Normal range*
WBC ($x10^3/\mu l$)	7.06±2.6	4.8-10.8
AST (IU/l)	19.8±10.8	<40
ALT (IU/l)	23.9±18.3	<35
fT3 (pg/ml)	2.8±0.5	1.7-4.1
fT4 (ng/dl)	1.1±0.2	0.7-1.6
TSH (μIU/ml)	1.5±1.3	0.3-4.7
TgAb (IU/ml)	47.4±100.8	<40
TPOAb (IU/ml)	109±344.5	<40

WBC, white blood cells; AST, aspartate aminotransferase; ALT, alanine aminotransferase; fT3, free tri-iodothyronine; fT4, free-thyroxine; TSH, thyrotropine; TgAb, anti-thryroglobulin antibody; TPOAb, anti-thyroid peroxidase antibody.

Although their white cell count, hepatic enzyme levels and thyroid hormones were within normal ranges, increased amount of anti-thyroglobulin (47.4±100.8IU/ml) and anti-thyroid peroxidase antibodies (109±344.5IU/ml) were observed.

Table 3-5 shows some of the most frequent medical conditions usually associated with MS.

Table 3-5. MS patients' personal history of other disorders

None	13 (21.6%0
Optic neuritis	50 (83.3%)
Autoimmune disease	20 (33.3%)
Inflammatory disease	18 (30%)
Viral infections (HIV, HTLV, Hepatitis B)	19 (31.6%)
Allergies	15 (25%)
Vaccinations (Influenza, Hepatitis B)	29 (48.3%)
Mental disorders, depression	12 (20%)

In our study population a high percentage of patients (eighty three comma three per cent) had symptoms of optic neuritis or other sensory symptoms specifically associated with the onset of disease. Confounding autoimmune or inflammatory diseases were also present in their personal clinical file (33.3% and 30% respectively). A significant

percentage of viral infections (31%), allergies (25%) and vaccinations (48.3%) were also reported.

Finally, mental disorders were also present, with a particular tendency for depressive symptomatology which was observed in twenty per cent of patients.

In order to obtain more information of the biochemical status of our study population, an evaluation of their lipid profile as well serum glucose levels was performed. Table 14 shows the baseline values of main lipids and glucose of MS patients upon their admittance to the study.

Table 3-6. Lipid profile and glucose levels of MS patients

	Mean ±SD	*Normal range*
Cholesterol (mg/dl)	199.6±40.9	<190
HDL Chol (mg/dl)	54.8±13.1	>35
Triglycerides (mg/dl)	185.2±57.1	<180
Glucose (mg/dl)	102±21.1	<120

Specifically, MS patients presented a high total cholesterol concentration in their serum (199.6±40.9mg/dl) as well as increased triglycerides (185.2±57 mg/dl). HDL-Cholesterol and glucose levels were found to be within the normal range.

3.3 Study I: Evaluation of cytokine secretion in MS patients and controls

Secretion of Th1 (IFN-γ, TNF-α, IL-12) and Th2 (IL-10, IL-4) type cytokines was evaluated in peripheral blood mononuclear cells of MS patients with or without treatment with IFNβ, using the ELISPOT methodology described in Chapter 2.

Table 15 summarizes the ELISPOT data on cytokine secretion levels from MS patients with or without IFNβ treatment as well from control subjects. Significant changes were observed for most cytokines between MS patients and controls as well as following treatment with the two types of IFNβ as described below.

Table 3-7. Number of cytokine-secreting cells per 10^5 blood MNC from patients with MS and healthy controls (Data are mean ±SD; n values are shown)

Cytokine secretion (no of spots/10^5 cells)	*Control (N=25)*	*MS w/o treatment (N=20)*	*MS with IFNβ-1a treatment (N=20)*	*MS with IFNβ-1β treatment (N=20)*	*P (contr vs MS w/o treat.)*	*P (contr vs MS IFNβ-1a)*	*P (contr vs MS IFNβ-1β)*	*P (MS w/o vs IFNβ-1a)*	*P (MS w/o vs MS IFNβ-1β*	*P (MS IFNβ-1a vs MS IFNβ-1β)*
IFN-γ	24±2.64	154.15±2.6	52.1±1.62	23.2±2.62	<0.01**	<0.01**	0.098	<0.01**	<0.01**	<0.01**
TNF-α	21.9±3.21	121.9±4.39	56.4±2.36	45.4±3.12	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**
IL-10	64.5±4.78	36.4±3.65	54±2.10	55.1±2.1	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	0.330
IL-12	106±8.9	159.6±6.1	109±5.7	122.5±1.3	0.05	0.485	0.096	0.08	0.548	0.785
IL-4	94.86±2.7	62.63±4.7	108±3.6	179.5±2.0	0.08	0.135	<0.01**	0.05	<0.01**	0.64

** Significant differences <0.01 level

3.3.1 Evaluation of IFN-γ secretion

Patients with MS receiving no treatment demonstrated significantly increased levels of IFN-γ -secreting PBMCs (154.15±2.6) compared to healthy controls (24±2.64, $P<0.01$). Treatment of MS patients with IFNβ-1α significantly reduced IFN-γ levels (52.1±1.62) but without reaching normal levels. However, treatment with IFNβ-1β decreased the number of IFN-γ cells even further, below normal levels (23.2±2.62, $P=0.098$), proving more efficient in reducing IFN-γ secretion levels than IFNβ-1α (Figure 3-1).

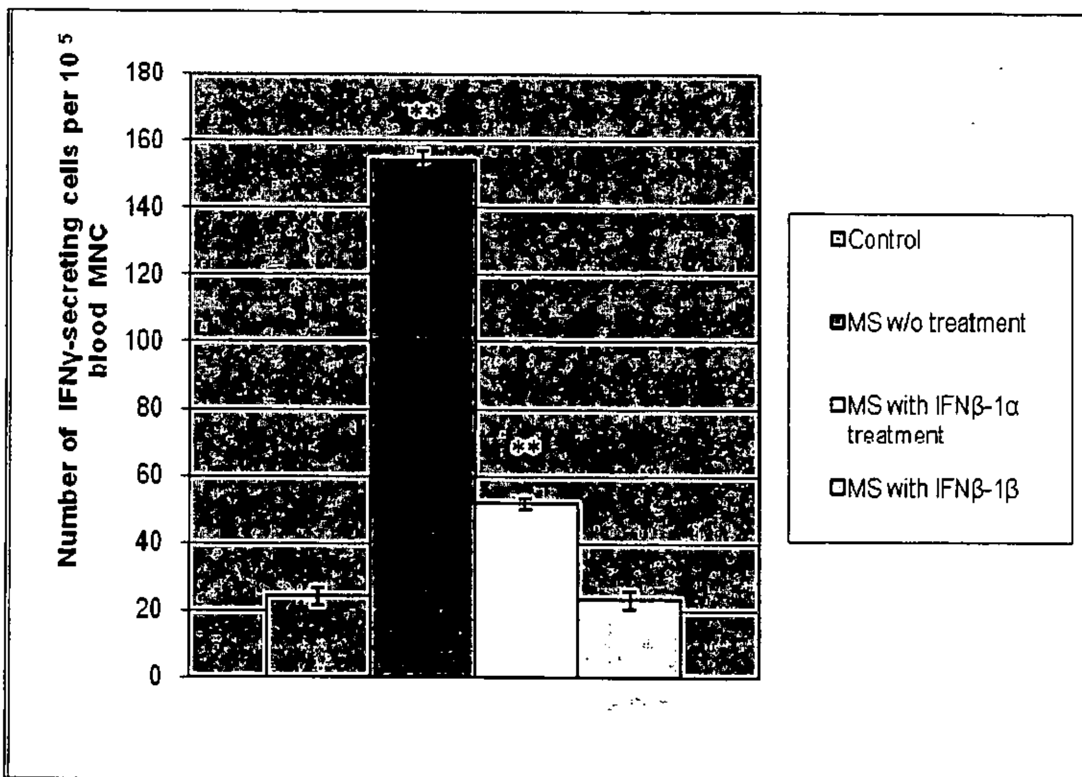

Figure 3-1. ELISPOT analysis of IFN-γ -secreting PBMCs of MS patients (n=60) with and without IFNβ treatment compared to controls (n=25, **$P<0.01$).

3.3.2 Evaluation of TNF-α secretion

The number of TNF-α–secreting lymphocytes was markedly increased (121.9±4.39) in MS patients compared to healthy subjects (21.9±3.21, $P<0.01$). Treatment with IFNβ-1α significantly reduced TNF-α secretion levels in MS patients (56.4±2.36, $P<0.01$). Additionally, IFNβ-1β administration was more effective than IFNβ-1α in decreasing TNF-α levels (45.4±3.12, $P<0.01$) but failed to reach normal levels (Figure 3-2).

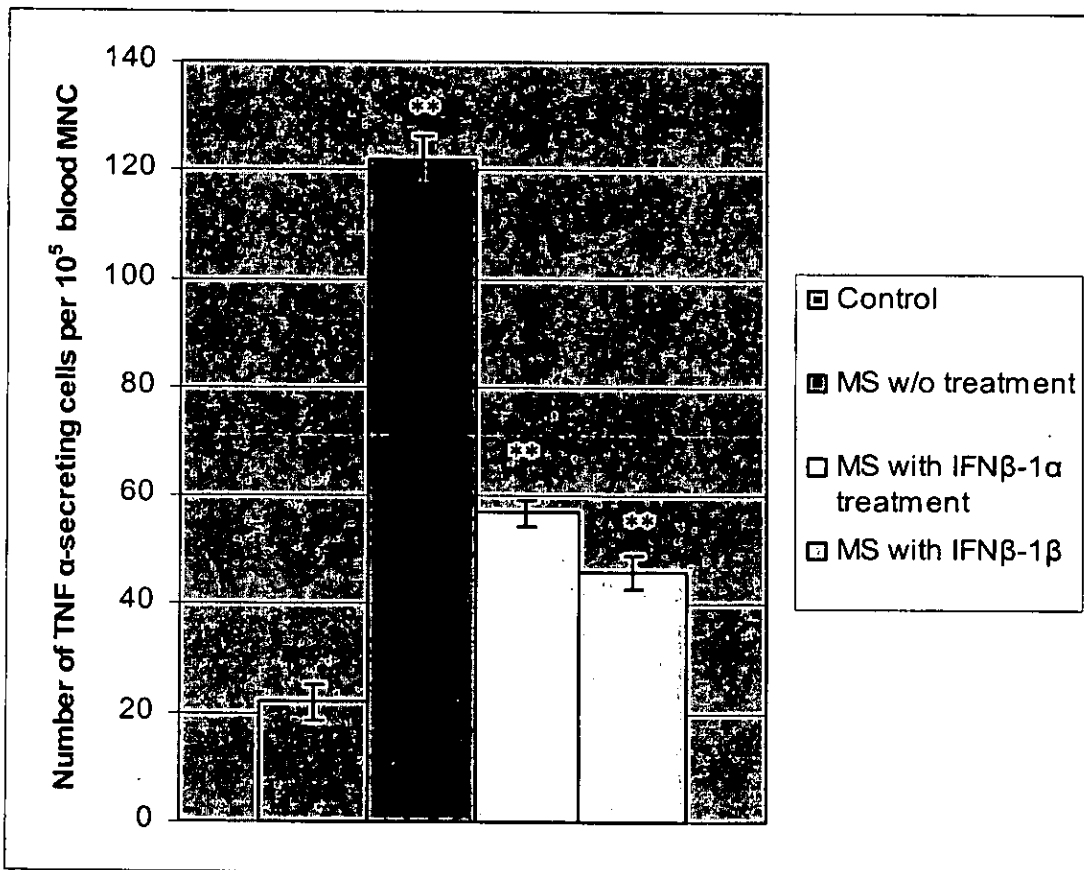

Figure 3-2. ELISPOT analysis of TNF-α-secreting PBMC of MS patients (n=60) with and without IFNβ treatment compared to controls (n=25, **$P<0.01$).

3.3.3 Evaluation of IL-10 secretion

Patients with MS presented decreased levels of IL-10-secreting PBMCs (36.4±3.65) compared to healthy controls (64.5±4.78, P<0.01). Treatment with IFNβ-1α significantly elevated IL-10 levels to 54±2.1 in MS patients. IFNβ-1β treatment was also effective in increasing the number of IL-10-secreting cells (55.1±2.1, P<0.01) (Figure 3-3).

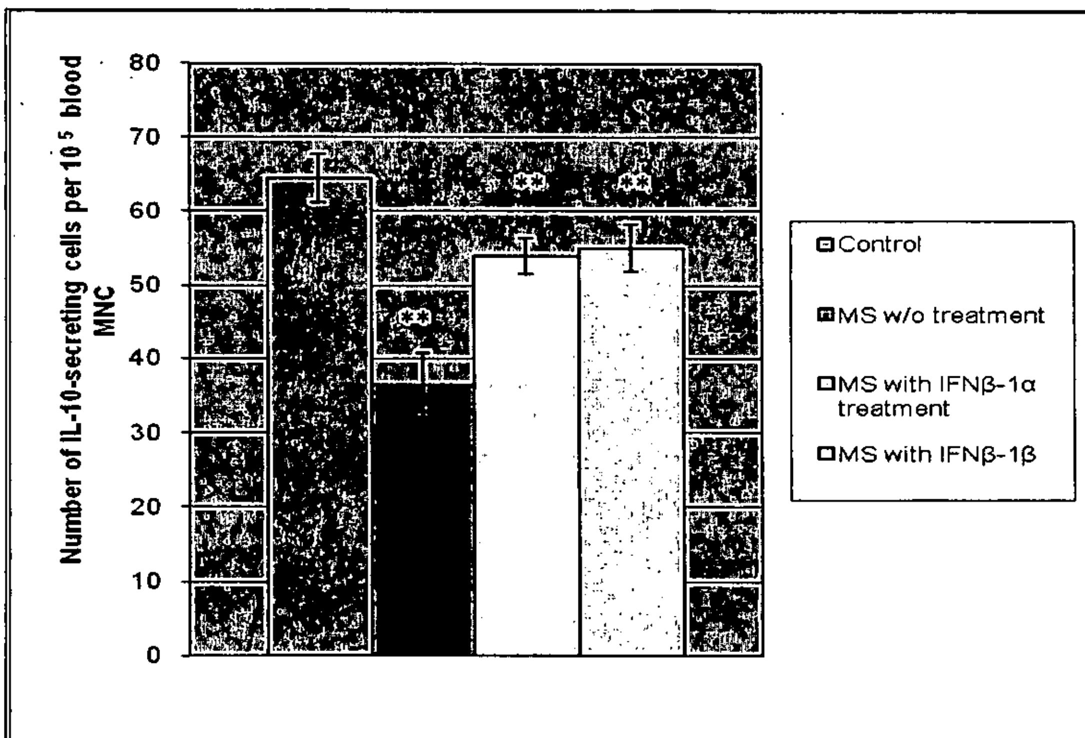

Figure 3-3. ELISPOT analysis of IL-10 -secreting PBMC of MS patients (n=60) with and without IFNβ treatment compared to controls (n=25, **P<0.01).

3.3.4 Evaluation of IL-12 secretion

IL-12 secreting PBMCs were detected in all patients with MS; either with or without treatment and healthy subjects. Increased levels of IL-12 were observed in patients with MS without receiving any treatment (159.6±6.1) compared to healthy subjects (106±8.9, P=0.05). These levels decreased after treatment with IFNβ-1α (109±5.7) as well as with IFNβ-1β (122.5±1.3) but did not attain statistical significance (Figure 3-4).

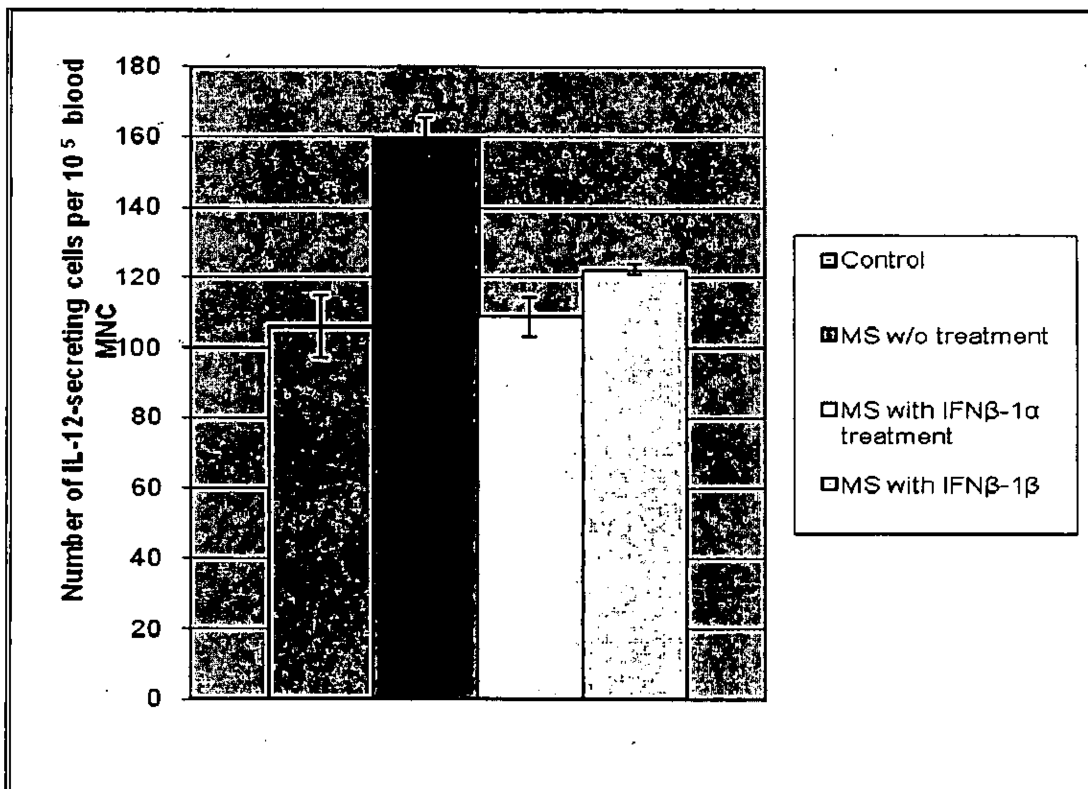

Figure 3-4. ELISPOT analysis of IL-12 -secreting PBMC of MS patients (n=60) with and without IFNβ treatment compared to controls (n=25).

