EVOLUTION OF THE ANTI-INTERFERON BETA (IFNß) ANTIBODY RESPONSE IN MULTIPLE SCLEROSIS PATIENTS

IgG Subclass Distribution, Affinity Maturation and Clinical Correlates

by

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Abstract

Multiple Sclerosis (MS), a chronic degenerative disease of the central nervous system, is characterized by demyelination, axonal damage, and inflammatory lesions in the white matter. Symptoms include neurological deficits, relapses and progressive disability. Three recombinant interferon beta (IFNβ) products and glatiramer acetate are licensed for treatment. They have been shown to reduce the frequency and severity of relapses and slow disease progression in about 30% of treated patients. Long-term administration of IFNβ can result in the development of anti- IFNβ antibodies. Binding antibodies (BAbs) bind IFNβ and neutralizing antibodies (NAbs) prevent interaction with its receptor, reducing IFNβ bioavailability and clinical efficacy.

The detection and characterization of anti- IFNβ antibodies does not adhere to any internationally recommended standards. A comprehensive strategy is required to elucidate the antibody properties that play a role in the immune response against IFNβ. To this extent, our objectives were: first, to investigate the IgG subclass-specificities of BAbs over time; second, to ascertain the affinity maturation pattern of BAbs and NAbs; and third, to investigate the effects of NAbs on clinical efficacy.

We used an enzyme-linked-immunosorbent assay (ELISA) to measure relative distribution of IgG subclass-specific BAbs and found that subclasses not only change over time, but their distribution varies between subcutaneous (SC) IFNβ-1a and SC IFNβ-1b. We also found that NAb+ patients tend to have higher levels of IgG4 subclass-specific BAbs than NAb- patients. To investigate the affinity maturation of anti-IFNβ antibodies, we utilized Biacore™, a biosensor device based on the optical phenomenon of Surface Plasmon Resonance (SPR).

Our results indicate that relative antibody affinities, as reflected by antibody dissociation rates, improve over time in NAb+ patients. Furthermore, we found a close parallel between antibody affinity and NAb levels.

Our investigation showed that the effects of NAbs on clinical efficacy are delayed, with an increase in relapse rates being more evident in NAb+ patients than in NAb- patients at year 3 (IFNβ-1b), and at year 3 and 4 (IFNβ-1a).

We conclude that there is a need for a quantitative and qualitative framework for monitoring anti-IFNβ antibodies that could prove valuable for better management of IFNβ-treated MS patients.

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ACTH	Adrenocorticotropic hormone
ADCC	Antibody dependent cell-mediated cytotoxicity
APCs	Antigen presenting cells
APL	Altered peptide ligand
BAbs	Binding antibodies
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BRMs	Biological response markers
BSA	Bovine serum albumin
Btk	B cell tyrosine kinase
CHO	Chinese hamster ovary
CM-D	Carboxymethyl dextran
CNPase	2' 3'-cyclic nucleotide 3' phosphodiesterase
CNS	Central Nervous System
CPE	Cytopathic effect
CSF	Cerebrospinal fluid
E. coli	*Eschericia coli*
EAE	Experimental allergic encephalomyelitis
EBV	Epstein-Barr virus
ECs	Endothelial cells
EDC	N-ethyl-N'-(3 diethylaminopropyl) carboiimide
EDSS	Expanded Disability Status Scale
ELISA	Enzyme-linked immunosorbent assay
EMC	Encephalomyocarditis virus
Fab	Variable fragment
Fc	Constant fragment/region
GA	Glatiramer acetate
GPCR	G protein-coupled receptor

HAM-TSP	Human T cell lymphotropic virus-1-associated myelopathy / tropical spastic paraparesis
HLA	Human leukocyte antigen
HRP	Horse radish peroxidise
HSV	Herpes simplex virus
HSV-1	Herpes simplex type-1
HTLV	Human T cell lymphotropic virus
ICAM-1	Intercellular adhesion molecule 1
IFNAR	IFNα/β Type 1 receptor
IgG	Immunoglobulin G
IIA	Interferon inhibitory activity
IL	Interleukin
IM	Intramuscular
INFβ	Interferon beta
INF-γ	Interferon gamma
IP	Interferon inducible protein
ISGF-3	Interferon-stimulated gene factor-3
ISGs	Interferon-stimulated genes
ISRE	Interferon-stimulated response element
ITIM	Immunoreceptor tyrosine-based inhibitory motif
IU	International Units
Jak PTKs	Janus protein-tyrosine kinases
Jak	Janus kinase
k_{off}	Dissociation constant
k_{on}	Association constant
LFA-1	Lymphocyte function associated antigen 1
LT	Lymphotoxin
LU	Laboratory Units
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MHC	Major histocompatibility complex

MIP	Macrophage inflammatory protein
MMPs	Matrix metalloproteinases
MOBP	Myelin-associated oligodendrocyte basic protein
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
MxA	Myxovirus A
NAbs	Neutralizing antibodies
NAWM	Normal appearing white matter
NGF	Nerve growth factor
NHS	*N*-hydroxysuccinimide
NK cells	Natural killer cells
OPD	*o*-phenylenediamine
OWIMS	Once Weekly Interferon for MS
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline, 0.05% Tween
PH	Pleckstrin homology
PLC	Phospholipase C
PLP	Proteolipid protein
PMBCs	Peripheral blood mononuclear cells
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive Multiple Sclerosis
RIPA	Radioimmunoprecipitation assay
RRMS	Relapsing-remitting Multiple Sclerosis
RU	Resonance Units
SC	Subcutaneous
SEM	Standard error of the mean
SH-2	Src-homology-2
sICAM-1	Soluble intercellular adhesion molecule-1
sIFNAR	Soluble INFα/β Type 1 receptors
SPMS	Secondary progressive Multiple Sclerosis

SPR	Surface Plasmon Resonance
SSPE	Subacute sclerosing panencephalitis
STAT	Signal transducer and activator of transcription
TAL-H	Transaldolase-H
T_C	T cytotoxic
TCR	T cell receptor
TGF	Transforming factor
T_H	T helper
TIMPS	Tissue inhibitors of MMPs
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor apoptosis inducing ligand
TRU	Ten-Fold Reduction Unit
TSH	Thyroid-stimulating hormone
TSP	Tropical spastic paraparesis
VCAM-1	Vascular cell adhesion molecule 1
VEP	Visual evoked potentials
V_H	Hypervariable region
V_L	Hypervariable region
VLA-4	Very late activation antigen 4
VSV	Vesicular stomatitis virus
WHO	World Health Organization
WISH	Wistar Institute Susan Hayflick

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Co-Authorship Statements

Manuscript 1

This research was designed by Ebrima Gibbs. All experiments were performed by E. Gibbs. All data analyses and manuscript preparation were done by E. Gibbs.

Manuscript 2

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Manuscript 3

In this research, E. Gibbs ensured liaison between all 4 co-authors. E. Gibbs maintained the UBC MS Serum Bank and performed all ELISA screens for BAbs. E. Gibbs identified all BAb+ samples and sent them out for NAb testing (Dr. Grossberg). E. Gibbs entered all BAb and NAb results. E. Gibbs identified and stratified all patients that fit criteria for this study and provided list of patients for patient chart review (Dr. Boz). E. Gibbs analysed data by categorizing patient samples according to treatment received and treatment duration. E. Gibbs wrote the Introduction section and aided in the revision of other sections.

Chapter 1

Introduction

1.1 MS the Disease

Multiple Sclerosis (MS) affects about 2.5 million people globally and is the commonest cause of neurological disability in young adults. It is a chronic inflammatory disease of the central nervous system (CNS), typically presenting between the ages of 15 and 40 years; the course is very variable from patient to patient. Pathologically, MS is characterized by focal inflammatory demyelination, axonal loss and astrocytic scar formation. This results in sclerotic plaques or lesions that are disseminated throughout the CNS, consisting of the brain, spinal cord and optic nerves, with no involvement of the peripheral nervous system. Myelin, the lipid-rich extension of the plasma membrane of oligodendrocytes, ensheaths and insulates the neurons of the CNS and spinal cord thus ensuring smooth high-speed conduction of electrical impulses: action potentials are propagated in voltage-gated channels accumulating at the unmyelinated nodes of Ranvier and are passively spread along the myelinated nerve segments (Compston and Coles, 2002). In MS, this saltatory transmission is blocked as a result of myelin loss and results in diminished or complete loss of nerve function.

The signs and symptoms of MS are dependent on the site of neuronal damage, and this random occurrence results in a wide range of clinical features. Commonly, MS starts with sensory disturbances, unilateral optic neuritis, diplopia, gait instability or ataxia. As the disease worsens, other symptoms follow such as Lhermittes sign (paresthesias invoked by neck flexion), limb weakness, clumsiness, tremor, nystagmus, bowel and bladder dysfunctions, spasticity, fatigue, sensory impairment and cognitive abnormalities (Noseworthy et al., 2000). Symptoms vary from patient to patient, and are also dependent on the time of the day.

The early clinical course of MS follows a relapsing-remitting sequence that manifests as attacks followed by partial or complete remissions of symptoms, with no accumulation of disability

between attacks but with increasing neurologic deficits. Approximately 85 to 90% of patients follow this clinical course, termed relapsing-remitting (RR) MS. After 10 years, 50% of RRMS patients will develop secondary progressive (SP) MS , and after 20 – 25 years, about 90% will develop SPMS (Trojano et al., 2003). SPMS is characterized by a chronic and progressive disability with or without occasional relief by remissions. Approximately 10% of MS patients exhibit a chronic progressive course from the onset, termed primary progressive (PP) MS, with no intervening episodes of remissions between exacerbations and no clinical improvement (McDonald and Ron, 1999; Lublin, 2005). Some of the RRMS patients, with an estimated prevalence of 5 -20%, are characterized as having benign MS which manifests as a few mild attacks that are widely dispersed in time, and are followed by complete recovery (McDonald et al., 2001) and do not lead to disability after 10 years of disease.

1.2 MS Etiology

1.2.1 Genetic Factors

The etiology of MS is not completely understood, although it is clear that genetic and environmental factors are involved. The genetic predisposition for MS is supported by a significant body of evidence. Family pedigree studies show that first degree relatives have a 20% higher incidence and a 2-3% increased risk of developing MS compared to the general population. As the closeness of relatives decreases, so too does the risk of developing MS: children of MS sufferers are at a greater risk than nephews and nieces (Sadovnick et al., 1993). Another strong evidence supporting the genetic component for MS is that the risk for developing MS is the same in adopted relatives of MS sufferers as in the general population (Ebers et al., 1995). Various twin studies have consistently demonstrated a concordance rate of 20 – 30% in

monozygotic twins compared to 2 – 5% in dizygotic twins (Ebers et al., 1986; Mumford et al., 1994; Robertson et al., 1996).

The methods used in the analysis of the genes that confer susceptibility to MS include linkage, association and candidate gene studies, or a combination of studies (Sawcer, 2006). Linkage studies seek to identify those genes that cosegregate with the disease in families, whereas in association studies the frequency of certain genes of unrelated individuals with and without the disease are compared. In candidate gene studies, variants of genes that may play a role in disease pathogenesis are examined.

Through linkage and association studies, the gene unambiguously associated with susceptibility to MS across multiple populations is the major histocompatibility complex (MHC) (Lincoln et al., 2005; Oksenberg and Barcellos, 2005; Sawcer et al., 2005). MHC is a complex of cell surface glycoproteins involved in self / nonself recognition, and in antigen binding and presentation to initiate the immune response. Human MHC is termed human leukocyte antigen (HLA) which is designated as HLA class I and class II, each of which are encoded by three separate genes: HLA-A, -B and -C for class I, and HLA-DP, -DQ and -DR for class II. The HLA gene cluster that is located on chromosome 6, consists of over 200 genes inherited in a Mendelian fashion with one copy from each parent. HLA genes are the most polymorphic in the human genome with multiple copies (alleles) of each gene being present in the population. Consequently, these polymorphisms result in differential binding of HLA to antigens thus leading to potentially different immune responses between individuals (Klein and Sato, 2000). In MS, MHC-associated susceptibility is conferred by HLA-DRB1 and HLA-DQB1. However, the exact mechanism by which these genes influence MS susceptibility are unknown, but are most likely to involve multiple epistatic interactions at these loci (Dyment et al., 2005).Candidate

genes associated with MS, but which have not withstood the test of time or are not confirmed, include TCRβ, CTLA-4, TNF-α and -β alleles, ICAM-1, CCR2, IL-10 receptor B, IFNβ, Notch4 and APOE4 (Sospedra and Martin, 2005).

Recently, an admixture mapping study was conducted in African-American patients. This approach is based on the observation that the prevalence rate of MS differs between European-Americans and Africans, and that African-Americans are an admixture of the two populations and have a prevalence of 40% that of European-Americans compared to 1% in the parent African population. Thus African-American MS patients are expected to have an increased proportion of susceptibility genes of European ancestry. Indeed, such a study has mapped a novel susceptibility gene on chromosome 1 (Reich et al., 2005).

