MEASUREMENT OF BIOACTIVITY OF IMMUNOMODULATORY TREATMENT OF RELAPSING MULTIPLE SCLEROSIS

WITH EMPHASIS ON MxA PROTEIN

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ABSTRACT

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Measurement of bioactivity of immunomodulatory treatment of relapsing multiple sclerosis with emphasis on MxA protein

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Background Multiple sclerosis (MS) is a demyelinating disease of the central nervous system, which mainly affects young adults. In Finland, approximately 2500 out of 6000 MS patients have relapsing MS and are treated with disease modifying drugs (DMD): interferon- β (INF-β-1a or INF-β-1b) and glatiramer acetate (GA). Depending on the used IFN-β preparation, 2 % to 40 % of patients develop neutralizing antibodies (NAbs), which abolish the biological effects of IFN-β, leading to reduced clinical and MRI detected efficacy. According to the Finnish Current Care Guidelines and European Federation of Neurological Societis (EFNS) guidelines, it is suggested to measure the presence of NAbs during the first 24 months of IFN-β therapy.

Aims The aim of this thesis was to measure the bioactivity of IFN-β therapy by focusing on the induction of MxA protein (myxovirus resistance protein A) and its correlation to neutralizing antibodies (NAb). A new MxA EIA assay was set up to offer an easier and rapid method for MxA protein detection in clinical practice. In addition, the tolerability and safety of GA were evaluated in patients who had discontinued IFN-β therapy due to side effects and lack of efficacy.

Results NAbs developed towards the end of 12 months of treatment, and binding antibodies were detectable before or parallel with them. The titer of NAb correlated negatively with the amount of MxA protein and the mean values of preinjection MxA levels never returned to true baseline in NAb negative patients, but tended to drop in the NAb positive group. The test results between MxA EIA and flow cytometric analysis showed significant correlation. GA reduced the relapse rate and was a safe and well-tolerated therapy in IFN-β-intolerant MS patients.

Conclusions NAbs inhibit the induction of MxA protein, which can be used as a surrogate marker of the bioactivity of IFN-β therapy. Compared to flow cytometric analysis and NAb assay, MxA-EIA seemed to be a sensitive and more practical method in clinical use to measure the actual bioactivity of IFN-β treatment, which is of value also from a cost-effective perspective.

Key words: Multiple Sclerosis, interferon-beta, glatiramer acetate, neutralizing antibodies, binding antibodies, immunogenicity, MxA

YHTEENVETO

Anna-Maija Vallittu

Immunomoduloivan hoidon biologisen vasteen mittaaminen aaltomaisessa multippeliskleroosissa käyttäen MxA-proteiinimääritystä

Taustaa Multippeliskleroosi (MS) on nuorten aikuisten merkittävin demyelinoiva keskushermoston sairaus. Suomessa on 6000 MS potilasta, joista noin 2500 saa aaltomaisesti etenevään MS-tautiin taudinkulkua muuntavaa lääkehoitoa: beeta-interferonia (INF-β-1a tai INF-β-1b) tai glatirameeriasetaattia (GA). Käytetystä IFN-β-valmisteesta riippuen 2-40 %:lle hoidetuista potilaista kehittyy IFN neutraloivia vasta-aineita (NAb), joiden on todettu vähentävän hoidon tehoa sekä kliinisen oirekuvan että aivojen magneettitutkimuksessa todettujen muutosten perusteella. Suomalaisen Käypä Hoito ja eurooppalaisen EFNS suosituksen mukaisesti NAb tulisi mitata ensimmäisten 24 kk aikana IFN-β-hoidon aloituksesta.

Tarkoitus Tämän väitöskirjatyön tarkoituksena oli mitata IFN-β-hoidon biologista vastetta mittaamalla IFN indusoimaa MxA proteiinia (myxovirus resistance protein A) ja arvioida sen korrelaatiota neutraloiviin vasta-aineisiin. Lisäksi kehitettiin aiempaa nopeampi ja yksinkertaisempi MxA proteiinia mittaava EIA-testi ja selvitettiin glatirameeriasetaatin siedettävyyttä ja turvallisuutta MS-potilailla, jotka olivat joutuneet keskeyttämään IFN-β-hoidon sivuvaikutusten ja tehon heikkenemisen vuoksi.

Tulokset NAbs kehittyivät ensimmäisten 12 hoitokuukauden aikana ja sitoutuvat vasta-aineet ilmaantuivat joko edeltävästi tai samanaikaisesti NAb kanssa. NAb tiitterien ja MxA proteiinitason välillä oli negatiivinen korrelaatio. MxA proteiinin perustaso laski NAb positiivisilla, mutta ei NAb negatiivisilla potilailla. MxA-EIA testin ja virtaussytometrianalyysin tulosten välillä oli merkitsevä korrelaatio. GA vähensi relapsien määrää ja osoittautui turvalliseksi ja hyvin siedetyksi hoidoksi potilailla, jotka eivät kyenneet käyttämään IFN-β-hoitoa.

Johtopäätökset NAb estävät MxA proteiini-induktiota, jota voidaan käyttää IFN-β-hoidon biologisen vasteen mittarina. Virtausytometrianalyysiin ja NAb testiin verrattuna MxA-EIA proteiinimääritys on helppokäyttöisempi ja kliiniseen käyttöön riittävän herkkä menetelmä mittaamaan IFN-β-hoidon biologista vastetta. Tällä on myös kustannus-vaikuttavuudellista merkitystä.

Avainsanat: multippeliskleroosi, beetainterferoni, glatirameeriasetaatti, neutraloivat vasta-aineet, sitoutuvat vasta-aineet, immunogeenisyys, MxA

CONTENTS

ABBREVIATIONS

AMs	adhesion molecules
APC	antigen presenting cell
ATCC	American Type Culture Collection
BAb	binding antibodies
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CD	cluster of differentiation
CDMS	clinically definite MS
CIS	clinically isolated syndrome
CNS	central nervous system
CPE	cytopathic effect
CSF	cerebrospinal fluid
DMD	disease-modifying drug
EAE	experimental allergic
EDSS	expanded disability status scale
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate
GA	glatiramer acetate
Gd	gadolinium
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ISG	interferon-stimulated gene
ISRE	interferon-stimulated response element
MBP	myelin basic protein
MMP	matrix metalloproteinase

MOG	myelin oligodendrocyte-glycoprotein
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MxA	myxovirus resistance protein A
NAb	neutralizing antibodies
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
RRMS	relapsing-remitting MS
SPMS	secondary progressive MS
TNF-α	tumor necrosis factor-α
TRAIL	tumor necrosis factor related apoptosis inducing ligand
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
VSV	vesicular stomatitis virus
XAF-1	x-linked inhibitor of apoptosis factor-1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV.

I   Vallittu A-M, Halminen M, Peltoniemi J, Ilonen J, Julkunen I, Salmi A, Erälinna J-P. Neutralizing antibodies reduce MxA protein induction in interferon-beta-1a-treated MS patients. Neurology 2002;58:1786-1790.

II   Vallittu A-M, Salmi AA, Erälinna J-P. MxA protein induction in MS patients treated with intramuscular IFN-β-1a**.** Neurol Sci 2006;26:438-443.

III   Vallittu A-M, Erälinna J-P, Ilonen J, Salmi AA, Waris M. MxA protein assay for optimal monitoring of IFN-β bioactivity in the treatment of MS patients (submitted)

IV   Vallittu A-M, Peltoniemi J, Elovaara I, Kuusisto H, Färkkilä M, Multanen J, Erälinna J-P. The efficacy of glatiramer acetate in beta-interferon intolerant MS-patients. Acta Neurol Scand 2005;112:234-237.

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1.   INTRODUCTION

Multiple Sclerosis (MS) is one of the most important inflammatory demyelinating diseases of the central nervous system (CNS), which mostly affects young adults causing remarkable disability. Its incidence in Europe and North America is approximately 0.1 % (Noseworthy et al. 2000). MS is characterized by various clinical disease courses, heterogenous genetic backgrounds and immunopathogenetic subtypes (Reindl et al. 2006). Clinical presentations vary from benign to classical relapsing-remitting MS (RRMS), to primary progressive MS (PPMS) and to the secondary progressive form of MS (SPMS). In addition, rare fulminant disease courses are possible.

Neuropathologically, MS is characterized by multiple foci of myelin sheath lesions that especially exhibit infiltrates of T lymphocytes and microglia/macrophages (Babbé et al. 2000, Jacobsen el al. 2002). Axonal lesions correlate with long-term irreversible disability, and recent studies have revealed that this destruction of axons begins early during the disease course (Trapp et al. 1999). In later stages of the disease, atrophy of grey and white matter is also involved in the accumulating disability of MS patients. The long-term treatment of MS should abolish the predominantly inflammation-induced demyelination and reduce ongoing axonal and neuronal loss. Interferon-beta (IFN-β) and glatiramer acetate (GA) are the most frequently applied therapies in MS, and an early start of the treatment is recommended (Jacobs et al. 2000, Comi et al. 2001, Kappos et al. 2006). Reduced relapse rates and delay in disability progression have been observed after initiation of immunomodulatory therapy together with reductions in MRI activity and accumulation of lesion burden (The IFN-beta MS study group 1993).

However, some patients have to discontinue the immunomodulatory therapy because of side effects. In addition, depending on the used IFN-β preparation, 2 to 40 % of patients develop neutralizing antibodies (NAb) to IFN-β, which is associated with reduced clinical and MRI detected efficacy (The IFNβ multiple sclerosis study group 1996, PRISMS 1998, Panitch et al. 2002). Recently published EFNS (European Federation of Neurological Societies) guidelines on the use of anti-IFN-β antibody measurements in MS recommend that tests for the presence of NAbs should be performed in all patients at 12 and 24 months of therapy. In patients with NAbs, measurements should be repeated at 3-6-months intervals, and if high titres of NAbs are maintaned the IFN-β therapy should be discontinued (Sørensen et al. 2005).

The aim of this thesis was to measure biological efficacy markers of IFN-β therapy by focusing on MxA protein expression and its correlation to NAbs. Due to the lack of a practical and low cost assay for measuring these markers, an MxA EIA assay was set up to replace flow cytometric analysis of MxA and antiviral bioassay detecting NAbs. In addition, tolerability and efficacy of GA were evaluated in IFN-β-intolerant MS patients.

2.   REVIEW OF THE LITERATURE

2.1   Multiple Sclerosis

MS is a chronic inflammatory demyelinating disease of the central nervous system (CNS) which affects 1.1 million people worldwide. It is one of the most common disability-causing neurological diseases of young adults and creates a high economic burden for affected individuals and society (Jönsson 2006). The etiology of MS is multifactorial with genetic and environmental influences, but specifically unknown. It is assumed that a number of infectious agents may trigger the disease in genetically susceptible individuals (Compston & Coles 2002, Sospedra & Martin 2005). A recent study revealed the presence of viral genomic sequences in the cerebrospinal fluid (CSF) of MS patients as markers of viral replication within the CNS (Mancuso et al. 2007). However, no causative pathogenic viruses have been isolated from the brains of MS patients despite attempts to identify such an agent.

Uneven geographic distribution is typical of MS. It is most common in populations of northern European descent, especially in Scandinavia, the British Isles, the northern parts of the United States and Southern Canada (Compston 1997). In addition, northwestern Sardinia (Sotgiu et al. 2002) and Sicily (Grimaldi et al. 2001), along with western Hungary (Guseo 2004) and northern Croatia (Materljan & Sepcic 2002), are small foci of higher prevalence. In Finland, the approximate prevalence of MS is 100/100 000 inhabitants, and the incidence 4-5/100 000/year, but regional differences both in prevalence and incidence are clear. According to epidemiologic studies, there are three foci in Finland with approximately double the prevalence of the estimated national average: in southern Ostrobothnia, western Finland (Sumelahti et al. 2000 and 2001), in southwestern Finland (Panelius 1969) and in southeastern Finland (Kinnunen et al. 1983). Especially in the Seinäjoki district the prevalence (219/100 000) and incidence (12/100 000) of MS have been exceptionally high (Sumelahti et al. 2000 and 2001). Women are affected twice as often as men, but in Seinäjoki an increased incidence trend has been observed for men (Sumelahti et al. 2000). In addition, the proportion of patients with primarily progressive disease, which is associated with poor prognosis, has been found to be higher in southern Ostrobothnia (Sumelahti et al. 2003). MS is rare in children. Only in 2-5 % of MS patients has the disease been diagnosed before 16 years of age (Ness et al. 2007).