3.3.5 Evaluation of IL-4 secretion

Lower levels of IL-4 were observed in MS patients (62.63 ±4.7) compared to controls (94.86±2.7, P=0.08), but increased after treatment with IFNβ. Specifically, IFNβ-1α treatment increased the number of IL-4-secreting cells to 108±3.6, whereas IFNβ-1β proved even more effective in significantly elevating the number of IL-4 cells to 179.5±2.0 (P<0.01, Figure 3-5)

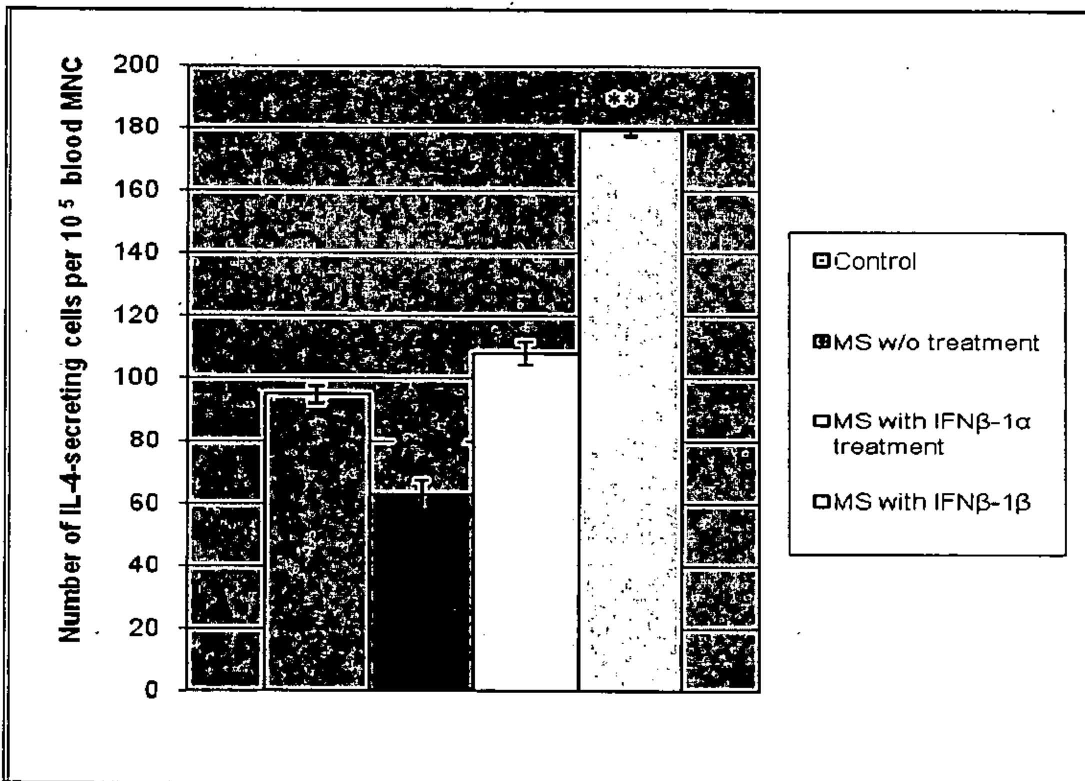

Figure 3-5. ELISPOT analysis of IL-4 -secreting PBMC of MS patients (n=60) with and without IFNβ treatment compared to controls (n=25, **P<0.01).

3.4 Study II: Percentages of peripheral lymphocytes of MS patients and controls expressing cytokine receptors

3.4.1 Flow cytometric analysis of percentages of IL-12 receptor-b1 (IL-12Rβ1) expressing T and NK cells from patients with MS and healthy subjects before and after PHA activation

Blood T cells and NK cells constitutively expressed IL-12Rβ1 *ex vivo* as shown in Table 16. Percentages of CD4 and CD8 T cells expressing IL-12Rβ1 did not differ between MS patients and healthy controls (Table 3-8). Percentages of IL-12Rβ1 expressing NK cells were higher in patients with MS, without or with treatment with IFNβ, compared to healthy individuals ($P<0.01$, $P<0.05$, respectively).

Three-day culture with PHA (2μg/ml) strongly augmented expression of IL-12Rβ1 on T cells (Table 3-8, Figure 3-6). The percentage of PHA-activated CD4 and CD8T cells expressing IL-12Rβ1 were higher in patients with MS ($P<0.05$) than in healthy subjects. NK cells from MS patients and controls expressed IL-12Rβ1 to a similar extent after PHA stimulation, without any difference between the groups (Table 3-9). IFNβ treatment further reduced IL-12Rβ1 levels of T and NK cells to that similar to normal levels, with IFNβ-1α being more effective in reducing IL-12Rβ1 levels than IFNβ-1β (Figure 3-7).

Table 3-8. Percentage of *in vitro* and PHA-activated T and NK cells from patients with MS and healthy subjects expressing IL-12Rβ1 as determined by flow cytometry

Percentage of positive cells (%)	*Control (N=25)*	*Control + PHA stimulation (N=25)*	*MS w/o treatment (N=20)*	*MS w/o treatment + PHA stimulation (N=20)*	*MS with IFNβ-1a treatment (N=20)*	*MS with IFNβ-1a treatment + PHA stimulation (N=20)*	*MS with IFNβ-1β treatment (N=20)*	*MS with IFNβ-1β treatment + PHA stimulation (N=20)*
CD4	*8*	*65*	*15*	*80*	*12*	*78*	*14*	*76*
CD8	*19*	*62*	*22*	*75*	*20*	*76*	*19*	*78*
NK	*25*	*44*	*58*	*56*	*38*	*41*	*43*	*45*

Table 3-9. Statistical analysis of percentages of T and NK cells expressing IL-12Rβ1

	P (contr vs MS w/o treat.)	*P (contr vs MS IFNβ-1a)*	*P (contr vs MS IFNβ-1β)*	*P (contr+PHA vs MS w/o treat. +PHA)*	*P (contr+PHA vs MS IFNβ-1a+PHA)*	*P (contr +PHA vs MS IFNβ-1β+PHA)*
CD4	NS	NS	NS	NS	NS	NS
CD8	NS	NS	NS	NS	NS	NS
NK	0.01**	0.05	0.05	NS	NS	NS

** Significant differences <0.01 level

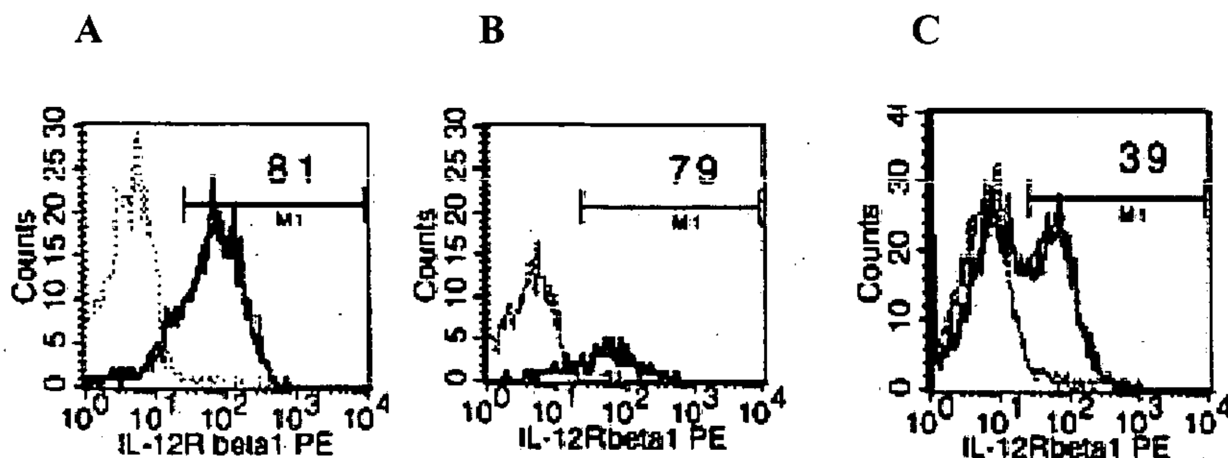

Figure 3-6. Percentage of PHA-activated CD4 (A), CD8 (B) T cells and NK (C) cells from a representative MS patient (w/o treatment) expressing IL-12Rβ1. Solid line, staining with anti-IL-12R mAb. Dashed line, control staining with an isotype-matched mAb. Numbers in each plot indicate percentages of positive cells after subtraction of control signal.

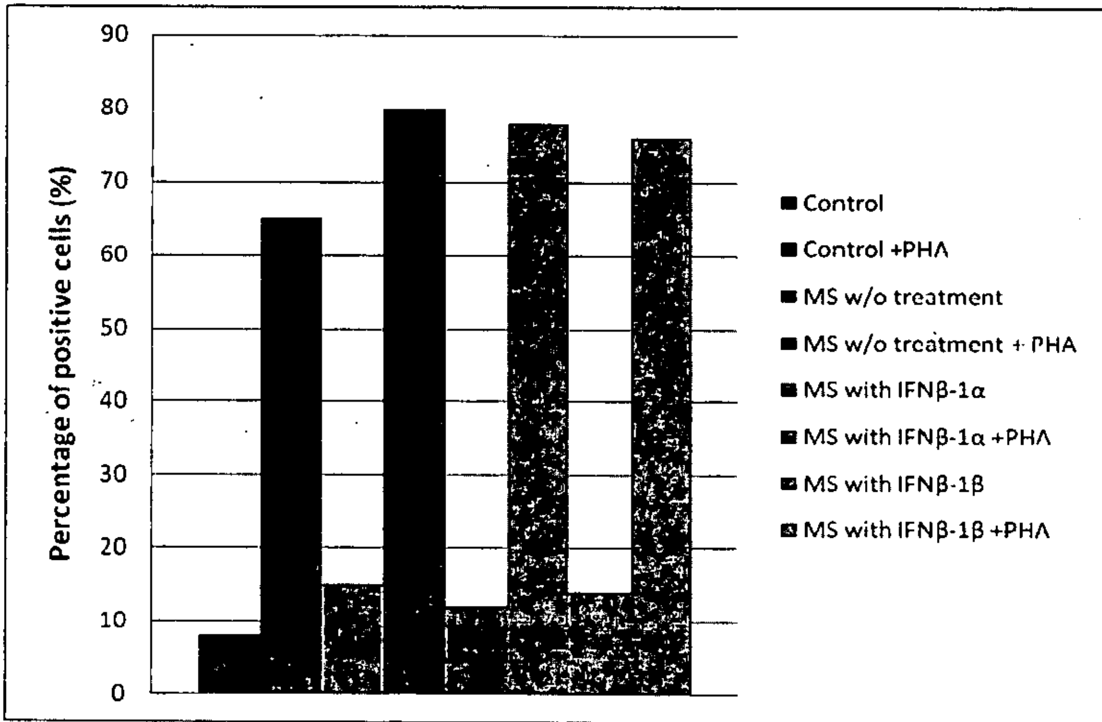

Figure 3-7A. Percentage of in *vitro* and PHA-activated CD4 cells from patients with MS (n=60) and healthy subjects (n=25) expressing IL-12Rβ1.

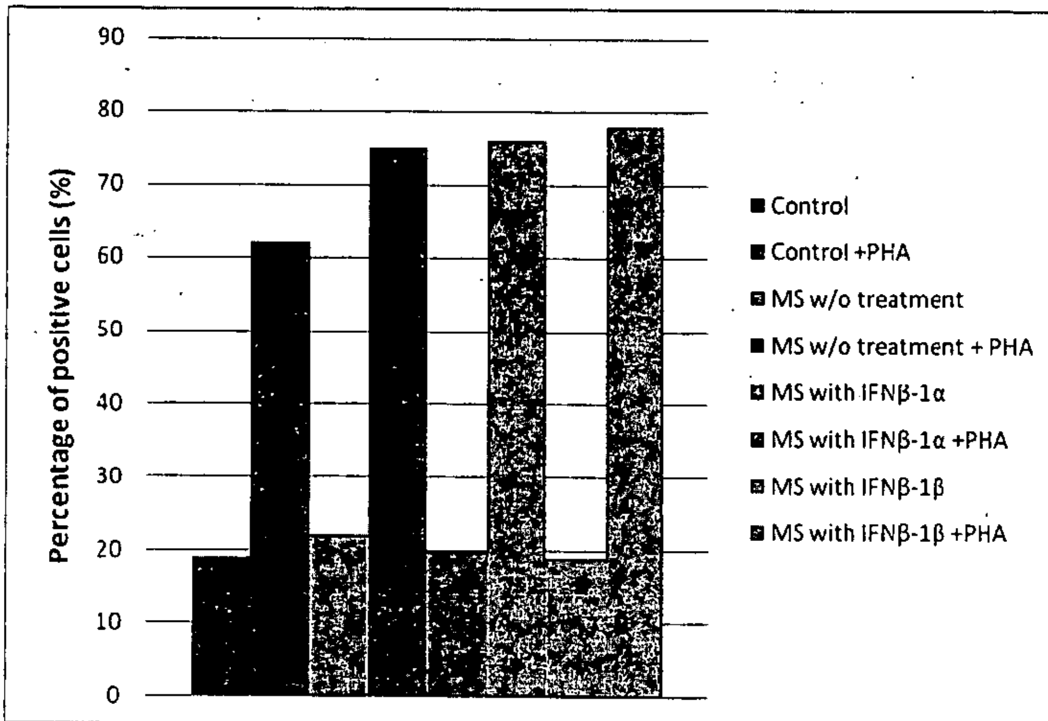

Figure 3-7B. Percentage of *in vitro* and PHA-activated CD8 cells from patients with MS (n=60) and healthy subjects (n=25) expressing IL-12Rβ1.

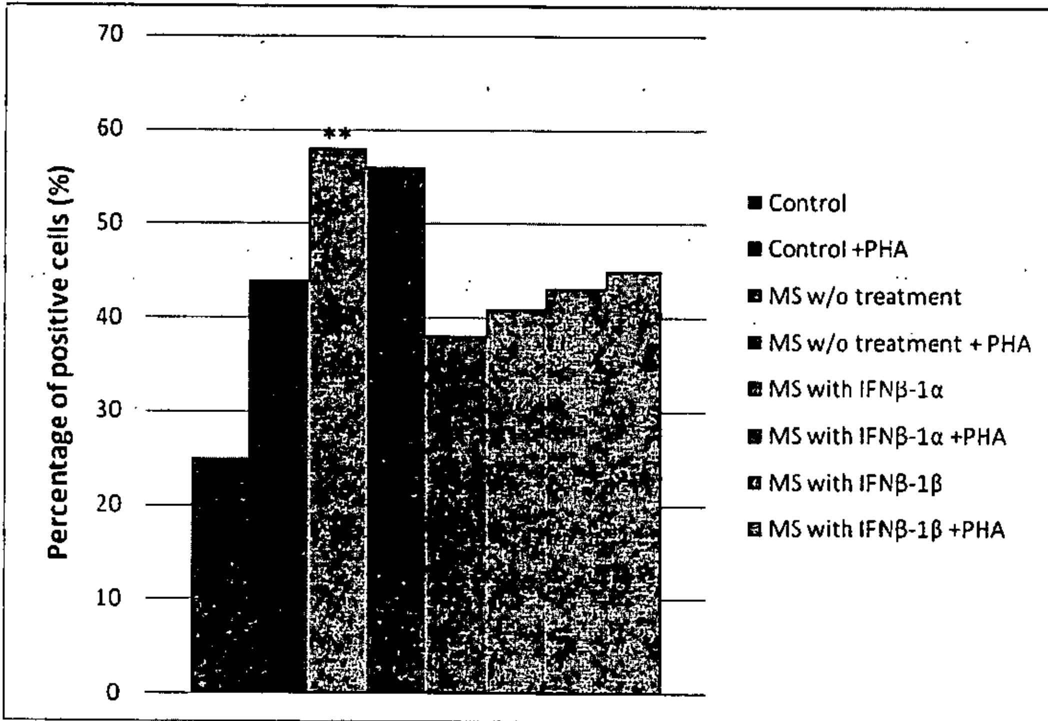

Figure 3-7C. Percentage of in *vitro* and PHA-activated NK cells from patients with MS (n=60) and healthy subjects (n=25) expressing IL-12Rβ1 (**P<0.01).

3.4.2 Flow cytometric analysis of percentages of T and NK cells from patients with MS and healthy subjects expressing IFN-γR , before and after PHA activation

Peripheral blood cells from untreated patients with MS had significantly smaller amounts of IFNγR than those from control subjects. Percentages of IFN-γR-expressing CD4 and CD8 cells differed significantly between MS patients and controls ($P<0.05$, $P<0.01$, respectively). The percentage of IFN-γR-expressing NK cells did not differ between MS and control subjects (Table 3-10). Percentages of PHA-activated CD4 and CD8T cells expressing IFNγR were higher in patients with MS ($P<0.05$) than in healthy subjects. NK cells from MS patients and controls expressed IFN-γR to a similar extent after PHA stimulation, without any significant difference between the groups (Table 3-11). PHA stimulation significantly increased expression of IFN-γR on T cells ($P=0.05$, Table 3-11). After 6 months of IFNβ treatment, T-cell IFN-γR expression was increased, being only slightly lower than that of the control subjects (Figure 3-8).

Table 3-10. Percentage of *in vitro* and PHA-activated T and NK cells from patients with MS and healthy subjects expressing IFN-γR , as determined by flow cytometry

Percentage of positive cells (%)	*Control (N=25)*	*Control + PHA stimulation (N=25)*	*MS w/o treatment (N=20)*	*MS w/o treatment + PHA stimulation (N=20)*	*MS with IFNβ-1a treatment (N=20)*	*MS with IFNβ-1a treatment + PHA stimulation (N=20)*	*MS with IFNβ-1β treatment (N=20)*	*MS with IFNβ-1β treatment + PHA stimulation (N=20)*
CD4	*27*	*45*	*10*	*50*	*26*	*48*	*15*	*42*
CD8	*23*	*52*	*9*	*57*	*20*	*56*	*21*	*49*
NK	*13*	*34*	*7*	*42*	*15*	*42*	*12*	*47*

Table 3-11. Statistical analysis of percentage of T and NK cells expressing IFNγR

	P (contr vs MS w/o treat.)	*P (contr vs MS IFNβ-1a)*	*P (contr vs MS IFNβ-1β)*	*P (contr+PHA vs MS w/o treat. +PHA)*	*P (contr+PHA vs MS IFNβ-1a+PHA)*	*P (contr +PHA vs MS IFNβ-1β+PHA)*
CD4	0.05	NS	NS	NS	NS	NS
CD8	0.01**	NS	NS	NS	NS	NS
NK	NS	NS	NS	NS	NS	0.05

** Significant differences < 0.01 level

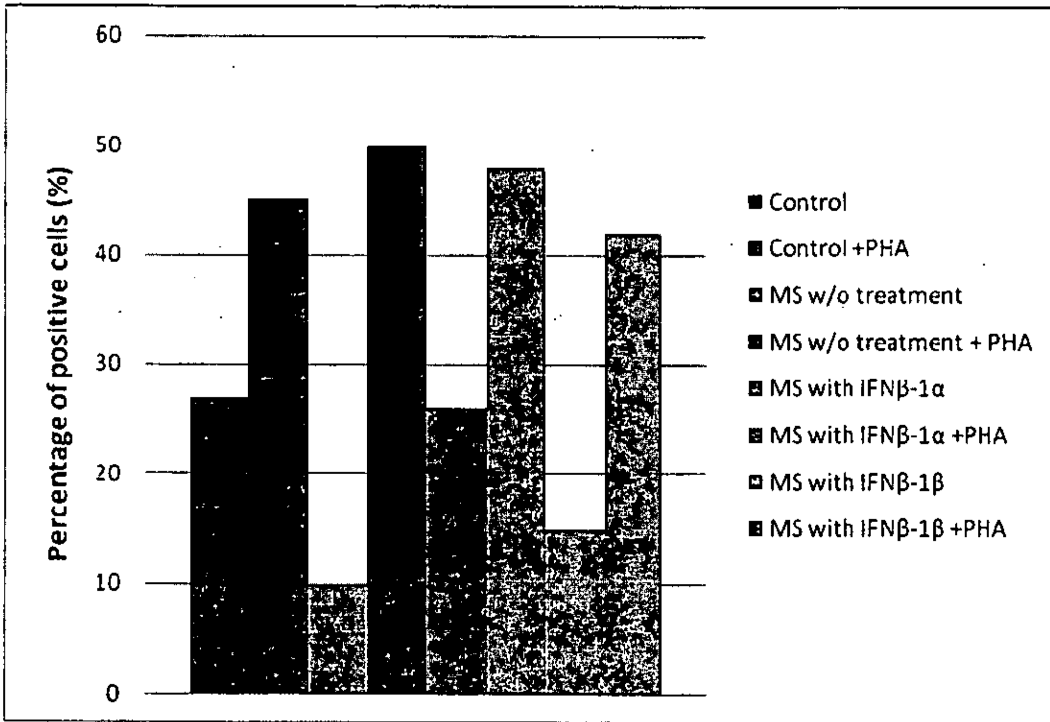

Figure 3-8A. Percentage of *in vitro* and PHA-activated CD4 cells from patients with MS (n=60) and healthy subjects (n=25) expressing IFN-γR.