1.2.2 Environmental Factors

The fact that about 70% of monozygotic twins are discordant for MS strongly implies that non-genetic factors are important in susceptibility to MS. Geographically, the prevalence of MS follows a specific distribution around the world. The disease prevalence increases with increasing distance from the equator. Thus people of northern European descent in North America, Europe, New Zealand and Australia are more likely to have MS than the inhabitants of Africa, Asia and South America (Compston, 1997). Geographical clusters or epidemics of MS have also been documented, as in the Faroe Islands where between the 1940's to the 1970's a significantly higher incidence of the disease was observed (Kurtzke and Hyllested, 1986). Other evidence that supports the significant role of the environment in MS has been obtained from migration studies which show that if one migrates before the age of 15 – 16, from a high to a low prevalence area, the risk of MS is lower than if one migrates after the age of 15 – 16 (Kurland,

1994). A proposed link between geography and MS prevalence is vitamin D biosynthesis which depends on sunlight exposure. Studies have demonstrated that high levels of vitamin D decrease the risk of MS, as well as relapses (Brown, 2006). Hormones are also potential risk factors considering that women are twice more likely to develop the disease than men. Similarly, during pregnancy, relapse rates are low, but rebound post-partum, and also MS symptoms worsen during menstruation (Confavreux and Vukusic, 2002).

Several infectious agents have been implicated in the pathogenesis of MS, but no conclusive evidence is forthcoming. Generally, MS patients exhibit abnormal immune responses to some viral antigens: antibodies against the Epstein-Barr virus (EBV) and T-cells specific to EBV nuclear antigen are present in significantly greater frequencies in MS patients than compared to control subjects (Wandinger et al., 2000; DeLorenze et al., 2006; Lunemann et al., 2006). Furthermore, the epidemiologies of MS and infectious mononucleosis, a common manifestation of EBV, are strikingly similar as individuals with a history of infectious mononucleosis have an increased risk for MS compared to individuals without such a history (Thacker et al., 2006). This putative association between EBV and MS is not surprising as other viruses are known to cause demyelinating diseases in humans. Examples of virus-induced demyelination include JC-induced progressive multifocal leukoencephalopathy (PML), subacute sclerosing panencephalitis (SSPE) and post-infectious demyelinating encephalomyelitis which follows infection with measles, varicella or vaccinia viruses, and human T cell lymphotropic virus (HTLV)-1-associated myelopathy / tropical spastic paraparesis (HAM-TSP) (Johnson, 1985). Pathologically, some viruses have also been detected in the CNS of MS patients and these include herpes simplex virus (HSV), measles and parainfluenza viruses. However, viral isolations have never been duplicated.

One plausible mechanism of how viruses may induce MS is molecular mimicry: homology exists between viral peptides and myelin antigens, such that anti-viral responses can also be directed at myelin. Another mechanism is super-antigenic stimulation of T cells by viral and bacterial proteins. Binding of these antigens to specific T cells via the T cell receptor (TCR) VB chain would result in non-antigen specific stimulation of whole populations of T cells, including myelin-reactive T cells (Zhang et al., 1995).

1.3 Diagnosis of MS

The diagnosis of MS is primarily clinical based on detailed neurological history and complete neurological examination, exclusion of other diseases and supported by paraclinical evidence. Over the years, criteria have been put forth to aid neurologists and researchers in the diagnosis of MS. As new technologies emerge and our understanding of the disease changes, the diagnostic criteria have been progressively refined with the most recent being an update to the McDonald Criteria (Polman et. al., 2005). The unifying concept of all criteria is evidence of multiple episodes of inflammatory lesions in the CNS which are disseminated in both space and time, i.e. more than one episode of neurological dysfunction involving more than one area of the CNS. Exclusion of other diagnoses involves laboratory examination of vitamin B-12 and thyroid-stimulating hormone (TSH) levels, syphilis serology and a complete blood cell count (Lublin, 2002).

Paraclinical evidence that aids in the diagnosis of MS include magnetic resonance imaging (MRI), visual evoked potentials (VEP) and cerebrospinal fluid (CSF) studies. MRI is an important radiologic tool that supports and improves the accuracy of MS diagnosis. MRI scanning of the brain and spinal cord is the most sensitive way of detecting MS lesions which

appear round or ovoid. Gadolinium enhancement, an indication of blood-brain barrier disruption, identifies active demyelinating lesions, whereas brain and spinal cord atrophy under MRI demonstrate cumulative tissue injury culminating in tissue destruction. VEPs assess sensory function and demonstrate that, consistent with demyelination, conduction of electrical impulses is altered.

Examination of the CSF and serum may reveal the presence of oligoclonal immunoglobulin (IgG) bands; the presence of these bands in CSF alone and not in serum indicates intrathecal IgG synthesis. CSF oligoclonal banding is the most consistent laboratory finding in MS patients appearing in over 90% of cases, though their role in the pathogenesis of MS remains elusive.

1.4 Immunopathology of MS

1.4.1 Pathological Hallmarks of MS

Based on clinical and MRI data, and on the observation that anti-inflammatory therapies are ineffective in PPMS, it is now recognized that MS manifests differently in RRMS and in PPMS. Neuroinflammation is the principal disease process in RRMS being characterized by inflammation and the appearance of new and active white matter lesions. The classic pathology of the chronic inflammatory lesion was first defined by Charcot and the disorder aptly named "la sclerose en plaques" (Hickey, 1999). The sharply demarcated plaques, with a predilection to the optic nerves, brain stem, periventricular white matter, cerebellum and spinal cord white matter, are characterized by myelin loss, axonal transections and astrocytic scars. There is a prominent infiltration by T cells, macrophages containing intracytoplasmic myelin degradation products and activated microglia. Also prominent is the disruption of the blood brain barrier (BBB), local expression of adhesion molecules (Cannella and Raine, 1995), costimulatory

molecules (Windhagen et al., 1995), and cytokines and chemokines and their cognate receptors (Sorensen et al., 1999).

In PPMS, the driving force behind the disease is neurodegeneration with diffuse atrophy of gray and white matter, changes in normal appearing white matter (NAWM) and the rare occurrence of new and active inflammatory lesions (Lassmann et al., 2007). The plaques are slow and show gradual expansions at the edges, and are accompanied by a moderate cellular infiltrate. Outside of the focal plaques, there is diffuse inflammation, microglial activation, axonal injury and loss, and secondary demyelination in the NAWM. The cortex also shows extensive demyelination. Investigators, analyzing a large series of brain biopsies and autopsies, have identified the pathological heterogeneity of plaques that are dependent on the degree of contribution of the different immune cells, antibodies and complement deposition to the lesion, the target of the immune-mediated attack (oligodendrocyte death versus myelin loss) and the amount of remyelination (Lucchinetti et al., 2000). Based on these characteristics, 4 pathological MS subtypes have been defined that vary interindividually but not intraindividually. Pattern 1 is predominated by T cell and macrophage infiltration, with a high level of remyelination as evidenced by shadow plaques (thinly remyelinated old lesions). Pattern 2 is characterized by a predominant antibody and complement mediated demyelination, but resembles pattern 1with T cell and macrophage infiltrates. Pattern 3 is characterized by profound oligodendrocyte apoptosis, a selective loss of myelin-associated glycoprotein (MAG) and a rim of myelin surrounding nerve fibres. Pattern 4 is marked by non-apoptotic oligodendrocyte loss with no selective MAG loss and a low potential for remyelination as evidenced by the lack of shadow plaques.

1.4.2 The Inflammatory Response in MS

Traditionally the CNS is considered as an immunologically priviledged site, being excluded from the cells and mediators of the immune system, and with limited immune response to infectious agents and minimal tissue injury. Two cardinal features of the CNS, an immunological and a physical barrier, are responsible for maintenance of this status. The immunological barrier consists of the lack of draining lymph nodes and immune surveillance, minimal local expression of MHC class I and class II molecules, costimulatory molecules, and few resident antigen presenting cells. Regulatory mechanisms are also at work, which actively suppress the immune response in the CNS. The BBB presents a physical barrier that separates the CNS from the peripheral circulation. It is a tightly sealed interface formed by capillary endothelial cells (ECs) of the brain and spinal cord, in association with pericytes, astrocytes, microglial cells and smooth muscle cell (Correale and Villa, 2007). These specialized ECs of the CNS possess unique features that distinguish them from ECs in peripheral tissues: they are non-fenestrated and connected through tight junctions at their adjacent margins and thus limiting transmigration across the brain to transcellular mechanisms. However, it is now known that the immunopriviledged status of the CNS is conditional because the BBB is not an absolute barrier as immune cells are present in the CNS under normal conditions, albeit in low numbers (Hickey, 1991). Under pathological conditions such as MS, activated lymphocytes can migrate across the BBB into the CNS which becomes an inflammatory milieu with a bidirectional communication between the invading immune cells and the resident cells (Lassmann et al., 1991)

While it's etiology remains elusive, it is generally accepted that MS is an autoimmune demyelinating and neurodegenerative disease initiated by T cells that are reactive against myelin

components such as myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG) (Steinman, 1996). This consensus stems largely from the strong resemblance of MS to chronic experimental allergic encephalomyelitis (EAE) which is the most intensively studied experimentally induced animal model of MS. Chronic EAE is an inflammatory demyelinating disease of the CNS and reflects many of the immunopathological aspects of MS, including the role of autoreactive T cells and antibodies, myelinotoxic cytokines like TNF-αand adhesion molecules and MMPs (Steinman, 1999). EAE was initially observed after repeated injections of monkeys with spinal cord material (Rivers and Schwentker, 1935). Kabat later proposed an autoimmune etiology and the presence in CNS white matter of the eliciting autoantigen (Kabat et al., 1942). EAE can also be induced in susceptible strains of mice, rats, rabbits and guinea pigs by immunization with whole spinal cord, MBP, PLP, MOG, or by experimental infection with neurotropic viruses such as Semliki Forest and Theiler's viruses (Fazakerley and Webb, 1987; Lipton, 1975). Another EAE animal model involves the adoptive transfer of MBP-specific CD4+ T-helper cells to naïve recipients (Constantinescu et al., 1998). Both healthy individuals and MS patients possess in their blood, circulating T cells that can react against self-antigens in the CNS, which indicates that these cells escape thymic negative selection (clonal deletion) during T cell development and become established as a component of the normal T cell repertoire (Pette et al., 1990; Markovic-Plese et al., 2004). However, these autoreactive T cells are more numerous and are at a higher state of activation in MS patients as compared to normal individuals (Olsson et al., 1992).

The CNS inflammatory response in MS is a multistep process consisting of activation, adhesion, attraction, migration and reactivation. Central to this process is a trimolecular complex consisting of the interaction of T cell receptor, MHC molecules on antigen presenting cells (APCs) and the

putative self-antigens. The inflammatory response is initiated by the activation of T cells of the CD4+ helper and CD8+ cytotoxic phenotypes in the periphery. The T cells are activated by APCs which present myelin and other cross-reactive microbial antigens in the context of MHC Class II and MHC I molecules. Expression of adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 as well as their cognate receptors are upregulated, and this allows activated T cells to adhere and extravasate through the endothelium and subendothelial basal lamina of the BBB into the interstitial matrix of the CNS (Hauser and Oksenberg, 2006). Chemokine signals induce and activate adhesion molecules, and attract activated T cells to the endothelium of the BBB, whilst matrix metalloproteinases (MMPs) are involved in the penetration of the BBB as they digest the extracellular matrix composed partly of type IV collagen (Hafler, 2004).

Once in the CNS, T cells are reactivated by macrophages, microglia and astrocytes which perpetuate the inflammatory responses and retain the activated T cells. Activated T cells provide help to B cells, and secrete proinflammatory cytokines and chemokines which recruit more T cells and other non-specific immune cells to initiate the inflammatory cascade (Ransohoff, 1999). Demyelination ensues by an inflammatory process that is consistent with a delayed-type hypersensitivity reaction: macrophages are in close contact with disintegrating myelin sheath and contain intracytoplasmic myelin breakdown products. Other factors that cause demyelination include CD8+ -mediated cytotoxicity, antibody- and complement-mediated cytolysis, reactive oxygen and nitrogen species, toxic effects of TNF-α and IFN-γ, excitatory amino acids, proteolytic and lipolytic enzymes, Fas-Fas ligand interaction and perforin (Noseworthy et al., 2000). Accompanying the inflammatory cascade is axonal injury and loss, the cause of which remains to be determined; it is not known whether axonal damage occurs as a result of

demyelination or whether it is the direct result of macrophage and cytotoxic molecules. It is also proposed that the loss of trophic support provided to axons by myelin and glia may result in axonal damage (Compston and Coles, 2002).

Subsequently, the inflammatory response subsides, as anti-inflammaory cytokines such as interleukin (IL)-4, IL-5, IL-10 and transforming growth factor (TGF) β down-regulate the autoimmune response. IL4 and neurotrophic factors such as brain derived neurotrophic factor (BDNF) also promote the remyelination of axons and the maintenance of neurogenesis (Hohlfeld et al., 2000; Ziv et al., 2006). Thus, paradoxically, inflammation has both destructive and neuroprotective (beneficial) properties and hence the term " the Janus face of inflammation" to describe this dichotomy (Ruffini et al., 2004).