MS begins most commonly with sporadic neurological deficits (relapses) separated by periods of remission. Pathologically, relapses are characterized by patchy loss of myelin particularly in the periventricular white matter, optic nerves, brainstem and

spinal cord. This demyelination disturbs nerve conduction, which manifests in visual, cerebellar, motor and sensory symptoms. Cognitive impairment, bladder and bowel incontinence and sexual difficulties are also common. About 85 % of patients experience this relapsing-remitting course of MS (RRMS). Within 10 to 15 years, 50 % of RRMS patients begin to experience gradual progression of disability with or without relapses, developing the secondary-progressive form of MS (SPMS) (Figure 1). In a part of the patients, the clinical course of MS is progressive from the beginning, which is called primary-progressive MS (Sospedra & Martin 2005).

Most of the patients with MS initially present with a clinically isolated syndrome (CIS) suggestive of demyelinating disease. A subgroup of these patients does not progress to MS but risk factors for conversion of CIS to clinically definite MS (CDMS) have been identified (Tintore et al. 2003, Selmaj et al. 2006, Thrower 2007). Abnormal baseline MRI with multiple lesions, motor, cerebellar or brainstem involvement, multifocal presentation and poor recovery after a first CIS event have been considered to raise the risk of conversion to CDMS (Selmaj et al. 2006). A European study looked at a long-term risk of abnormalities on brain MRI for the development of MS in different manifestations of CIS including optic neuritis, transverse myelitis, and brainstem syndromes. An abnormal brain MRI was associated with an 83 % risk of MS within 10 years compared with an 11 % risk with a normal MRI (O´Riordan et al. 1998).
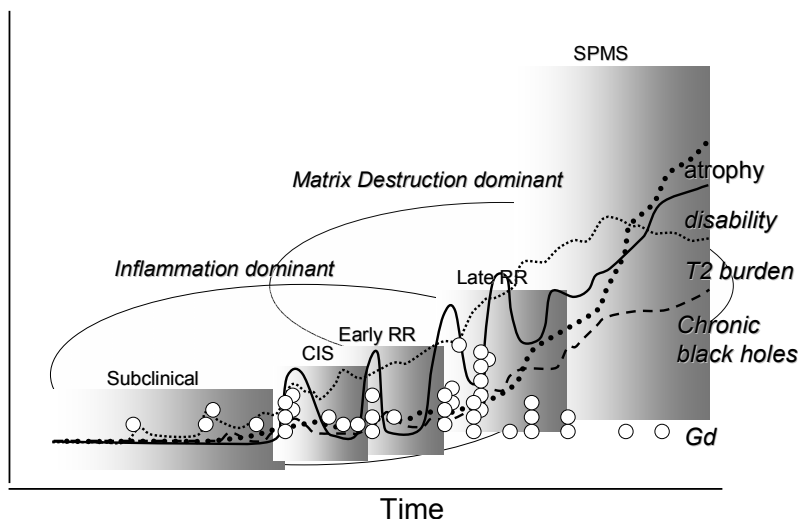
Figure1. The clinical course of RRMS. Inflammation is a dominant feature in the subclinical and relapsing phase of the disease, when MRI is characterized by Gd-enhancing lesions as a marker of active disease. However, axonal damage occurs early in the disease course. The later phase and secondary progressive form of the disease (SPMS) are characterized by increased disability and matrix destruction with typical MRI findings such as brain atrophy, increased T2 lesion burden and chronic black holes with rare enhancing lesions. CIS=clinically isolated syndrome; Gd=gadolinium.

The diagnosis of MS has included a combination of both clinical and paraclinical studies (imaging, CSF analysis, visual evoked potentials and so forth), because no single clinical feature or diagnostic test is sufficient for MS diagnosis. Poser´s criteria, introduced in 1983, were used until 2001, when new diagnostic criteria of McDonald were introduced (McDonald et al. 2001). The criteria were revised in 2005 to simplify and speed the diagnosis, while maintaining adequate sensitivity and specifity (Polman et al. 2005). The core concept in MS diagnosis is to determine dissemination of lesions in time and space, in which the role of MRI is emphasized (Barkhof et al. 1997, Tintoré et al. 2000) (Table 1). In the study of Tintoré et al. the new criteria showed a sensitivity of 74 %, a specificity of 86 %, and an accuracy of 80 % in predicting conversion to CDMS. One year after symptom onset, more than three times as many

Table 1. The revised McDonald diagnostic criteria for MS

MRI dissemination in time	1) detection of Gd enhancement at least 3 months after the onset of initial clinical symptoms, if not at the site corresponding to the initial event or 2) detection of new T 2 lesion if it appears at any time compared with reference scan done at least 30 days after the onset of clinical event
MRI dissemination in space	**Three of the following:** 1) At least one Gd+ lesion or nine T2 hyperintense lesions if there is no Gd+ lesion 2) At least one infratentorial lesion 3) At least one juxtacortical lesion 4) At least three periventricular lesions A spinal cord lesion can be considered equivalent to a brain infratentorial lesion.
Clinical Presentation: -Two or more attacks, objective clinical evidence of two or more lesions	**Additional data needed for MS diagnosis:** none
-Two or more attacks, objective clinical evidence of one lesion	Dissemination in space in -MRI or -two or more MRI-detected lesions consistent with MS and positive CSF (oligoclonal bands or increased IgG index) or -new clinical attack implicating a different site
-One attack, objective clinical evidence of two or more lesions	Dissemination in time demonstrated by -MRI or -second clinical attack
-One attack, objective clinical evidence of one lesion (CIS)	Dissemination in time demonstrated by -MRI or -second clinical attack and Dissemination in space demonstrated by -MRI or -two or more MRI-detected lesions consistent with MS and positive CSF
Insidious neurological progression suggestive of MS	One year disease progression (retrospectively or prospectively determined) and Two of the following: -Positive brain MRI (nine T2 lesions or four or more T2 lesions with positive VEP) -Positive spinal cord MRI (two focal T2 lesions) -positive CSF

VEP=visual evoked potentials; CSF=cerebrospinal fluid; Gd=gadolinium
MRI criteria are based on data from Barkhof et al. and Tintoré et al.
(Polman et al. 2005)

patients with CIS were diagnosed with MS using new diagnostic criteria incorporating MRI results compared to older criteria. Thus, these criteria have enabled earlier diagnosis of MS, even after a single demyelinating event (Tintoré et al. 2003).

2.1.1 Pathogenesis of MS

Inflammatory demyelination and axonal transection are the main pathological causes of neurological disability in MS. Based on studies in experimental autoimmune encephalomyelitis (EAE), an animal model for MS, several hypotheses have been introduced to explain the relationship between inflammation and neurodegeneration. Theories exist in which inflammation causes neurodegeneration, neurodegeneration causes inflammation, inflammation and neurodegeneration participate in a cycle where they augment one another and in which inflammation protects against neurodegeneration (Peterson & Fujinami 2007). Pathophysiological processes, which include inflammation, demyelination, axonal damage, glial scarring and repair mechanisms, are not uniformly represented in MS patients, contributing to the heterogeneity in phenotypic expression of the disease and its prognosis (Reindl et al. 2006). On the basis of myelin protein loss, the geography and extension of plaques, the patterns of oligodenrocyte destruction, and the immunopathological evidence of compliment activation, four fundamentally different patterns of demyelination have been introduced (Lucchinetti et al. 2000). Pattern I shows T cell/macrophage-mediated demyelination and pattern II similarities to antibody/ complement-mediated demyelination. These two patterns (I and II) dominate in RRMS patients. Pattern III displays oligodendrocyte dystrophy with myelin protein dysregulation and oligodendrocyte apoptosis, while pattern IV correlates to PPMS and primary oligodendrocyte degeneration, having features similar to viral infection or toxic oligodendrocyte damage, but not to autoimmunity (Lucchinetti et al. 2000, Brück 2005).

Two main mechanisms have been proposed to explain how infections could induce MS and autoimmune responses against myelin components: molecular mimicry, i.e., the activation of autoreactive cells by cross-reactivity between self-antigens and foreign agents and bystander activation, which assumes that autoreactive cells are activated because of nonspecific inflammatory events that occur during infections (Sospedra & Martin 2005). Epitope spreading leads to a wider range of antigens capable of inducing immune response and progression of MS (Vanderlugt & Miller 2002).

Myelin-specific CD4^{+} T- cells are considered to be the initiators of the disease both in EAE and MS, but also clonal expansion of CD8^{+} T cells has been detected in both MS and EAE lesions (Babbé et al. 2000, Jacobsen el al. 2002). Trafficking of these inflammatory T cells into the CNS are crucial in the pathogenesis of MS. Adhesion molecules (AMs) and the interaction between chemokines and their receptors are

critical to this trafficking and to the selective recruitment of inflammatory cells to foci of inflammation. Very Late Antigen-4 (VLA-4) on the T-cell surface and vascular cell adhesion molecule-1 (VCAM-1) on the brain vascular endothelium are important in regulating lymphocyte adhesion and migration across the blood brain barrier (BBB) to initiate MS lesions (Chofflon 2005). Similarly, abundant data suggest that CXCR3 and CCR5 chemokine receptors are operative in the development of CNS inflammation in MS (Balashov et al. 1999, Sørensen et al. 1999). The supporting compounds, the matrix metalloproteinases MMP-7, MMP-9, MMP-2, MMP-12 and MMP-14 have been shown to enhance the permeability of BBB in MS and thus the migration of inflammatory cells to the CNS (Prat & Antel 2005).

Interferon-γ (IFN- γ) and tumor necrosis factor-α (TNF-α) produced by CD4^{+} T helper (Th) 1 cells, are the major effector cytokines that exert different effects in EAE and MS. IFN- γ exacerbates disease in MS patients although it has been shown to be protective in rodent models of EAE. Similarly, overexpression of TNF-α induces demyelination and neurodegeneration in EAE while neutralization of TNF-α is protective, but in MS patients neutralization of TNF-α exacerbates the disease (Lassman & Ransohoff 2004).

Cytotoxic CD8^{+} T cells are involved in axonal damage, which occurs early in the disease course (Trapp et al. 1999), but because of compensatory mechanisms within the CNS, it remains silent until a threshold level of axonal loss is achieved (Confavreux et al. 2000). Cytotoxic CD8^{+} T cells can identify antigens which are presented by HLA I molecules and microglia. This, in turn, induces the production of cytokines, resulting in the death of neurons and oligodendrocytes.

Microglia are considered to be the resident macrophages of the CNS, which upon activation can proliferate, upregulate MHC molecules and secrete cytokines, chemokines, nitric oxide and reactive oxygen species (Bezzi & Volterra 2001). Astrocytes produce extracellular matrix molecules that are compounds of the supporting framework in the CNS. Astrocytes can be activated by inflammatory stimuli to proliferate and migrate towards sites of injury in order to form a tight glial scar (reviewed in Peterson & Fujinami 2007).

Humoral immunity plays also a role in MS pathogenesis, as suggested by intrathecal Ab synthesis and oligoclonal B cell expansion. Antibodies produced by clonally expanded B cells from the CSF of MS patients and patients with CIS have been found to react with axons in acute MS lesions. Myelin oligodendrocyte-glycoprotein (MOG) is a target myelin antigen for both humoral and cellular CNS-directed immune response (reviewed in Reindl et al. 2006). Its expression is sequestered at the outermost

surface of the myelin sheath and oligodendrocyte plasmamembrane. A single immunoglobulin domain is exposed to the extracellular space accessible for autoantibodies (reviewed in Reindl et al. 2006 and Kroepfl et al. 1996).