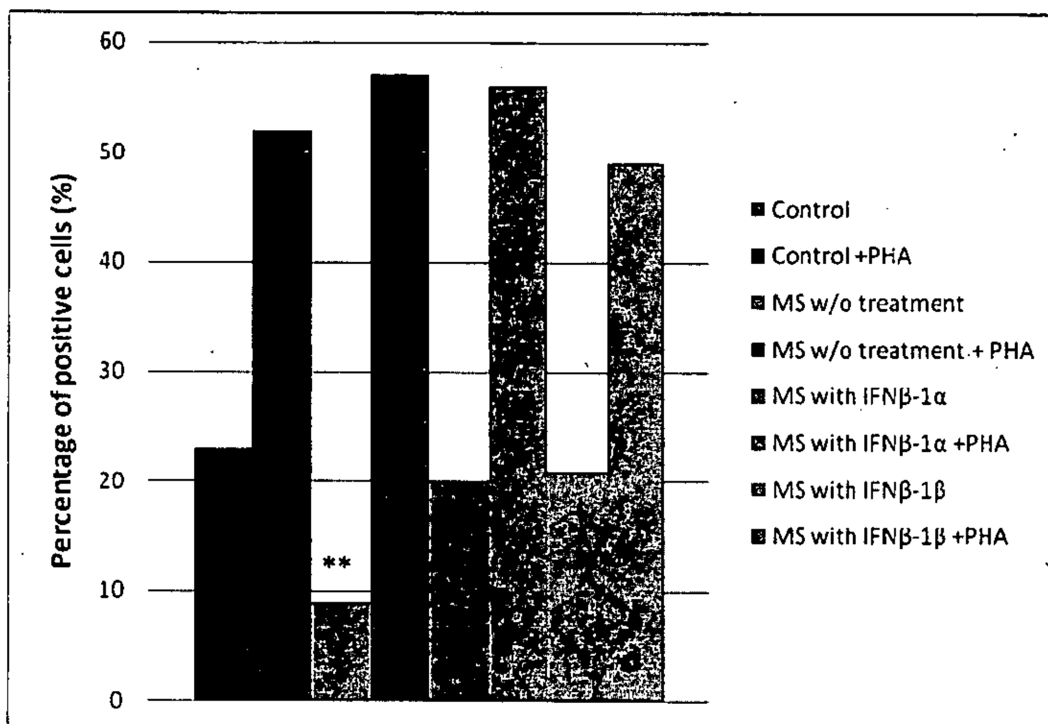

Figure 3-8B. Percentage of *in vitro* and PHA-activated CD8 cells from patients with MS (n=60) and healthy subjects (n=25) expressing IFN-γR (**P<0.01).

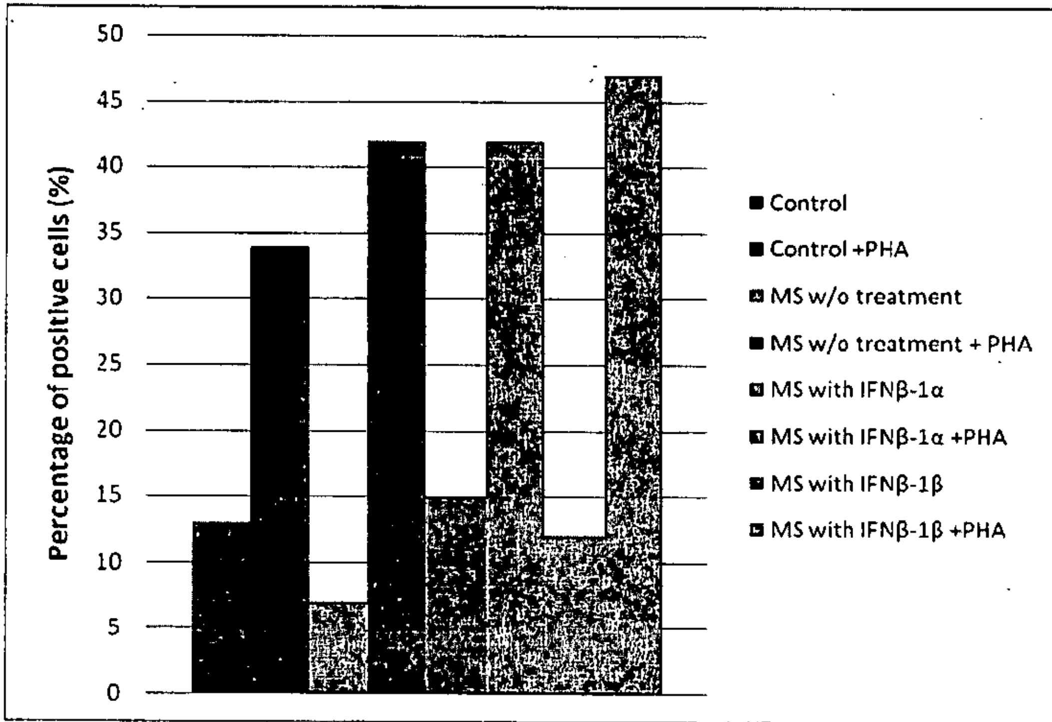

Figure 3-8C. Percentage of *in vitro* and PHA-activated NK cells from patients with MS (n=60) and healthy subjects (n=25) expressing IFN-γR.

3.4.3 Flow cytometric analysis of percentages of T and NK cells from patients with MS and healthy subjects expressing TNF-α receptor (TNF-αR), before and after PHA activation

Blood T cells and NK cells constitutively expressed TNF-αR *in vitro* as shown in Table 3-12. Percentages of CD4 and CD8 T cells expressing TNF-αR did not differ between MS patients and healthy controls (Table 3-12). Percentages of TNF-αR expressing NK cells were higher in patients with MS, without or with treatment with IFNβ, compared to healthy individuals ($P<0.01$, $P<0.05$, respectively).

Three-day culture with PHA strongly augmented expression of TNF-αR on T cells (Tables 3-12, 3-13). Percentages of PHA-activated CD4 and CD8T cells expressing TNF-αR were significantly higher in patients with MS ($P<0.05$) than in healthy subjects. NK cells from MS patients and controls expressed TNF-αR to a similar extent after PHA stimulation, without any difference between the groups (Figure 3-9).

Table 3-12. Percentage of *in vitro* and PHA-activated T and NK cells from patients with MS and healthy subjects expressing TNFαR , as determined by flow cytometry

Percentage of positive cells (%)	*Control (N=25)*	*Control + PHA stimulation (N=25)*	*MS w/o treatment (N=20)*	*MS w/o treatment + PHA stimulation (N=20)*	*MS with IFNβ-1a treatment (N=20)*	*MS with IFNβ-1a treatment + PHA stimulation (N=20)*	*MS with IFNβ-1β treatment (N=20)*	*MS with IFNβ-1β treatment + PHA stimulation (N=20)*
CD4	*20*	*57*	*26*	*82*	*18*	*76*	*13*	*73*
CD8	*24*	*61*	*34*	*73*	*23*	*74*	*18*	*79*
NK	*24*	*52*	*43*	*58*	*36*	*43*	*45*	*42*

Table 3-13. Statistical analysis of percentage of T and NK cells expressing TNFαR

	P (contr vs MS w/o treat.)	*P (contr vs MS IFNβ-1a)*	*P (contr vs MS IFNβ-1β)*	*P (contr+PHA vs MS w/o treat. +PHA)*	*P (contr+PHA vs MS IFNβ-1a+PHA)*	*P (contr +PHA vs MS IFNβ-1β+PHA)*
CD4	NS	NS	NS	NS	NS	NS
CD8	NS	NS	NS	NS	NS	NS
NK	0.01**	0.05	0.05	NS	NS	NS

** Significant differences < 0.01 level

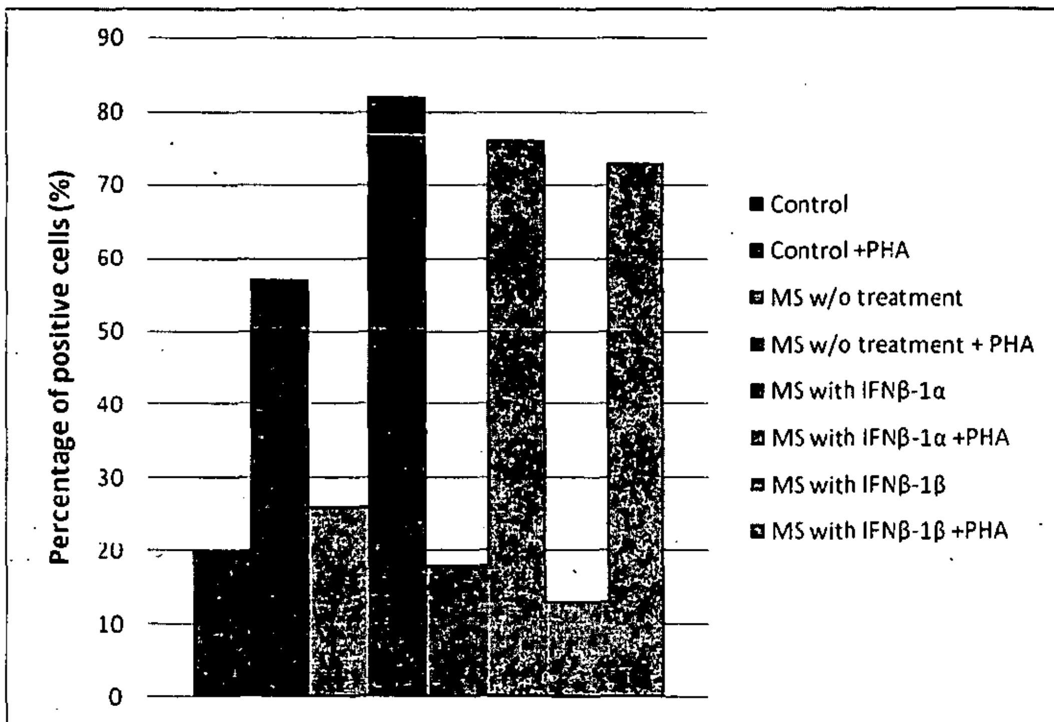

Figure 3-9A. Percentage of *in vitro* and PHA-activated CD4 cells from patients with MS (n=60) and healthy subjects (n=25) expressing TNF-αR.

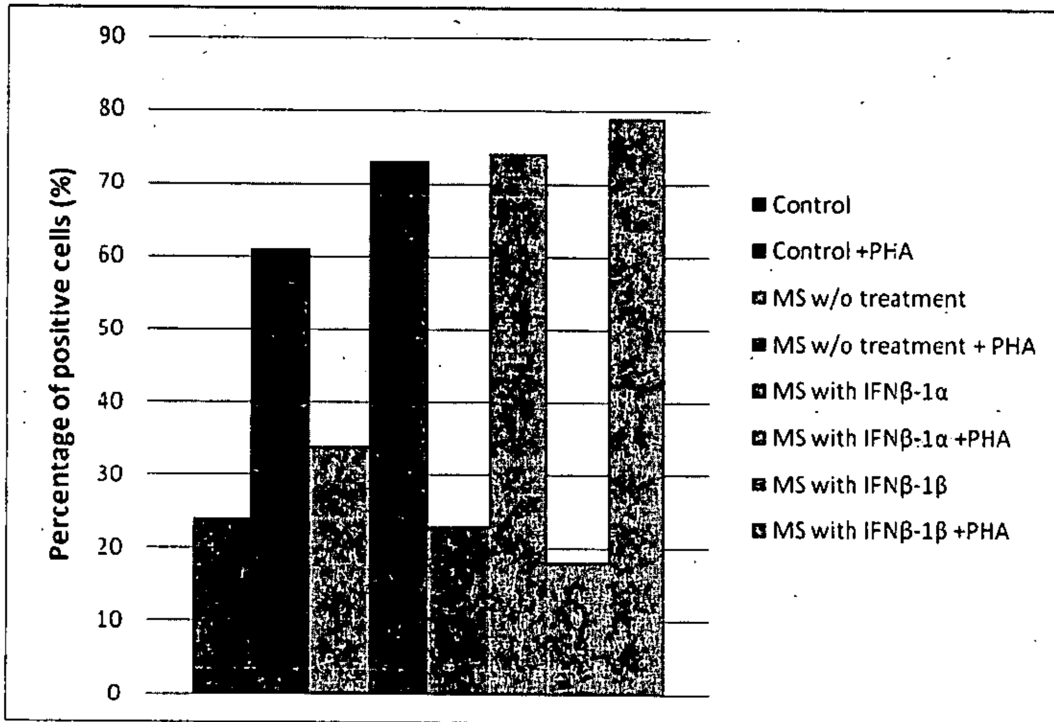

Figure 3-9B. Percentage of *in vitro* and PHA-activated CD8 cells from patients with MS (n=60) and healthy subjects (n=25) expressing TNF-αR.

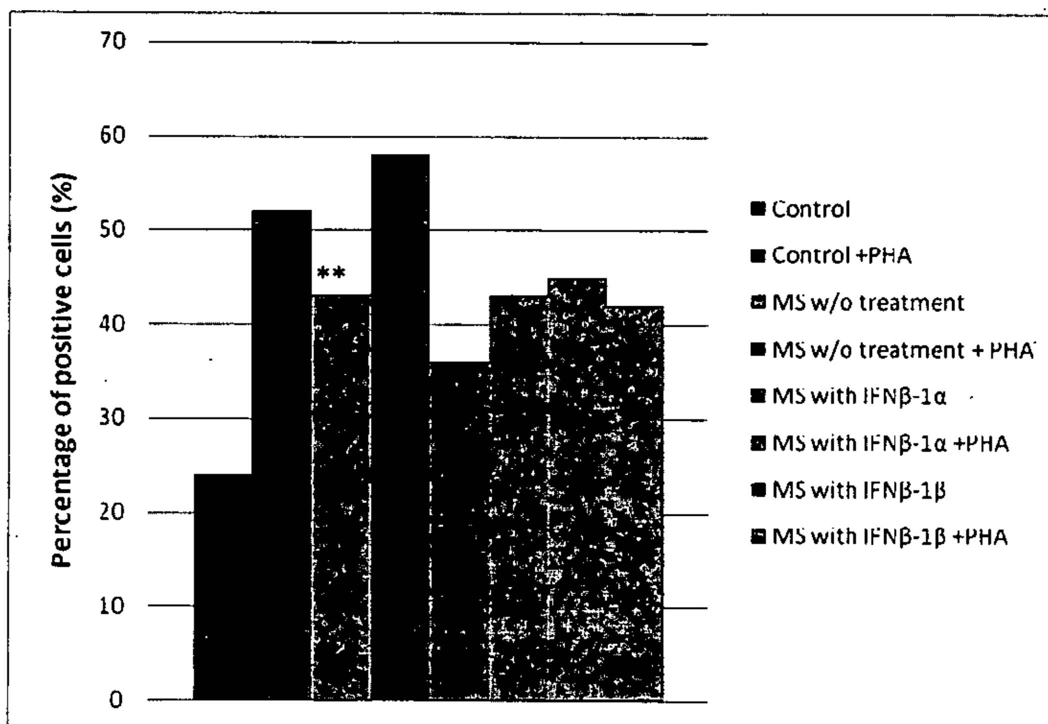

Figure 3-9C. Percentage of in *vitro* and PHA-activated NK cells from patients with MS (n=60) and healthy subjects (n=25) expressing TNF-αR (**P<0.01).

3.4.4 Flow cytometric analysis of percentage of T and NK cells from patients with MS and healthy subjects expressing IL-10R, before and after PHA activation

Peripheral blood cells from untreated patients with MS had significantly smaller amounts of IL-10R than those from control subjects. Percentages of IL-10R -expressing CD4 and CD8 cells differed significantly between MS patients and controls ($P<0.05$, $P<0.01$, respectively). The percentage of IL-10R -expressing NK cells did not differ between MS and control subjects (Table 3-14). PHA stimulation increased expression of IL-10R on T cells (Table 3-14). Percentages of PHA-activated CD4 and CD8T cells expressing IL-10R were higher in patients with MS ($P<0.05$) than in healthy subjects. NK cells from MS patients and controls expressed IL-10R to a similar extent after PHA stimulation, without any difference between the groups (Table 3-15).

After 6 months of IFNβ treatment, T-cell IL-10R expression was increased with IFNβ-1a treatment showing a better effect on elevation of IL-10R levels than IFNβ-1b (Figure 3-10).

Table 3-14. Percentage of *in vitro* and PHA-activated T and NK cells from patients with MS and healthy subjects expressing IL-10R , as determined by flow cytometry

Percentage of positive cells (%)	*Control (N=25)*	*Control + PHA stimulation (N=25)*	*MS w/o treatment (N=20)*	*MS w/o treatment + PHA stimulation (N=20)*	*MS with IFNβ-1a treatment (N=20)*	*MS with IFNβ-1a treatment + PHA stimulation (N=20)*	*MS with IFNβ-1β treatment (N=20)*	*MS with IFNβ-1β treatment + PHA stimulation (N=20)*
CD4	*25*	*48*	*11*	*52*	*23*	*49*	*19*	*44*
CD8	*27*	*56*	*10*	*56*	*25*	*57*	*26*	*48*
NK	*19*	*39*	*9*	*44*	*20*	*44*	*18*	*45*

Table 3-15. Statistical analysis of percentage of T and NK cells experessing IL-10R

	P (contr vs MS w/o treat.)	*P (contr vs MS IFNβ-1a)*	*P (contr vs MS IFNβ-1β)*	*P (contr+PHA vs MS w/o treat. +PHA)*	*P (contr+PHA vs MS IFNβ-1a+PHA)*	*P (contr +PHA vs MS IFNβ-1β+PHA)*
CD4	0.05	NS	NS	NS	NS	NS
CD8	0.01**	NS	NS	NS	NS	NS
NK	NS	NS	NS	NS	NS	0.05

** Significant differences < 0.01 level

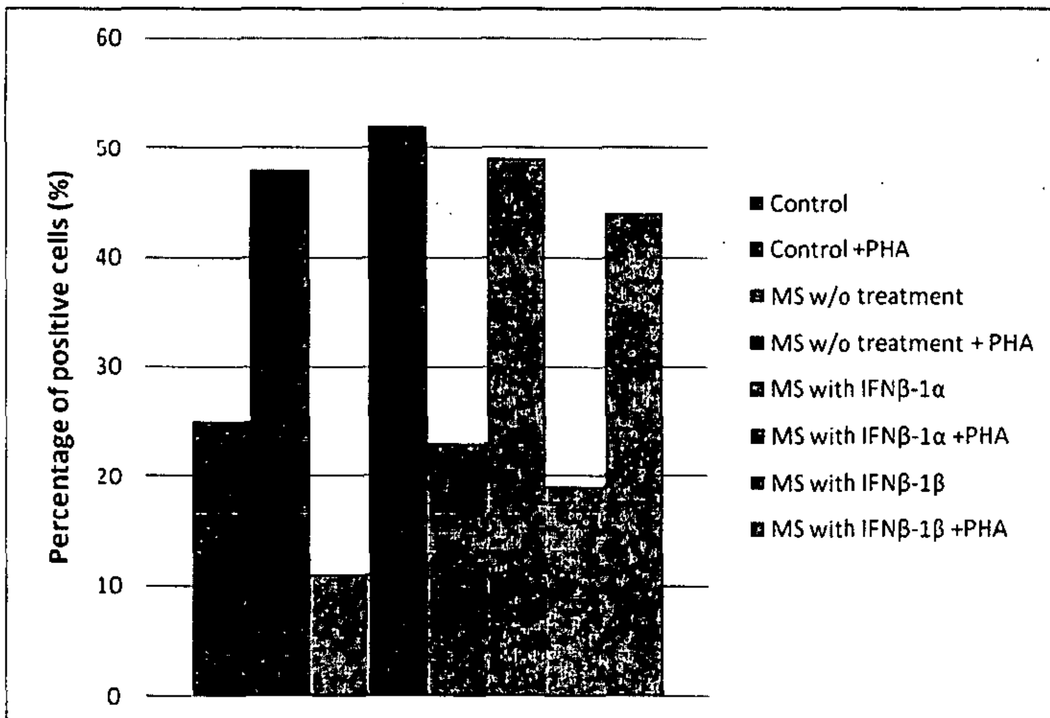

Figure 3-10A. Percentage of *in vitro* and PHA-activated CD4 cells from patients with MS (n=60) and healthy subjects (n=25) expressing IL-10R.