1.4.3 Cellular and Humoral Immune Responses in MS

Albeit simplistic, T cells are still believed to be central to the pathogenesis of MS. T cells are categorized into 2 subtypes, αβ and γδ, according to their T cell receptor (TCR) variable gene (Vβ), with αβ cells constituting over 95% of T cells in the blood. Based on the surface proteins they express and which MHC protein they recognize, αβ cells can be further divided into T helper (T_H) CD4+ and T cytotoxic (T_C) CD8+ T cells. CD4+ cells recognize antigen in the context of MHC class II protein and are involved in delayed type hypersensitivity reactions and in antibody responses, whereas CD8+ recognize antigen in the context of MHC class I protein and are involved in cell-mediated cytotoxicity. CD4+ cells can be divided into T_H1 and T_H2 subsets which produce proinflammatory cytokines like IL-2, TNF-α and IFN-γ, and anti-inflammatory cytokines like IL-4, IL-5, IL-10 and IL-13, respectively, and as T_H0 that are neither pro- nor anti- inflammatory, with the prevailing intercellular milieu dictating which

subset is induced (Imitola et al., 2005). In MS, the immune deviation is suspected to be towards a T_H1 differentation (Martino and Hartung, 1999).

The pivotal contribution of CD4+ T cells to MS is amply demonstrated. Besides the cardinal observation that EAE can be induced by the adoptive transfer of myelin-reactive CD4+ T cells, numerous myelin and non-myelin antigens have been found to be targets of CD4+ T cells, and to be encephalitogenic in animals, and immunogenic in MS and in normal individuals; MBP, PLP, MOG, MAG, myelin-associated oligodendrocyte basic protein (MOBP), oligodendrocyte-specific protein, α-B crystallin, 2' 3'-cyclic nucleotide 3' phosphodiesterase (CNPase), S-100b protein, transaldolase-H (TAL-H) and gangliosides (Prat and Martin, 2002). Other evidence include the presence of CD4+ T cells in the CNS and CSF cellular infiltrates, MHC class II molecules (HLA-DR and –DQ) conferring an increased risk to MS, expression of MS-associated HLA-DR and MS-associated MBP-specific TCR chains as transgenes in mice resulting in the spontaneous development of EAE, and in a phase II study of an altered peptide ligand (APL) of MBP in MS patients, CD4+ T cells reacting against both MBP and APL worsened the disease (Sospedra and Martin, 2005). CD8+ T cells are also strongly implicated in the pathogenesis of MS, stemming in part from the fact that they dominate the inflammatory infiltrates in MS lesion at all stages of their development (Bitsch et al., 2001), and that myelin- and MBP-specific CD8+ T cells can induce EAE which mimics MS more than CD4+-mediated EAE (Sun et al., 2001). Under inflammatory conditions, there is an increased expression of MHC I, but not MHC II, in functionally compromised neurons, consistent with a prominent CD8+ T cell role in neuronal injury. Most importantly, the CD8+ response to MBP is increased in MS patients (Zang et al., 2004). CD8+ T cells can contribute to the pathology of MS in various ways. Through engagement of their Fas ligand to Fas antigen on oligodendrocytes, they can kill the latter by the

process of programmed cell death (apoptosis). By secreting enzymes such as perforin and granzymes, they are also directly cytotoxic to oligodendrocytes expressing myelin epitopes in the context of MHC I (Jurewicz et al., 1998). In another capacity, CD8+ myelin-specific T cells secrete chemoattractants such as macrophage inflammatory protein (MIP)-1α and MIP-1β, IL-16 and interferon inducible protein (IP)-10 to recruit CD4+ myelin-specific T cells to the site of injury (Biddison et al., 1998).

γδ T cells, which are MHC-unrestricted, are also involved in the pathogenesis of MS, and are found in excess in blood and CSF of MS patients. Furthermore, depletion of these cells has been shown to reduce disease activity in EAE. *In vitro*, γδ T cells can bind to stress-induced heat shock proteins on oligodendrocytes, and lyse them via perforin (Selmaj et al., 1991; Battistini et al., 1995) .

The MS plaque also contains B cells, plasma cells, antibodies and Ig transcripts (Genain et al., 1999; Lock et al., 2002). Within the CNS, phenotypic and molecular analyses reveal that the B cells and plasma cells undergo a T cell-mediated antigen-driven clonal expansion, resulting in raised intrathecal Ig synthesis and the detection of oligoclonal bands (Qin et al., 2003). The precise antigenic specificities of these CSF Igs have not been fully established, but reactivity against several infectious agents including measles, mumps, herpes simplex type-1 (HSV-1), varicella-zoster virus, cytomegalovirus, rotavirus and Epstein-Barr virus and Chlamydia pneumonia, have been reported in MS patients (Hafler et al., 2005). Antibodies with specificities for MOG, MBP and other myelin proteins have been also detected in the serum and CSF of MS patients.

The involvement of B cells and autoantibodies in the pathogenesis of MS is further substantiated, in part, by the finding that culturing nerve cells in serum results in their demyelination by

humoral factors identified as anti-myelin antibodies (Bornstein and Appel, 1959). Also administration of anti-MOG antibodies can exacerbate disease in EAE disease (Raine et al., 1999), but B cells and antibodies alone cannot initiate the disease, in addition to the observation that B cell-deficient mice can still develop EAE (Wolf et al., 1996). This indicates that B cells and autoantibodies are not an absolute requirement for EAE. In patients with clinically isolated syndromes, anti-MOG antibodies were found to be predictive of subsequent exacerbations and the development of clinically definite MS (Berger et al., 2003), although these findings could not be confirmed by another group of investigators (Lampasona et al., 2004). As the subtyping of MS lesions revealed (Lucchinetti et al., 2000), Pattern II is characterized by an abundant accumulation of antibodies and complement on the myelin sheath, thus implicating the destruction of the myelin by antibody / complement deposit. Plasma exchange, a procedure to remove serum components, in MS patients effectively reversed severe neurological deficits, again implicating antibodies as effector molecules in MS (Weinshenker et al., 1999). A potential role of B cells in MS includes the production of autoantibodies against myelin that can cause demyelination through antibody dependent cell-mediated cytotoxicity (ADCC). Autoantibodies may also act as opsonin promoting phagocytosis by macrophages or they may activate complement that can lyse oligodendrocytes and microglia (Archelos et al., 2000). As B cell and T cell epitopes share sequence homology, B cells can act as APCs presenting antigens to T cells, in addition to providing costimulatory signals (Sospedra and Martin, 2005). For a naïve T cell to be fully activated, it requires two signals; signal 1 is provided when TCR recognizes the MHC II / antigen complex on APCs, and signal 2, a costimulatory or secondary signal, occurs when APCs expressing the B7 family of cell surface molecules, CD80 and CD86, and CD 40 engage the cognate receptors, CD28, CTLA-4 and CD40L respectively, on T cells. In general, CD80-

CD28 interactions are involved in stimulating a T_H1 response, while the CD86-CTLA-4 interaction is important in the downregulation of the immune response (Racke et al., 2000). As such, the increased frequency of B cells expressing CD80 molecule and CD86-bearing monocytes have been noted in MS patients in clinically active patients and during relapses (Genc et al., 1997; Boylan et al., 1999).

Macrophages are some of the most abundant infiltrating cells and outnumber lymphocytes by greater than 10-fold in demyelinating lesions (Lucchinetti et al., 2000). They appear at the edges of active demyelinating plaques, and indeed demonstration of intracytoplasmic myelin-degradation products is the best way of identifying these plaques (Lassmann et al., 1998). Evidence for the role of macrophages in MS comes from studies in EAE where demyelination is mediated primarily by macrophages, and animals devoid of macrophages do not develop EAE (Brosnan et al., 1981). Besides scavenging and phagocytosing myelin, macrophages are the major APCs in MS lesions, and when activated they secrete neurotoxins like IL-1, IL-6, TNF-α and nitric oxide which damage myelin (Al-Omaishi et al., 1999).

1.4.4 Cytokines in MS

Cytokines are soluble factors secreted by immune cells during an antigenic challenge and are involved in modulating a wide variety of immune responses including immune activation, cell movement, antigenic recognition and effector functions. They are broadly divided into 2 categories. The proinflammatory cytokines are secreted by Th1 cells and serve to upregulate the inflammatory response, while anti-inflammatory cytokines are secreted by Th2 cells and serve to downregulate inflammatory responses. Under homeostatic conditions there is a dynamic balance between pro-inflammatory and anti-inflammatory cytokines, and an imbalance in the cytokine

network is a prelude to autoimmune diseases such as MS (Kuchroo et al., 2002). Binding of cytokines to their cognate receptors initiates a cascade of intracellular signals that culminates in cellular functions. Signal transduction of cytokines commonly involves Janus kinase and signal transducer and activator of transcription (Jak / STAT) pathways, with different cytokines utilizing different Jak / STAT proteins.

Cytokines are becoming increasingly recognized as key players in the pathogenesis of MS with the development of novel detection systems with high sensitivities, and on the basis of both EAE and MS studies, even though findings are often contradictory (Sospedra and Martin, 2005). In general, proinflammatory cytokines exacerbate disease in EAE and MS, while anti-inflammatory cytokines ameliorate disease. Prior to clinical relapses in RRMS patients, the mRNA levels of the proinflammatory cytokines TNF-α and lymphotoxin (LTα) or TNF-β increase compared to the decreased levels of the anti-inflammatory cytokines TGF-β and IL-10, in peripheral blood mononuclear cells (PBMCs) (Rieckmann et al., 1995). A growing number of cytokines have been identified in active MS lesions, CSF and blood (Navikas and Link, 1996). During relapses, there is a preferential augmentation of pro-inflammatory cytokines whilst anti-inflammatory cytokines dominate during remissions. It is hypothesized that the genetic basis of MS can be explained, at least in part, by the possibility that MHC II genes command differential expression of cytokines as seen in other autoimmune disease (Imitola et al., 2005). TNF-α is one of the most critical cytokines in MS and its levels in serum and CSF have been shown to correlate with clinical disease activity (Bitsch et al., 1998), and in actively demyelinating lesions its expression is increased as compared to inactive lesions (Bitsch et al., 2000). It is produced by activated macrophages, and following binding to its receptor, mediates a plethora of events. Most importantly, it is cytotoxic to oligodendrocytes and damages myelin in nervous tissue cultures

(Conlon et al., 1999). TNF-α can also stimulate endothelial cells to express ICAM-1, and levels of TNF-α correlate well with BBB damage. In synergy with IFN-γ, TNF-α induces MHC II molecules and therefore increases antigen presentation to autoreactive T cells and promotes the inflammatory process. In EAE studies antibodies to TNF-α and TNF-α receptor ameliorate the disease (Selmaj et al., 1991). Nonetheless, in TNF-α-deficient mice the EAE disease is worsened (Liu et al., 1998), and treatment of MS patients with TNF-α receptor Ig fusion protein or anti-TNF-α antibodies resulted in worsening of the disease (van Oosten et al., 1996; The Lenercept Study Group and the University of British Columbia, 1999).

Another pro-inflammatory cytokine that plays an important role in MS is interferon gamma (IFN-γ), the prototypical marker of a T_H1 response, produced by T cells and natural killer (NK cells). IFN-γ is a potent activator of macrophages leading to upregulation of MHC and adhesion molecules, enhanced cytokine production, and induction of nitric oxide synthase. The latter stimulates the release of nitric oxide that is a major mediator of myelin and oligodendrocyte damage, and it further stimulates phagocytosis of myelin fragments by macrophages. IFN-γ also stimulates B cell switching, differentiation of T cells to the T_H1 subset and the apoptosis of T cells. As with TNF-α, IFN-γ levels correlate with MS disease activity. However, the precise role that it plays is contradictory: increased production of IFN-γ by PBMCs have been found in MS patients, whereas others have found no such associations (Becher et al., 1999; Nguyen et al., 1999). Not surprising, a clinical trial to ascertain the efficacy of IFN-γ in MS was discontinued as it exacerbated the disease (Panitch et al., 1987).

1.4.5 Chemokines in MS

Chemokines are a superfamily of soluble proteins that are secreted by various cell types

responding to tissue injury and other stimuli. To date, approximately 50 chemokines have been identified, and these are categorized into 4 families on the basis of the number and spacing of the first 2 conserved cysteine residues at the amino terminus: α (C-X-C), β (C-C), γ (C) and fractalkine (C-X3-C) families (Esche et al., 2005). Chemokines function by binding to chemokine receptors that are members of the seven-transmembrane G protein-coupled receptor (GPCR) family. They activate a multitude of signaling pathways resulting in various responses including chemotaxis, cell activation and polarization of TH cells towards the T_H1 or T_H2 phenotypes. On the basis of their functional activities, chemokines can be grouped into inducible or inflammatory chemokines and constituitively expressed or homeostatic chemokines (Kapsenberg, 2003). A prominent characteristic of the inflammatory chemokines is their polygamous receptor usage, thus explaining the redundancy of the chemokine-chemokine receptor network. The homeostatic chemokines are less redundant and are responsible for lymphocyte homing.