In conclusion, activated myelin-specific T cells can cross the BBB into the CNS where they proliferate and secrete pro-inflammatory cytokines. In addition, these infiltrating T-cells increase the permeability of the BBB by MMPs, which in turn degrade the extracellular matrix. Activated immune cells in the brain interact with their responsive antigen presented by macrophages or microglia and secrete cytokines and chemokines that further permeabilize the BBB. In addition, cytokines stimulate microglia, macrophages and astrocytes and recruit B cells, resulting in damage to myelin, oligodendrocytes and axons (Zamvil & Steinman 2003, Markowitz 2007) (Figure 2).
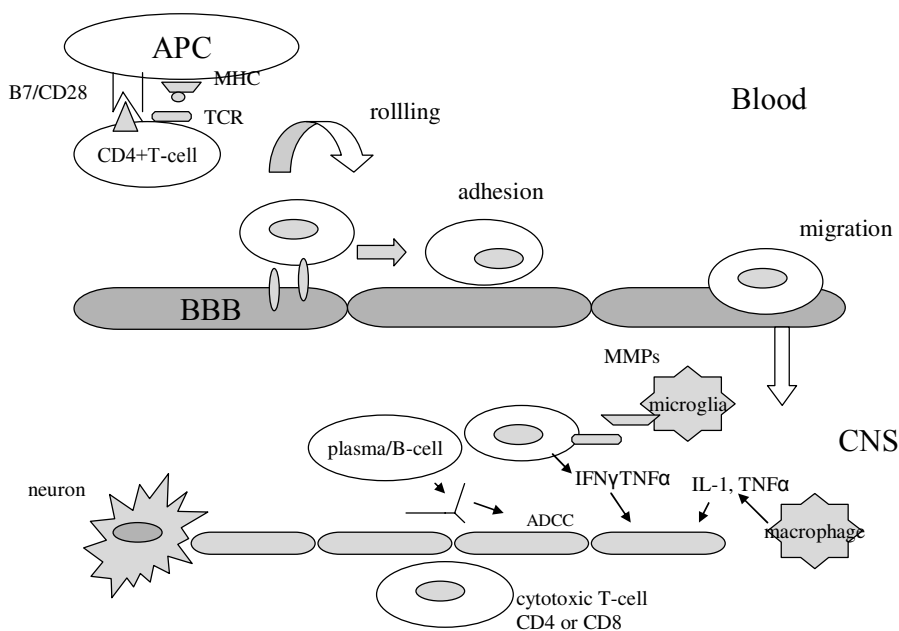
Figure 2. Mechanisms of immune-mediated neurodegeneration. After activation in periphery, T-cells adhere to endothelial cells and transmigrate through the blood-brain barrier (BBB) into the CNS. In the CNS, T-cells proliferate in response to antigen re-stimulation. These activated T-cells secrete pro-inflammatory cytokines that activate resident microglia, macrophages and B-cells. ADCC=antibody-dependent cellular cytotoxicity; APC=antigen presenting cell; BBB=blood brain barrier; TNF=tumor necrosis factor; IL=interleukin. *(Modified from Giuliani F and Young VW, 2003)*

2.2   Immunomodulatory therapy of MS

Disease-modifying drugs (DMD) for MS have been available since 1993, when IFN-β-1b was shown to alter the natural history of RRMS (The IFN-beta MS study group

1993, UCB MS/MRI study group 1993). This was followed by beneficial trials with IFN-β-1a (Jacobs et al. 1996, PRISMS study group 1998) and GA (Jacobs et al. 1995). In Finland, IFN-β has been used for the treatment of MS since 1996. Recent multicentre studies (CHAMPS and ETOMS) have shown beneficial effects with once-weekly administered IFN-β-1a on the rate of conversion from CIS to clinically definite MS (CDMS) (Jacobs et al. 2000, Comi et al. 2001). The 3-year CHAMPS trial was extended for 2 years as the CHAMPIONS trial, with placebo patients crossed over to active therapy. At 5 years, the risk of CDMS in the group on immediate therapy was 36 % versus a 49 % risk for those on delayed therapy (Kinkel et al. 2006). In a more recent BENEFIT study, IFN-β-1b 250 μg subcutaneously every other day reduced progression to CDMS from 45 % to 28 % during a 2-year follow-up (Kappos et al. 2006). However, the efficacy of these immunomodulatory therapies and their effect on the progression of disability are only partial, due to the immunopathogenetically and clinically heterogeneous background of MS. Thus, there is a need for biological markers of disease progression and prognosis, as well as for therapy monitoring.

2.2.1   Interferons

Rational therapies for MS include strategies to decrease the generation of autoreactive Th1 cells, and immune deviation in favour of Th2 environment. IFN-β affects antigen presentation and the cytokine milieu. It acts on its receptor on T-cells and APC, which in turn decreases the expression of molecules needed for antigen presentation. Due to its effects on T-cell expansion and survival, the generation of antigen-specific T-cells decreases (Yong 2002). Many studies have shown that the Th1 cytokines, IFN-γ, IL-12 and TNF-α, are decreased by IFN-β (Yong et al. 1998), but the net effect indicates more general suppression of both Th1 and Th2 subsets (Furlan et al. 2000). IFN-β also affects BBB by inhibiting the trafficking of inflammatory cells into the CNS with different mechanisms. Adhesion molecules on T-cells interact with their counter receptors on endothelial cells and regulate lymphocyte adhesion and migration across the BBB to initiate MS lesions. IFN-β has been shown, e.g. to increase soluble VCAM-1 and decrease VLA-4 expression on peripheral blood lymphocytes (PBL), which decreases the ability of T-cells to adhere to the endothelium (Calabresi et al. 1997). In addition, IFN-β inhibits the production of certain MMPs, e.g. MMP-9 (Trojano et al. 1999, Stuve et al. 1996), which in turn decreases the capacity of T-cells to migrate across a matrix barrier.

Three different IFN-β products have been available for the treatment of MS: IFN-β-1b (Betaferon®, Schering AG, Berlin, Germany), 250 μg administered subcutaneously (s.c.) every other day, IFN-β-1a (Avonex®, Biogen, Cambridge, MA, USA) 30 μg administered intramuscularly (i.m.) once weekly, and s.c. three times weekly

administered IFN-β-1a 22 μg or 44 μg (Rebif®, Serono International, Geneva Switzerland). IFN-β-1a is a recombinant 23 kDa peptide that is produced in a Chinese hamster ovarian cell line, is glycosylated at position 80, and contains the natural human IFN-β amino acid sequence. IFN-β-1b, 18.5 kDa in weight, is produced as a nonglycosylated molecule in Esterichia coli bacterial cells with a genetically engineered cysteine to serine substitution at position 17, and there is no methionine at the N terminal end (reviewed in Hemmer et al. 2005).

Both IFN-β-1a and IFN-β-1b have been shown to reduce the relapse rate, the progression of disability and MRI-assessed disease activity compared with placebo (The IFN-beta MS study group 1993, The IFN-beta MS study group and UCB MS/MRI study group 1995, PRISMS study group 1998, 2001). Two comparative studies between different IFN-β preparations have been published. The EVIDENCE trial compared the efficacy and safety of three times weekly s.c. administered IFN-β-1a 44 μg (Rebif®) and once weekly i.m. administered IFN-β-1a 30 μg (Avonex®). The number of relapse-free patients was higher and MRI-detected disease activity lower in the Rebif® group. The INCOMIN study showed more benefit in terms of the amount of MRI lesions and relapse-free patients after one year of IFN-β-1b (Betaferon®) treatment compared to i.m. administered IFN-β-1a (Avonex®) (Durelli et al. 2002). However, in a recent study, IFN-β-1b (Betaferon®) did not prove to be clinically superior when compared to s.c. 22 μg once-a-week administered IFN-β-1a Rebif® (Koch-Henriksen et al. 2006).

Treatment with IFN-β-1b and secondarily with IFN-β-1a can be continued if relapses still occur in the secondary progressive phase of the disease. For the primary progressive form of MS, no effective immunomodulatory therapy is available.

Some of the side effects emerging during the IFN-β therapy are considered to be natural effects of interferon-inducible proteins. The most common adverse effects include injection site reactions (40-85 % of patients) and flu-like symptoms (headache, fever, myalgia). In a retrospective chart review, the highest proportions of interruptions of IFN-β therapy (27 %) occurred in the first six months. The three most common reasons for interruption were lack of efficacy (30 %), injection-site reactions (12 %) and flu-like symptoms (10 %) (Tremlett & Oger 2003). Leukopenia, elevation of liver enzymes, headache and depression can also occur.

2.2.2 Glatiramer acetate

Glatiramer acetate (Copaxone®) (GA) is a synthetic copolymer with an amino acid composition based on the structure of myelin basic protein. It is a standardized

mixture of synthetic polypeptides consisting of L-glutamic acid, L-alanine and L-tyrosine and L-lysine. GA has been shown to have protective and suppressive effects in EAE. The mechanism of action and clinical effects of GA in MS patients are thought to be largely mediated by a shift from Th 1 to Th 2 GA-reactive regulatory cells, including CD4+ and CD8+ T-cells (Neuhaus et al. 2000). Glatiramer-reactive Th2 cells are believed to enter the brain and, through cross-reactivity with myelin antigens, produce neuroprotection and anti-inflammatory effects (Dhib-Jalbut 2003, Wee Yong 2002). Release of anti-inflammatory cytokines such as interleukin (IL)-4, IL-5 and IL-10 inhibit neighbouring inflammatory cells by a mechanism termed bystander suppression, and a recent study has demonstrated that both GA-specific Th2 and Th1 cells produce the neurotrophin brain-derived neurotrophic factor (BDNF), which has profound effects on neuronal survival and repair, especially by inducing axonal outgrowth, remyelination, regeneration and neurogenesis (Ziemssen et al. 2002, Aharoni et al. 2005).

Clinical studies have confirmed the effectiveness of GA in reducing relapse rate and disability accumulation in RRMS, at a magnitude comparable to that of other immunomodulating therapies (Boneschi et al. 2003, Mancardi et al. 1998). The benefits of early GA therapy compared with delayed therapy have also been reported. GA has been used in the USA as a disease-modifying therapy for MS since 1996 and in Finland since 2002. Despite a Cochrane review showing controversial results for GA without any beneficial effect on the disease progression and the risk of clinical relapses over time (Munari et al. 2003), recently published long-term follow-up studies have shown a lower expanded disability status scale (EDSS) change after six and 10 years of GA treatment compared to patients who had discontinued the treatment (Johnson et al. 2000, Ford et al. 2006). Recent MRI and magnetic resonance spectroscopic (MRS) studies suggest potential neuroprotective effects of GA and thus its effect on decreasing the rate of brain atrophy (Khan et al. 2005, Ge et al. 2000).

GA is administered daily by subcutaneous injections of 20 mg. The most common side-effects have been flushing, chest tightness, sweating, palpitations, anxiety and injection site reactions (Munari et al. 2003, Munari & Filippini 2004). No regular laboratory screening is needed. MS patients treated with GA develop GA reactive antibodies, which peak after three months of treatment and decrease at six months (Brenner et al. 2001). None of the antibodies has been shown to interfere with GA activities either in vitro (binding to MHC molecules and T-cell stimulation) or in vivo (blocking of EAE) (Teitelbaum et al. 2003). A recent study has demonstrated that, initially, GA-reactive antibodies of the IgG1 subclass predominate, but after nine months IgG1 decreases and IgG4 subclass increases, remaining high for the 3-year follow-up. This supports a shift from Th_1 to Th_2 immune response (Basile et al. 2006).

As the relapse-free patients have been shown to develop higher antibody titres (Brenner at al. 2001) the GA-reactive antibodies may even have a beneficial effect.

The combination of a type I IFN with GA has also been studied, because both drugs may have an additive effect in blocking the activation of autoreactive T-cells. On the other hand, IFN-β could block the generation of GA-reactive T-cells and their migration into the CNS due to its mechanism of action. In EAE, the combination therapy was not beneficial and was considered even counterproductive (Brod et al. 2000). In further studies, the cellular response has been observed to remain predominantly Th2-polarized with combined therapy (Dhib-Jalbut et al. 2002), but the combination is not used in clinical practice.

2.2.3 Other treatment options

Natalizumab (Tysabri[®], Biogen Idec and Elan Pharmaceuticals) is a $\alpha_4$integrin antagonist and belongs to a new class of selective adhesion-molecule inhibitors. It binds to the α_4 subunit of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ and blocks binding to their endothelial receptors. Thus, it inhibits leukocyte migration into the brain and reduces inflammation. The risk of sustained progression of disability has been reduced by 42 % over a 2-year period, and the rate of clinical relapse by 68 % at one year. An 83 % reduction in the accumulation of MRI-detected MS lesions has also been observed over two years (Polman et al. 2006). About 6 % of the patients develop antibodies to natalizumab which have led to allergic reactions and to reduced clinical efficacy. The risk of progressive multifocal leukoencephalopathy (PML) is 1.0 per 1000 treated patients, and limits its use with other immunomodulatory or immunosuppressive therapies (Yousry et al. 2006). However, natalizumab is recommended if IFNβ or GA has become ineffective in the treatment of active MS.

Immunosuppressive drugs have been used in the treatment of MS for over 30 years. According to a recent international questionnaire in Europe, approximately 10 % of patients are undergoing immunosuppressive therapy (Hommes & Weiner 2002). Sytostatic drugs are an alternative to immunomodulatory treatment, but only azathioprine and mitoxantrone have shown evidence of an effect on MS. Mitoxanthrone inhibits T-cell activation, reduces proliferation of B- and T-cells and antibody production, and deactivates macrophages. It is recommended for use in SPMS and in the severe, active relapsing progressive form of MS, which does not react to disease-modifying drugs. Mitoxanthrone can be administered intravenously (12 mg/m^2) every third month, but because of its cardiotoxicity the maximum total dose cannot exceed 120 mg. It is generally well tolerated and has reduced the progression of disability and clinical exacerbations (Hartung et al. 2002).

Azatioprine is administered at a dose of 2.5 mg/kg orally as continuous treatment. Although it has slightly reduced the relapse rate, it has had no effect on the disease progression (Yudkin & Ellison 1991, Bryant et al. 2001). However, a recent study showed reduction in new brain inflammatory lesions (Massacesi et al. 2005).