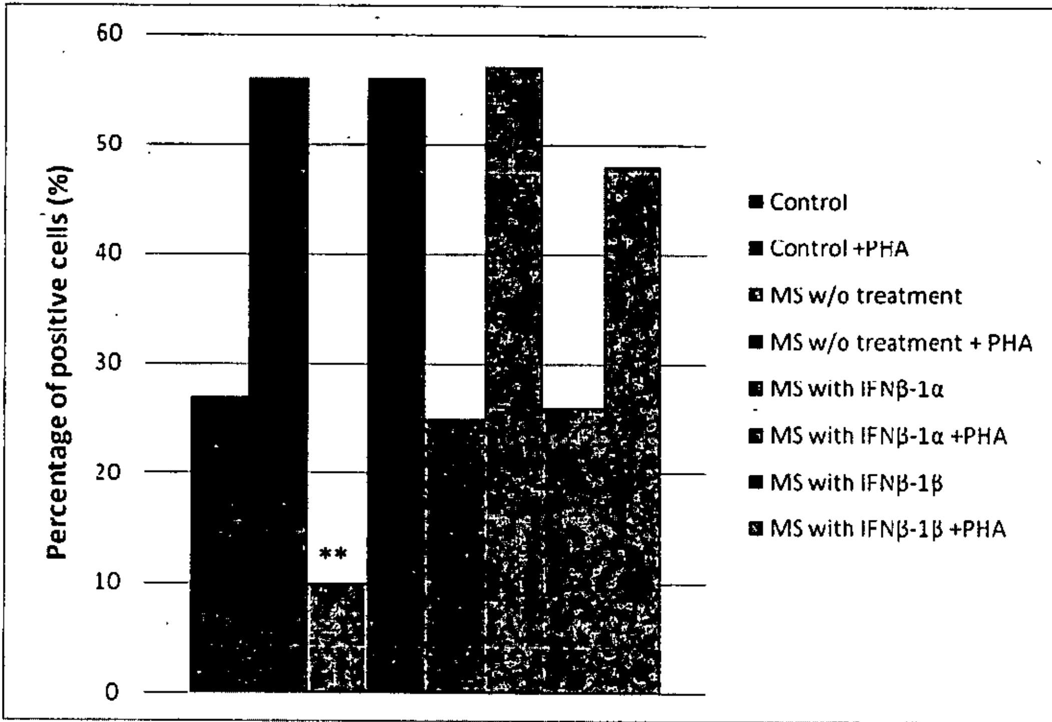

Figure 3-10B. Percentage of *in vitro* and PHA-activated CD8 cells from patients with MS (n=60) and healthy subjects (n=25) expressing IL-10R (**P<0.01).

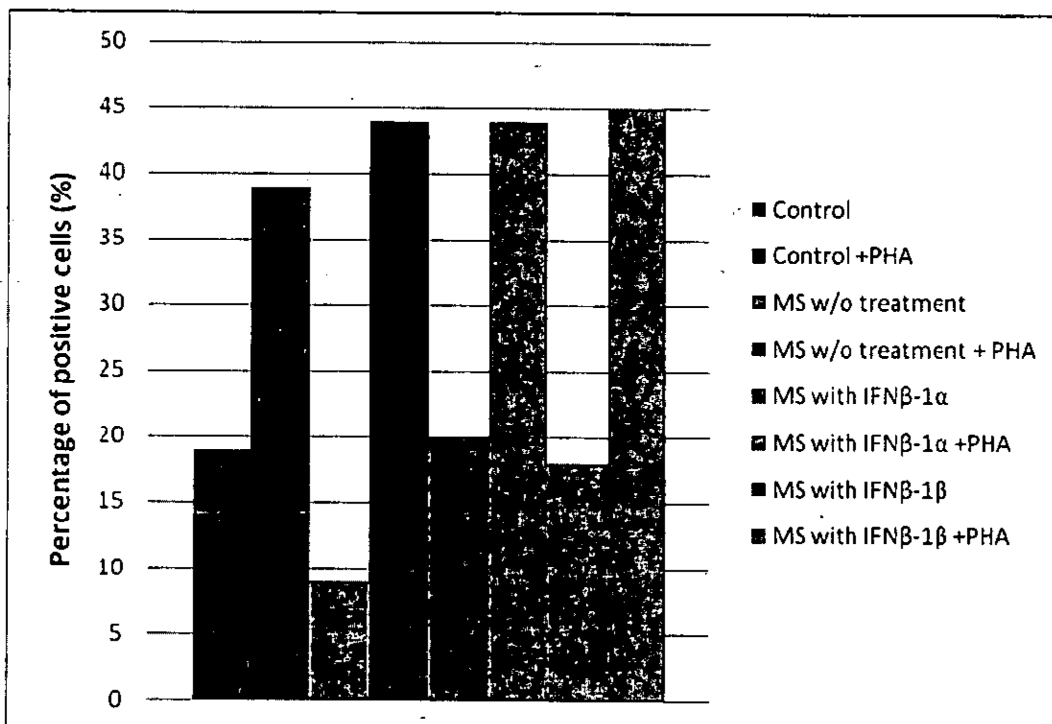

Figure 3-10C. Percentage of by *in vitro* and PHA-activated NK cells from patients with MS (n=60) and healthy subjects (n=25) expressing IL-10 R.

3.5 Study III: Estimation of the apoptotic marker sCD95 (Fas) expression

sCD95 levels were detectable in sera of MS patients, with or without treatment and healthy controls with significant differences (Table 3-16).

MS patients without treatment demonstrated increased levels of sCD95 in their sera (1447±125) compared to healthy individuals (517.79±256.19, $P<0.01$). Treatment with IFNβ-1α further induced CD95-mediated apoptosis, as shown by significantly elevated CD95 levels (2695±134, $P<0.01$). The same pattern of increased sCD95 levels was also observed in MS patients receiving IFNβ-1β treatment (2711±104, $P<0.01$, Figure 3-11).

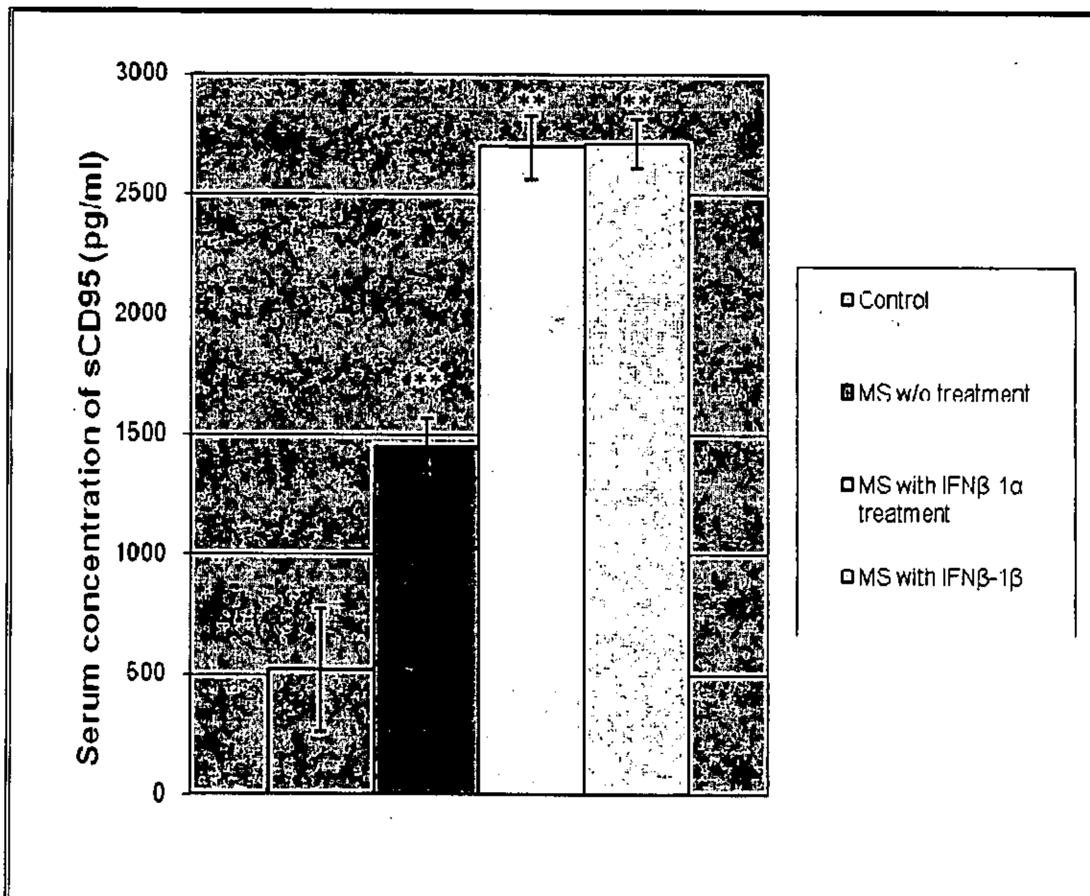

Figure 3-11. Serum sCD95 levels of MS patients (n=60) with and without IFNβ treatment compared to controls (n=25, **P<0.01).

Table 3-16. Serum levels of sCD95 in MS patients and healthy controls (Data are mean ±SD; n values are shown)

Soluble CD95 (pg/ml)	*Control (N=25)*	*MS w/o treatment (N=20)*	*MS with IFNβ-1a treatment (N=20)*	*MS with IFNβ-1β treatment (N=20)*	*P (contr vs MS w/o treat.)*	*P (contr vs MS IFNβ-1a)*	*P (contr vs MS IFNβ-1β)*	*P (MS w/o vs IFNβ-1a)*	*P (MS w/o vs MS IFNβ-1β*	*P (MS IFNβ-1a vs MS IFNβ-1β)*
sCD95 (pg/ml)	517.79±256.19	1447±125	2695±134	2711±104	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	0.425

** Significant differences at the P<0.01 level

3.6 Study IV: Estimation of the adhesion molecules VCAM-1 and ICAM-1 expression

The expression of the soluble forms of adhesion molecules (VCAM-1 and ICAM-1) in MS patients compared to controls is summarized in Table 3-17.

MS patients presented increased levels of sVCAM-1 (564.14±127.20) compared to controls (453.04±241.27, P=0.05). IFNβ-1α/1β treatments significantly elevated VCAM-1 levels to 661.64±169.76 and 685±104.2 respectively (Figure 3-12).

No differences were observed in sICAM-1 levels between MS patients (442.15±125) and healthy subjects (427.05±247.2). Furthermore, treatment with IFNβ-1α/1β failed to induce any significant change in ICAM-1 levels of MS individuals (values were 489±216 after IFNβ-1α and 472.1±202 after IFNβ-1β respectively, Figure 3-13).

Table 3-17. Serum levels of VCAM-1 and ICAM-1 of patients with MS and healthy controls (Data are mean ±SD; n values are shown)

	Control (N=25)	*MS w/o treatment (N=20)*	*MS with IFNβ-1a treatment (N=20)*	*MS with IFNβ-1β treatment (N=20)*	*P (contr vs MS w/o treat.)*	*P (contr vs MS IFNβ-1a)*	*P (contr vs MS IFNβ-1β)*	*P (MS w/o vs IFNβ-1a)*	*P (MS w/o vs MS IFNβ-1β*	*P (MS IFNβ-1a vs MS IFNβ-1β)*
sVCAM-1 (ng/ml)	453.04±241.27	564.14±127.20	661.64±169.76	685±104.2	0.05	<0.01**	<0.01**	0.168	0.125	0.327
sICAM-1 (ng/ml)	427.05±247.2	442.15±125	489±216	472.1±202	0.320	0.126	0.231	0.426	0.514	0.52

** Significant differences < 0.01 level

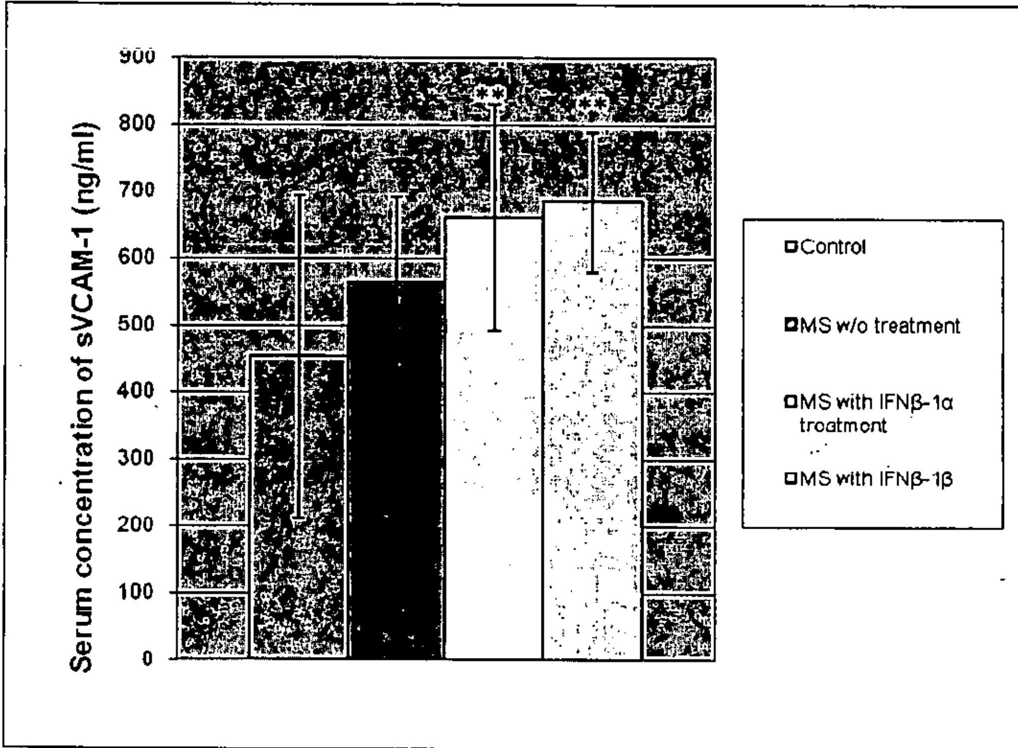

Figure 3-12. Serum sVCAM-1 levels of MS patients (n=60) with and without IFNβ treatment compared to controls (n=25, **P<0.01).

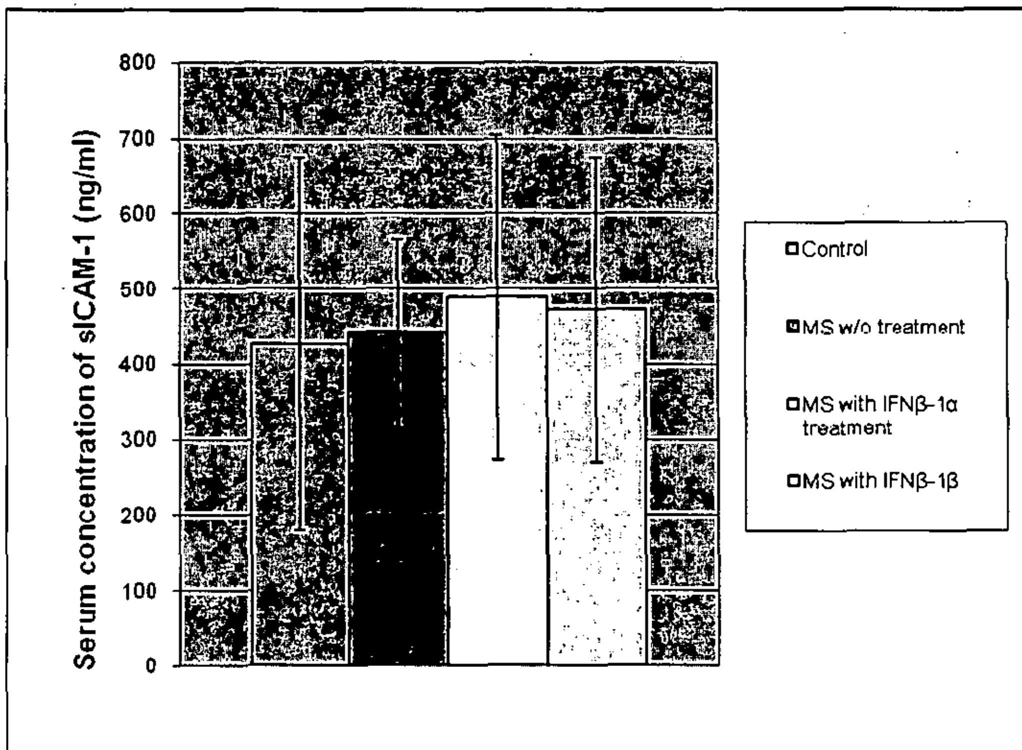

Figure 3-13. Serum sICAM-1 levels of MS patients (n=60) with and without IFNβ treatment compared to controls (n=25).

3.7 Study V: Estimation of levels of metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in serum samples

MS patients without treatment showed significantly increased sMMP-9 levels (226.06±5.1), decreased sTIMP-1 levels (796.3±1.1) and a higher sMMP-9/sTIMP-1 ratio (0.35±0.07) compared to controls (161.90±8.1, 964.4±2.4, 0.17±0.11 respectively, Table 3-18).

However, treatment with IFNβ decreased significantly sMMP-9 levels ($P<0.01$ for IFNβ-1α and $P=0.05$ for IFNβ-1β), increased sTIMP-1 levels and reduced the sMMP-9/sTIMP-1 ratio at MS patients.

Notably, IFNβ-1α decreased sMMP-9 levels of MS patients to 187.73±6.3 (Figure 3-14) and increased sTIMP concentration to normal levels (967±3.2, Figure 3-15). The sMMP-9/sTIMP-1 ratio was also reduced to 0.21±0.17 following IFNβ-1α treatment.

Furthermore, treatment of MS patients with IFNβ-1β reduced sMMP-9 levels (195.33±8.3), increased sTIMP-1 levels (883±3.2) and decreased the sMMP-9/sTIMP-1 ratio (0.25±0.23).