Central to the pathogenesis of MS is the recruitment of inflammatory T cells and other cell types across the BBB, and it is now appreciated that chemokines are involved in this process. They induce the expression of adhesion molecules and their corresponding receptors that allows for the firm adherence of T cells to the endothelial monolayer of the BBB, and establish a chemotactic gradient that enables migration into the CNS. Proinflammatory TH1 cells express a set of receptors (CCR5 and CXCR3) distinct from those expressed on the anti-inflammatory TH2 cells (CCR3 and CCR4) (Correale and Villa, 2007). Observations from EAE studies strongly support the importance of chemokines in MS. Elevated levels of CCL2, CCL3, CCL5 and IP-10 have been reported in animals during the progressive phase of EAE, and removal of these chemokines resulted in disease amelioration (Elhofy et al., 2002). During the preclinical and

recovery or subclinal phases of EAE, minimal or significantly decreased levels of chemokines were reported (Miyagishi et al., 1997). In MS patients, increased expression of chemokines and chemokine receptors have been observed in CNS lesions (Sindern, 2004). Studies of the CSF during relapses have also found the overexpression of chemokines including MIP1-a, CCL3, CCL5, CXCL9 and IP-10 compared to controls (Miyagishi et al., 1995; Sorensen et al., 1999; Mahad et al., 2002). Similar results were observed in another study that found significantly elevated levels of IP-10 and CCL17, but significantly lower levels of CCL2 in MS patients, than in controls (Narikawa et al., 2004). Peripheral T cells have been reported to have increased expression of CCR5 and CXCR3 during relapses (Balashov et al., 1999). Finally, in a study of PLP-specific CD8+ T cell lines generated from MS patients, the T cells were shown to secrete MIP1-a, IL-16 and IP-10 (Biddison et al., 1997). Thus ample evidence supports the active involvement of chemokines / chemokine receptors in MS.

1.4.6 Adhesion Molecules in MS

The transmigration of inflammatory cells across the BBB into the CNS is an essential step in the pathogenesis of MS and is mediated by adhesion molecules that are expressed on cells of the inflamed endothelium and on activated leukocytes (Springer, 1994). Cell adhesion molecules are cell surface glycoproteins that mediate specific cell-to-cell interactions and are divided into 3 families depending on their structure: selectins, integrins and members of the immunoglobulin (Ig) superfamily. From the perspective of MS pathogenesis, two molecular pairs have been identified as being crucial in the transmigration of inflammatory cells. The intercellular adhesion molecule 1 (ICAM-1), a member of the Ig superfamily expressed on cells of the inflamed endothelium and on leukocytes, recognizes its ligand lymphocyte function associated antigen 1

(LFA-1) a member of the intergrin family which is expressed on leukocytes, and the vascular cell adhesion molecule 1 (VCAM-1) another Ig superfamily member expressed on endothelial cell and macrophages recognizes its ligand, the very late activation antigen 4 (VLA-4) another intergrin expressed on monocytes and lymphocytes (Correale and Villa, 2007). Under homeostasis, cerebrovascular endothelium expresses minimal or no adhesion molecules, but under inflammatory conditions endothelial cells and immune cells are activated by pro-inflammatory cytokines such as TNF-α resulting in the upregulation of adhesion molecules (Elovaara et al., 2000). In addition, chemokines increase the affinity of adhesion molecules for their respective ligands.

Adhesion molecules are shed from the cell surface and secreted into bodily fluids such as serum and CSF, following interactions with their ligands. Consequently, quantitation of circulating adhesion molecule levels have been correlated to disease activity and used as surrogate markers for MS disease progression (Bitsch et al., 1998). Studies in MS patients have also indicated significantly increased serum levels of VCAM-1 and L-Selectin and of ICAM –1 and E-Selectin, all of which are associated with varying disease activity and clinical course (Dore-Duffy et al., 1995; Hartung et al., 1995; Kraus et al., 1998).

1.4.7 Matrix Metalloproteinases in MS

Once T cells have passed through the endothelium by the process of diapedesis, they must penetrate the subendothelial basement membrane, which consists primarily of type IV collagen. This involves the degradation of the basement membrane and the process is mediated by matrix metalloproteinases (MMPs). MMPs are family of 23 structurally and functionally related proteolytic enzymes that have a characteristic Zn^{2+} ion at their active site. They are involved in

the degradation of all components of the extracellular matrix, a process important during embryogenesis, osteogenesis and wound healing, and are also important in cellular signaling and survival (Hartung and Kieseier, 2000; McCawley and Matrisian, 2001). Abnormal expression or overexpression of MMPs, however, leads to excessive degradation of the extracellular matrix which could result in diseases such as autoimmune diseases (Yong et al., 2001). Thus MMP expression and activity are closely controlled at different levels. At the gene level, transcription is regulated by growth factors, chemokines and cytokines, with TNF-α being a potent inducer of MMPs. Posttranscriptional regulation entails the secretion of MMPs as inactive proenzymes that are activated by proteolytic cleavage. A group of 4 endogenous antagonists, the tissue inhibitors of MMPs (TIMPS) bind to the catalytic site of MMPs and tightly regulate the proteolytic activity of MMPs by binding to their catalytic site (Correale and Villa, 2007).

MMPs are critical factors that have been implicated in the pathogenesis of MS, being intricately involved in the breakdown of the BBB and migration of cells into the CNS. Evidence supporting their role comes from EAE studies and from MS patients (Hartung and Kieseier, 2000). In EAE, enhanced expression of MMPs correlates with increased disease activity (Yong et al., 2001) and inhibition of these MMPs alleviates the severity of the disease (Norga et al., 1995). MMP-deficient mice are more resistant to EAE than wild-type mice. In MS patients, enhanced expression of MMP-2, MMP-3, MMP-7, MMP-9 and MMP-12 have been reported in brain tissues (Cuzner and Opdenakker, 1999; Lindberg et al., 2001; Vos et al., 2003). Elevated levels of MMP-9 in serum and CSF have also been associated with increased disease activity as evidenced in gadolinium-enhancing lesions on magnetic-resonance imaging (Waubant et al., 2003). Furthermore, in a comprehensive analysis of leukocyte subsets, different cell populations exhibited characteristic patterns of MMP expression: MMP-11, MMP-26 and MMP-27 were

overexpressed in B cells, MMP-15, MMP-16, MMP-24 and MMP-28 were enriched in T cells, and MMP-1, MMP-3, MMP-9, MMP-10, MMP-14, MMP-19 and MMP-25 were prominently expressed in monocytes / macrophages (Bar-Or et al., 2003). Inhibitors of MMPs have also been demonstrated to be able to inhibit the transmigration of T cells across BBB in in vitro BBB systems (Brundula et al., 2002). In addition to remodeling of extracellular matrices, MMPs are involved in the proteolysis of CNS demyelination and axonal injury. MMPs also have TNF-α convertase activity, being able to cleave cell-bound TNF-α to soluble forms (Conlon et al., 1999).

1.5 Therapy of MS

Though MS is presently not medically curable, three parallel approaches are used in the treatment of affected patients. These include symptomatic management of clinical and neurological complaints, management of acute attacks, and disease modifying therapies. The rationale behind current therapeutic approaches is based on the increased understanding of the etiology and pathogenesis of the disease, and on the hypothesis that MS is an organ-specific autoimmune disease. Furthermore, elucidation of the mechanisms of action of these therapies has widened the knowledge on the etiology of MS (Hafler, 2004).

1.5.1 Symptomatic Treatment

Management of symptoms involves a multidisciplinary approach that utilizes pharmacological and non-pharmacological methods (Metz, 1998). Common complaints and discomforts can be alleviated with medications such as baclofen for spasticity and sildenafil acetate for sexual dysfunction. Intense rehabilation to improve function and the quality of life include physical,

occupational, speech, cognitive and recreational therapies, social intervention and psychological counselling.

1.5.2 Management of Acute Attacks

An attack consists of the appearance of new neurological symptoms or worsening of preexisting symptoms, with a duration of ≥24 hours and without concommittant infection. Mild attacks do not usually require intervention as they resolve spontaneously. However, moderate to severe attacks do not resolve spontaneously and cause functional impairment (Keegan and Noseworthy, 2002). These are normally treated with corticosteroids, which include adrenocorticotropic hormone (ACTH), prednisolone, methylprednisolone and dexamethasone. They act by accelerating the body's natural recovery process and shortening the duration of relapses. These drugs have immunomodulatory and anti-inflammatory effects that decrease the production of pro-inflammatory cytokines, inhibit T_H1 cells and macrophages, reduce brain edema, restore BBB integrity and improve neuronal condition (Pozzilli et al., 2004). However, corticosteroids are only used for short-term and not long-term management of acute attacks, as they are associated with numerous adverse side effects such as bone degeneration, cataracts, and salt and water imbalances (Rudick et al., 1997). Finally, in steroid-resistant relapses, plasma exchange in MS patients has been shown to be beneficial in reducing acute neurological attacks (Kieseier et al., 2007).

1.5.3 Disease-Modifying Therapies

Disease-modifying therapies consist of immunosuppressants and immunomodulators. They impact on the biology of MS to reduce the frequency and severity of relapses, and at the same

time delaying or preventing relapse-related disease progression.

1.5.3.1 Immunosuppressive Therapy

Immunosuppressants are chemotherapeutic agents, which are typically used in conservative doses to treat MS patients who are at advanced stages of the disease or who are not responding to the currently approved traditional immunomodulatory therapies. They act globally by dampening the autoreactive mechanisms in MS, and consequently reducing the frequency and severity of relapses. However, immunosuppressants have not gained widespread acceptance because of their non-selective nature, considerable toxicity, limited efficacy and the requirement for frequent hematologic monitoring. The scant availability of supporting MRI data also adds to their limited use.

Mitoxantrone, an immunosuppressive agent, is indicated for treating worsening RRMS and SPMS patients for whom it was shown to be effective in reducing relapses and disease progression (Hartung et al., 2002). It is a synthetic anthracenedione that inhibits DNA synthesis in both proliferating and non-proliferating cells by interfering with DNA topoisomerase. In MS, it acts by suppressing T cells, B cells and macrophages (Neuhaus et al., 2004). Azathioprine suppresses cell-mediated and humoral immunity, and has been shown to reduce relapse rates, but it has no significant effect on disease progression. Methothrexate also inhibits cell mediated and humoral immunity, and appears to be marginally effective in reducing progression of disease. Cyclosporine A has a selective inhibitory effect on T_H cells, but it is minimally effective in reducing relapses or disease progression. Other immunosuppressive agents with marginal effects include cladribine, mycophenolate and cyclophosphamide, an alkylating agent that has potentially life-threatening side effects.

1.5.3.2 Immunomodulatory Therapy

The main goal of therapy in patients with RRMS is to reduce the frequency and severity of relapses and to prevent or postpone the progressive worsening of the disease. In the past, immunosuppressive drugs have been used, but owing to their unimpressive efficacy and potential toxicity, they have been largely replaced by immunomodulatory agents. Presently, 6 medications are approved for the treatment of RRMS, including the three interferon betas (IFNβ, glatiramer acetate, mitoxantrone and natalizumab). The IFNβs and glatiramer acetate are first- line therapies in use for over a decade, and with proven efficacy in reducing relapses and MRI-measured disease activity, and with acceptable safety and tolerability records. Mitoxantrone and natalizumab are second-line therapies having only recently been approved. They are similarly efficacious, but have potential toxicity issues with Natalizumab having to be temporarily withdrawn because of the risk of developing progressive multifocal leukoencephalopathy (PML) (Costello et al., 2007).

1.5.3.2.1 Glatiramer Acetate (GA)

Glatiramer acetate (GA; Copaxone™) is a pool of random synthetic polypeptides composed of the most prevalent amino acids in MBP namely L-alanine, L-glutamate, L-lysine and L-tyrosine, in a defined molar ratio and with lengths of 40- 100 amino acid residues. It is thus an immunochemical mimic of MBP and was initially shown to inhibit EAE in various species including guinea pig, rabbit, mouse, rhesus monkey and baboon (Arnon and Aharoni, 2004). A small trial of 50 patients that led to the approval of the agent, indicated efficacy in the treatment of RRMS (Bornstein et al., 1987). Subsequently, in a pivotal multicenter trial of 251 patients and

in longer-term follow-up studies, daily subcutaneous GA was shown to reduce annual relapse rates by about 30% in mildly affected MS patients (Johnson et al., 1995; Johnson et al., 2000). GA does not seem to be active orally: in a recent double-blinded, randomized, multicentre study (CORAL), GA administered orally did not have any effect on RRMS patients treated over 14 months (Filippi et al., 2006).

GA binds strongly to MHC II and is presented by APCs resulting in the generation of GA-reactive T cells, mainly of the T_H2 lineage, which are central to the mechanisms of action of GA. These include competitive inhibition of MBP-reactive T cells, the production of anti-inflammatory cytokines which suppress all autoreactive T cells in the vicinity (bystander immune suppression), modulation of myelin-reactive T cells and inhibition of monocytes (Hemmer et al., 2006). In addition, GA-reactive T cells can produce neurotropic factors such as BDNF which promote neuroregeneration. GA is generally well tolerated with only mild transient systemic reactions including flushing, chest tightness, palpitations, dyspnea and anxiety. Chronic administration results in the development of serum anti-Copaxone antibodies, but it is not clear whether the presence of these antibodies compromises the clinical benefits (Farina et al., 2005; Basile et.al., 2006).

1.5.3.2.2 Interferon Beta (IFNβ)

IFNβ was the first therapeutic agent shown to alter the natural history of MS in RRMS patients and is the first line of treatment in patients with RRMS. Three different IFNβ preparations have been approved for MS therapy: IFNβ-1b (Betaseron®- Bayer), IFNβ-1a (Avonex®- Biogen-IDEC), IFNβ-1a (Rebif®- EMD-Serono). IFNβ-1b is a non-glycosylated fibroblast-derived IFNβ that is produced in *Eschericia coli (E. coli)* with a Met-1 deletion and a Cys-17 to Ser amino acid

substitution in the 165 amino-acid peptide (Markowitz, 2007). IFNβ-1a is a glycosylated IFNβ produced in mammalian (Chinese hamster ovary) cells and whose amino acid sequence (166 amino acids) is identical to the naturally occurring human IFNβ.