2.3 Immune response to interferon therapy

2.3.1 Interferon-inducible proteins

IFNs are able to suppress viral replication in cells, but beside this antiviral action, all IFNs have pronounced antiproliferative, immunomodulating, and differentiating effects. All type I IFNs (alpha, beta and omega) share the same surface receptor, which is composed of two subunits, IFNAR1 and IFNAR2. Each of them is formed by an extracellular transmembrane and intracytoplasmic domains (Oritani, Kincade et al. 2001; Oliver, Mayorga et al. 2006). All type I IFNs activate receptor-associated Janus family tyrosine kinases, (Jak 1, Tyk 2), which in turn activate the signal transducer and activator of transcription proteins (Stats). This translocates to the nucleus, where activated transcriptional elements interact with the interferon-stimulated response element (ISRE), leading to transcription of interferon-stimulated genes (ISGs) (Rudick & Ransohoff 1995, Melén et al. 2000) and to the induction of several proteins responsible for the biological functions of interferons such as 2´,5´-oligoadenylate synthetase, neopterin, β2-microglobulin and Mx proteins (Figure 3).
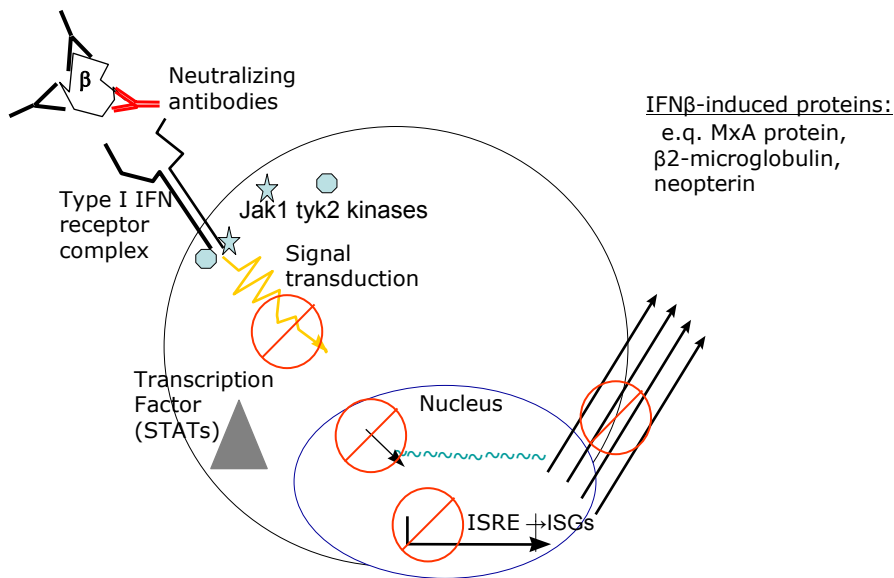
Figure 3. The IFN-β signalling pathway and the effect of NAbs. NAbs block the binding of IFN-β to its receptor and the subsequent activation of intracellular signal transduction and production of proteins.

Humans have two Mx proteins, MxA and MxB. MxA is a cytoplasmic protein with a molecular weight of approximately 75 kD. It has an intrinsic GTPase activity to block viral replication and antiviral activity against influenza A, measles, vesicular stomatitis virus, and bunyaviruses. Thus, its value as a marker of viral infections has been studied during recent years (Halminen et al. 1997, Chieux et al. 1999). However, no antiviral functions have been observed for MxB.

Neopterin is a product of IFN-γ activated macrophages and it is also augmented by TNF-α. Human monocyte-derived dendritic cells have also been identified as a source of neopterin production (Wirleitner et al. 2002). Neopterin concentrations are significantly increased in several autoimmune and neoplastic diseases and in infections, especially in HIV (Wirleitner 2005). Elevated levels of neopterin have also been found in the CSF (Shaw et al. 1995) and serum (Ott et al. 1993) of MS patients and urinary neopterin excretion has been shown to be increased in both the relapsing and progressive form of MS (Giovannoni et al. 1997). Therefore, it has been considered to be a potential surrogate marker of the inflammatory component of MS disease activity.

β2-microblobulin is present in serum as a small protein and forms the light chain of the HLA class 1 antigen molecule. It is expressed on the surface of most somatic cells, including B-/T-lymphocytes and macrophages (Karlsson et al. 1980). A recent study did not demonstrate its value as a surrogate marker of disease activity in MS (Bagnato et al. 2004), but another study showed increased serum β2-microblobulin levels over a one year IFNβ-1a (Avonex) treatment. Simultaneously neopterin levels decreased, probably due to long-term reduction in IFN-γ expression and macrophage activation. However, neopterin and β2-microblobulin serum concentrations remained elevated 24 and 48 hours after IFNβ-1a injection compared to predose values (Bagnato et al. 2002).

These IFN-induced proteins can be used as surrogate markers of biologic responsiveness during IFN-β treatment (Williams &Witt 1998, Rothuizen et al. 1999, Rudick et al. 1998, Pachner et al. 2006, Hoffman et al. 2007). Especially MxA protein has become a standard because of its specificity for type I IFNs. According to a recent study, it proved to be the most sensitive gene to detect decreased bioavailability due to NAbs compared to other IFN-stimulated genes, TRAIL (tumor necrosis factor related apoptosis inducing ligand) and XAF-1 (x-linked inhibitor of apoptosis factor-1) (Gilli et al. 2006).

Although MxA protein does not have any functional role in either the pathogenesis of MS or the therapeutic effect of IFN-β, there are differences in biologic response between IFN-β preparations. IFN-β-1b has induced higher levels of MxA protein than

either of the two available IFN-β-1a preparations, and there has been a slight difference between the two IFN-β-1a preparations in favour of subcutaneous IFN-β-1a (Deisenhammer et al. 2000). In a comparative study of the pharmacodynamic and pharmacologic effects of IFN-β-1b every other day and IFN-β-1a i.m. once weekly, IFN-β-1b induced significantly greater neopterin and β2-microblobulin levels than IFN-β-1a (Williams & Witt 1998).

2.3.2   Antibody formation during IFN-β treatment

Many factors influence the immunogenicity of recombinant protein treatments, e.g. dose, route of administration, and nature and formulation of the antigen. Large proteins (relative molecular mass > 2500), complexed, particulate and denatured antigens are more likely to be immunogenic than small, soluble and naive proteins. The closer a product is to the species` natural antigen the less likely it is to provoke antibodies. Bacterial adjuvant, aggregation, slow release of antigen and effective binding to host MHC molecules also enhance immune response (reviewed in Hemmer et al. 2005). The route of administration is of importance because intravenous, nasal, and oral administrations induce a weaker response than intraperitoneal, intramuscular and subcutaneous routes.

The acquired immune system can initiate a specific immune response to an antigen which is taken up by dendritic cells and carried to regional lymph nodes. Naive B- cells can interact with proteins or linear peptides bound to cell membranes of dendritic cells, or they encounter soluble proteins in the blood stream or spleen. Interaction with antigen-specific T-cells is also required. During antigenic challenges, B-cells undergo affinity maturation, expansion and phenotype changes and evolve into memory B-cells and plasma cells, which release immunoglobulin specific to the protein antigen (reviewed in Hemmer et al. 2005).

NAbs can be induced by two different immunological mechanisms. If the therapeutic agent is a foreign protein, a classical immune response may occur, which leads to high and persisting levels of antibodies. The reaction to these proteins is comparable with an immune reaction to a vaccine. Human homologues, such as IFN, induce antibody formation by breaking B-cell tolerance. This process needs prolonged treatment but, according to the literature, antibodies tend to disappear shortly after stopping treatment (Polman et al. 2006, Schellekens 2003).

The three commercially available IFN-β products have different immunogenicity. The frequencies of NAbs against IFN-β in MS have varied from approximately 2% to 40% (The IFNβ multiple sclerosis study group 1996, PRISMS 1998, Panitch et al 2002).

IFN-β-1a is less immunogenic than IFN-β-1b, due to its identical primary and secondary structure compared to the naive form and to its production in mammalian cells leading to fewer host cell contaminants. However, its immunogenicity has increased with high-dose and high-frequency administration and with subcutaneous injections (Ross et al. 2000). A single dose of IFN-β-1b, which differs in its amino acid sequence from natural IFN, has about a 10-fold increase in weight of protein compared to IFN-β-1a. This probably increases the possibility of aggregation and leads to enhanced antigenicity (Runkel et al. 1998). In addition, the lack of glycosylation of IFN-β-1b leads to decreased solubility and the presence of high levels of aggregates. Although in the study of Sominanda et al., Betaferon® was the most seroprevalent, it showed lower immunogenicity by inducing lower NAb titres compared to Rebif® (Sominanda et al. 2007). Earlier studies have also shown that reversion to NAb seronegativity occurs more frequently in IFN-β-1b-treated patients compared to IFN-β-1a-treated patients (Rice et al. 1999, Sorensen et al. 2005). Since the persistence of NAbs is associated with high NAb titre levels, the lower immunogenicity can be one explanation why the disappearance of NAbs is more likely with IFN-β-1b (Sorensen et al. 2005). Also high-affinity antibodies, which occur especially with IFN-β-1a therapy, may account for the persistence of the NAbs, since in a study by Gneiss et al., binding of NAbs to the first 12 amino acids of IFN was much lower with IFN-β-1b than with IFN-β-1a (Gneiss et al. 2004). Because the N-terminal methionine is deleted from IFN-β-1b, NAbs would be expected to bind less avidly to the N-terminal region of IFN-β-1b than to native IFN or IFN-β-1a (Noronha 2007).

In immune tolerant human IFN-β transgenic mice only the non-glycosylated IFN-β has been shown to break B-cell tolerance (Hermeling et al. 2005). Thus, the different mechanisms of NAb formation can also explain the difference in the persistence of NAbs between IFN-β-1a and IFN-β-1b (Polman et al. 2006, Rice et al. 1999, Sørensen at al. 2003). In addition, the frequency of injections seems to be important (Ross et al. 2000), while, according to recent reports, the influence of the intramuscular versus the subcutaneous route has been considered to be less clear (Bertolotto et al. 2002, Sørensen et al. 2005).

2.3.3 Effect of neutralizing antibodies on clinical efficacy of IFN-β therapy

NAbs interfere with the interaction between IFN and its receptor blocking downstream IFN signalling, transcription of IFN-stimulated genes and gene products (Weinstock-Guttman et al. 1995) (Figure1). They are associated with reduced clinical efficacy as measured by relapse rate and MRI activity (Rudick et al. 1998, PRISMS 2001, The IFNβ multiple sclerosis study group 1996, Sørensen et al. 2003). However, the clinical

manifestations due to NAbs become evident only after 12 to 18 months of therapy and several months after the appeareance of NAbs (Figure 4).
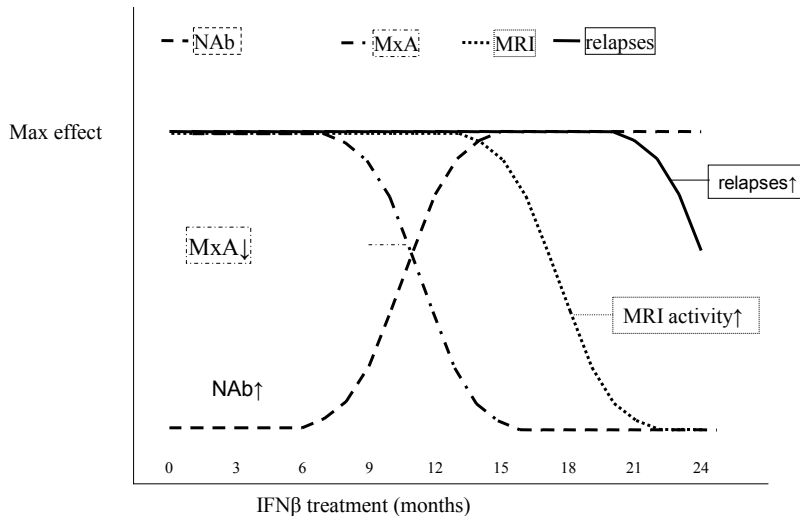
Figure 4. The effect of NAbs on the MxA levels and disease activity according to the published literature.