IFNβ-1α treatment was shown to be more effective (Figure 3-14) in reducing sMMP-9 levels and increasing sTIMP-1 compared to IFNβ-1β (Figure 3-15).

Table 3-18. Serum levels of MMP-9 and TIMP-1 of patients with MS and healthy controls (Data are mean ±SD; n values are shown)

	Control (N=25)	*MS w/o treatment (N=20)*	*MS with IFNβ-1a treatment (N=20)*	*MS with IFNβ-1β treatment (N=20)*	*P (contr vs MS w/o treat.)*	*P (contr vs MS IFNβ-1a)*	*P (contr vs MS IFNβ-1β)*	*P (MS w/o vs IFNβ-1a)*	*P (MS w/o vs MS IFNβ-1β*	*P (MS IFNβ-1a vs MS IFNβ-1β)*
MMP-9 (ng/ml)	161.90±8.1	226.06±5.1	187.73±6.3	195.33±8.3	0.004**	0.05	0.05	0.05	0.07	0.09
TIMP-1 (ng/ml)	964.4±2.4	796.3±1.1	967±3.2	883±3.2	0.07	0.12	0.11	0.06	0.10	0.15
MMP-9/TIMP-1 ratio	0.17±0.11	0.35±0.07	0.21±0.17	0.25±0.23	0.003**	0.01**	0.01**	0.05	0.05	0.09

** Significant differences < 0.01 level

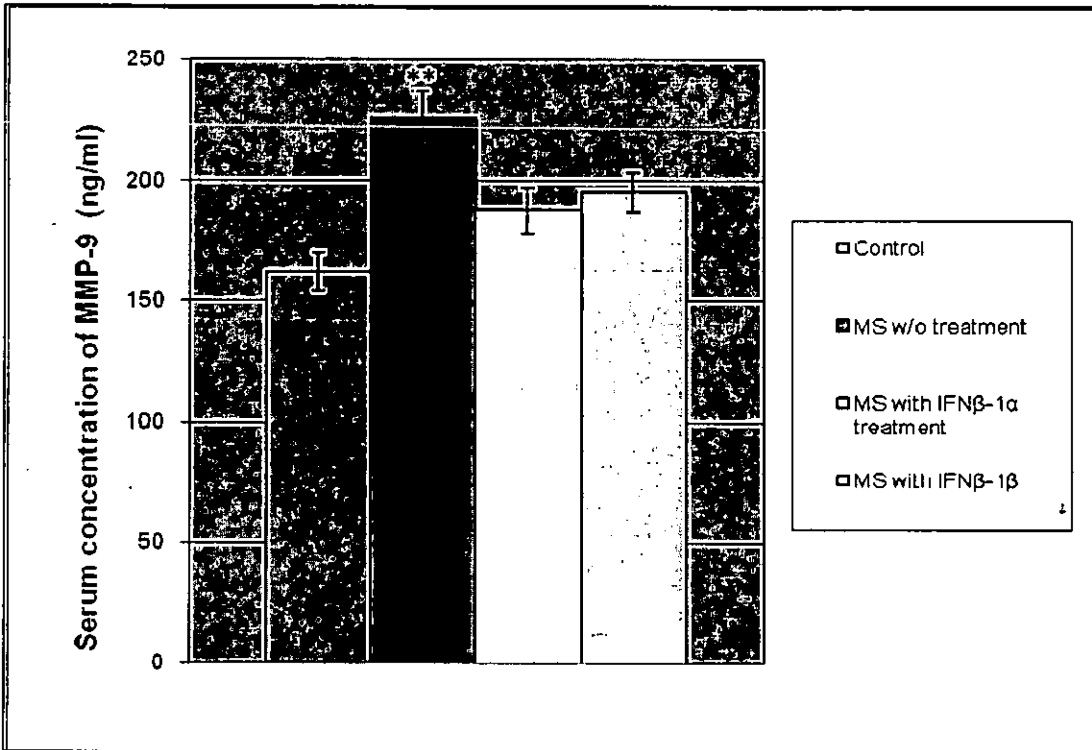

Figure 3-14. Serum MMP-9 levels of MS patients (n=60) with and without IFNβ treatment compared to controls (n=25, **P<0.01).

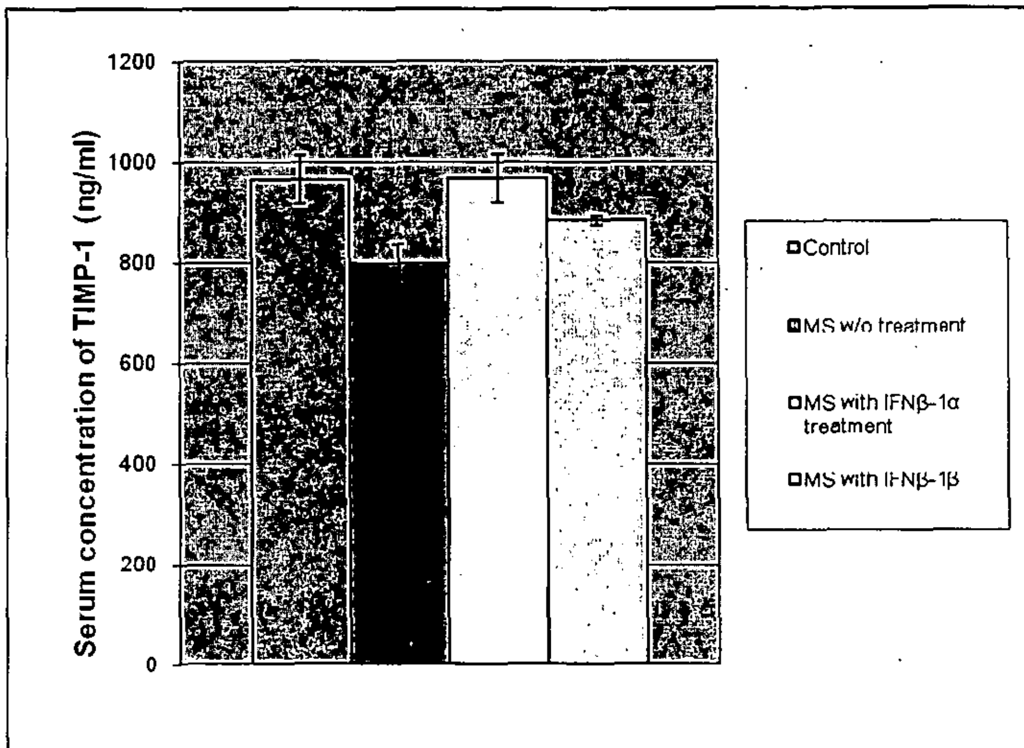

Figure 3-15. Serum TIMP-1 levels of MS patients (n=60) with and without IFNβ treatment compared to controls (n=25).

CHAPTER 4: DISCUSSION

The present study was designed to characterize the regulation of the inflammatory background underlying MS pathophysiology mediated by cytokines as well as investigating the mode of action and biological role of IFNβ treatment for MS patients Elucidation of the mechanisms of action of the different forms of IFNβ treatment (IFNβ-1α/1β) on cytokine secretion as well as on other immunoregulatory molecules (adhesion molecules, metalloproteinases and apoptotic markers) may prove of clinical importance for the choice of treatment and disease monitoring as well as in the follow up of patients.

MS represents the most common inflammatory demyelinating disorder of the human central nervous system, and is the leading cause of non-traumatic neurological disability in young adults in Europe and North America. The estimated European mean annual MS incidence rate is 4.3 cases per 100,000, whilst almost 1 million people are affected worldwide every year (Kumar & Clark 2002; Rich *et al.*, 2001).

The burden for young adults caused by the disease is multiple: personal, social, and financial and is particularly heavy for their caregivers. Early and accurate diagnosis together with a better selection of appropriate therapy for each disease type is, thus, a desirable combination for an improved outcome.

Today, there are several possible treatments for MS including copaxone (glatiramer acetate), mitoxandrone and interferon-beta (IFNβ-1α/1β) (Rizvi and Aqius 2004; Flackenecker 2004; Fox 2004). The exact mode of action and the biological role for none of these drugs are known, therefore making selection and appropriate dose very difficult, whilst creating debate and disagreement among clinicians (Durelli 2004). However, the

evidence to date suggests that high-dose, high-frequency regimens of IFNβ might offer superior efficacy over a low-dose, low-frequency regimen in terms of relapse rates and MRI measures of disease.

IFNβ has been used for more than 10 years in the treatment of MS and has proved to exhibit better clinical efficacy compared to other therapies (Pozzilli *et al.*,2006). Although initially designed as an antiviral drug, the therapeutic benefit of IFNβ appears to be based on immunomodulatory mechanisms, several of which still need to be elucidated (Takaoka & Yanai, 2006).

Two different recombinant IFNβ's, IFNβ-1α (Avonex and Rebief) and IFNβ-1β (Betaferon) are approved for the treatment of relapsing and remitting form of MS (Ann Marie and Rudick, 2006; Montalban and Rio, 2006). They differ in recommended dosage, and in route and frequency of administration, and consequently most probably also in clinical effectiveness.

The present study attempts a comprehensive approach for the characterization of the inflammatory profile contributing to the autoimmune defects of MS patients. The main regulators of inflammation, cytokines, as well as their receptors, were extensively studied in the peripheral blood of MS patients, together with other soluble mediators and apoptotic markers. The immunomodulatory activity of both forms of IFNβ upon these parameters was also investigated so as to elucidate their mechanism of action and suggest a preferable selection between the two forms for therapy.

The study group was composed of 85 individuals, 60 MS patients diagnosed with the remitting relapsing form of the disease and 25 controls, carefully selected based on their

clinical diagnosis, laboratory examination and MRI results so as to form two well-matched groups. All individuals had Greek nationality, shared similar genetic and environmental elements and occupational habits.

The study population had a narrow age-span: 30–45 at the time of the study. The mean age in the MS group was 34±3.2 years and is in accordance with epidemiological studies in MS where the highest prevalence rates have been estimated for the 35-54 years age group in both sexes and all European countries (Pugliatti *et al.*, 2006).

Based on the age of MS patients, the control group was carefully selected (mean age: 38.8±6.9 years) so that there was no statistically significant age difference between the two groups ($p>0.05$). However, this fact does reduce the ability to extrapolate these results to either younger or older age groups.

4.1.1 Female gender is a risk factor for MS

Previous studies have suggested that female gender may be an established risk factor for MS and this was also consistent with the present study data (Pugliatti *et al.*, 2006).

Available MS epidemiological estimates indicate that total estimated prevalence rate of MS in Europe for the past three decades is 83 per 100,000 with higher rates in northern countries and a female: male ratio of approximately 2.0. Prevalence rates are higher for women in all countries considered in the study.

In this present study, from the MS group, 43 participants were female and 17 male. Marital status, level of education and form of employment did not show any significant differences between the two groups ($p>0.05$).

4.1.2 Cigarette smoking may worsen disease activity

Cigarette smoking and low alcohol consumption was related to MS presence. Smokers were outnumbered by the non-smokers (61.6%) in the MS population but not in controls (24% were smokers). The majority of MS (58.3%) and control subjects (60%) consumed low quantities of alcohol (<2 drinks weekly). Recent studies have shown that patients with MS, angina and asthma had slightly higher rates of current smoking than the general population (Nortvedt *et al.*, 2005) and that smoking may present a risk factor for MS (Hernan *et al.*, 2001; Riise *et al.*, 2003). In the same study, although many MS patients had quit smoking, as many as 40% were still smoking. An earlier study of a similar general population showed that smoking was related to a markedly reduced health-related quality of life and that previous smokers had almost the same score as people who had never smoked (Riise *et al.*, 2003). This might suggest that ceasing smoking may help MS patients.

4.1.3 Leisure physical activity is vital for MS patients

Another lifestyle factor known to strongly influence the health-related quality of life is physical leisure activity (Riise *et al.*, 2003). MS patients reported spending markedly less time on both strenuous and light physical leisure activity. An increased percentage of MS patients (75%) were virtually inactive whereas only 8.3% exercised between 2-3 hours per week. This is probably related, in part, to the physical disability many of these patients had. However, the possible effects of vigorous training on the disease process in MS as yet remain unknown.

A concern for patients' thermosensibility might influence advice given regarding physical activity. Nevertheless, two studies have shown that aerobic training positively influences the quality of life of MS patients, and further, that the more disabled group appeared to profit most from the training programme (Petajan *et al.*, 1996; Mostert & Kesselring 2002).

In addition to a positive effect on quality of life, the result of increased physical activity gives, a general benefit in the reduction of weakness, fatigue and other health risk factors associated with detraining and deconditioning (Mostert & Kesselring 2002).

Despite low physical leisure activity, MS patients had a lower mean BMI than the general population. Previous studies have reported that almost three times as many MS patients as the general population were below the recommended range (Nortvedt *et al.*, 2005). This may be related to a change in metabolism after the onset of disease or muscle atrophy as a result of reduced muscle activity resulting from neural impairment, although this is less likely in our study, as most of the patients were still ambulatory and working.

Regarding their clinical characteristics, all MS patients in this study had the relapsing-remitting form of the disease with a relative early age of onset (27.3±8.8 years) and a mean disease duration of 4 years. Their degree of disability was reported as moderate in the majority of cases (71.6%) and their treatment with IFNβ lasted for six months for the under treatment subgroup.

Various factors such as genetic, autoimmune and environmental have previously been considered in the etiopathogenesis of MS (Dijkstra *et al.*, 1993) and many studies emphasize the role of hepatic and hormonal disturbances in patients with MS (Ajuebor *et*

al., 2006; de Seze *et al.*, 2005; Petek-Balsi *et al.*, 2005; Kotilainen *et al.*, 2005; Edwards and Constantinescu, 2004). Therefore, a variety of laboratory parameters were evaluated in MS patients including white blood cell level, hepatic enzyme levels and thyroid hormone concentrations.

4.1.4 Thyroid dysfucntion may be related with MS

Baseline laboratory data for hepatic function were within normal range values as well as thyroid hormonal values, however, anti-thyroglobulin antibody and anti-thyroid peroxidase antibody titers were markedly elevated in these patients compared to normal. These observations are in accordance with previous studies where thyroid dysfunction is much more common in patients with MS as compared to the general population. There are reports for both hyper-and hypothyroidism in MS patients (Iwasaki *et al.*, 1988; Kiessling *et al.*, 1980; Klappas *et al.*, 1992). Furthermore, antithyroid antibodies, mainly those for the thyroglobulin and microsomal fraction of thyroid cells are frequently found in many patients with MS (De Keyser *et al.*, 1988; Ioppoli *et al.*, 1990; Kiessling *et al.*, 1980) and their elevated presence suggests the involvement of autoimmune mechanisms in the development of thyroid dysfunction in these patients.

4.1.5 Optic neuritis is a common disorder among MS patients

Co-existence of other medical conditions which either lead to or are associated to MS status was also evaluated in the MS population of the present study. The personal history data for other disorders revealed that there was a prevalence of optic neuritis in MS patients (83.3%). This is in agreement with earlier studies where MS was commonly

associated with visual system problems and MS patients may have initially presented with ophthalmologic symptoms (Cerovski *et al.*, 2005).

Manifestations of multiple sclerosis in the eye include both afferent and efferent visual pathways. Optic neuritis, the most common ocular indication of multiple sclerosis, may be the initial symptom of clinical disease. Recent long-term follow-up data show that most patients with demyelinating optic neuritis have an excellent prognosis for recovery of central visual acuity. Evidence is emerging, however, for a significant and broad reduction in both contrast sensitivity and color perception in multiple sclerosis patients despite near-normal visual acuities (Sisto *et al.*, 2005). Ocular motor deficits in multiple sclerosis include internuclear ophthalmoplegia and nystagmus, resulting in diplopia, oscillopsia, blurred visual, loss of stereopsis, and reading fatigue. Multiple sclerosis may also be associated with ocular inflammatory diseases, in particular pars planitis and retinal periphlebitis (Cerovski *et al.*, 2005).

In summary, it is possible that observations of ocular problems may be an initial manifestation of multiple sclerosis and may predict additional demyelinating events. Therefore, recognizing these syndromes and signs will help clinicians to properly evaluate the patients, and help develop an effective therapeutic plan.

4.1.6 History of vaccination, autoimmunity and inflammatory disorders underlies MS manifestation

The history of childhood and early life vaccination has previously been associated with an increased risk of MS exacerbation (Zingg, 2005). Although there is strong evidence against an increased risk of MS exacerbation after influenza immunization, contradictory

data exist relative to hepatitis B, varicella, tetanus, or Bacille Calmette-Guerin vaccinations and MS exacerbation (Rutschmann *et al.*, 2002).

In the present study, the history of vaccination, autoimmunity and inflammatory disorders ranged from 30-40% of MS patients which is consistent with previous studies which have shown that the risk of MS increases with a positive history of all the above (Demichelli *et al.*, 2003; Hernan *et al.*, 2006; Tischler and Shoenfeld, 2004; Vial and Descotes, 2004). A potential association between vaccination and autoimmune diseases has been questioned in the past few years (Vial and Descotes, 2004), and is largely based on case reports. The available evidence, derived from several negative epidemiological studies, is reassuring and indicates that vaccines are not a major cause of autoimmune diseases (Salleras *et al.*, 2006). However, there are still uncertainties as to whether a susceptible subpopulation may be at a higher risk of developing an autoimmune disease without causing an overall increase in disease incidence.

4.1.7 Viral infections are implicated in MS aetiopathogenesis

It has been proposed that multiple sclerosis is caused by an interaction of genetic and environmental factors. Previous viral infections, particularly Epstein-Barr virus were reported by 31.6% of MS patients in this present study. Previous epidemiological and basic research studies have demonstrated a possible association between Epstein-Barr virus infection and the risk of MS (Haahr and Hollsberg, 2006) and several physiopathologic mechanisms, including molecular mimicry and bystander activation, have been proposed for this association (Alonso *et al.*, 2006; Thacker *et al.*, 2006).

The human neurotropic polyomavirus--JC virus (JCV) has also been implicated in MS pathology, as a possible etiologic agent of demyelination of the central nervous system

(Pietropaolo *et al.,* 2005). JCV is also responsible for progressive multifocal leukoencephalopathy, a fatal demyelinating disease of the central nervous system that occurs mainly in immunosuppressed patients, especially those with HIV/AIDS (Khalili and White, 2006).

4.1.8 Mental disorders co-exist with MS pathology

Regarding the co-existence of mental disorders with MS, the patients in this study reported numerous symptoms, including spasticity, fatigue, cognitive dysfunction, pain , and predominantly, in 20% of the patients, depression (Crayton and Rossman 2006). Other studies have also presented depression as the most frequent psychiatric disorder in MS patients (Buchanan, 2006; Gottberg *et al.,* 2006; Wallin *et al.,* 2006). Its etiology is multifactorial and is likely associated with psychosocial stress, focal demyelinating lesions, and immune dysfunction (Gold and Irwin, 2006). Proper diagnosis and severity assessment are critical prior to initiation of therapy and patients with suicidal tendencies should be referred for immediate psychiatric consultation and be closely monitored. Depression may exacerbate cognitive dysfunction in MS patients and early intervention has been reported to prevent decline in the quality of life of these patients (Wallin *et al* 2006).