The original rationale for IFNβ therapy in MS patients was based on the concept that the disease might be caused by a viral infection and on the fact that IFNs had antiviral activity. In a multicentre, double-blinded, randomized, placebo- controlled study, MS patients that received natural fibroblast IFNβ intrathecally had a significant reduction in relapse rates compared to placebo controls (Jacobs et al., 1985; Jacobs et al., 1986). However, trials were limited due to the difficulties in obtaining natural IFNs. With the advent of genetic engineering, large quantities of recombinant IFNs became available for Phase III clinical trials, which were initiated to assess the safety and efficacy of the recombinant IFNβs. In a large multicenter, double-blinded, randomize, placebo- controlled study Betaseron®, given subcutaneously at a dose of 8 x 10^6 International Units (IU) every other day, significantly reduced relapse rates by about 30% compared to placebo controls. A non-significant trend towards reduced disability was also noted in this study (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1995). Similarly, a randomized, double-blinded, placebo-controlled study was used to assess intramuscularly administered Avonex given at a dose of 6 x 10^6 IU once weekly. Avonex®-treated patients had a significantly longer time to onset of sustained progression in disability together with a 32% reduction in relapse rates compared to placebo controls (Jacobs et al., 1996). The Rebif® trial was also a randomized, double-blinded, placebo-controlled study. Patients were treated with either 6 x 10^6IU or 12 x 10^6 IU of Rebif given subcutaneously three times weekly. Compared to placebo controls, Rebif –treated patients had a significantly reduced relapse rate (32%) and an equally significant reduction in disease progression (The PRISMS Study Group

and the University of British Columbia, 1998). Thus IFNβ has been conclusively shown to reduce clinical relapses, decrease brain MRI activity and slow the progression of the disease. However, IFNβ is only partially effective and a great number of patients are refractory to treatment. This inter-individual variability in IFNβ responsiveness has been investigated by analyzing polymorphisms in the IFNβ receptor, as it is well established that in treatments of other diseases, polymorphisms in drug targets or receptors confer these differences. In the case of the IFNβ receptor, genetic heterogeneity did not affect IFNβ treatment response (Sriram et al., 2003; Leyva et al., 2005). However, unresponsiveness to IFNβ therapy in various malignancies has been linked to circulating inhibitory factors, independent of IFN-induced antibodies, including free soluble IFNα/β Type 1 receptors (sIFNAR), a protein termed interferon inhibitory activity (IIA), prostaglandin E2 and high levels of cAMP phosphodiesterase (Chadha et al., 2004). Indeed, recently the role of these inhibitors was investigated in IFNβ-treated MS patients, who were negative for anti-IFNβ antibodies. It was found that generally, MS patients had significantly elevated levels of IIA and sIFNAR compared to healthy controls. There were also significantly elevated levels of IIA in partial responders (active disease) in comparison to good responders (stable disease). However, the sIFNAR levels did not differ between the partial and good responders. Thus the investigators concluded that unresponsiveness to IFNβ and increased disease activity are linked to IIA (Chadha et al., 2006).

1.6 Anti-IFN*β* Antibodies in MS

1.6.1 IFNβ Mechanisms of Action

An absolute prerequisite to the understanding of the immunogenicity of IFNβ and the induction of antibodies against IFNβ, is the elucidation of the mechanisms of action of IFNβ. The IFNs

were first discovered in 1957 by Isaac and Lindemann who observed that a soluble factor, produced by virus-infected cell cultures derived from chick chorioallantoic membranes, could transfer protection against subsequent viral infection to other cells (Isaacs and Lindenmann, 1957). They are a family of naturally occurring cytokines that have antiviral, antiproliferative, antiangiogenic, antitumor and immunomodulatory activities. IFNs are produced by cells in response to biological stimuli such as viruses, bacteria, mycoplasma, mitogens, double-stranded RNA, polypeptides and other cytokines. Originally they were classified based on their cells of origin (IFNα in leukocytes, IFNβ in fibroblasts and epithelial cells, and IFNγ in T-lymphocytes), separation profiles on high-pressure liquid chromatography and antigenicity (Parmar and Platanias, 2003). There are two major groups of IFN, designated as Type I and Type II IFNs. Type I IFNs are encoded by a cluster of intronless genes on chromosome 9, exhibit a high degree of sequence homology and are heat stable at pH 2.0. They consist of IFNα, IFNB, IFNτ and IFNω, with IFNα being further subdivided into 14 different subtypes, while IFNβ, IFNτ and IFNω exist as single proteins. Type II consists only of IFNγ, which is encoded by a gene located on chromosome 12, is heat labile at pH 2.0 and exists as a single protein.

The biological effects of IFNβ are mediated through binding to a common Type I IFN receptor, which is expressed by all vertebrate cells. The Type I IFN receptor is a heterodimer consisting of the two subunits IFNAR1 and IFNAR2 that are encoded by a gene cluster located on chromosome 21q22.1. The two subunits are transmembrane glycoproteins belonging to the class II helical cytokine receptors that are characterized by conserved fibronectin type III-like repeats on the extracellular domain (Bekisz et al., 2004). The cytoplasmic domains of IFNAR1 and IFNAR2 are constituitively associated with Janus protein-tyrosine kinases (Jak PTKs) Tyk2 and Jak1 , respectively, and are involved in signal transduction. Binding of IFNβ to the extracellular

domains results in dimerization of the two chains into a functional receptor complex, and initiation of the signal transduction pathway. Tyk2 and Jak1 become cross-activated and they phosphorylate tyrosine residues on the receptor, which become docking sites for src-homology-2 (SH-2) domains of signal transducer and activator of transcription (STAT) proteins. STAT1 and STAT2 then heterodimerize to form a STAT1-STAT2 dimer, which is released from the receptor and becomes associated with DNA binding protein, p48, to form a complex termed interferon-stimulated gene factor-3 (ISGF-3). ISGF-3 translocates to the nucleus and binds specific DNA sequences called interferon-stimulated response elements (ISRE) that are located in the promoters of certain interferon-stimulated genes (ISG). ISGs become transcriptionally activated to evoke the biological activities of IFNβ (Pestka et al., 2004). Over 600 genes and gene products are induced as a result of the IFNβ signal transduction cascade, a majority of which have no known functions in IFNβ-treatment effects. Some of these IFNβ-induced products include neopterin, B_2-microglobulin, oligoadenylate synthetase, tumor necrosis factor apoptosis inducing ligand (TRAIL) and Myxovirus A (MxA), all of which have been used as biological response markers (BRMs) of IFNβ. However, with the exception of MxA, which is specifically induced by Type I IFNs and HIV, these BRMs are not exclusive to IFNβ (Pachner et al., 2003a; Farrell and Giovannoni, 2007).

In MS, treatment with IFNβ has many purported mechanisms of action that mediate its therapeutic clinical effects. These are complex and not well understood, but basically entail the downregulation and upregulation of a variety of genes that are involved in the immune responses in MS. The therapeutic benefits of IFNβ stem in part from its ability to antagonize the stimulatory effects of IFNγ, a potent mediator and activator of the disease process in MS. IFNβ downregulates the expression of IFNγ-induced MHC II on APCs, a consequence of which is a

decrease in antigen presentation to T cells, and ultimately a reduction of T cell activation (Chofflon, 2005). This IFNβ-mediated inhibition of MHC II antigens is thought to occur by suppression of class II transactivator, a transcription factor involved in MHC II transcription (Baron et al., 1991). In addition, IFNβ modulates the expression of co-stimulatory molecules that are essential for the optimal activation and clonal expansion of T cells. IFNβ has been shown to decrease expression CD80 (B7-1) on lymphocytes, in effect decreasing the induction of proinflammatory T_H1 cells, whereas it increases the expression of CD86 (B-7) on monocytes which upregulates the anti-inflammatory T_H2 cells (Genc et al., 1997; Zhang et al., 2002). IFNβ has an anti-proliferative effect on T cells and in addition reduces the expression of T cell activation markers like CD2, and transferrin and IL-2 receptors (Noronha et al., 1993; Rudick et al., 1993). It also inhibits IFNγ production by T_H1 cells, and other proinflammatory cytokines such as TNFα, lymphotoxin, IL-2 and IL-12. Furthermore, it skews the immune response towards a T_H2 response and the upregulation of anti-inflammatory cytokines such as TGFβ, IL-4 and IL-10 (Hartung et al., 2004).

IFNβ is known to have a significant effect in reducing GAD-enhanced MRI lesions, which are a measure of BBB leakage or disruption. Indeed in one study it was demonstrated that IFNβ reduced lesion frequency in 13 of 14 patients (Stone et al., 1995). This is partly attributable to the selective downregulation of VLA-4 on T cell surfaces and an upregulation of soluble VCAM-1 (Milo and Panitch, 1999). IFNβ also downregulates MMP9 secretion, and simultaneously upregulates TIMP-1, the inhibitor of MMP9 (Waubant et al., 2003). All of these molecular mechanisms help in maintaining the integrity of the BBB and prevent T cell entry into the CNS.

There is also evidence that IFNβ restores the non-specific suppressor cell activity of PBMCs

isolated from MS patients (Noronha et al., 1990). This effect of IFNβ is particularly beneficial, as defective suppressor cell function is associated with increased disease activity in MS (Antel et al., 1988). Another potential therapeutic effect of IFNβ is on natural killer (NK) cells; significant decreases in a subset NK cells, CD57+ cells, have been observed in MS patients during treatment with IFNβ (Kastrukoff et al., 1999; Perini et al., 2000). NK cells are implicated in the immunopathogenesis of MS, as increases in CD57+ cells have been positively correlated with increases in total number of active lesions. Another subset of NK cells, $CD56^{Bright}$, are also potential therapeutic targets of IFNβ. However, they play an immunoregulatory role, as they are efficient producers of the anti-inflammatory cytokine IL-10 and also express high levels of Fas, factors which can negatively regulate activated lymphocytes and are implicated in maintaining clinical remission in MS patients (Takahashi et al., 2004). Recently IFNβ was shown to selectively expand the proportion of these immunoregulatory $CD56^{Bright}$ NK cells in the peripheral blood of treated MS patients (Saraste et al., 2007).

Finally, it is noteworthy to mention the recent findings supporting a neuroprotective role for IFNβ in MS. In MOG-induced EAE, IFNβ-treated rats had an increased survival of retinal ganglion cells, thus indicating a neuroprotective effect (Sattler et al., 2006). Another study, demonstrated that pretreatment of MS patient-derived lymphocytes with IFNβ potentiated the secretion of nerve growth factor (NGF) by endothelial cells, when these two cell populations were co-cultured. The study also showed an inverse relationship between IFNβ-induced NGF production and MRI activity as measured by brain atrophy and axonal injury (Biernacki et al., 2005).

1.6.2 Immunogenicity of Biotherapeutic Proteins

Over the past few decades, it has been documented that natural antibodies or auto antibodies reacting with cytokines and other self antigens can be detected in sera of normal, non-immunized individuals, and in patients suffering from various autoimmune and infectious diseases (Avrameas, 1991; Bendtzen et al., 1998). However, with the advent of recombinant DNA technology for wide-scale production of therapeutic proteins, this occurrence has tremendously increased as self-antigens are administered at therapeutically high doses. Presently, with over 80 therapeutic proteins already approved for clinical use in a wide variety of diseases, their potential immunogenicity has become of major concern. Immunogenicity refers to the ability of a protein to evoke an immune response against it, specifically detectable antibodies against the protein. These antibodies can have negative impacts on therapy, such as an increased elimination rate and decreased efficacy of the drug. Most importantly, antibodies against therapeutic proteins can pose some serious safety issues especially when they induce a severe hypersentivity or anaphylactic response, or when the antibodies bind to the endogenously produced protein (Porter, 2001). Some documented examples of antibody responses against therapeutic proteins include insulin in diabetic patients (Meager, 1994), erythropoietin in patients with chronic renal failure (Casadevall et al., 2002), granulocyte-macrophage colony stimulating factor in cancer (Gribben et al., 1990), factor VIII in hemophilia patients (Ehrenforth et al., 1992), TNFα in MS patients (Francis G.S., 1998), IFNα in the treatment of chronic viral hepatitis and various malignant diseases (Oberg and Mckenna, 1996), and botulinum toxin (Goschel et al., 1997).