The evaluation of progression of disability due to NAbs needs longer follow-up emphasizing the importance of trials of sufficient duration (over 3 years) (Sørensen et al. 2003, SPECTRIMS study group 2001, Rudick 2003). This became evident especially in the PRISMS study where the effect of NAbs on relapse rate could be seen only after a 2-year extension of the trial. However, the effect of NAbs on MRI activity can be seen earlier as in the PRISMS study where there was an almost five-fold increase in the median number of T2 active lesions in the NAb+ group (PRISMS study group 2001). In a Danish nationwide prospective 5-year study, NAbs had a significant effect on relapse rates, increasing them by more than 50% compared to NAb-negative periods. In addition, the median time to first relapse was significantly reduced by 244 days in NAb-positive patients. The same study showed an obvious trend towards a higher mean EDSS at months 42 and 48 in NAb-positive patients, and a shorter time to disease progression (Sørensen et al. 2003). Although the SPECTRIMS study and the Betaferon study in SPMS patients found no significant difference between NAb+ and NAb- patients, a recent Avonex study showed that NAb+ patients had more progression on EDSS than NAb– patients (Kappos et al. 2005) (Table 2).

For future clinical trials, a longitudinal analysis of NAb positivity and titres in correlation with clinical and MRI parameters could be recommended, since a longitudinal analysis of the IFN-β-1b pivotal trial data showed that not all NAb+ patients have increased relapse rates (Petkau et al. 2004). In addition, the possible

disappearance of NAbs should be considered in long-term therapy because NAbs can decline or disappear even after several years of continuous IFN-β therapy (Sorensen et al. 2005) despite the fact that high NAb titres tend to persist for a long time after discontinuation of IFN-β therapy (Petersen et al. 2007).

Table 2. The frequency and effect of NAbs on clinical outcome of IFN-β therapy in long-term MS trials.

Study	Duration (years)	IFNβ therapy	NAb+(%) patients	Relapse rate	EDSS	MRI
Kappos et al. 2005	4	Avonex 30µg and 60µg i.m.	1.8 % 4.8 %	+	+	+
Perini et al. 2004	4	Avonex 30 µg i.m. Rebif 22 µg s.c. Betaferon 8MIU s.c.	6.7 % 26.7 % 40 %	+	+	ND
Sorensen et al. 2003	5	Avonex 30µg i.m. Rebif 22 µg s.c. once weekly/3 times weekly	7-42 %	+	n.s.	ND
Polman et al. 2003	3	Betaferon 250 µg	28 %	+	n.s.	+
Durelli et al. 2002, INCOMIN	2	Betaferon 250 µg Avonex 30µg i.m	22 % 6 %	n.s.	ND	ND
PRISMS Study Group 2001	4	Rebif 22 µg and 44 µg	24 % 14 %	+		+
SPECTRIMS Study Group 2001	3	Rebif 22 µg and 44 µg	21 % 15 %	+ (n.s.)	n.s.	ND
The IFNβ Study group 1996	3	Betaferon 1.6 MIU and 8.0 MIU	38 % 35 %	+	n.s.	n.s.

+ outcome worse in NAb-positive group
ND not done
n.s. statistically not significant

2.4 Antibody measurements during IFN-β treatment

2.4.1 Binding antibodies

Binding antibodies have been found in up to 78% of IFN-β-treated MS patients (The IFNβ multiple sclerosis study group and the University of British Columbia MS/MRI analysis group 1996, Kivisäkk et al. 1997). Because BAbs bind to those parts of the IFN molecule that are not involved in IFN receptor interaction they seem not to impair the therapeutic effects of IFN-β (Rudick et al. 1998). BAbs do not necessarily predict the later formation of NAb, but about 50% of BAb-positive patients also develop NAbs. Thus, simple binding assays have been used for screening BAb positivity and only positive samples have been further analysed to detect NAbs.

Three different basic methods have been used for measurement of BAbs: ELISA, Western blotting (WB) and radioimmunoprecipitation (RIPA) or affinity chromatography (ACA). Direct binding ELISA assay (dELISA) has been the most

commonly used method where the test wells are directly coated with IFNβ (Deisenhammer et al. 1999, Vallittu et al. 2002). In capture ELISA (cELISA) the wells are coated with a capture anti-IFN-β antibody (Pachner et al. 2004).

The Western blot method has given similar results compared to the ELISA method (Deisenhammer et al. 1999). The usage of radioactive isotopes limits the use of ACA and RIPA, although ACA has been very sensitive to detect BAbs (Ross et al. 2000). By using RIPA a moderate correlation has been detected between BAb and NAb (Lawrence et al. 2003) although BAb titres usually correlate only weakly with NAb titres.

2.4.2 Neutralizing antibodies

The most often used methods to detect NAbs are the cytopathic effect assay (CPE) and the MxA induction assay. In the CPE method, the virus is added to virus-susceptible cells in the presence of IFN-β and serum of the patient. If the patient´s serum contains NAb, IFN is neutralized, resulting in viral infection and death of the cells. The lowest serum concentration that leads to occurrence of cytopathic effects indicates the NAb titre (Abdul-Ahad et al. 1997, Vallittu et al. 2002). Standardisation of this method has been difficult because of the different cell lines, viruses and assay protocols used by different laboratories, which has made the precise comparison of data impossible.

The MxA induction assay in vitro has been widely used for determination of NAbs. IFN-β responsive cells are cultured in a plate, to which IFN-β and patients´ serum are added. Normally, IFN-β causes MxA production in the cells but in the presence of NAbs, IFN is neutralized and MxA induction does not occur or is diminished. The MxA concentration induced by IFN can be determined from lysed cells by a chemoluminescence assay (Deisenhammer et al. 1999).

Both of the above-mentioned NAb assays require cell cultures, long incubation periods and, in the case of CPE, also handling of viruses. A simpler method for NAb detection is a whole blood MxA stimulation assay, where IFN is directly incubated with whole blood samples from IFN-β-treated MS patients (Kob et al. 2003). After lysis of cells, the determination of MxA has been performed by ELISA (Deisenhammer et al. 1999, 2000). This type of MxA stimulation assay has been virtually as sensitive as the conventional NAb assay but less specific (Kob et al. 2003).

According to recent recommendations (Sørensen et al. 2005), a validated CPE assay is the gold standard to measure NAbs. The use of A549 cells is recommended with a fixed amount of IFN-β preparations used by the patient. The stimulated cells can be

challenged with EMC viruses or MxA production determined, and the NAb titres should be calculated using the Kawade formula (Kawade 1980). This method calculates the serum dilution that reduces IFN potency from 10 to 1 LU/ml (Grossberg et al. 2001 a,b). An overview of assays used for NAb and MxA detection in earlier trials is presented in Table 3.

Table 3. Overview of assays used for NAb and MxA detection in earlier trials.

Type of assay	Cells/Virus	IFNβ type/ concentration	Titer calculation/ Cut-off for Nab positivity	validation/ QC	Reference
MxA protein	Human whole blood	Betaferon/ 1000 IU/ml	MxA increase < 22 ng/ml	NAb assay (Pungor et al., 1998) / standard curve with rMxA	Kob et al., 2003
MxA RNA	Human PBMC	Avonex, Rebif, Betaferon/therapeutic dos	MxA RNA<132fg/pgGAPDH	CPE assay (Bertolotto 2000)	Bertolotto et al., 2003
MxA protein	Human lung carcinoma cells(A549)	Betaferon 10 IU	> 20 neutralizing unit	CPE assay (Pungor et al., 1998)	Polman et al., 2003
MxA protein	A549	IFNβ-1b 10 LU	Kawade > 20	CPE assay using EMCV	Pungor et al., 1998
CPE/MxA protein by FACS	WISH/VSV PBMC	IFNβ-1a/ 10 experimental units	titre > 20 for bioassay MxA protein < 2 x mean of baseline	internal positive/ negative control for bioass	Vallittu et al., 2002
CPE	A549/ EMCV	Avonex, Rebif, Betaferon/ 10 IU/ml	Kawade titre > 20	internal positive/ negative control	Bertolotto et al., 2000
CPE	A549/ EMCV	IFNβ-1a	Kawade / 50 % CPE	-	Zang et al., 2000
CPE	WISH/VSV	IFNβ-1b/ 100 IU/ml	Kawade titre > 20	Reference ab G038-501-572	Kivisakk et al., 2000
CPE	Sindbis virus	IFNβ-1a and -1b/ 20 U/ml	Kawade/10 LU	-	Antonelli et al., 1999
CPE	Human fibroblasts/ VS	IFNβ-1a and -1b/ 100 U/ml	50 % of CPE	-	Khan and Dhib-Jalbut, 1998
CPE	A549/ EMCV	IFNβ-1a/ 10 IU	Kawade, different cut-off values compared to neopterin as bioactivity marker	internal positive/ negative control	Rudick et al., 1998
CPE	WISH/VSV	IFNβ-1a/ 10 EU/ml	Kawade/ titre > 4	controlled for cell survival	Abdul-Ahad et al., 1997
CPE	FS-4 fibroblast EMCV	Fiblaferon (natural IFNβ)/100 U/ml	neutralizing unit= one unit of neutralized IFN	-	Dummer et al., 1991
CPE	A549/ EMCV	rIFNβ-1a and -1b/ 10 LU	Kawade titre > 80	internal positive/ negative controls	Monzani et al., 2002
Cell proliferation	Daudi cells	IFNβ 10 IU	50 % inhibition of proliferation	CPE	Prummer et al., 1996

VSV: vesicular stomatitis virus; EMCV: encephalomyocarditis virus; LU: laboratory unit; PBMC: peripheral blood mononuclear cells; FACS: fluorescence-activated cell sorter. Modified from EFNS official guidelines (Sørensen et al., 2005).

2.5   Quantification of MxA in peripheral blood leukocytes as a marker of IFN-β bioactivity

Measurement of MxA production can be used as a surrogate marker for the presence of NAbs. Induction of MxA indicates binding of IFN to its receptor and reflects activity of the cytokine. MxA can be measured in vivo either in protein or mRNA level in the blood of IFN-β-treated MS patients.

Three different PCR assays have been described for MxA mRNA quantification: quantitative competitive-PCR (qc-PCR) (Bertolotto et al. 2001), real-time-PCR (RT-PCR) (Pachner et al. 2003), and semi-quantitative-PCR (sq-PCR) (Gilli et al. 2004). In qc-PCR, PCR amplification products are separated and visualized by agarose electrophoresis against competitor cDNA (co-MxA) and quantification of MxA-mRNA is done in relation to a housekeeping gene (GAPDH) by densitometry. The advantage of RT-PCR is that the increase of DNA can be measured directly by fluorescence (Pachner et al. 2003). In a comparison trial, RT-PCR gave the highest specifity (93%) and qc-PCR the highest sensitivity (97%). sq-PCR proved to be useful for routine screening purposes, because it is easy to perform and does not need specialized laboratories (Gilli et al 2004). Because mRNA levels increase rather sharply, peak at 9 to 13 hours after IFN injection, the timing of blood samples is restricted (Pachner et al. 2005, Ronni et al. 1993). However, PCR is considered a very sensitive technique, because the MxA mRNA levels correlate strongly with the interaction between IFN-β and its receptor, and is not influenced by the post-transcription phenomena (Bertolotto et al. 2001). However, it requires expensive equipment and good quality RNA, which can be labile.

MxA induction as protein level has been quantified by a capture sandwich ELISA (Deisenhammer et al. 1999), a chemoluminescence assay (Chieux et al. 1998), and by flow cytometry (Vallittu et al. 2002). The ELISA technique is fast and simple, but the blood cells have to be lysed, because MxA is an intracytoplasmic protein. Both ELISA and chemoluminescence assays require monoclonal MxA antibodies, and quantification is performed using a standard curve derived from recombinant MxA protein. The disadvantage of this method is that anti-MxA monoclonal antibodies are not commercially available (reviewed in Pachner et al. 2003). Because the half-life of protein is several days longer than its mRNA (Kracke et al. 2000), it is assumed that the level of MxA protein is influenced by all events taking place in the week preceding the blood collection (Bertolotto et al. 2001). However, the long half-life and the accumulation of MxA protein in cells (4 h after the injection and maximum at 24-48 h) (Ronni et al. 1993) are also an advantage, because the time interval between IFN-β injection and blood collection can vary.

Flow cytometry requires isolation of PBMC and a specific anti-MxA-antibody for indirect immunofluorescence staining. Timing of blood samples is not very restricted, although the time between blood collection and FACS cell preparation and analysis is short to preserve cell integrity. It also needs a specialized laboratory with expensive equipment.

3. AIMS OF THE STUDY

This study was designed to examine and measure the biological effects of IFNβ and glatiramer acetate treatments in relapsing MS patients.

The specific aims of the investigation were:

I   To evaluate MxA protein induction in comparison with the development of binding and neutralizing antibodies during the first year of subcutaneous IFN-β-1a (Rebif) treatment.

II   To measure MxA protein production during the first year and after one to three years of weekly intramuscular IFN-β-1a (Avonex) treatment, and to evaluate possible differences in MxA protein induction between intramuscular and subcutaneous IFN-β-1a preparation.