4.1.9 Dyslipidemia is a common feature of MS patients

The evaluation of the lipid profile of MS patients indicates that the mean value for both total cholesterol and triglycerides concentration were increased in MS patients compared to normal. Recently, it has been suggested that total cholesterol and low density

lipoproteins (LDL) levels can behave as biological markers of activity in demyelinating diseases (Giubilei *et al.*, 2002). It has been proposed that a lipid peroxidation disturbance, caused by free radicals is involved in the breakdown of the myelin sheath. Several subsequent studies have also demonstrated the role of increased free radical production and/or a decreased antioxidant defense in the central nervous system as causal factors for MS (Bo *et al.*, 1994; Hunter *et al.*, 1985; Langeman *et al.*, 1992). While the principal site of pathology is the CNS, lipid status and membrane properties of platelets and erythrocytes in the peripheral blood are also altered (Leoni *et al.*, 2002; Wheeler and Ford, 1998). Increased lipid peroxide levels have also been previously observed both in cerebrospinal fluid and in the blood of MS patients (Hunter *et al.*, 1985). However, although there is accumulating evidence for the importance of lipids in MS pathology, further studies need to be designed so as to determine whether measurement of plasma cholesterol and triglycerides levels are of practical use in monitoring the disease course.

4.1.10 Cytokines regulate immune response in MS

Cytokine production is considered an important factor in determining disease activity in chronic autoimmune and inflammatory diseases (Chen *et al.*, 2006). The prevailing paradigm in the pathogenesis of MS and its animal model, experimental allergic encephalomyelitis (EAE), suggests that these diseases have their origin in the disruption of self-tolerance for CNS antigens. This disruption results in manifestation of cell-mediated autoreactivity that is mainly governed by activated T cells with a Th1 phenotype, i.e., those associated with a relatively high level production of so-called Th1

cytokines (e.g., IFN-γ) and a relatively low-level production of Th2 cytokines (e.g., IL-10) (Navikas and Link., 1996; Ozenci *et al.,* 1999).

Based on the present data it is clearly evident that aberrant immune responses occur in MS, and it is likely that the spectrum of cytokines produced decisively influences disease outcome. The detrimental consequences of IFN-γ and the beneficial effects of IFNβ-treatment in MS support this hypothesis (Goodin, 2006; Ozenci *et al.*, 2000). However, there are still major gaps in our knowledge of the involvement of cytokines in MS. Numerous studies have addressed the question of cytokine levels in MS, often with conflicting results; elevated, normal and decreased levels of almost all cytokines have been reported (Crucian *et al.*,1996; Drulovic *et al.*,1997; Link , 1998; Link *et al.*,1994; Matusevicious *et al.,* 1996; Matusevicious *et al.,* 1998; Ozenci *et al.,* 2000; Rieckman *et al.,* 1994; Rohowsky-Kohan *et al.*,1999; Musette *et al.* 1996).

4.1.11 ELISPOT assay presents a valuable tool for estimation of cytokine secretion in MS

The present study was designed to better characterize the cytokine profile of a well-defined population of MS patients with the relapsing remitting form of the disease both with and without treatment with IFNβ through the use of a more sophisticated assay, the enzyme-linked immunospot (ELISPOT), than has previously been used. This assay allows real-time cytokine detection by identifying the cells actively secreting cytokines at a given time point and is sensitive enough to detect cytokine-secreting cells in the absence of stimulant. Instead of measuring mRNA or the accumulation of cytokine in the cytoplasm of pharmacologically treated cells, ELISPOT assays detect immunologically relevant secretion of the cytokine (Karulin *et al.,* 2000).

Until recently, data from ELISPOT assays performed to investigate cytokines in MS had been limited to studies of IFN-γ. The recent availability of suitable pairs of antibodies has now made possible the application of ELISPOT assays to the study of other cytokines as well, revealing new insights in the involvement of cytokines in MS. However, in the present study, the ELISPOT assay was succesfully applied, permitting accurate estimation of the understudy cytokines, and giving valuable and clear informations about the cytokine levels in MS.

4.1.12 Th1/Th2 cytokine imbalance in MS: role of IFN-γ and TNF-α

In the present study, the secretion levels of a selection of both proinflammatory (IFN-γ, TNF-α and IL-12) as well as anti-inflammatory (IL-4, IL-10) cytokines were evaluated in MS patients compared to healthy controls, in order to reveal any possible imbalance between Th1 and Th2 immune response. The data clearly show an increased secretion of Th1 cytokines and reduced secretion of the Th2 cytokines compared to controls, thus indicating an important role for these cytokines in the activation of the immune system in MS.

More specifically, increased levels of IFN-γ-secreting cells were observed in MS patients compared to controls. IFN-γ has potent proinflammatory properties, including the capacity to induce the production of other proinflammatory cytokines, such as IL-12 (Ozenci *et al.,* 2000) and the expression of MHC class II on monocytes/macrophages (Kudinov *et al.,* 2003). Furthermore, IFN-γ-mediated signaling leads to increased intracellular Ca^{2+} levels, thereby lowering the excitability threshold of T cells (Martino *et al.,* 1998).

Elevated numbers of IFN-γ mRNA-expressing blood mononuclear cells (Link *et al.*, 1994) and increased serum IFN-γ levels, compared to findings in controls, (Hohnoki *et al.*, 1998) have been reported in MS patients. However, other studies have found no difference between MS patients and controls with regard to either the percentage of IFN-γ-expressing cells in blood detected by flow cytometry (Nguyen *et al.*, 1999) or the blood levels of IFN-γ mRNA (Gayo *et al.*, 1999; Huang *et al.*, 1999). These studies did, however, clearly show a dramatic accumulation of spontaneously IFN-γ-secreting MNC in MS patients' CSF. Therefore, it can be concluded that there is a possible local overproduction of IFN-γ in CNS and a stronger Th1 autoimmune reaction in CNS than in peripheral tissues. This reaction is responsible for myelin destruction in the pathology of MS, since the peripheral tissues are not affected although blood cells seem to contribute to autoimmune activation.

TNF-α is another potent pro-inflammatory cytokine found elevated in our study with effects ranging from the promotion of proliferation to cell apoptosis. TNF-α induces CD40 (Salgado *et al.*, 1999) and MHC class II expression, which are important in T cell activation. It has been shown to stimulate production of IL-12, (Ozenci *et al.*, 2000) which in turn induces IFN-γ production. Also it has the ability to induce expression of adhesion molecules at the blood-brain-barrier facilitating entry of immune cells into the CNS.

Our data are in agreement with a few studies which report elevation of TNF-α in patients with MS compared to control levels. Numbers of TNF-α mRNA-expressing blood cells, (Navikas *et al.*, 1996) serum TNF-α concentrations (Hohnoki *et al.*, 1998) and levels of

TNF-α -secreting blood MNCs (Ozenci *et al.*, 2000b) were found higher in MS patients than in controls.

Elevated levels of TNF-α -secreting cells might be important for the induction of proinflammatory immune responses in MS. However, a randomized double-blind placebo controlled study showed that inhibition of TNF-α by recombinant soluble TNF-α receptor p55 immunoglobulin fusion protein (Lenercept) treatment was not beneficial in MS (LMSSG 1999).

As a consequence of its potent proinflammatory properties, it has been hypothesized that TNF-α may be detrimental in autoimmune diseases and this has already been demonstrated in rheumatoid arthritis, (Feldman *et al.*, 1991) where anti-TNF-α treatment is now part of the standard therapeutic arsenal for this disease (Moreland *et al.*, 1997).

4.1.13 IL-12 is an important inducer of Th1 immune response

An important cytokine favoring the deviation of (auto)immune response in the Th1 direction is IL-12. IL-12 is a powerful inducer of IFN-γ production by T cells and natural killer cells (Trinchieri, 1995). IL-12 also induces cell-mediated cytotoxicity and exerts co mitogenic effects on T cells (Storkus *et al.*, 1998). IL-12 acts through its receptor, which is composed of two subunits, IL-12Rβ1 and IL-12Rβ2. The interaction of IL-12 with IL-12R represents a pivotal crossroad in the development of Th1-type immune responses (Gately *et al.*, 1998).

The number of IL-12-secreting cells was elevated in our MS patients compared to controls. Conflicting results regarding IL-12 levels have been reported in MS. Increases, compared to findings in controls, both in the number of IL-12 p40 mRNA-expressing blood MNC (Matusevicius *et al.*, 1998) and of IL-12 p40 mRNA levels in blood, (van

Boxel-Dezaire *et al.,* 1999) have been described. In contrast, serum levels of IL-12 p40 were similar in MS patients and controls (Fassbender *et al.,* 1998; Heesen *et al.,* 1999). A recent study, evaluating the IL-12-IL-12R system in MS and employing ELISPOT assays showed no difference in the numbers of IL-12-secreting blood MNCs between patients and controls, although the percentages of PHA-activated $CD4^+$ T cells expressing IL-12Rβ2 were elevated in MS compared to healthy subjects, indicating that $CD4^+$ T cells from MS patients are more prone to Th1-type activation (Ozenci *et al.,* 2001).

IL-12 is thought to play a central role in the pathogenesis of a group of other organ-specific autoimmune diseases including uveitis, type I diabetes mellitus and rheumatoid arthritis (Kang and Kim, 2006; Petrovic-Rackov, 2005). IL-12Rβ2 is central molecule to these responses as it has been shown *in vitro* that IL-12Rβ2 is a marker for Th1 cells and that it determines IL-12 responsiveness (Rogge *et al.,* 1997; 1999). It has also recently been shown that pulmonary T cells from patients with sarcoidosis, but not from patients with asthma, exhibit enhanced IL-12Rβ2 expression, again suggesting the importance of IL-12Rβ2 as a marker for Th1-type autoimmune disease (Rogge *et al.,* 1999).

High IL-12 production at the time of initiation of the immune response can be due to exposure to microbial agents possessing molecules called pathogen-associated molecular patterns (PAMPs) recognized by TOLL-like receptors on antigen-presenting cells (Obonyo *et al.,* 2006; Wang *et al.,* 2006).

4.1.14 IL-10, as a potent inhibitor of proinflammatory cytokines

IL-10 is the most important anti-inflammatory cytokine described to date and, in this present study, the level of IL-10 secreting cells in the peripheral blood of MS patients

was markedly reduced compared to controls. Since IL-10 is known to exert inhibitory effects over proinflammatory cytokines (IL-2, IL-12, TNF-α and IFN-γ), low IL-10 levels in blood may further enhance Th1 immune responses, which are harmful for disease progression (Ozenci *et al.*, 1999; Rep *et al.*, 1999). Long considered as a Th2 cytokine, IL-10 is now seen as an overall counter-regulator for the production of other cytokines (de Waal-Malefyt *et al.*, 1991; Fiorentino *et al.*, 1991). The inhibitory effects of IL-10 on cytokine production are mainly mediated *via* suppression of the expression of MHC class II and of adhesion and co stimulatory molecules on monocytes/ macrophages and dendritic cells (de Waal Malefyt and Moore, 1998). A potential therapeutic role for IL-10 in autoimmune diseases, such as rheumatoid arthritis, (Isomaki *et al.*, 1996) type 1 diabetes mellitus (Pennline *et al.*, 1994) and psoriasis, (Asadullah *et al.*, 1998) has been proposed.

A number of studies have evaluated IL-10 in MS, with partly contradictory results. Elevated numbers of IL-10 mRNA-expressing blood MNCs, compared to numbers in controls, (Navikas *et al.*, 1995) were reported in patients with MS. However, decreased IL-10 levels and IL-10 blood MNCs mRNA have been observed in MS (Huang *et al.*, 1999; Salmaggi *et al.*, 1996). In this present study, the numbers of IL-10-secreting blood MNCs were also lower in MS patients than in controls. Such a decrease of IL-10 in blood might further enhance Th1-type immune responses which are harmful for disease activity. More specifically, the presence of low IL-10 peripheral blood levels in MS patients may theoretically be either the cause or the result of Th1 immune response activation observed in these patients. This can be possibly due to the inhibitory effects of IL-10 over the production of Th1 cytokines since Th1 and Th2-type cytokines have been

reported to exert mutual inhibitory effects over each other (Ozenci *et al.*,1999; Rep *et al.*,1999).

4.1.15 Low IL-4-producing T cells are associated with the stable stage of MS

Another Th2 cytokine that contributes to the stable stage of the disease is IL-4 which, similarly to IL-10, was found to be secreted in reduced levels compared to normal controls in the present study. This finding is in agreement with a previous study employing an intracellular cytokine staining technique. This enabled the analysis of $CD3^+$, $CD8^-$, and $CD8^+$ T cells, which differentiated into IL-4-producing (Th2) subsets after short-term stimulation, in peripheral blood samples from MS patients and healthy subjects. Significantly lower percentages of IL-4-producing T cells were found in stable MS patients than in controls, and in active than in stable patients. It was further shown that IL-4 can respectively drive $CD4^+$ T cells to Th1 and Th2, and $CD8^+$ T cells to Tc1 and Tc2 polarization (Franciotta, 2000).

Previous studies have also shown a downregulation of IL-4 in MS patients during relapses (Urcelay *et al.,* 2005). However, contradictory studies exist showing that IL-4 levels in CSF during the stable stage as well as during the relapse were significantly higher than that in the control group (Bartosik-Psujek and Stelmasiak, 2005). Also *in situ* hybridization studies with complementary DNA oligonucleotide probes for human IL-4 and expression of IL-4 mRNA found elevated levels of expressing MNC in peripheral blood from untreated patients with MS compared to patients with other neurological diseases and healthy subjects (Link *et al.,* 1994). In another study, expression of IL-4 was reduced in patients still in the relapsing-remitting phase compared to patients with secondary chronic progressive MS (Soderstrom, 1995). The present data indicate that

decreased levels of IL-4 secreting blood mononuclear cells are present in the relapsing-remitting form of the disease further leading to a polarisation of $CD4^{+}$ T cells to Th1 response through this cytokine.

Overall the cytokine data of our study indicate that IFN-γ, TNF-α , IL-12 and IL-10 may be used as candidate complementary diagnostic markers for MS and, moreover, as possible therapeutic agents or targets (IFN-γ, and TNF-α) or therapeutic modulators (IL-10, IL-4). The present study has the advantage over the previous ones in that measurements have been performed in parallel on the same lymphocyte pools of MS patients presenting their cytokinetic profile at the given period. Previous studies have investigated the same cytokines in different patients or treatment times with a variety of techniques thus resulting in contradictory data, making very difficult to predict the state (or pathway) of the immunological response.

4.1.16 IFNβ treatment affects the Th1/Th2 cytokine imbalance

Since clinical studies have shown that treatment of MS patients with IFNβ reduces the frequency and severity of clinical relapses in relapsing-remitting MS (Meier *et al.*,2006), the basis of these beneficial effects merited extensive investigation. The present study indicates that treatment with IFNβ-1α/1β significantly reduces pro-inflammatory cytokine secretion and elevates the anti-inflammatory one. Furthermore, IFNβ-1β was found more beneficial than IFNβ-1α in reducing proinflammatory cytokine levels (IFN-γ, TNF-α), and in elevating anti-inflammatory cytokines (IL-4, IL-10) whereas IFNβ-1α was more efficient in reducing IL-12 levels(Gayo *et al.*,1999; Weber *et al.*,1999). Therefore, the immunological effects of IFNβ treatment in multiple sclerosis include reduction of Th1

cells secretion while maintaining the potentially beneficial effects of the Th2 response (Duddy *et al.*, 1999; Ochi *et al.*, 2004). This observation is in accordance with a previous study where IFNβ-1α inhibited IFN-γ secretion and enhanced IL-4 in MS patients (Jansson *et al.*, 2003). Other studies have also been performed regarding the benefits of IFNβ treatment particularly in the clinical aspects of the disease and especially in reduction of relapses and disease progression. Specifically, a reduction was shown in the Expanded Disability Status Scale, in the rate of relapses and in the T2 burden of the disease in patients recieving IFNβ-1α therapy during an 8 years-period compared to a group of MS patients with a 2 years later onset of therapy. Also, a reduced annual relapse rate was observed in MS patients after 3 years of treatment compared to baseline (Coppola *et al.*, 2006; Kappos *et al.*, 2006).

Consistent with the results of the present study, previous studies have reported modulation of high serum levels of IL-12 and TNF-α after IFNβ treatment (Fillion *et al.*, 2003; Brod *et al.*, 1996; Wang, 2000; Losy *et al.*, 2002), potentiation of IL-10 production by blood mononuclear cells of MS patients after IFNβ treatment (Chabot et Yong, 2000; Porrini *et al.*,1995; Rudick *et al.*,1998), and increase of IL-4 serum levels after IFNβ treatment using ELISA assay methodology (Rudick *et al.*, 1998; Cebria-Perez *et al.*, 2000). However, decreased numbers of blood mononuclear cells secreting TNF-α and increased numbers of IL-10 secreting blood mononuclear cells were also detected in MS patients after IFNβ treatment using the ELISPOT assay (Ozenci *et al.*, 1999; 2000). Finally, enhancement of IL-10 expression and reduction of IFN-γ producing T cells in MS patients was also observed after IFNβ treatment using a flow cytometric approach

(Rep *et al.*, 1999; Rudick *et al.*, 1996). Therefore, the present data show clearly that IFNβ treatment acts by inhibiting the Th1 immune overexpression underlying MS and at the same time it enhances the reduced Th2 immune response. However, it is still uncertain whether IFNβ treatment acts simultaneously at both Th1 and Th2 immune response or whether it acts only in one of them and this indirectly modulates the other.

Previous studies have reported contradictory results regarding the effect of IFNβ treatment in cytokine levels of MS patients (Furlan *et al*, 2000; Khademi *et al.* 2000; Bahner *et al.*, 2002; Rothuizen *et al.*, 1999; Andersson *et al.*, 1993). Specifically, a transient increase of IFN-γ blood levels after a short-term treatment with IFNβ-1β was observed by solid-phase ELISA without significant modification of IL-4 or TNF-α levels (Nicoletti *et al.*, 2000). In another study no sustained alteration in the plasma levels of IL-12, TNF-α and IL-10 cytokines was found in MS patients after a 3 month treatment period with IFNβ-1α (Duddy *et al.*, 1999).

The differences obtained between our data and other studies may be mainly attributed to the different assay methods used in a variable number of patients with different forms and at different stages of the disease. The ELISPOT assay provides a more precise and valuable assay for the estimation of the secretion of cytokines compared to the other methods since it permits a real time and high sensitivity estimation of the cells that secrete a certain cytokine (Stott, 2000; Sedwick *et al.*,1983). Since other studies (Jansson *et al.*, 2003; Ozenci *et al.*, 2000) using the same assay for cytokine levels estimation, present similar findings and the fact that our data agree with the established course of the

disease (enhancement of Th1 response and weakening of the Th2 immune response during relapses and exacerbations, and reversibility of this status after IFNβ treatment) suggest the credibility of our data. Moreover the present study has the advantage over others (Bienvenu *et al.*, 2000; Drulovic *et al.*, 1997) that cytokine measurements have been performed in parallel from same lymphocyte pools of a satisfactory number of MS patients presenting their cytokinetic profile at a given period.

It is not certain whether overexpression of the Th1 immune response is the cause or the result of the inflammatory lesions in the CNS of MS patients, but it is obvious that this mechanism plays a key role in the pathogenesis of the disease. Evidence in favor to this statement is the clinical improvement observed in these patients after IFNβ treatment which is accompanied by a shift of Th1/Th2 balance towards Th2. This Th1-cytokine overexpression may possibly lead to activation of other immunological molecules (such as matrix metalloproteinases, apoptotic markers, cytokine receptors, adhesion molecules) which are discussed later in this chapter (Contasta *et al.*, 1999; Karni *et al.*, 2006; Rep *et al.*, 1999).