1.6.3 Immunogenicity of IFNβ in MS Patients

With MS being a chronic disease, long-term administration of IFNβ is necessary, and as with

other therapeutic proteins, antibodies can be elicited against IFNβ. Indeed, Vallbracht et al. in 1981 were the first to report the development of antibodies against IFNβ in a nasopharyngeal carcinoma patient treated with human fibroblast IFN (Vallbracht et al., 1981). This was contrary to the hypothesis at that time that IFNs would not be immunogenic in homologous systems as antibodies are not normally generated against self-antigens. Since then, it has become well-established that Type 1 IFNs administered exogenously as therapeutic agents in several neoplastic and infectious diseases elicit antibodies in patients (Antonelli and Dianzani, 1999). Anti-IFNβ antibodies are generally classified as binding antibodies (BAbs), which encompass all the antibodies that can recognize and bind IFNβ, and neutralizing antibodies (NAbs), a subset of BAbs that can abrogate or neutralize a biological activity of IFNβ. BAbs do not neutralize IFNβ but they can affect its pharmacokinetics by forming immune complexes which are rapidly cleared from the circulation by the reticuloendothelial system (Perini et al., 2001). NAbs interfere with the binding of the IFNβ molecule to its target receptor, thus inhibiting IFNAR activation and the ensuing transcellular signal transduction and expression of IFNβ-induced gene products (Hartung et al., 2005). BAbs and NAbs are of the immunoglobulin G (IgG) isotype, but a recent study detected low levels of IgM-reactive antibodies against IFNβ during the first three months of treatment in a minority of patients. However, IgM antibodies became undetectable after 6 months of therapy being replaced by IgG antibodies (Di Marco et al., 2006b).

1.6.3.1 Factors Affecting Immunogenicity

The extent to which a therapeutic protein can elicit an immune response is dependent on a variety of factors. These can be broadly defined as the disease being treated, inherent patient characteristics, concomitant therapy, the intrinsic and extrinsic characteristics of the therapeutic

protein, and the sensitivity of the assay (Koren et al., 2002; Schellekens, 2002b). Disease and patient characteristics that can influence immunogenicity include immune status and genetic profile. As an example, cancer patients who are usually immunocompromised, are less likely to mount an immune response against a therapeutic protein compared to patients with viral infections or autoimmune diseases who have a more activated immune system (Ryff, 1997; Meager et al., 1999). The genetic makeup of an individual also plays a very important part in an immune response. In a groundbreaking study to ascertain how the immunogenicity of a biotherapeutic is influenced by genetic make-up and MHC class II-binding epitopes, investigators have found an association between a particular HLA haplotype, DRB1*0701, and the development of antibodies against IFNβ in MS patients (Barbosa et al., 2006). Other concomitant medications can also affect the immunogenicity of the therapeutic protein. This was exemplified in the study of Pozilli et al. (2002) in which it was shown that concomitant therapy of MS patients with IFNβ and methylprednisolone significantly delayed the development of anti-IFNβ antibodies, in addition to reducing the antibody titres. Intrinsic characteristics of the therapeutic protein that can affect immunogenicity include the sequence and overall structure of the protein, and host cell in which the protein was produced. IFNβ-1b is produced in *E. coli* cells, which unlike mammalian cells lack the machinery for glycosylation. Thus IFNβ-1b is non-glycosylated as opposed to the 2 IFNβ-1as, which are produced in Chinese hamster ovary (CHO) cells and are glycosylated. Non-glycosylation decreases solubility and increases the formation of molecular clumps, such that only 40% of IFNβ-1b exists in soluble monomeric form compared to greater than 98% of IFNβ-1a that appears in soluble monomeric form (Runkel et al., 1998). This increased aggregation of IFNβ -1b molecules increases immunogenicity as the aggregates are more easily recognizable by the immune system. Glycosylation also masks epitopes that

would otherwise be exposed in the non-glycosylated IFNβ-1b, further increasing immunogenicity. Extrinsic factors include the route, dose and frequency of administration, and type of formulation. It is well established that in general, the hierarchy of immunogenicity is subcutaneous (SC) > intramuscular (IM) > intravenous, oral, intranasal, intradermal. As such, in IFNβ therapy, SC IFNβ-1b and IFNβ-1a are more immunogenic than IM IFNβ-1a. This was well demonstrated in the Danish National Study and later confirmed by Perini et al. (Ross et al., 2000; Perini et al., 2001). Opponents of this view, may however argue that the observed higher frequency of NAbs in the SC group could be attributed to higher dose frequency and cumulative weekly dose in this group compared to the IM group (Farrell and Giovannoni, 2007). The Danish Study also demonstrated that NAb frequency was higher in patients treated with SC IFNβ-1b every other day (98%) compared to patients treated with SC IFNβ-1a three times weekly (89%), SC IFNβ-1a once weekly (58%) and IM IFNβ-1a once weekly (33%). Thus increased dosing frequency resulted in increased immunogenicity and higher NAb positivity. However, this conclusion is refuted by studies of Bertolotto et al. (2002) showing that patients treated IM with 22 or 44 μg IFNβ-1a (Rebif®) weekly, did not differ in the incidence of NAbs as those treated SC with 66 μg IFNβ-1a (Rebif®) weekly. They hypothesize that immunogenicity differences between SC IFNβ-1a (Rebif®) and IM IFNβ-1a (Avonex®) may be attributable to differences in the products such as excipients.

The actual IFNβ dose has also been shown to affect the frequency of antibodies. In the European Dose-Comparison Study of IFNβ-1a (Avonex®) and in the Once Weekly Interferon for MS (OWIMS) study of IFNβ-1a (Rebif®), increases in dose resulted in increases in NAb positivity (The Once Weekly Interferon for MS Study Group, 1999; Clanet et al., 2002). Paradoxically, in the PRISMS and in the PRISMS extension trials, NAb frequency was higher in the 22 ug

treatment arm (23.8%, 23.7%) than in the 44 ug treatment arm (12.5%, 14.3%) (The PRISMS Study Group and the University of British Columbia, 1998; The PRISMS Study Group and the University of British Columbia, 2001). The host cells in which the IFNβis produced may also add contaminants and impurities, which can conceivably act as antigens or adjuvants and increase the immunogenicity of the product. During manufacturing, processes such as deamidation and oxidation can all increase immunogenicity. In the pivotal phase III trial of IM IFNβ-1a (Avonex®), the batch used (BG9015) resulted in 22% of patients developing NAbs (Jacobs et al., 1996). However, with changes in manufacturing, purification and formulation, the immunogenicity decreased substatially to between 1.8 to 4% NAb positivity in later clinical trials (Goelz and Walt, 2007).

1.6.3.2 Binding and Neutralizing Antibodies Against IFNβ

Anti-IFNβ antibodies are classified as BAbs and NAbs depending on the technique used for detecting them. BAbs represent all the antibodies that are elicited by and that bind to IFNβ. BAbs can be either neutralizing or non-neutralizing. However, it is thought that all BAbs may be biologically significant as they can bind IFNβ to form immune complexes that can rapidly be eliminated by cells of the reticulo-endothelial system. NAbs are a subset of BAbs, which block the effect of IFNβ in *in vitro* assays probably by interfering with the interaction between the IFNβ molecule and the IFNAR. Depending on the type of detection assay, BAbs can occur in up to 80% of treated patients, whereas NAbs can occur in up to 44% of treated patients. Chronologically, BAbs emerge and peak earlier (3 to 6 months) than NAbs (6 to 18 months). However, it has been argued that a thin line exists between BAbs and NAbs, and that assay

sensitivity determines which antibodies are detected; using a high-sensitivity NAb assay, Ross et al. (2002) showed that the number of NAb+ patients approximates the number of BAb+ patients. The phase III pivotal trials revealed differences in the incidence of BAbs and NAbs between the different IFNβ formulations. However, NAb testing during the pivotal trials were performed independently and differently by the three different companies, in terms of study design and assay methodology. Hence comparisons of the incidence of NAbs are not entirely valid across IFNβ products. However, results from recent comparative studies are in strong agreement with pivotal trial results. They reaffirm the hierarchy of immunogenicity of the three IFNβs: SC IFNβ-1b > SC IFNβ-1a > IM IFNβ-1a (Ross et al., 2000). But over the long term, the titres of the antibodies have been shown to decline, and some patients revert to NAb- status. This seroreversion is a function of both NAb titre and the IFNβ being received; a longitudinal analysis of 57 patients showed that patients whose NAb titres peaked earlier and patients who had lower peak titres were more likely to serorevert compared to those patients with persistent NAb titres (Gneiss et al., 2004). Additionally, more SC IFNβ-1b – treated patients seroreverted (58%) compared to SC IFNβ-1a – treated patients (27%).

1.6.3.3 Measurement of Anti-IFNβ Antibodies

Different methods are used for the detection and measurement of anti-IFNβ antibodies. These are broadly defined as immunoassays for measuring BAbs and functional bioassays for NAbs. The strategy generally used is to screen for the presence of BAbs, and subsequently analyzing only BAb+ samples for NAbs. This is based on the premise that NAbs are a subset of BAbs.

1.6.3.3.1 BAbs

BAbs are detected using classical immunoassays that depend on the binding of serum antibodies to IFNβ antigen. Immunoassays that have been used to measure BAbs include enzyme linked immunosorbent assay (ELISA), radioimmunoprecipitation assay (RIPA), column-based assays and Western blot. ELISAs are the most commonly used method, and in essence consist of directly immobilizing the IFNβ antigen onto microtitre plates (direct ELISA) or precoating the plates with monoclonal antibody that is specific for IFNβ and then adding IFNβ (capture ELISA) (Brickelmaier et al., 1999). Diluted test sera are then added, and bound antibody is detected by an enzyme-labelled secondary antibody with specificity for human IgG. Addition of a chromogenic substrate results in a colorometric reaction which is quantified by a spectrophotometer. The cutoff for BAb positivity is usually defined as the mean +2 or 3 standard deviations of healthy conrols included in each assay.

Immunoassays have the advantage of being rapid and easy to perform, and less laborious than bioassays. However, they are not designed to distinguish between neutralizing and non-neutralizing antibodies, and in addition could be fraught with false positive results (Hartung et al., 2005).

1.6.3.3.2 NAbs

NAbs are measured using both *in vitro* and *in vivo* functional bioassays. *In vitro* bioassays include the CPE, MxA induction, luciferase reporter gene and antiproliferative assays, all of which are based on the inhibition of IFNβ bioactivity by NAbs.

1.6.3.3.2.1 CPE Assay

The CPE assay is the WHO recommended method for the measurement of NAbs (World Health Organization Expert Committee on Biological Standardization, 1985). The assay is based on the principle that NAbs present in a patient's serum can neutralize the protective, antiviral effect of IFNβ on virus-challenged cell culture lines. It consists of adding known amounts of IFNβ to diluted sera. The diluted serum / IFNβ mixtures are then added to an IFNβ responsive cell line such as the human lung carcinoma cell line A459 or the Wistar Institute Susan Hayflick (WISH) cell line which are seeded in microtitre plates. Controls for serum toxicity are included for each serum sample to rule out serum viral or antiviral activities. Following 12 – 24 hours incubation, the cell cultures are challenged with vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMC), and incubated for a further 18 – 24 hours. The cytopathic effects of the virus are then determined by staining the cells with crystal violet, eluting with acetic acid and reading absorbance in a spectrophotometer. Cells incubated with sera that have no NAbs are protected from the virus-mediated CPE, whereas cells incubated with sera that contain NAbs are killed by virus.

Initially, the sera are screened for NAbs and NAb+ sera are further subjected to serial 2-fold dilutions to determine the titre. The Kawade method is used to calculate the NAb titre, i.e. the highest dilution of sera resulting in a reduction of IFNβ activity from 10 Laboratory Units (LU) to 1 LU, with 1 LU providing 50% protection against viral challenge (Kawade, 1986). This WHO recommended way of reporting NAb titres has been modified. Grossberg et al. upon analyses of the interactions between anti-IFNβ antibodies and IFNβ in several different laboratories and using different bioassay systems, have proposed a new general formula to report the neutralization titre (t): $t = f(n-1) / 9$, where f is the reciprocal antibody dilution achieving

endpoint and n is the IFNβ concentration in LU/ml as determined for that particular day (Grossberg et al., 2001a; Grossberg et al., 2001b). This results in a neutralization unitage of Ten-Fold Reduction Unit (TRU) that can be universally applied across the different available NAb assays. Of all NAb assays, the CPE assay is considered the gold standard (Sorensen et al., 2005a).

1.6.3.3.2.2 MxA Induction Assay

This assay also uses an IFNβ-responsive cell line, but unlike the CPE, it does not require viral infection. The assay quantifies the induction of serum levels of MxA protein or of peripheral blood mononuclear cells (PBMC) MxA mRNA, following incubation of the cells with diluted sera / IFNβ mixtures. In NAb- sera or patients, high MxA levels are detected, whereas in NAb+ patients low MxA levels are detected (Pachner et al., 2003a). This assay has the inherent advantage of being highly specific, as MxA is only induced by type I IFNs and by HIV.

1.6.3.4 Biological Significance of Anti-IFNβ Antibodies

The biological effects of NAbs can be elucidated by measuring the BRMs that are upregulated or down-regulated by IFNβ. In the Phase III study of Avonex®, NAb positive (NAb+) patients had significantly lower levels of both serum neopterin and β_2-microglobulin levels compared to NAb negative (NAb-) patients (Rudick et al., 1998). Deisenhammer et al. (1999) measured the blood MxA protein levels in IFNβ-1b treated patients and concluded that once NAbs develop, the bioavailability of the IFNβ is completely inhibited. Persistently NAb+ patients treated with SC IFNβ-1a were also shown to have significantly reduced lymphocyte MxA protein levels

($p < 0.001$) (Vallittu et al., 2002). At the mRNA level, MxA was found to be significantly lower in the persistently NAb+ and isolated NAb+ patients (a single NAb+ sample or sporadic NAb positivity) compared to NAb- patients (Bertolotto et al., 2003; Pachner et al., 2003b; Santos et al., 2006). NAb+ patients also tended to have higher levels of soluble intercellular adhesion molecule-1 (sICAM-1) that is normally downregulated by IFNβ therapy (Trojano et al., 1999). NAbs have also been implicated in the inhibition of IFNβ-mediated suppression of MMPs (Gilli et al., 2004). Other IFNβ-inducible genes, including TRAIL and STAT1, have been shown to be substantially reduced or abolished in NAb+ patients (Wandinger et al., 2003; Santos et al., 2006). At the cellular level, Perini et al. (2000) have shown that in IFNβ-treated RRMS patients, NK cells decrease with initiation of therapy but with the appearance of anti-IFNβ antibodies, they return to pre-treatment levels.