III To set up a simple MxA EIA assay for measurement of MxA protein, and to evaluate the EIA test by comparing the results with flow cytometric analysis and measurements of NAbs.

IV   To assess prospectively the safety and efficacy of glatiramer acetate compared with the annual relapse rate and EDSS score in patients who had earlier been IFN-β-intolerant, and to evaluate whether GA has any effect on the level of MxA protein production in PBMC.

4. MATERIALS AND METHODS

4.1 Patients

Study I. A total of 20 patients (12 women, eight men; mean age 37 years, range 21 to 54 years) from eight Finnish hospitals were included in this one-year prospective study. They began to receive IFN-β-1a treatment (Rebif 22 μg s.c. three times a week) for relapsing-remitting MS from July 1998 to May 1999. Blood samples (both sera and whole blood) were collected before the treatment and at the control visits at three, six and 12 months of therapy. IFN-β injections were performed in the morning. Whole blood samples for evaluation of the MxA induction were obtained from the patients before (non-induced MxA values) and 24 hours after the injection (induced MxA values). Serum samples were collected before administration of interferon to avoid the possible interference of interferon with NAb titre determination. In addition, whole blood samples and sera were obtained from 10 healthy volunteers to evaluate baseline MxA protein expression in leukocytes, and to detect the possible presence of IFN-β-specific BAb and NAb.

Study II. Eighteen patients were included in the study. Prospectively studied nine RRMS patients (5 women, 4 men; mean age 37 years, range 22 to 51 years) were enrolled from July 1998 to May 1999 when they had begun to receive IFN-β-1a treatment (Avonex®; 30 μg i.m, once weekly). Peripheral blood samples (both whole blood and serum) were collected before the treatment and at the control visits at 3, 6 and 12 months of therapy. The samples were obtained just before (noninduced MxA values) and 24 hours after (induced MxA values) the IFN-β-1a injection. Sera were collected at the time of drawing the uninduced sample and studied for possible presence of BAb and NAb.

A retrospectively studied group consisted of another nine RRMS patients (5 women, 4 men; mean age 34 years, range 21 to 54 years) who had received IFNβ-1a treatment for one year one month to 3.5 years (mean 2.8 years). Six out of nine patients had been treated for over three years and one patient for two years 11 months. The other two patients had been treated for 13 and 15 months. Patients were enrolled during 2001 from nine Finnish hospitals. Peripheral blood samples for measurement of BAb, NAb and MxA protein expression were taken only at one time point. IFN-β-1a NAb positive control samples were provided by Biogen Inc. (Cambridge, MA, USA).

Study III. A total of 15 RRMS patients (12 women, 3 men, mean age 38 years, range 25 to 59 years) from three Finnish University Hospitals (Helsinki, Tampere and Turku)

were included in this open one-year prospective study. The patients had to have RRMS, no progression between relapses and to be able to walk at least 20 metres with or without aid. They had to have been previously treated with IFN-β but had discontinued the treatment because of side-effects or lack of efficacy at least one month before initiating GA treatment. The exclusion criteria were secondary progressive and primary progressive forms of MS, pregnancy, breast-feeding or non-compliance with daily injections. Glatiramer acetate (20 mg/day) was administered by daily subcutaneous self-injections. Neurologic examinations, including Expanded Disability Status Scale (EDSS), were performed every three months by an examining neurologist who had been trained several times for EDSS scoring in the context of clinical trials of MS. Laboratory assessments and the incidence of adverse events and relapses were documented at each study visit. Blood samples were obtained 24 hours after the GA injection for measurement of liver enzymes (ALAT, GT, AFOS), leukocytes and platelet counts as well as MxA protein levels. At the beginning of the study, MxA protein levels were measured just before the first injection of GA and 24 hours later to determine the baseline MxA protein level and the possible MxA protein induction. Later measurements were taken from single peripheral blood samples taken 24 hours after the GA injection. The measurement of MxA protein in PBMC was done by indirect immunofluorescence staining and fluorescence intensity ≥ 100 channels was determined as positive MxA induction. Three days of intravenous methyl prednisolone was allowed for treatment of relapses.

Study IV. A total of 51 RRMS patients were included in the study. Thirty-eight patients were enrolled from nine Finnish hospitals between July 2003 and May 2005. A total of 51 blood samples were taken from them for MxA protein detection as a part of normal treatment procedure, when they manifested signs of reduced clinical efficacy of IFN-β therapy. Neither clinical data nor information on the used IFN-β preparation were collected.

Thirteen patients participated in the RECOVER trial, a multi-centre (Finland, Denmark, Norway, Sweden), open-label, non-comparative trial investigating the recovery of IFN-β efficacy in relapsing-remitting MS patients with neutralizing IFN-β antibodies (NAb). The RECOVER patients had a diagnosis of RRMS and they had been treated with either subcutaneous IFN-β-1b (Betaferon®) or IFN-β-1a (Rebif®) for at least 24 consecutive months prior to enrolment. Blood samples were collected at screening/baseline (month -1/0) and after 3, 6, 9, 12 and 15 months, but only the samples of the screening/baseline phase (n= 26) were included in this study. The NAb tests were done in a blinded fashion in two different laboratories in Austria and Sweden.

The procedures (I-IV) were approved by the Ethics Committee of Turku University Hospital and by the Ethics Committees in each other participating country (IV). All patients gave theirinformed consent prior to the study.

4.2   Interferon-beta ELISA for binding antibodies

IFNβ binding antibodies were tested by enzyme-linked immunosorbent assay, ELISA (Abdul-Ahad et al. 1997). Microtitre plate wells were coated with Rebif (purified recombinant human IFN-β-1a 0.349 mg/ml, Serono International S.A.) diluted to one μg/ml in coating buffer (0.05 M sodium carbonate, pH 9.6) and using overnight incubation at room temperature. After washing (0.05% Tween 20 in saline), the wells were further blocked for nonspecific binding by incubating the plates for two hours at room temperature in a buffer containing 0.5% bovine serum albumin, phosphate-buffered saline (PBS) and 0.05% Tween 20. After three washes, serum samples and positive (sera of determined titre) and negative control samples (sera from untreated patient) in serial dilutions (1:10 to 1:640) were added to duplicate wells and incubated for one hour. After three washes, peroxidase-conjugated rabbit anti-human IgG was added for one hour, followed by washing and incubation of peroxidase substrate (citrate phosphate buffer, OPD-tablets, H_2O_2) for 20 minutes in the dark. Stop solution (1M HCl) was added and the optical density values were determined at 492 nm. A cut-off optical density was defined as three times the background optical density.

4.3   Interferon-beta neutralizing activity assay

Serum samples (both BAb negative and positive) were tested for their ability to neutralize the antiviral activity of IFN-β. WISH human amnion cells were placed into 96-well plates with 40000 cells in 100 μl/well, and incubated at 37 °C for 24 hours. The supernatants were discarded and replaced with serial dilutions (1:10 – 1:640 and further dilutions up to 1:10240 when needed) of the sera pre-incubated in sterile test-tubes at 37° C for one hour with a fixed concentration of approximately 10 experimental units/ml (10 EU/ml) IFN-β-1a. One EU/ml is the IFN concentration (0.004 ng/ml in our test) that protects 50% of the cells from viral cytopathic effect. Each serum dilution was tested in triplicate. As a control, 1:10 dilution was pre-incubated only with medium. After 24 h incubation at 37°C, the supernatants were discarded and vesicular stomatitis virus (VSV indiana) suspension of predetermined potency was added. In each analysis, a group of wells received cell culture medium instead of virus to provide 100% survival control for WISH cells, and a group of wells received only virus without any pre-incubation with interferon to control that the virus was growing properly. A titration of the IFN preparation was also included in each test

to determinate the actual EU/ml used. After 24 hours at 37 °C, a 5 µl /well of MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) at 5 mg/ml was added, and the plates were incubated for a further two hours at 37 °C. Finally, the supernatants were removed, cells were fixed with pure ethanol, and the plates were maintained in agitation for five minutes before measuring the optical density at 590 nm. The optical density is known to be proportional to the number of living cells and the cut-off value was defined as three times the background optical density. NAB positivity was determined as NAb titre ≥20 and titres 10-20 were regarded as borderline titres.

## 4.4   Flow cytometric analysis of MxA protein expression in lymphocytes

Heparinised venous blood was collected and layered on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient to separate peripheral blood mononuclear cells (PBMC). After centrifugation at 1500 RPM for 30 min, PBMCs were collected from the interphase layer and resuspended in RPMI 1640. After another centrifugation for 10 minutes, the cells were resuspended in 1 ml AB-serum and stored at +4°C. The measurement of MxA protein in PBMC was done by indirect immunofluorescence staining. A total of 0.65-1.0 × 10^6 cells were washed, pelleted and suspended in one ml of PBS. One ml of 6% paraformaldehyde solution was added for 15 minutes and the cells were permeabilized by adding 200 µl of 1% Triton 100-X (in PBS) for five minutes. After the cells were washed, intracellular MxA protein was stained with a rabbit anti-MxA serum and incubated at 37°C for 30 min. After washes, a secondary antibody, conjugated with fluorescein (FITC) was added and incubated with the cells at 37°C for 30 minutes. Finally, cells were washed again and suspended in FACS Flow buffer fixative. Cells were refrigerated, stored (+8°C), and protected from direct light and analysed using a fluorescence activated cell sorter (FACScan) cytometer (Becton Dickinson) within 24 hours after the staining. Values used to indicate the MxA level are the median channel of logarithmic fluorescence counts from the cytometer (Vallittu, Halminen et al. 2002). Positive MxA induction was determined as ≥ 100 (two times the mean baseline).

4.5   Enzyme immunoassay (EIA) for MxA protein expression in whole blood samples

The heparinized whole blood samples were lysed by diluting 1:20 in hypotonic buffer containing 1.5% bovine serum albumin (BSA), 1% ascorbic acid, 0.5% $NaHCO_3$, and 0.05% NaN_3. Diluted specimens were frozen and stored at -70˚C until assayed.

Standards for the assay were prepared from A549 cells induced with IFN-β to express MxA protein and diluted into the hypotonic buffer. The concentration of MxA protein in the standard was calibrated using purified recombinant MxA protein (from prof. O. Haller, Freiburg, Germany). Microtitre strip wells were coated by overnight incubation with a 0.2 µg/well of purified rat 2D12 monoclonal antibody (Biogen) in 0.1 M carbonate, pH 9.6. The strips were washed twice with 5 mM TRIS, 0.15 M NaCl, 0.05% Tween 20, pH 7.75, and saturated for one hour with the assay buffer containing 0.5% BSA, 0.05% Tween 20, and 0.1 mM merthiolate in phosphate buffered saline (PBS). Strips were washed four times, and 50 µl each of the sample and biotinylated mouse 4E5 monoclonal antibody (Biogen), 0.5 µg/ml in assay buffer, were added to the duplicate wells. After overnight incubation with shaking at 4°C, strips were washed 6 times, and 100 µl of strepavidin-peroxidase (Pierce, #21127) diluted 1:15000 in assay buffer was added to the wells. Strips were shaken for 15 min, washed eight times and incubated with 100 µl of tetramethylbenzidine peroxidase substrate solution (Ani Labsystems, Espoo, Finland) for 5 min in the dark. The colour development was stopped by adding 100 µl of 1 N H_2SO_4, and the absorbance values were measured at 450 nm. MxA concentrations (µg/l) of the specimens were read from a master curve plotted with the standard values using polynomial curve fitting and Multisoft software (Labsystems, Helsinki, Finland). Samples containing only hypotonic buffer were used to control the reagent backround and one negative and one positive control sample was included in each assay (Figure 5).