In summary, evaluation of the specificity and sensitivity of the ELISPOT assay in identifying even a small number of cytokine-secreting cells based on our study, provides a promising tool for monitoring IFNβ treatment response in patients with multiple sclerosis. However, before conclusions about exact pathogenesis or suggestion of therapeutic targets can be made, the differences between the two components of IFNβ-(1α and 1β) relating to the regulation of cytokine profile and of other immunological markers need further investigation.

4.1.17 Percentages of cells expressing cytokine receptors in MS

An imbalance in cytokine homeostasis is considered to play a major part in the pathogenesis of immuno-inflammatory diseases (Louis, 2001;Campell and Harrison, 1990; Dayer *et al.*, 2005) and determination of cytokine levels in body fluids is important in defining their role in health and disease. However, the level of a certain cytokine reflects only one aspect of its net effect. Cytokines act mainly through their receptors in order to regulate immune responses and they are not functional in the absence of their receptor on the target cell membrane. Therapeutic approaches are usually developed with the purpose of impeding the interaction between the ligand (cytokine) and its specific receptor, or interactions that involve the use of anti-inflammatory cytokines to switch off inflammation (Moller and Villiger, 2006; Pennline *et al.*, 1994).

While a number of studies have described changes in cytokine levels in MS, expression of cytokine receptors has received little attention (Gimenez *et al.*, 2006; Karni *et al.*, 2006).

In the present study, we estimated percentage of T and NK cells expressing cytokine receptors on their surface in MS patients, with and without IFNβ treatment, using flow cytometry.

4.1.18 Percentage of T and NK cells expressing IL-12R may regulate the cytokine network in MS

MS is associated with elevated levels of IL-12Rβ1 compared to controls (Li *et al.,* 2003; van Boxel-Dezaire *et al.,* 2001). In this study, although the percentages of CD4 and CD8 T cells expressing IL-12Rβ1 did not differ significantly between MS patients and healthy controls, the percentage of IL-12Rβ1 expressing NK cells were significantly higher in patients with MS without or with treatment with IFNβ compared to healthy individuals. Furthermore, PHA-stimulation strongly augmented IL-12Rβ1 in all cell types. Therefore, it is reasonable to suggest that, even in absence of increased levels of IL-12, the net effect of IL-12 on T cells might be augmented by elevated percentage of cells expressing its receptor in MS.

The IL-12/IL-12R system is a turning point for the type of immune response that will develop during activation (Gately *et al.,* 1998; Ozenci *et al.,* 2001). IL-12R seems to be a central molecule in the regulation of the cytokine network that shifts the immune response to Th1 type (Adorini, 1999; Seder *et al.,* 1993).. In vitro, it has been repetitively shown that IL-12R is a marker for Th1 cells (Rogge *et al.,* 1997, 1999). It has also been recently also reported that pulmonary T cells from patients with sarcoidosis, but not asthma, show enhanced IL-12R expression, suggesting the importance of IL-12Rβ1 as a marker for Th1-type autoimmune disease (Chang *et al.,*2000;Rogge *et al.,* 1999; Skarsvik *et al.,* 2005). The present data on percentage of cells expressing IL-12R indicate a clear elevation in percentage of NK cells expressing IL-12Rβ1 and an increased suspectibility of Th1 ($CD4^+$ and $CD8^+$) cells of MS patients compared to controls at expressing higher percentage of IL-12Rβ2 after PHA stimulation. These two types of cells are critical coefficients of the Th1 immune response and since they are shown to be activated by IL-

12 binding to IL-12 receptors, it is indicated that T cells from MS patients are more prone to Th1 type of activation.

Treatment with both forms of IFNβ was proved efficient in decreasing percentage of all cell types expressing IL-12R. Therefore, this implies that IFNβ inhibits the characteristic Th1 immune response by acting through modulation of percentage of cells expressing IL-12R. It is not however, clear if this is a direct action of IFNβ in percentage of cells expressing IL-12R, or indirect through the reduction of IL-12 secretion which furthers leads to downregulation of percentage of cells expressing IL-12R.

4.1.19 Percentage of T and NK cells expressing IFN-γ R has a dual regulatory role on IFN-γ effects

Percentage of peripheral blood cells expressing IFNγR on their surfaces was diminished in untreated patients with MS compared to control subjects. Although the percentage of IFNγR-expressing CD4 and CD8 cells differed significantly between MS patients and controls, IFNγR-expressing NK cells did not show any difference between the two groups. These findings are in accordance with previous studies where expression of the IFNγR-beta chain was low during active untreated disease (Ahn *et al.*, 2004). After IFNβ therapy, the IFNγR-beta/alpha ratio increased after 3 months but had fell at 12 months (Ahn *et al.*, 2004). Increased susceptibility to apoptosis with high IFNγR-beta chain expression at 3 months is likely to remove activated T cells during IFNβ therapy (Ahn *et al.*, 2004; Bongioanni *et al.*, 1999). This can be explained by the fact that IFN-γ R at low concentrations favors the pro-inflammatory actions of IFN-γ, whereas at high concentrations it induces its' apoptotic actions. There is evidence that the elevation of

IFN-γR-beta chain expression after IFNβ therapy enforces the apoptotic action of IFN-γ to activated Th1 lymphocytes (despite the inhibition of its secretion by IFNβ) and by this way affects positively the course of the disease (Bongioanni *et al.*, 1999).

IFNβ therapy ameliorates MS, possibly by lowering IFN-γ secretion and inhibiting responses to IFN-γ (Rep *et al.*, 1999; Yasuda *et al.*, 1999). IFN-γ effects are regulated by IFN-γR expression and it has been shown that IFN-γ is pro-inflammatory at low IFN-γR levels, but induces apoptosis in cells with high IFN-γR levels (Ahn *et al.*, 2004). Given that reduced IFN-γ binding after IFNβ treatment may be related to lymphocyte activation, it is possible that the major effect of IFNβ treatment is a decrease in T-cell activation. T cells are activated at the Th1 immune response mainly by IL-12, IFN-γ, and TNF-α and they are regulated through binding of these cytokines to their receptors. Since IFNβ affects all of these components it raises the possibility of exerting its actions mainly through this mechanism.

4.1.20 Role of percentage of T and NK cells expressing TNFαR in MS

Percentage of NK cells expressing TNFαR was significantly elevated in MS patients compared to normal controls. TNF-α in MS is responsible for peripheral blood leukocyte priming (Dowling *et al.*, 1997). The present findings are consistent with the study of Ziaber *et al.*, 2000 who showed higher serum levels of TNF soluble receptors sp55 and sp75 in MS patients during MS acute exacerbation and in chronic progressive forms as compared to MS remission and other neurological diseases (Ziaber *et al.*, 2000). IFNβ therapy reduced the percentage of cells expressing TNF-αR, further reducing TNF-α

activity during the disease process. This finding suggests that IFNβ exerts of its action by reducing the percentage of cells expressing TNF-αR, thus inhibiting the action of TNF-α at T lymphocytes. Therefore, TNF-α-TNF-α R binding at T lymphocytes is an important mechanism for the development of Th1 immune response in MS as well as a promising selective therapeutic target.

Furthermore, a study in the EAE model showed that immunosuppression by TNF and protection against EAE does not require the p55 TNFR, whereas the same receptor is necessary for the detrimental effects of TNF during the acute phase of the disease (Kassiotis and Kollias, 2001). Thus, blocking the function of the p55 TNFR in autoimmune demyelination may inhibit the noxious proinflammatory activities of TNF without compromising its immunosuppressive properties (Kassiotis and Kollias, 2001).

4.1.21Percentage of T and NK cells expressing IL-10R are involved in suppression of Th2 immune response in MS

Percentage of peripheral blood cells expressing IL-10R was significantly reduced in MS patients compared to controls. Low percentage of all cell types, CD4, CD8 and NK cells, presented IL-10R expression. This was partly expected based on the reduced secretion levels of this cytokine by MS patients. However, PHA stimulation leads to increased expression of IL-10R in these cells up to normal levels. Our data are in agreement with the study of Vandenbark *et al.,* (2001) where reduced frequencies of IL-10-secreting PBMCs were established by ELISPOT in untreated MS patients and healthy controls. Furthermore, these data show clearly the suppression of Th2 immune response in MS

which is partly expected since Th2 activation is inhibited by Thl cytokines which are overexpressed in MS.

IL-10 is a potent inhibitor of IL-12 and IFN-γ and its effects are mediated by the high-affinity IL-10 receptor which is structurally related to IFN receptors (Ho *et al.*, 1993, Liu *et al.*, 1994). The inhibitory role of IL-10 in the pathogenesis of MS and EAE is based on the finding that IL-10 can suppress the expression of inflammatory mediators by activated immune cells and glial cells (Ledeboer *et al.*, 2000; Moore *et al.*, 2001). Furthermore, IL-10R has been shown to recruit the signaling pathways of IL-6-type cytokine receptors, and many IL-10 responses are also induced by IL-6 (Hirano *et al.*, 1998).

Not many studies exist regarding IL-10R expression in peripheral blood of MS patients. Most studies involve the immunohistochemical localization of IL-10R in postmortem brain tissue of MS patients, showing strongest IL-10 immunoreactivity in reactive astrocytes within active demyelinating lesions and the hypercellular rim of chronic active MS lesions (Cannella and Raine, 2004; Hulshof *et al.*, 2002). Moreover, strong immunoreactivity for IL-10R was detected on macrophages in both parenchymal and perivascular areas and on reactive astrocytes in active and chronic MS lesions. These results indicate that IL-10 has an active role in CNS immune responses whilst the specific patterns ofn protein localization and protein expression of IL-10R in MS lesions at different stages of development suggest that IL-10 and its receptor participate in processes leading to the formation of chronic MS lesions (Hulshof *et al.*, 2002).

The present data of IL-10 decreased secretion and suppressed percentage of peripheral T-lymphocytes expressing IL-10R in MS patients, verifies the theory of peripheral Th1 activation and supression of Th2 response in MS and indicates a different role of IL-10 and its receptor at the peripheral immune response compared to its inflammatory actions in the cells of the central nervous system.

IFNβ treatment increased the percentage of cells expressing IL-10R in MS patients after a six month period. Although the exact mechanism of action of IFNβ on IL-10 secretion is not known, there is evidence that it may enhance the capacity of dendritic cells to stimulate autologous T-cells to secrete IL-10 and further upregulate their receptors (Wiesemann *et al.,* 2002). Regarding the differential effects of the two forms of IFNβ treatment, it is clear from our study that IFNβ-1α was more effective in normalizing the percentage of cells expressing the cytokine receptors under study compared to IFNβ-1β, suggesting a different mechanism of action between the two forms of IFNβ, and providing a more promising new therapeutic approach.

4.1.22 Impaired T-cell apoptotic mechanisms in MS: role of CD95

Failure of CD95-mediated apoptosis of autoreactive T cells has been postulated as a major disease mechanism in MS (Pender, 1998). CD95 (Fas) is originally described as a cell surface molecule that belongs to the family of TNF receptors mediating cytolytic cell death. It can be detected in a membrane-bound and a soluble form (Zipp *et al.,* 1998a). CD95 transmits an apoptotic signal in susceptible target cells when induced to trimerize by ligation of agonistic antibody or the natural CD95 ligands (CD95L), another member

of the TNF superfamily (Zurak, 1997; Dhein *et al.,* 1995). The soluble form of CD95 has apoptosis blocking properties, and the function of soluble CD95 as apoptosis inhibitor or promoter is still controversially discussed (Zipp *et al.,* 1999). Activation induced apoptosis may occur in the central nervous system in normal individuals to delete naturally occurring autoreactive T cells (Gold *et al.,* 1997; Zipp *et al.,* 1999). In MS a genetically determined failure of this mechanism may lead to survival of autoreactive T cells and may induce CNS damage (Bilinska *et al.,* 2002; Pender, 1998; Okuda *et al.* 2006). Furthermore, recovery from EAE is associated with apoptosis of T cells, leading to the conclusion that the impaired apoptotic mechanism is a major pathogenic pathway in MS (Macci *et al.,* 1999).

Also, CD95 positive microglial and T cells are infrequently found in brain tissue of patients with MS (Dowling *et al.,* 1996; Kohji and Matsumoto, 2000). Therefore, it is still an open question whether T cells or microglial destructive effects dominate the effects of apoptosis in MS. This section of the present study aimed to clarify whether soluble CD95 may be detected in the sera of MS patients and to investigate the effect of IFNβ treatment upon CD95 expression.

The present data showed that sCD95 levels were detectable and increased significantly in the sera of MS patients compared to healthy controls. This is in contrast to Giusani *et al.,* (1998) who failed to find any differences in sCD95 serum levels of MS patients in different disease stages not classified by disease activity.

However, the present findings are in accordance with the results of Zipp *et al.,* (1998b), who reported elevated serum levels in the relapsing remitting type of MS. However, in

this study they found no correlation with activity as defined by lesion formation using MRI. This observation indicates that the elevation of sCD95 and the consequent activation or inhibition of apoptosis- through binding to CD95L (thus preventing its binding to membrane CD95 and apoptosis), does not affect the CNS cells, but rather additional apoptotic mechanisms may contribute to the impaired apoptotic process in MS. Additionally, Inoue et al., (1997) have demonstrated significantly elevated sCD95 levels in CSF of 40 active RR MS patients compared to 10 patients with other inflammatory neurological diseases but not in comparison with controls. Giusani *et al.*, (1998) showed that the sCD95 CSF/serum ratio was increased in MS patients and concluded that they may reflect an increased sCD95 mediated apoptosis of encephalitogenic autoreactive T cells in the active stage of MS. As it is difficult to draw functional conclusions from the detection of soluble receptors, further studies have dealt with cell-surface molecules and functional *in vitro* assays. Zipp *et al.*, (1998a) has showed that sera of MS patients protected a glioma cell line from CD95 mediated apoptosis leading to the hypothesis of an anti-apoptotic activity in MS.

On the other hand, Alcazar *et al.*, (1998) demonstrated a neuronal apoptosis-inducing potential of CSF specimens of active primary-progressive MS patients. Increased CD95 cell surface expression in T cells in MS was postulated by Ichikawa *et al.*, (1996). However, Zipp *et al.*, (1998a) could not confirm their findings by analysing T-cell surface expression of CD95 and CD95 mediated functional apoptosis in MS versus healthy controls. Macchi *et al.*, (1999) demonstrated *in vitro* that PHA-stimulated peripheral blood mononuclear cells from MS patients were less sensitive to apoptotic signals than cells from controls and are in general accordance with the present data.

Contradictions between the various reports may be due to methodological differences in sCD95 quantitation, as the different commercial kits used probably had different sensitivities and specificities (Mahovic *et al.,* 2004). Since several different functional forms of sCD95 have been described (Cascino *et al.,* 1995), the same primary antibody should be used for all comparative studies. In addition, patient and controls groups in the different studies may not be exactly the same in terms of activity, stage, and duration of disease.

However, due to these contradictory data, sCD95 cannot at present be justified as a MS activity marker.

The mechanism whereby IFNβ elicits its beneficial clinical effects in MS patients is not clear although induction of CD95-mediated T cell apoptosis may play a role (Zipp, 2000; Sharief *et al.,* 2002). In this study, treatment with IFNβ induced an elevation in circulating sCD95 after a six-month period. Information on neutralising antibody status of the patients in the study was not available to allow a similar evaluative analysis to be performed. Therefore, it can be concluded that IFNβ acts by elevating sCD95 at the onset of therapy, thus supporting a beneficial role of this molecule at the course of MS. The subsequent reduction in sCD95 levels can be explained by the immunoregulatory results of long-term IFNβ treatment. The present data are in agreement with this observation (i.e. elevation of sCD95 in short term after the onset of IFNβ treatment) and indicate this effect is a very important mechanism of action of IFNβ in MS.

The results demonstrated in this thesis show that IFNβ-1β was slightly more efficient than 1a at increasing sCD95 levels, reflecting different action mechanisms between the two forms. Although no previous comparative data exist between the two forms of IFNβ, this finding needs further investigation.

In conclusion, the elevated serum sCD95 levels may be a reflection of the immune dysregulation underlying the MS disease process and may explain, to some extent, the paradox of the presence of unequal frequencies of MBP-specific T cells in the peripheral blood of MS patients and healthy individuals (Zhang *et al.*, 1994). Since differences in serum and CSF sCD95 levels were noted in MS clinical studies, sCD95 does not appear to be a definite disease marker in MS (Boylan *et al.*, 2001).

4.1.23 Altered T lymphocyte trafficking in MS due to increased levels of adhesion molecules

Multiple sclerosis is thought to be caused by a T cell-mediated attack on CNS myelin and axons (Brosnan and Raine, 1996; Traugott and Lebon, 1988). The transmigration of leukocytes from the peripheral nervous system into the CNS is a multistep process that occurs in an ordered sequential fashion. A co-ordinated expression of vascular (selectins, ICAM-1, VCAM-1) and leukocyte (LFA-1, VLA-4) adhesion molecules facilitates the adhesive interactions between leukocytes and endothelial cells (Shimizu *et al.*, 1991; Jia *et al.*, 1999). The expression of the selectins, ICAM-1 and VCAM-1 in endothelial cells is upregulated by proinflammatory cytokines IFN-γ and TNF-α (Dhib-Jalbut *et al.*, 1996). Therefore, upregulated TNF-α in MS may play a role in increasing the expression of adhesion molecules, which in turn results in the docking of activated leukocytes to vessel walls.

The expression of the soluble forms of adhesion molecules (VCAM-1 and ICAM-1) in this study was increased in MS patients compared to controls. Contradictory data exist regarding evaluation of serum sICAM-1 and sVCAM-1 in MS. Some studies show an increase in sVCAM-1 and sICAM-1 levels in MS patients compared to controls (Baraczka *et al.*, 2001; Dore-Duffy *et al.*, 1995; Mc Donell *et al.*, 1999), and others show no significant difference between serum s ICAM-1 and s VCAM-1 levels of MS patients and controls (Correale *et al.*, 2003; Droogan *et al.*, 1996). The observation of increased levels of adhesion molecules is in accordance with the current theory of Th1 immune activation in MS, which induces an altered expression of adhesion molecules, further affecting the trafficking of activated Th1 cells into the CNS.

However, the presence of contradictory data which show no difference in the levels of adhesion molecules between MS patients and controls indicate that these molecules may not be of vital importance for the demonstration of Th1 immune response or that Th1 immune response is mainly intrathecal (Kraus *et al.*, 2004; Robinson Agramonde *et al.*, 2001). The differences between the studies may also be attributed to the number of samples that were examined, and to the form and the stage of the disease of the patients under investigation.

Treatment of MS patients with IFNβ resulted in an increase in the plasma concentration of sVCAM-1, and in elevation of the plasma concentrations of ICAM-1. sVCAM-1 has been reported to block the function of the VLA-4 molecule at physiological concentrations, and its production may be induced in brain endothelial cells by IFN-γ and TNF (Kallmann *et al.*, 2000).The effects on plasma concentrations of sVCAM-1 are in

agreement with previous studies (Calabresi *et al.*, 1997; Muraro *et al.*, 2000; Calabresi *et al.*, 2001; Matusevicious *et al.*, 1998).

However, contradictory data exist regarding sICAM-1 concentration where no change was found in plasma levels during treatment with IFNβ (Gelati *et al.*, 1999; Jiang *et al.*, 1997), which agrees with other studies finding either no effect or a transient increase in sICAM-1 concentrations in MS patients treated with IFNβ (Corsini *et al.*, 1997; Kilinc *et al.*, 2003).