1.6.3.5 Clinical Significance of Anti-IFNβ Antibodies

1.6.3.5.1 Relapse Rates

The primary effect ascribed to the presence of NAbs is a poor clinical response. This is not surprising as NAbs to IFNβ and other type I IFNs have been consistently associated with a negative impact on therapeutic efficacy in diseases other than MS (Schellekens, 2002a ; Scagnolari et al., 2003). Strong support for this association between NAbs and decreased IFNβ clinical efficacy stems largely from observations from the pivotal phase III trials and European post-marketing studies, all of which demonstrated that NAb+ patients had a significantly greater number of relapses when compared to NAb- patients (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996; Rudick et al., 1998; The PRISMS Study Group and

the University of British Columbia, 2001; Polman et al., 2003; Sorensen et al., 2003; Francis et al., 2005).

In the pivotal trial of SC IFNβ-1b, patients who received 8 mIU of IFNβ-1b and were NAb+ patients had significantly greater mean relapse rates (1.08) than NAb- patients (0.56) during months 18 – 36. Indeed the relapse rates in the NAb+ patients approximated that of placebo patients (1.06) (The IFNb Multiple Sclerosis Group and the University of British Columbia , 1996). Similarly, in the extension of the PRISMS pivotal phase III trial, the negative effects of NAbs became apparent between months 24 and 36. The mean relapse rate of patients who received 44 ug SC IFNβ-1a and who were NAb+ patients was 38% higher than that of NAb- patients (0.81 vs 0.50, $p = 0.002$) (The PRISMS Study Group and the University of British Columbia, 2001). Polman et al. (2003) also investigated the effects of NAbs on clinical efficacy of SC IFNβ-1b in SPMS patients, using detailed longitudinal and cross-sectional analyses. When analyzed cross-sectionally, NAbs did not have any impact on relapse rates. In the longitudinal analyses, when NAb positivity was defined as "once positive, always positive", patients had significantly higher rate of relapses (45% increase) during NAb+ periods in comparison to NAb- periods (Polman et al., 2003). The most convincing data on the effects of NAbs on clinical efficacy is provided by results from the Danish National Study, which followed patients for up to 5 years. The annual relapse rates of patients during NAb+ periods increased more than 50% compared to NAb- periods (0.64 – 0.70 vs 0.43 – 0.46, $p< 0.03$). This study also revealed the delay of time to first relapse (median) by 241 days in the NAb- patients compared to NAb+ patients; median time to first relapse was 361 days in NAb- patients, and 604 days in NAb+ patients ($p= 0.0009$) (Sorensen et al., 2003).

1.6.3.5.2 MRI Disease Activity and Disease Progression

In addition to relapse rates, data from the pivotal trials also showed that NAbs had a negative impact on MRI measures of disease activity including increases in the number of enlarging (active) or new lesions, burden of disease on MRI , and on disability progression as measured by the Expanded Disability Status Scale (EDSS) scores. Enlarging lesions were significantly greater in NAb+ patients compared to NAb- patients in the pivotal SC IFNβ-1b trial (months 12-24, 0.41; months 24-36, 0.59 vs months 12-24, 0.19; months 24-36, 0.26, $p<0.03$) (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996). The 2-year extension of the SC IFNβ-1a pivotal trial also showed a significant difference in active lesions between NAb+ and NAb- patients receiving 44 ug IFNβ-1a (1.4 vs 0.3 $p< 0.001$). The same study demonstrated a 17.6% increase from baseline of disease burden on MR among NAb+ patients, in contrast to an 8.5% decrease among NAb- patients ($p< 0.001$) (The PRISMS Study Group and the University of British Columbia, 2001). In the European study of IM IFNβ-1a, NAb+ patients had significantly higher rate of mean change (worsening) in disability from baseline to month 48 ($p = 0.01$), greater number of T1 gadolinium-enhanced lesions at months 24 and 36 ($p = 0.02$ and 0.03), and greater number of new or enlarging T2 lesions from month 12 to months 24 and 36 ($p = 0.05$ and 0.09) (Kappos et al., 2005). All these findings confirm the deleterious effects of NAbs on IFNβ therapeutic efficacy. However, other studies have found contradictory results that failed to demonstrate any effect of NAbs on therapeutic efficacy (Durelli et al., 2002; Panitch et al., 2002). These results were from short-term studies of less than 2 years, and could not detect the effects of NAbs mainly because NAbs generally appear 6 – 18 after the start of IFNβ therapy, and their clinical impacts are further delayed being observed after month 18.

In spite of their detrimental effects, patients who become NAb+ may respond better to IFNβ

during months 6 – 12 compared to patients who never develop NAbs (persistent NAb-). It is hypothesized that low affinity antibodies during this period increase the half-life of IFNβ (Sorensen et al., 2007). Similarly, Kappos et al. (2005) have shown that patients destined to become NAb+ have a greater reduction in gadolinium enhancing lesions during the first year of treatment. In addition, NAb+ patients have been shown to have less IFNβ-associated side effects particularly skin reactions compared to NAb- patients, implying the abrogation of the biological effects of IFNβ in the presence of NAbs (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996).

1.7 Dilemma Regarding IFNβ and Anti-IFNβ Antibodies

Despite numerous studies and publications, the issue of anti-IFNβ antibodies remains complex and highly debatable. There is no consensus regarding the clinical utility of NAb testing, the optimum treatment time-point to collect sera for testing, and which NAb assay to use. The optimum cut-off point that would be of predictive value of a NAb+ titre is also not clear. In essence, there is no methodological standardization for antibody testing, and no definitions for the biological and clinical activity of these antibodies.

Regarding the biological activity of anti-IFNβ antibodies, it is generally accepted that MxA is the most specific IFNβ-inducible gene (Deisenhammer et al., 2000; Bertolotto et al., 2001; Pachner et al., 2005; Capra et al., 2007), and thus the effects of anti-IFNβ antibodies can best be monitored by measuring MxA. However, IFNα also induces MxA (Scagnolari et al., 2003) and it is also probable that subclinical viral infections may result in increased IFNa and MxA induction (Capra et al., 2007).This would complicate the correlation of NAbs with MxA measurements. It has also been reported that repetitive stimulation of the IFNAR, as found during the long-term

administration of IFNβ in MS, can result in the release of the receptors into the circulation (Kiessling and Gordon, 1998).These soluble receptors can bind IFNβ and thus potentially reduce or completely abrogate MxA induction. Moreover, the mechanism of action of IFNβ in MS, and the *in vivo* function of MxA, is poorly understood. In addition, IFNβ is only modestly effective. There is also a lag time between the initiation of IFNβ therapy and the appearance of its clinical effects, and between NAb onset and appearance of its detrimental effects (Farrell and Giovannoni, 2007).

Controversy also exists over the relative titres of NAbs elicited against the different IFNβ products, with SC IFNβ-1b treated patients typically manifesting low NAb titres in contrast to the higher NAb titres in SC IFNβ-1a-treated patients. This will impact on what is deemed to be the clinically and biologically relevant NAb titre; the different IFNβ products will likely have different cut-offs representing a 'positive' or 'high' result. Also, BAb- samples are not tested for NAbs, and this presents the problem of under-reporting of NAbs if the screening ELISAs are not sensitive enough. Additionally, the Danish MS group contends that all antibodies that bind IFNβ as measured by immunoassays can neutralize IFNβ and as such all BAbs are NAbs (Bendtzen, 2003). This premise is based on their experience in manipulating the sensitivity of the NAb assays. However, this is in stark contrast to the generally held belief that NAbs are a subset of BAbs and that BAbs can also be non-neutralizing (Hartung et al., 2005).

In certain instances, it has been reported that NAb+ samples test negative for BAbs. Gilli et al. (2006) postulate that non-immunoglobulin inhibitory factors may be responsible for such a phenomenom and that NAb assays may not only be measuring antibodies. In contrast, ELISAs are selective for immunoglobulins, such that it has been suggested that ELISAs may not be suitable for screening but should instead be used to confirm that NAb+ sera are due to

antibodies. A follow-up study by the same investigators showed that out of 256 patients, 11(4.3%) exhibited non-antibody mediated inhibitory activity (Gilli et al., 2007). The existence of these non-antibody inhibitors has been demonstrated by others (Mizukoshi et al., 1999; Ambrus et al., 2003; Lampasona et al., 2003; Chadha et al., 2006). Another possibility exists that anti-cytokine antibodies, as in the case of NAbs against IL-4 and IL-7, can prolong the in vivo cytokine activity (Finkelman et al., 1993). Thus the "Janus face of anti-IFNβ antibodies", with these antibodies being able to both enhance and reduce IFNβ bioactivity, a function that is concentration and affinity dependent. Indeed, this paradoxical finding has been documented in the pivotal IFNβ trials (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996; The PRISMS Study Group and the University of British Columbia, 2001) and by the Danish MS group (Sorensen et al., 2007): patients who eventually become NAb+ do better clinically with fewer relapses, during the first 6 –12 months of IFNβ therapy. Sorensen et al. (2007) suggest that such a phenomenom can be attributed to low affinity antibodies that increase the half-life of IFNβ. As affinity maturation ensues, the high affinity antibodies reverse the benefits endowed by low affinity antibodies and NAb+ patients lose the clinical effects of IFNβ as manifested by higher relapse rates and more disease activity.

In light of the incomplete characterization of anti-IFNβ antibodies, we set out to address some of these outstanding issues with the ultimate aim of increasing our understanding of these phenomena.

1.8 Rationale, Hypotheses and Objectives

1.8.1 Studies of the IgG-subclass Distribution of Anti-IFNβ Antibodies in IFNβ-treated MS Patients

The immune response typically starts with IgM antibodies that are replaced gradually over time by IgG. In humans, IgG consists of 4 subclasses that have distinct physicochemical properties, and markedly different functions that are imparted by the constant (Fc) region which interacts with effector molecules such as complement in serum or Fc receptors on immune cells. Antigen-specific antibodies are not randomly distributed among the IgG subclasses, with IgG 1 and IgG3 being generally elicited by viral and bacterial protein antigens, whilst carbohydrate antigens are mainly restricted to IgG2. IgG4 responses occur as a result of prolonged, repetitive antigenic stimulation.

It is generally accepted that anti-IFNβ antibodies are of the IgG isotype (Deisenhammer et al., 2001; Bendtzen, 2003) even though some IgM-specific antibodies have been observed within the first 3 months of treatment (Di Marco et al., 2006b). In a cross-sectional study of 59 IFNβ-1b-treated patients, Deisenhammer et al. (2001) evaluated the IgG subclasses of anti-IFNβ antibodies in individual serum samples and found a higher frequency of IgG2 and IgG4 in NAb+ patients in comparison to NAb- patients. Furthermore, they reported significantly higher levels of IgG1 and IgG4 in NAb+ patients than in NAb- patients. However, the major drawback of such a study is that it does not address the temporal development and dynamics of the IgG subclasses. This led us to question whether the IgG subclass distribution pattern differs between NAb+ and NAb- patients, and whether there are patterns peculiar to IFNβ-1a and IFNβ-1b-treated patients. To this extent, we undertook to longitudinally analyse the IgG subclasses of anti-IFNβ antibodies. We hope that elucidating these patterns will provide an insight into the nature of the

immune response in NAb+ versus NAb- patients, and in IFNβ-1a versus IFNβ-1b-treated patients. In addition, if differences exist between IFNβ-1a and IFNβ-1b-treated patients, this may account for the occurrence of higher NAb titres in IFNβ-1a treated patients (Gneiss et al., 2006b), the higher rate of seroreversion in IFNβ-1btreated patients (Bellomi et al., 2003; Sorensen et al., 2005b) and more NAb persistence in IFNβ-1a-treated patients (Hesse and Sorensen, 2007). Our results may also give an indication of whether different mechanisms are involved in the induction of antibodies against IFNβ-1a and IFNβ-1b. Because cytokines are key regulators of IgG subclass responses (Pene et al., 2004), and since the distribution of IgG subclasses is an important factor in B cell development (Reding et al., 2002), our study may provide a perspective into the mechanisms driving the immune response against IFNβ, and such information could be of tremendous value in finding ways to reduce IFNβ immunogenicity.