• Whole blood samples lysed in hypotonic buffer (1:20)
• 50 µl of lysate used per well
• MAb coated microtiter wells
• Biotinylated second MAb
• Streptavidin-peroxidase
• Color reaction with TMB

LDL=mean+3sd of blanc (<10 µg/l)
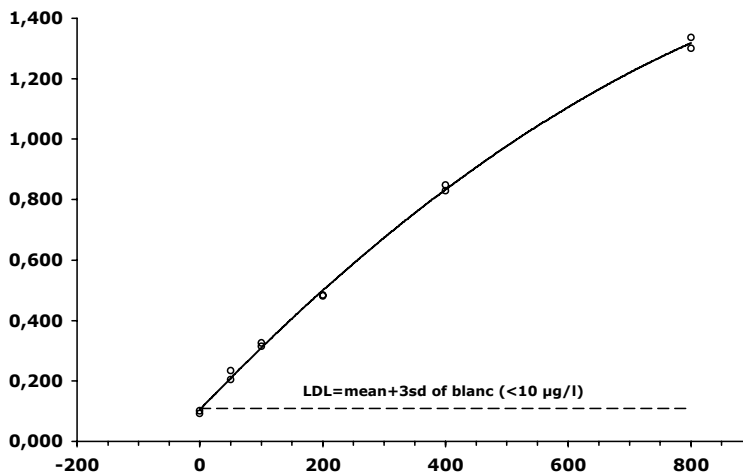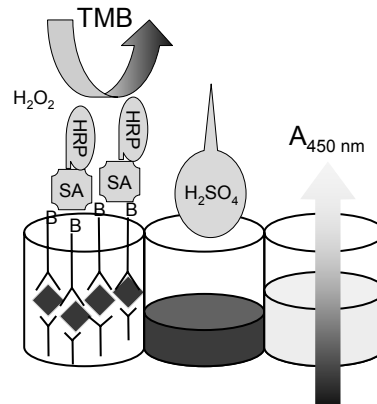
Figure 5. The principle of MxA EIA and the standard curve with reference MxA preparations

4.6 Statistical analysis

Statistical analysis was perfomed using statistical package SPSS version 10.0 for Windows. MxA protein values of NAb + and NAb - groups were compared with the Mann-Whitney U test. Paired values (pre and postinjection MxA levels) were compared using the Wilcoxon test, and Spearman´s correlation was used to quantify the relationship of MxA to BAb and NAb, and the relationship of BAb to NAb (Studies I, II, III). MxA levels between two IFN-β treatment groups were compared with the one-sample t-test (Study II). Friedman´s test was performed for comparison of relapse rates between GA and IFN-β therapies (Study IV). Spearman´s Correlation coefficient was calculated for evaluation of the test results between ELISA and flow cytometric analysis (Study III).

Differences between groups were considered significant when the p-value was less than 0.05.

5. RESULTS

5.1 Neutralizing antibodies and MxA protein induction during IFN-β-1a therapy

In the Rebif® treated group, 11 patients out of 20 developed BAb and nine patients developed NAb during the first 12 months of treatment. BAb developed before or at the same time as NAb of which the latter developed mostly toward the end of 12 months. In the Avonex® group, none of the 18 patients developed NAb, but three patients, who had been treated for over one year, were BAb-positive in the retrospectively studied group (n=9).

Median MxA value before treatment was 47 MFI in Rebif®-treated patients and 63 MFI in patients treated with Avonex®. In healthy controls, the median MxA level was 52 MFI. There was no difference in pre-treatment MxA values between patients developing and not developing NAb.

IFN-β treatment raised the baseline MxA protein level. The mean preinjection MxA values in the NAb-negative group after 3, 6 and 12 months of treatment were 265, 211 and 231, respectively, in Rebif®-treated patients. In patients who tested NAb- positive after 12 months of treatment the corresponding mean values of MxA were 299, 166 and 110 (Table 2).

In the Avonex® group, the mean MxA level rose remarkably after the first injection, 356 MFI (p=0.008), but the preinjection levels remained lower compared to the Rebif®-treated group. The preinjection MxA levels after 3, 6 and 12 months of treatment were 117, 108, 142 MFI, and the corresponding MxA values 24 h after the IFN injection were 246, 211 and 243 MFI, respectively. The difference was clear at each time point (p=0.012 after 3 months, p=0.036 after 6 months, and p=0.015 after 12 months of treatment).When the patients had been treated with Avonex® for over one year (mean 2.8 years), the mean preinjection MxA level of 66 MFI was similar to pre-treatment values. However, the mean postinjection MxA level was increased (150 MFI) and the difference between pre- and postinjection MxA levels was significant (p=0.015).

In Rebif®-treated patients, the MxA values never returned to true baseline in NAb-negative patients, but tended to drop in NAb-positive patients. The relative MxA response to IFN-β stimulation decreased during the follow-up. The MxA stimulation index (MxA after IFN injection/before IFN injection) at the onset of Rebif® treatment was 4.7, and after 12 months of treatment 1.2. The MxA stimulation index in both NAb- positive and -negative patients was 1.2 at the end of the study. Thus, there were two parallel effects, tachyphylaxis and NAb inhibition of MxA production. In addition,

a decrease in MxA protein expression level preceded the detection of NAb in sera. Also in Avonex®-treated patients the mean stimulation decreased during the first year of follow-up but it remained at a higher level compared to the Rebif® treatment. The stimulation index was 6.1 at the onset of treatment, 1.7 after 12 months of treatment, and 3.3 in the long- term treatment group.

Table 4. Comparison of MxA induction (MFI) between two IFNβ-1a preparations

mean MxA uninduced (MFI)	REBIF NAb-	REBIF NAb+	Avonex
0 months	55 (all patients)		58
3 months	265	269	117
6 months	211	166	108
12 months	231	110	142
mean MxA induced			
3 months	212	87	246
6 months	356	165	211
12 months	250	110	243
stimulation index			
at onset	4.7. (all patients)		6.1
after 12 months	1.2		1.7
1-3 years			3.3

Seven out of the nine NAb-positive Rebif® patients had a clear reduction in both baseline and induced MxA values starting after three months of treatment and prior to NAb formation. Two patients had an elevated MxA protein level despite their positive NAb titres 1:160 and 1:40, respectively. The corresponding MxA protein expression values were 371 and 148. There was a clear difference in induced (24 h after injection) MxA values between NAb + and NAb – groups after six ($p=0.026$) and 12 months of treatment ($p=0.021$) and in non-induced (48 h after injection) MxA values after 12 months of treatment ($p=0.005$). The development of NAb correlated with a reduction in induced MxA protein expression values ($rs= -0.438$, $p<0.001$), and the difference between all non-induced and induced MxA values was clear at all time points. However, the difference was smaller during the follow-up period ($p<0.001$ before the treatment and $p=0.049$ after 12 months of treatment). There was a strong correlation between NAb and BAb titres ($r=0.643$, $p<0.001$) and also a weaker correlation between BAb titres and MxA protein expression levels ($r =-0.289$, $p=0.013$).

5.2 Correlation of MxA-EIA to flow cytometric analysis and measurement of NAbs

For the EIA the reference values were determined in preliminary tests and based on the normal distribution of the values obtained from healthy young individuals (n=81). Positive MxA protein induction was determined as > 100 µg/l. Values 50-100 µg/l were considered as a weak response to IFNβ, and values < 50 µg/l were defined as a

loss of MxA protein induction, indicating the presence of NAbs. The inter-assay and intra-assay variation of the EIA test was 13% - 21%. In study IV, the cut-off value of FACS analysis ≥ 50 MFI was determined as two times the average baseline MxA level of non-induced samples.

The correlation between EIA and flow cytometric analysis was significant in both patient groups (Figure 6). Spearman´s Rho was 0.64 (p<0.01) for all blood samples (n=51) from the group of MS patients (n=38) undergoing a normal treatment schedule and 0.506 (p<0.01) for blood samples in the group of 13 RECOVER patients. NAb titres varied between 40-19600 and were associated with low MxA levels, but three patients had elevated MxA values of 105, 129, and 312 µg/l, respectively, despite NAb-titers 57, 19607 and 113 respectively.

The range of measured responses was wider in the EIA, and MxA protein induction was seen more frequently by EIA compared to flow cytometry (Figure 6). In nine (24%) of the 38 patients undergoing normal treatment, 11 postinjection blood samples were negative according to both assays indicating the presence of NAbs. In five patients, an apparent MxA response could be detected by EIA (range 114-275 µg/l), but not by flow cytometry (range 27-45 MFI).
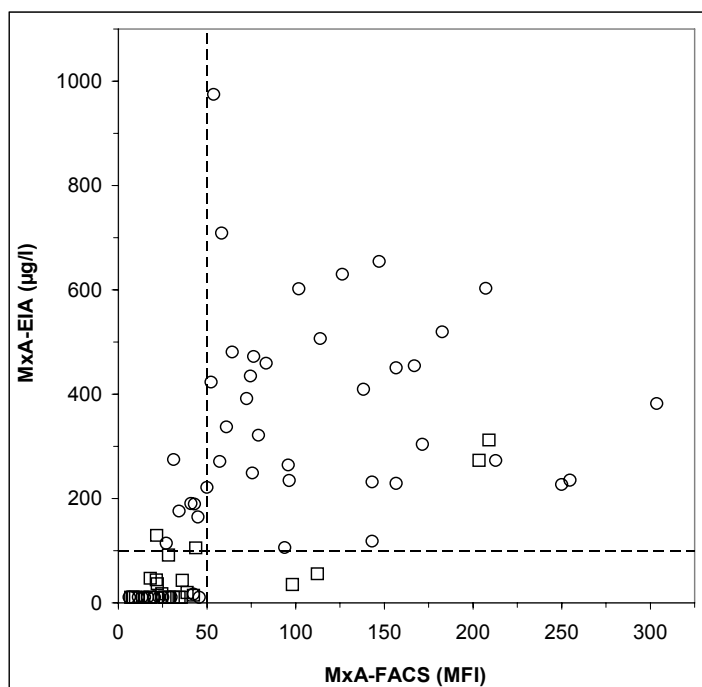
Figure 6. Correlation of EIA and flow cytometry (FACS) analysis of MxA protein levels. Results from 51 blood samples from 38 patients undergoing a normal treatment regimen (circles), and 26 samples from 13 NAb positive RECOVER patients (squares). Values above 100 µg/l for EIA and 50 MFI for flow cytometry show a good response to IFNβ.

For paired samples from eight patients in the normal treatment group and from all 13 patients in the RECOVER group, the correlation between EIA and flow cytometry test results was significant for both pre- (Rho 0.711, p<0.01) and postinjection (Rho 0.714, p<0.01) MxA values. The difference between pre- and postinjection MxA levels was also significant (p=0.009) in EIA, but not in flow cytometry (p=0.449). Thus, the stimulation indices (postinjection/preinjection MxA levels) were more clearly elevated, when measured by EIA.

5.3   Safety and efficacy of glatiramer acetate in IFN-β-intolerant MS patients

All the 15 patients included in study III had had severe adverse effects during IFNβ treatment. The most common causes of withdrawal in the Betaferon® group (n=7) were injection site reactions, headache, leucopenia and elevation of liver enzymes. One patient had taken Betaferon® for one year but due to injection site reactions it was replaced by Avonex® which, in turn, was discontinued because of hepatitis after one year. Elevated liver enzymes were also a cause of discontinuation of Avonex treatment in another patient. In Rebif®-treated patients (n=7) nausea, tremor, fatigue, injection site reactions, myalgia and depression were the most common causes of discontinuation.

Eleven out of fifteen (73 %) patients completed the study successfully. Four patients (one man, three women) discontinued GA treatment (27 %) during the first six months of the one-year follow-up because of chest tightness (n=2), insomnia (n=1), local injection site reactions (n=3) and non-compliance (n=1). No elevation of liver enzymes and neither thrombocytopenia nor leukopenia were seen during GA therapy. One patient had idiopathic thrombocytopenia but no change in the level of thrombocytes could be seen (thrombocytes 59 E^9/l before GA treatment and 54 E^9/l after 12 months of treatment).

The relapse rate diminished from an average of 1.86/year to 0.91/year (p=0.035) among patients who completed the study. Six of these patients were relapse-free and five patients had one to three relapses. Patients with the most relapse activity (2.5-4.5/year prior to study, EDSS score 1.0 - 6.0) completed the study, showing a decrease in relapse rate in three out of four patients. The patients who discontinued GA therapy had 0-1.5 relapses/year two years prior to this study. They had neither relapses nor significant worsening of disability during the follow-up.

MxA protein was not induced by GA. The mean MxA protein level was 54 MFI before the first GA injection and 53 MFI after the injection. After 12 months of treatment the mean MxA level was 39 MFI.

6.   DISCUSSION

6.1   Measurement of MxA induction and the bioactivity of IFN-β

The need for clinically useful and standardized screening assays is clear, but especially standardisation of the NAb test has been difficult because of interfering factors influencing the growth of the cells and virus. It is also difficult to compare bioassay data from different laboratories, because assay protocols vary with respect to the amount and type of virus, cell lines, and the dose and type of IFN-β used in the assay. However, measurement of NAbs with a validated CPE assay is still considered to be the gold standard. This recent recommendation consists of the use of A549 cells with a fixed amount of IFN-β for stimulation and serial dilution of sera. The stimulated cells can either be challenged with encephalomyocarditis (EMC) virus or determined MxA production (Sørensen et al. 2005).

In mononuclear cells, MxA expression is dose-dependently controlled by type I IFN. Monocytes have a Mx protein content about eight times higher than lymphocytes (Towbin et al.1992), and also higher basal and induced MxA protein levels, but the dose-dependency of MxA expression is similar in both cell types (Ronni et al. 1993). This phenomenon can be explained by the fact that increased MxA protein levels were more easily found with the EIA compared to flow cytometry, in which MxA was measured only from lymphocytes to avoid non-specific and background staining.