There is evidence that IFNβ facilitates the conversion of cell-associated adhesion molecules (such as VCAM-1) into its soluble form, which interacts with its receptor in T cells and, as such, prevents the interaction of the latter with endothelial cells (Calabresi *et al.*, 1997). Several studies indicate that IFNβ also decreases the expression of chemokines and their receptors, reducing the chemotactic signal guiding the way into the CNS for inflammatory cells (Zang *et al.*, 2001; Comabella *et al.*, 2002). These inhibitory actions of IFNβ are crucial for the development of the disease, since they block the transmigration of inflammatory T-cells into the CNS and therefore their destructive effects on myelin sheaths.

It has been previously reported that sVCAM-1 can down-regulate leukocyte expression of CD49d/VLA-4 *in vitro* (Calabresi *et al.*, 1997). However, other studies did not observe any correlation between plasma concentrations of CD49d/ VLA-4 and sVCAM-1, and plasma concentrations of sVCAM-1 were maximally increased already after 1 month of treatment, whereas T cell expression of CD49d/VLA-4 decreased further from 1 month to 3 months of treatment, suggesting that there is no simple relationship between increased

plasma concentrations of sVCAM-1 and decreased T cell expression of CD49d/VLA-4 (Jensen *et al.*, 2005; Muraro *et al.*, 2000).

IFNβ-1β proved slightly more efficient at increasing sVCAM-1 levels, and IFNβ-1α at increasing sICAM-1 levels, supporting the presence of different mechanisms of action between the two components of IFNβ.

4.1.24 Role of MMPs in regulating T lymphocyte migration through BBB and tissue destruction in MS

Matrix metalloproteinases (MMPs) comprise a family of at least 23 zinc-containing endopeptidases that can degrade extracellular proteins (Leppert *et al.*, 2001). MMPs control cell migration across the blood–brain barrier (BBB), by disrupting the sub-endothelial basement membrane and other components of the extracellular matrix (ECM), and eventually affect tissue destruction in MS (Chandler *et al.*, 1997; Lee *et al.*, 1999). MMP activities are tightly regulated in on least three different levels (Hartung and Kieseier, 2000; Ries and Petrides, 1995; Yong *et al.*, 2001): gene transcription, proenzyme activation and activity of tissue inhibitors of metalloproteinases (TIMPs). TIMPs, which are widely expressed in the extracellular milieu, form complexes with either activated MMPs or with their proforms after their secretion and so reduce their activity (Ogata *et al.*, 1995; Shapiro *et al.*, 1995; Yong *et al.*, 2001). The role of MMPs in controlling transmigration of activated T lymphocytes through the blood-brain-barrier,

makes interesting the study of their expression-and of their inhibitors–in MS patients in order to detect their possible role in the developement of the disease.

MS patients without treatment showed significantly increased MMP-9 levels, compared to healthy controls. Increased expression of various MMPs (MMP-2, -3, -7 and -9) have previously been demonstrated in postmortem MS brains (Anthony *et al.*, 1997; Cuzner *et al.*, 1996), and MMP-9 has been detected in acute MS lesions and localized in macrophages and astrocytes and also in white matter perivascular mononuclear cells (Cuzner *et al.*, 1996; Maeda and Sobel, 1996). In EAE, the animal model for MS, both MMP-9 and MMP-7 had their expression peak at the same time as the appearance of clinical signs (Kieseier *et al.*, 1998). Also, in MS, high gelatinase B (MMP-9) activity has been shown in cerebrospinal fluid (CSF) (Gijbels *et al.*, 1992). Increased MMP-9 CSF levels in MS patients were associated with BBB damage using gadolinium (Gd)-magnetic resonance imaging (MRI), and methylprednisolone treatment, by downregulating MMP transcription, reduced both Gd enhancement and CSF MMP-9 (Rosenberg *et al.*, 1996). Elevated MMP-9 mRNA levels in peripheral blood mononuclear cells (PBMNCs) (Kouwenhoven *et al.*, 2001; Lichtinghagen *et al.*, 1999; Ozenci *et al.*, 1999) and raised MMP-9 levels in serum and CSF from MS patients have also been detected (Lee *et al.*, 1999; Leppert *et al.*, 1998; Lichtinghagen *et al.*, 1999; Avolio *et al.*,2003), and they were associated with clinical or MRI disease activity (Lee *et al.*, 1999; Waubant *et al.*, 1999). The present study shows increased MMP-9 levels in the serum of MS patients without treatment compared to controls, potentiating further the involvement of MMPs in the regulation of cell migration across the BBB, by disrupting the extracellular matrix, and affecting tissue destruction in these patients.

Tissue MMP activity reflects a balance between MMPs and TIMPs. In the present study MS patients showed decreased TIMP-1 levels compared to controls. It has been hypothesized (Bever and Rosenberg, 1999) that early in MS lesions, increased MMP-9 is followed by BBB damage, but later, during repair processes, TIMPs and MMP-2 may prevail by remodeling of the extracellular matrix, which may correspond with the chronic progressive stage of MS (Wjtowicz-Praya *et al.,* 1997). These changes in the MMPs action may be partly attributed to the immunomodulatory therapy, but on the other hand they may reflect a physical remodeling attempt including TIMPs actions, related to the physiological roles of metalloproteinases.

The MMP-9/TIMP-1 ratio in serum is higher in MS than normal controls in this study. The increase is due to MMP-9 activity which results in higher values in MS patients. More interestingly, a higher serum MMP-9/ TIMP-1 ratio is linked to the phase of activity of the disease evaluated either clinically or from the Gd-MRI, compared to inactive disease. This increase in the ratio would be compatible with a prevalence of protease over its inhibitor contributing, on pathological grounds, to disease activity. These findings are in agreement with previous reports (Lee *et al.,* 1999; Waubant *et al.,* 1999; Fainardi *et al.,* 2006) showing that high levels of MMP-9 were associated to clinical or MRI disease activity. Lichtinghagen *et al.* (1999) reported a higher MMP-9/TIMP-1 ratio in active MS patients even compared to healthy controls. This indicates that there is a shift of the balance between MMP-9 and TIMP-1 towards MMP-9 in MS patients, thus facilitating the entry of T lymphocytes in to the CNS through the BBB according to our hypotesis.

Treatment with IFNβ decreased MMP-9 levels, increased TIMP-1 levels and reduced the MMP-9/TIMP-1 ratio in MS patients. Strong evidence is emerging that MMPs initiate early proteolytic activity on the integrity of blood brain barrier (BBB) (Lukes *et al.*, 1999; Rosenberg *et al.*, 1992; 1996; Opdenakker *et al.*, 2003) and that IFNβ, through its effects on MMPs, might contribute to restoration of the damaged BBB (Leppert *et al.*, 1996; Stuve *et al.*, 1996; Ma *et al.*, 2001; Bartholome *et al.*, 2001; Galboiz *et al.*,2002; Nelissen *et al.*,2002; Uhm *et al.* 1999; Waubant *et al.*,2003).

Trojano et al. reported that IFNβ1β treatment decreased MMP-9 expression in RRMS patients (Trojano *et al.*, 1999). A follow-up study of 6 patients for 12 months has shown that IFNβ treatment was associated with suppression of MMP-9 and MMP-7 mRNA in RRMS patients (Galboiz *et al.*, 2001). However, no significant changes were observed in secondary progressive MS group in this study. Therefore it can be concluded that IFNβ treatment reduces MMP-9 expression in MS pariens with the relapsing-remitting form of the disease, without having such an effect in the secondary progressive form of the disease. This observation indicates that either MMP's have no significant role in the pathogenic mechanisms of secondary progressive MS, or that IFNβ has not for some reason (due to anti-IFNβ antibodies, different pathogenic mechanisms) immunomodulatory effects on MMPs in the secondary progressive form of the disease.

Two studies have also evaluated the effects of IFNβ on TIMP-1 levels longitudinally. The numbers of TIMP-1 mRNA expressing blood mononuclear cells were higher after 6–12 months of IFNβ treatment compared with pre-treatment values where MMP-9 and 3 expressing cells were decreased (Ozenci *et al.*, 2000). In 38 patients with MS, MMP-2

and TIMP-1 levels were found similar during 9 months of IFN β therapy, whereas MMP-9 levels significantly decreased and TIMP-2 levels significantly increased in comparison with values obtained before treatment (Giannelli *et al.*, 2002).

Finally it has been suggested that the more pronounced effects of IFNβ might be via increasing TIMP-1 levels rather than changing MMP-9 levels (Karabudak *et al.*, 2004; Waubant *et al.*, 2003). Taken into account that TIMP-1 decreases activated T cell migration; and MMP/TIMP balance restores BBB integrity; the data of this study are in accordance with the clinical stability of MS patients and the present study data showing an increase in TIMP-1 and decrease in MMP-9 after IFNβ treatment of MS patients, are in agreement with these results

4.2 Conclusions

According to the experimental data presented in this thesis (increased TNF-α, IL-12 and IFN-γ secretion, and decreased IL-10, IL-4 secretion from PBMCs of multiple sclerosis patients), the basic conclusion is the presence of a strong stimulation of Th1 immune response and a suppression of Th2 immune response in multiple sclerosis patients (i.e. a shift of the immune response towards the Th1 pathway and activation of the cell-mediated immunity).

The changes observed in cytokine levels are accompanied by an altered cytokine receptor expression profile in key immune cells. In particular, it was observed an increased expression of IL-12β1R and TNF-αR by NK cells, and a reduced expression of IFN-γR and IL-10R by T cells. These alterations lead to an overexpression of the Th1 immune response which results to increased production of the sCD95 apoptotic marker, followed

by an increased production of sVCAM-1 and sICAM-1 adhesion molecules, increased expression of MMP-9 and reduced levels of TIMP-1. Consequently, all these molecules participate towards an infiltration process of activated T-lymphocytes in the CNS, possibly contributing to the initiation or the preservation of the inflammatory process that causes damage to the oligodendrocytes and their myelin sheaths, resulting in the clinical phenotype of MS.

Therefore, especially the increased pro-inflammatory cytokines seem to act mainly by: a) regulating the levels of their receptors in T and NK cells, b) increasing the sCD95 levels and thus reducing the apoptotic rates of T-lymphocytes, c) increasing the membrane and soluble forms of adhesion molecules VCAM-1 and ICAM-1, facilitating by this way the interactions between the surface adhesion molecules of the activated T lymphocytes and VCAM-1 and ICAM-1, and therefore, the passage of these cells through the BBB and their entry into the CNS, d) increasing MMP-9 levels, reducing TIMP-1 levels, and increasing the MMP/TIMP-1 ratio, thus inducing the disruption of the BBB and the adhesion of activated T lymphocytes at the vascular endothelium, facilitating the entry of these cells in the CNS.

This activation of Th1 immune response appears to be reversed by IFNβ treatment resulting in clinical improvement (prevention of the relapses) of these patients. Th1 stimulation may be either directly the cause of the myelin damage by direct cytokine action at myelin or cytokine induced apoptosis of oligodendrocytes or the immune reaction against the already damaged myelin by another cause (a virus for example). In both cases an antigenic stimulation, endogenous or exogenous or both is necessary for the

start and the demonstration of Th1 lymphocyte response at central nervous system and peripheral blood. Stimulated Th1 lymphocytes of the peripheral blood probably avoid regulatory apoptotic mechanisms and enter in the CNS under the influence of chemotactic agents enforcing the inflammatory process. Induction of adhesion molecules at the endothelium of the blood brain barrier by cytokines, and disruption of the blood brain barrier by matrix metalloproteinase's which are secreted by T cells and monocytes/macrophages facilitates the entry of Th1 cells in the CNS. In parallel to the activation of cellular immunity by the Th1 immune response, it seems that activation of humoral immunity occurs, since IFN-γ may induce the differentiation of B-cells to plasma cells secreting IgG immunoglobulins (antibodies) against myelin components in the CNS.

IFNβ has a strong immunomodulatory action in the MS patient. Based on the present data and the experimental material of other authors, IFNβ possibly enhances the Th2 immune response and at the same time inhibits the Th1 immune response. The present hypothesis regarding this phenomenon is that IFNβ exerts it's action mainly by stimulating the secretion of IL-10, thus inhibiting the secretion of IL-12 which is the first step for the demonstration of Th1 immune response (our data show that IFNβ-1a is more effective at decreasing IL-12 and that IFNβ-1β is more effective at decreasing TNF-α and IFN-γ and elevating IL-4 and IL-10) (Soilu-Hanninen *et al.*, 1995). Other interesting actions of IFNβ which may contribute to their immunomodulatory action at MS patients are: inhibition of expression of MHC II molecules and induction of expression of MHC I molecules, inhibition of cellular multiplication, and especially the inhibition of Th1 cells migration into the CNS. According to this hypothesis IFNβ may act at other components of Th1

immune response too. Indeed, IFN-β was shown to affect all the main players of the MS immune response. In particular, it reduces the levels of the serum pro-inflammatory cytokines (IFN-γ, TNF- α and IL-12), increases the levels of serum anti-inflammatory cytokines (IL-4 and IL-10), decreases the expression of IL-12β1 R and TNF-αR, increases IFN-γR and IL-10R expression levels, raises serum CD95, ICAM-1 and VCAM-1 levels, as well as TIMP-1, and lowers serum MMP-9 levels and the MMP-9/TIMP-1 ratio. IFNβ-1α was proved more effective at decreasing IL-12 levels and its receptor IL-12β1R expression in T and NK cells, increasing sICAM-1 levels, decreasing MMP-9 levels, increasing TIMP-1 levels, and decreasing MMP-9/s TIMP-1 ratio; whereas IFNβ-1β was more effective in decreasing IFN-γ and TNF-α levels, increasing IL-4 and IL-10 levels, decreasing TNF-αR levels in T cells, increasing s CD95 levels, and sVCAM-1 levels.

Although there are some contradictory data, mainly due to differences in the number of samples, the type of methods, as well as the phase and type of the disease, our present data of MS immunopathology and its potential modulation by IFNβ therapy provide valuable evidence since they are in accordance with the disease process and are accompanied by clinical improvement of MS patients. Moreover this immunopathological model based on our data and the potential different actions of IFNβ-1α and -1β forms over the disease process, are very promising areas for further investigation. The formulation of a therapeutic approach targeting the cytokine network in parallel with the immunomodulatory influence of IFNβ (1α and 1β) in MS may provide a novel diagnostic and prognostic prospective for effective disease management based on its autoimmune background.

Furthermore, the ELISPOT method proves to be a useful, and a very sensitive assay for cytokine detection by comparison of its results with other currently available methods.

4.3 Scope for future studies

Future studies that would allow further elucidation of the immunopathological mechanisms underlying MS should involve estimation of the frequency of IL-1, IL-6 and IL-10 group of pro-inflammatory cytokine-secreting cells, namely IL-19, and IL-22, as well as of the Th3 cytokine, TGF-β at MS patients with and without treatment with IFNβ (-1α and -1β) and controls using the ELISPOT assay. This would provide a full picture of the cytokine status of MS patients as well as of the effect of IFNβ.

Furthermore, estimation of IL-1, IL-4, IL-6, IL-10, 1L-12, 1L-19, 1L-22, IFN-γ, TNF-α, and TGF-β levels in the CSF of MS patients of untreated patients compared to treated with IFNβ would be very important in order to investigate the generalization of the Th1 immune response into the CNS or just in the periphery of these patients.

The study may also be expanded by the evaluation of further cytokine receptor expression in peripheral blood cells as well by the estimation of additional metalloproteinases, MMP-2, and MMP-3 in the serum and CSF of MS patients before and after treatment.

Additional molecular studies for the presence of HHV-1, HHV-6, HLTV-1 and measles viral components in the DNA of MS patients would provide important information on the

involvement of possible viral exogenous agents, participating in the triggering of the immune reactivity observed in multiple sclerosis.

Appendix I

Questionnaire

I. Demographic data

Marital status. The participants were divided into two sub-groups: married and single

Educational level. Three groups were formed: up to 6 years of education, between 6 and 12 years, over 12 years of education.

Employment. Two groups were formed: currently employed and unemployed.

II. Life-style features

Cigarette-smoking. Two sub-groups were formed: non smokers and smokers

Alcohol. Three subgroups were formed: Heavy drinkers (more than 1 drink per day), moderate drinkers (3 times weekly up to 1 drink daily), light or non drinkers (less than 2 times weekly, less than two drinks).

Leisure physical activity. Four groups were formed: no activity, less than 1 hour per week, between 2-3 hours per week and 3hours or more per week.

III. Personal history

Incidence of optic neuritis.

History of autoimmune disease.

History of inflammatory disease.

History of viral infections. The participants were asked specifically for previous HIV, HTLV and Hepatistis V infections

Vaccinations. The participants were asked specifically for Influenza and hepatitis B vaccinations

Family history of mental disorders. The participants were asked specifically specifically for incidences of depression, anxiety disorders and psychotic disorders.

Appendix II

Suppliers

Glycerol	Sigma-Aldrich, Hellas
Triglyceride monoreagent mixture	Linear Chemicals, Spain
Glucose 100mg/dl	Linear Chemicals, Spain
Glucose monoreagent mixture	Linear Chemicals, Spain
HDL-C enzyme reagent	Linear Chemicals, Spain
POD/4-AA reagent	Linear Chemicals, Spain
Cholesterol monoreagent	Linear Chemicals, Spain
Cholesterol 200 mg/dL	Linear Chemicals, Spain
sCD95 (Fas) ELISA kit	Diaclone Research,France
sVCAM-1 ELISA kit	Diaclone Research,France
sICAM-1 ELISA kit	Diaclone Research,France
MMP-9 ELISA kit	R&D systems, Minneapolis, MA
TIMP-1 ELISA kit	R&D system Minneapolis, MA
ELISPOT plates	Diaclone Research,France
Antibodies (primary and secondary)	Becton-Dickinson Mountain View, CA

Materials

Solutions and Buffers

Triglyceride nonoreagent mixture	PIPES buffer 50 mmol/L pH 6.8, LPL $\geq$ 12 KU/L, GK $\geq$ 1 KU/L, GPO $\geq$ 10 KU/L, ATP 2.0 mmol/L, Mg^{2+} 40 mmol/L, POD $\geq$ 2.5 KU/L, 4-AA 0.5 mmol/L, phenol 3 mmol/L, non-icon tensionactives 2 g/L (w/v)
Glucose monoreagent mixture	Phosphate 70mmol/L, Phenol 5 mmol/L, Glucose Oxidase 10 U/mL, Peroxidase 1 U/mL, 4-aminoantipyrine 0.4 mmol/L
HDL-C enzyme reagent	GOOD'S buffer 100mmol/L pH 7.0, $MgCl_2$ 18 mmol/L, CE 800 U/L, CO 500 U/L, catalase 100 KU/L, HDAOS 0.7 mmol/L
POD/4-AA reagent	POD 4 KU/L, 4-AA 4 mmol/L, N_3Na < 0.1 %, specific surfactants < 1.5 % (v/v)
Cholesterol monoreagent mixture	PIPES 200 mmol/L pH 7.0, sodium cholate 1 mmol/L, cholesterol esterase > 250 U/L, cholesterol oxidase > 250 U/L, peroxidase > 1 KU/L, 4-aminiantipyrine 0.33 mmol/L, ADPS 0.4 mmol/L, non-ionic tensioactives 2 g/L (w/v)

Triglycerides standard	Glycerol 2.26 mmol/L, equivalent to 200 mg/dL of glycerol trioleate
Glucose standard solution:	Glucose 100 mg/dL
	Cholesterol standard. Cholesterol 200 mg/dL

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