MxA protein appears to be very stable in cells and starts to accumulate at 4 h postinduction reaching its maximum at 24-48 h. When gene expression of MxA has been used as the analysing method, mRNA levels peak at 13 h after injection and return to basal level at a time when the MxA protein level is still rising (Pachner et al. 2005, Ronni et al. 1993). However, it has recently been confirmed that the mRNA changes occur as early as at 3 h (Gilli et al. 2006, Weinstock-Guttman et al. 2003). In the study of Santos et al., the maximum effect for Stat-1, MxA, TRAIL and MxB mRNA occured at 4 h and mRNA levels were already attenuated by 8 h (Santos et al. 2006). The discrepancy between results is probably caused by methodological differences, which confirms the difficulty to compare results from different laboratories.

The used IFNβ preparation and its dosing can affect to the biological response. While in the study of Dupont et al. (Dupont et al. 2002), T-cells regained full responsiveness to IFN treatment by one week after stimulation, another study showed that MxA gene

can be restimulated already 24 h after the initial IFN induction to levels similar as those in primary induction. MxA half-life is determined to be 2.3 days, although experiments with patients having viral infections suggest that MxA levels are elevated longer than the suggested theoretical half-life (Ronni et al. 1993).

Although some NAb positive patients showed biological response to IFN, it is possible that in repeated measurements the MxA protein levels would have decreased. Thus, the antibody-mediated decreased bioactivity can be a graduated phenomenon as previously reported (Pachner et al. 2005). It is also suggested that low and moderate NAb levels (20 to 100) correlate poorly with loss of bioactivity and a direct measurement of IFN-β bioavailability is reliable in these situations (Pachner et al. 2006). Differencies in individual cellular response to IFN-β can also partly explain why a part of patients have measurable bioactivity, while others have not with a similar NAb titre (McKay et al. 2006).

The measurement of MxA mRNA is considered to be a sensitive method for evaluating the biological response of IFNβ therapy by reflecting best the interaction between IFNβ and its receptor and second messenger activation. The disadvantage of using PCR is that it requires specialized laboratories and accurate blood sampling. Antiviral neutralization assays for NAb also need specialized laboratory and the number of positive patients can vary with respect to the amount of added IFN in the assay (Bendtzen 2003). The problem of unclear cut-off values in NAb assays can be overcome by direct determination of induced MxA protein levels. Thus, the measurement of the actual bioactivity of IFN-β seems to be more informative to evaluate the effects of IFN-β therapy and the simplified MxA EIA a sensitive and practical method for large-scale analysis of the bioavailability of IFN-β treatment in a single sample.

6.2   MxA protein induction and the development of NAbs

As shown in earlier trials (Bertolotto et al. 2004, Deisenhammer et al. 2000, Kracke et al. 2000, Gilli et al. 2005), lymphocyte MxA protein expression levels clearly increased after initiation of IFN-β-1a treatment, and NAbs inhibited the biological activity of IFN-β-1a in most patients. Accordingly, the MxA stimulation index decreased during the follow-up, indicating tachyphylaxis of MxA protein expression levels. However, two Rebif®-treated patients had increased MxA levels despite the presence of NAbs, and a similar phenomenon has also been discovered in other studies (Kracke et al. 2000, Bertolotto et al. 2004). It seems that there is a kind of transitional period, when MxA induction continues to occur despite NAbs. Especially patients with

persistently low NAb titres have shown MxA induction, although at significantly lower levels, but in the presence of high NAb titres, the biological response is always abolished (Bertolotto et al. 2004).

One Avonex®-treated patient demonstrated clearly reduced MxA protein levels without detectable NAb. Recent studies have suggested that neutralization is not entirely mediated by antibody. A soluble form of IFNα/β receptor (sIFNAR) has been present at high levels in serum samples, which are BAb-negative but show inhibition of IFN-β in the CPE assay (Bertolotto et al. 2006). In xenografted SCID mice, sIFNAR-2 could neutralize the bioactivity of type I IFN at high concentrations, and at lower concentrations caused an enhancement of IFN-β- mediated antiviral activity (McKenna et al. 2004). Another explanation for the reduced bioavailability without NAb could be desensitisation of PBMCs in response to repeated exposure to IFN. In the study of Bertolotto et al., all Avonex® injections once weekly were effective as evaluated by MxA mRNA induction, but only 82% of three times a week administered IFN-β injections were considered effective (Bertolotto et al. 2004). It has been also shown that in vitro T-cells become desensitised as a result of persistent IFN-β-1a stimulation and regain full responsiveness to treatment by one week after stimulation (Dupont et al. 2002). This might also explain the more clearly elevated stimulation indices in long-term Avonex® therapy compared to Rebif®.

Two criteria to evaluate the biological responsiveness to IFN-β have been suggested: an increase in MxA mRNA expression three-fold higher than the baseline MxA value of the individual patient, and MxA expression over the mean baseline expression of untreated MS patients +3 S.D (Gilli et al. 2004). According to the study of Gilli et al., the cumulative biological response is similar among different IFN-β products/dosing regimens, and even lower doses can induce the maximal biological response after the first injection of IFNβ (Gilli et al. 2005). However, the results are not fully comparable to long-term IFN-β therapy, and the used IFN-β preparation has to be taken into account, if the uninduced MxA levels are considered over a long period of therapy. It is obvious that more frequent administration gives a higher level of MxA protein during the whole week as was shown in this study. The uninduced MxA values were at the pre-treatment level after one year of Avonex® therapy although induced MxA levels were clearly elevated. The measurement of both baseline and induced MxA levels could give more information about the biological response, especially if the induced MxA level is moderate. However, in clinical practice, the measurement of only induced MxA protein (24 h after the IFN-β injection) seems to be adequate and practical.

A reduction in MxA protein expression levels tended to precede NAb formation. This can be considered as a transitional period, when the bioavailability of IFN-β is not totally abolished. In addition, it indicates that analysing MxA protein expression is a sensitive indicator of truly IFN-β neutralizing activity. Because the elimination of the biological effect of IFN-β has been seen even with low NAb titres (Deisenhammer et al. 1999), and NAbs are usually detected in in vitro tests, there is a need for other markers than NAbs for measurement of the in vivo consequences of antibodies (Rudick et al 1998). In addition, MxA expression or NAbs should be measured repetitively during the first two years of IFN-β treatment, because NAbs are shown to develop mostly within this period (Sorensen et al. 2005). Also in this study NAbs developed towards the end of 12 months of treatment, and BAbs were detectable before or at the same time as NAbs. BAbs do not impair the therapeutic effectiveness of IFN-β since they may bind to those parts of the IFN molecule, which are not involved in interferon receptor interaction (Pachner 1997). However, conflicting opinions have been introduced, suggesting that appropriately measured BAbs are probably always neutralizing, when NAbs are measured by a high-sensitivity assay (Bendtzen 2003). According to the study of Ross et al., NAbs initially seemed to bind with low affinity, but with longer treatment, high affinity NAbs appeared along with decreased binding capacity (Ross et al. 2000).

There is an ongoing debate about the clinical impact of NAbs and the methods to detect them. A recent report of the American Academy of Neurology (Goodin et al. 2007) stated that there is insufficient information on the utilization of NAb testing to provide specific recommendations regarding the indications and frequency of testing, different test methods, or application of cut-off titres. One of the problems is a lack of standardization between methods of NAb measurement. In addition, NAbs may disappear in many patients even with continued therapy, especially in those with low-titre NAbs (Goodin et al. 2007). Thus, the in vivo markers such as IFN-β- induced MxA protein or MxA-mRNA expression can offer a more reliable method to measure the actual bioactivity of IFN-β therapy.

6.3   The tolerability and efficacy of GA in IFN-β-intolerant MS patients

The results showed good tolerability of GA in individual patients who had previously not been able to use IFN-β. The most commonly reported adverse effects, injection-site reactions and chest tightness, were similar to those in earlier trials with GA. Considering the highly selected patient population in this study, the discontinuation rate of 27 % was at approximately the same level as in the six-year GA trial (Johnson et al. 2000). No laboratory abnormalities linked to GA use could be found, which is in

accordance with earlier trials, and confirms the clinical practice that no regular laboratory screening is needed. GA was also used safely in a patient with idiopathic thrombocytopenia.

No significant changes in disability were recorded as expected due to the short follow-up time and limited number of patients. The relapse rate was reduced in most of the patients after switching therapy, but evaluation of the long-term effects of GA on the disease progression needs a longer follow-up and a large patient population. NAbs to IFN-β could be one explanation for the poorer efficacy during IFN-β treatment and for the reduced relapse rates after initiation of GA therapy. However, all the patients in this study had side-effects during IFN-β therapy, which is not suggestive of the presence of NAbs. As a result of diminished IFN-β activity, the incidence of side-effects such as injection site reactions, lymphopenia and elevated liver enzymes has been found to be lower in persistently NAb-positive patients (Francis et al. 2005). The MxA protein levels did not change with GA therapy as expected, thus confirming the different mechanisms of action of GA and IFN-β.

6.4   Future prospects

A recent study of the costs of disorders of the brain in Europe estimated the number of patients with MS in Europe at 380 000. MS had the second lowest prevalence among the 12 disorders of the brain included in the study, but the mean annual cost per case was the second largest (Andlin-Sobocki et al. 2005). Thus, there is a need for an evaluation of the different treatments´ health benefits in relation to their costs. However, in a chronic disease such as MS this involves a long-term view, as total costs increase steeply with increasing severity of the disease. In addition, DMDs are costly and their major health benefit is not evident in the short-term perspective (Kobelt et al. 2006).

As early treatment of MS is recommended, possible markers for disease activity and tissue destruction have been studied. Autoreactive antibodies are of interest, because they occur more frequently in MS patients than in the general population. However, prospective studies are needed to better define their association with CIS and MS disease course (Garg et al. 2007).

To provide an inexpensive, easy and standardized method for measurement of bioactivity of IFN-β therapy is a challenge for the future. As new treatment options become available in clinical practice, such as oral VLA-4-antagonists, it would also be important to have measurable biological markers to evaluate their efficacy and

bioactivity. However, the long-term impact of NAbs and the discrepancy between NAb positivity and variability of clinical effects are still unclear. Also the phenomenon of high-dose IFN tolerance, in which NAbs decrease as the dose of IFN is increased (PRISMS study group 1998), requires confirmation.The design of adequate studies to answer these questions is crucial (Cutter 2003). Randomized controlled trials are intrinsically limited when determining long-term treatment outcomes in a chronic disease, while observational cohort studies could offer a better tool to assess this. Databases or registers are made up of individual patient records containing information on e.q. socio-economic data, disease course, medication and test results. Currently, there are a number of regional and national registers dedicated to acquiring prospective data for MS (Butzkueven et al. 2006). These kinds of datasets have been utilized in the development of a life- table analysis of prognosis of MS and an analysis of the relationship between early disease activity and long-term disability (Cottrell et al. 1999 a,b).

Along with MS registers, it is important to have uniform guidelines for the treatment of MS patients. After the introduction of the revised Finnish Current Care Guidelines for the treatment and rehabilitation of MS in July 2006, the number of MxA tests performed rose remarkably. During the last two years, over 1000 MxA-EIA tests have been performed compared to 50 MxA FACS analyses before this time. One natural explanation for this is the development and availability of a more rapid and practical test method for MxA protein detection, which is suitable for clinical use. Also the EFNS guidelines have had an important impact on defining the clinical use of the measurements of NAbs.

7.   CONCLUSIONS

The purpose of the present dissertation was to investigate the bioactivity of IFNβ therapy in MS patients by focusing on the measurement of neutralizing antibodies and its correlation to MxA protein induction. The following conclusions can be drawn according to the studies.

I In most patients NAbs inhibit the in vivo function of IFNβ. Analysis of MxA protein seems to be a sensitive method to reflect the biological response of IFNβ treatment and its actual bioavailability.

II The baseline MxA protein levels rose during intramuscular IFNβ-1a (Avonex[®]) therapy but the induction was weaker when compared to subcutaneously, three times a week, administered IFNβ-1a (Rebif[®]). However, the stimulation index was higher with Avonex[®], suggesting that weekly intramuscular dosing of IFNβ provides a sustained effect on lymphocytes.

III There was a positive correlation between MxA expression levels measured by EIA and flow cytometry with a wider range of measured responses in the EIA. The simplified MxA EIA assay seems to be a sensitive and practical method for large- scale analysis of the bioavailability of IFNβ treatment in a single sample.

IV Glatiramer actetate can be considered a well-tolerated, effective and safe treatment alternative in IFNβ-intolerant MS patients, although some patients are not able to use any available immunomodulatory treatment. This emphasizes the need for other therapeutic options. MxA protein was not induced by glatiramer acetate.

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10. ORIGINAL PUBLICATIONS