1.8.2 Studies of the Affinity Maturation of the Anti-IFNβ Antibody Response

Antibody affinity, together with antigen epitope specificity and antibody titre, determine the biological activity of an antibody. The affinity is the binding strength of a single antigen-binding site of the antibody to the antigenic epitope (Steward and Lew, 1985). Generally, during the early part of the immune response low-affinity antibody clones usually predominate. With continuous exposure to antigen, high-affinity antibodies dominate the immune response. This progressive increase in antibody affinity, affinity maturation, occurs as a result of somatic hypermutations in the hypervariable regions (V_H and V_L) of antibodies, and the selection of high affinity B-cells by competition for antigen in critical conditions (Nossal, 1992). However, as demonstrated by exhaustive analyses, and in agreement with the constant proportion hypothesis of antibody neutralization (antibody reduces the ratio of added IFN/residual active IFN molecules), it would seem that anti-IFNβ antibodies are of low affinity (Grossberg et al., 2001a; Grossberg et al.,

2001b).

In this context, we will evaluate the relative antibody affinities of serial serum samples at different time points. We hypothesize that overtime, the relative antibody affinity will increase more so in NAb+ patients than in NAb-. As previous reports have indicated that NAb+ patients generally have higher affinities than NAb- patients (Gneiss et al., 2006a), we also anticipate that the affinity maturation profile will parallel that of NAb titres and to this effect, we will correlate NAb titres to relative antibody affinities. Assessment of antibody affinity will increase the understanding of the parameters that define the biological potencies of anti-IFNβ antibodies. Our study of affinities could also help in identifying the *in vivo* dynamics of the B-cell populations specific for IFNβ. These B-cells subsets could then be identified and potentially blocked (e.g. co-stimulatory blockade) to prevent the immune response against IFNβ.

1.8.3 Studies of the Impact of Anti-IFNβ Antibodies on IFNβ Clinical Efficacy

The utility of NAbs in clinical practice remains highly complicating; the North American approach seems to base treatment decisions on clinical rather than NAb status (Goodin et al., 2007), in contrast to the recommendations put forth by the EFNS which suggests that if high titre NAbs are sustained, treatment should be discontinued (Sorensen et al., 2005a). These opposing guidelines are partly attributable to the lack of post-marketing studies in North America that can also demonstrate the detrimental effects of NAbs as documented in clinical trials, whereas in Europe the loss of clinical efficacy due to NAbs has been amply demonstrated outside of clinical trials (Sorensen et al., 2003; Malucchi et al., 2004). Other confounding factors include: the occurrence of a large number of patients who do not have high NAb titres but have active disease; from our experience with the UBC Routine Monitoring of Anti-IFNβ Antibodies

Program, patients exist who have very high NAb titres but who are not clinically different from patients with medium to high NAb titres (Dr. Oger, personal communication).

The above considerations led us to retrospectively investigate a cohort of Canadian patients by reviewing patient charts and correlating clinical outcomes with NAbs, and comparing IFNβ-1a- and IFNβ-1b-treated patients. As the effects of NAbs are evident only after 18 months of treatment and as a larger number of patients are needed to demonstrate these affects, we will cross-sectionally analyse a cohort of patients obtained from our UBC MS Database. With this strategy, we intend to use patients who are representative of the general MS population. We hope that this re-evaluation will corroborate previous reports and provide further evidence of the importance of anti-IFNβ antibodies in the treatment of MS patients.

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Chapter 2

The IgG subclass-specificities of anti-IFNβ antibodies change with time and differ between the IFNβ products in relapsing remitting multiple sclerosis patients[1]

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2.1 Introduction

An unwanted event in the treatment of MS patients with interferon beta is the development of anti-IFNβ antibodies. These antibodies are globally termed binding antibodies (BAb), a subset of which, neutralizing antibodies (NAb), can neutralize the biological effects of IFNβ; NAbs have been shown to reduce or completely abrogate IFNβ-induced biomarkers such as Myxovirus (Mx) A protein, β2-microglobulin and neopterin (Deisenhammer et al., 1999; Vallittu et al., 2002; Bertolotto et al., 2003; Pachner et al., 2003a). Clinically, NAb positive (+) patients have been shown to have higher relapse rates approximating those of placebo- treated patients, and to have higher MRI activity than NAb negative (-) (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996; Rudick et al., 1998; The PRISMS Study Group and the University of British Columbia, 2001; Sorensen et al., 2003; Francis et al., 2005; Kappos et al., 2005; Sorensen et al., 2006).

There are four human immunoglobulin G (IgG) subclasses (IgG1, IgG2, IgG3 and IgG4) that differ in structure based on unique sequences in their heavy chain constant regions. In healthy individuals, the proportion of each subclass is maintained within relatively narrow ranges; IgG1, 60-65%; IgG2, 20-25%; IgG3, 5-10%; IgG4, 3-6% (French and Harrison, 1984). However, in an antigen specific response, the distribution of IgG subclasses differs from their proportions in normal sera. This IgG distribution depends in part on the nature of the antigen, with proteins generally eliciting IgG1 and IgG3 subclasses, while IgG2 predominates in responses against carbohydrates (Siber et al., 1980; Ferrante et al., 1990). The prevailing cytokine microenvironment also influences the IgG subclass distribution. Immune responses with substantial IL-4 production result in IgG4 induction, whilst IFNγ and IL-10 induce the

production of IgG2 and IgG1/IgG3, respectively (Briere et al., 1994; Kawano and Noma, 1996) IgG subclasses vary in their biological activities, the main effector functions being complement activation and opsonization. These effector functions are mediated by the constant (Fc) fragment following interaction of the antibody, via its variable (Fab) fragment, with antigen. Differences in IgG effector capacities are related to differences in hinge regions, which ultimately determine the interaction between the Fc and Fab fragments. Loosely, subclass effector activity can be ranked as IgG3 = IgG1 > IgG4 = IgG2 (Papadea and Check, 1989).

We report here the IgG subclass distribution of the anti-IFNβ antibody response in MS patients receiving IFNβ therapy. The aim was to determine whether over time, the IgG subclass distribution differed between SC IFNβ-1b- and SC IFNβ-1a-treated patients, and between NAb- and NAb+ patients.

2.2 Materials and Methods

2.2.1 Patients

In the present study, patients were selected from the UBC MS Clinics Database based on the following criteria: 1) patients were receiving one single type of IFNβ therapy, 2) completion of at least 60 months of IFNβ therapy, 3) at least 3 serum samples collected with a period of at least 6 months separating these sera, 4) availability of results for BAbs and NAbs. Twenty-one patients were selected, with duration of treatment ranging from 66 to 198 months. Ten patients received SC IFNβ-1b (4 BAb+ / NAb-, 6 BAb+ / NAb+) and 11 received SC IFNβ-1a (5 BAb+ / NAb-, 6 BAb+ / NAb+). All patients originally had relapsing remitting MS and some of these became secondary progressive.

2.2.2 Total and Subclass-Specific IgG Antibodies to IFNβ

Total IgG and IgG subclass-specific antibodies against IFNβ were analyzed using a capture ELISA. Briefly, microtitre plates (Costar 3576, Cambridge, MA) were coated with 1ug/ml mouse anti-human IFNβ monoclonal antibody (Chemicon MAb 416, Temecula, CA.) in carbonate/bicarbonate buffer overnight at 4°C. The plates were then washed 3 times with phosphate buffered saline, 0.05% Tween (PBST) and blocked for 1 hour (hr) at 37°C with PBS, 1% bovine serum albumin (BSA). After 3 washes with PBST, the plates were coated with 10 μg/ml IFNβ-1b (Berlex, Canada) or 10ug/ml IFNβ-1a (Serono, Canada) in PBS at 4°C overnight, followed by 3 washes with PBST. Patient serum samples were then added in duplicate at a 1:100 dilution (in PBS, 0.1% normal human pooled serum) and incubated for 1hr at 37°C. All samples of each patient were assayed together for total IgG and IgG subclass antibodies to IFNβ. Plates were washed 3 times and for the total IgG ELISA, goat anti-human IgG-horse radish peroxidase (HRP) conjugate (Fc-specific, Sigma A-0176, Sigma Chemical Corporation, St. Louis, MO.) was added at a 1:12,000 dilution for 1 hr at 37°C. In the IgG subclass ELISA, HRP conjugated mouse monoclonal anti-human IgG1, Fc region-specific (clone HP6069), anti-human IgG2, heavy chain Fd region-specific (clone HP6014), anti-human IgG3, heavy chain hinge region-specific (clone HP6047) and antihuman IgG4, heavy chain Fc-region-specific (clone HP6025), all obtained from Zymed Laboratories (San Francisco, CA.), were diluted 1:1000 in PBS and added in the appropriate wells for 1 hr 37°C. These monoclonal antibodies were chosen because of their validated specificities (Reimer et al., 1984; Papadea et al., 1985; Hamilton, 1987; Hamilton and Morrison, 1993). Finally after 3 washes, 0.4 mg/ml *o*-phenylenediamine (OPD) and 0.1 μl /ml H_2O_2 were added to each well and color development was allowed to proceed for 15 minutes. The reaction was stopped with 2 M sulphuric acid and optical densities were measured at 490 nm

wavelength in an MRX ELISA Microplate Reader (Dynex Technologies, Chantilly, VA.). Known positive and negative sera from IFNβ-treated patients were included in each assay together with sera from normal healthy controls. OD values were blanked on wells containing only serum diluent, and subsequently for each patient pre-treatment serum OD values were subtracted from treated serum OD values to obtain corrected ODs.

2.2.3 Statistical Analysis

Patients were categorized according to the IFNβ treatment received and the BAb / NAb status into the following: 1) IFNβ-1b BAb+ / NAb-; 2) IFNβ-1b BAb+ / NAb+; 3) IFNβ-1a BAb+ / NAb-; 4) IFNβ-1a BAb+ / NAb+). Means ± SEM were calculated for each patient category. The different categories were compared using the Mann-Whitney test. Any p-values less than 0.05 were considered to be statistically significant.

2.3 Results

2.3.1 Kinetics of Total IgG and IgG Subclass-specific Antibodies Against IFNβ

The total IgG antibodies against IFNβ differs according to the IFNβ product, with antibodies being detected earlier and at higher levels in IFNβ-1b treated patients compared to IFNβ-1a treated patients (Figure 2.1). Figure 2.2 provides a longitudinal description of IgG subclass specificities of the anti-IFNβ antibodies in the 4 different patient categories, namely IFNβ-1b BAb+ / NAb-, IFNβ-1b BAb+ / NAb+, IFNβ-1a BAb+ / NAb- and IFNβ-1a BAb+ / NAb+. The mean levels of the IgG subclasses varied with the IFNβ treatment received, the treatment duration and the NAb status. Generally, all IgG subclasses were more prominent in IFNβ-1b-treated than in the IFNβ-1a-treated patients. In IFNβ-1b treated patients, IgG1 and IgG3 peaked

within the first 6 months of treatment followed by a progressive decline, while IgG4 levels increased and peaked later after 24 months. In IFNβ-1a-treated patients, the IgG subclass response was subdued particularly during the first 12 months of treatment. Patients began to show increases in the levels of IgG1, 2 and 4 at month 18, a period that coincided with their peak levels. Thereafter, levels progressively declined, though the IgG4 levels plateaued in the NAb+ patients. Regardless of the treatment received, NAb+ patients had higher levels of IgG4 subclass-specific antibodies than NAb- patients.

2.3.2 Analysis of the Correlations Between IgG3 Antibodies that Bind IFNβ1-a and IgG3 Antibodies that Bind IFNβ-1b

Though transiently emerging in the NAb- subgroup, IgG3 was barely detected in most IFNβ-1a-treated patients. To confirm the absence of IgG3 antibodies in IFNβ-1a treated patients, and to rule out the technical possibility that IFNβ-1a used as antigen did not bind properly to IgG3 antibodies in the ELISA, we reassayed 18 sera from IFNβ-1a treated patients for IgG antibodies against IFNβ-1a and IFNβ-1b, taking into consideration the crossreactivities of anti-IFNβ antibodies. Included in both plates as positive controls were 19 sera from IFNβ-1b treated patients known to be IgG3 antibody positive. Sera from IFNβ-1a treated patients bound at very low levels to both IFNβ-1a and IFNβ-1b, whilst sera from IFNβ-1b treated patients showed higher binding to both IFNβs (Figure 2.3), confirming that in IFNβ-1a treated patients IgG3 reactivity to IFNβ1a (and to IFNβ1b) is very low.

2.3.3 Correlations Between Patient Categories

Among IFNβ-1b treated patients, NAb+ patients had significantly higher IgG2 and IgG4 levels

(month12) compared to NAb- patients. Among IFNβ-1a treated patients, the only significant difference between the NAb+ and NAb- patients were higher IgG2 levels at months 30-60. Among the NAb+ patients (IFNβ-1b BAb+ / NAb+ and IFNβ-1a BAb+ / NAb+), IFNβ-1b treated patients demonstrated higher levels of IgG1 (months 6, 12), IgG2 (months 6, 12, 24), IgG3 (months 6, 12, 24, 30-60) and IgG4 (months 6, 12) than IFNβ-1a treated patients.

2.4 Discussion

Data presented here demonstrate a wider distribution of IgG subclass antibodies to IFNβ in IFNβ-1b-treated patients, with antibodies of all 4 subclasses being induced. IgG1 and IgG3 peaked during the first year of treatment with a progressive switch to IgG4 in the latter periods of treatment, especially in the NAb+ patients. A more restricted distribution in IFNβ-1a treated patients was observed and these had a delayed emergence and peak. IgG4 levels were higher compared to other IgG subclasses in the NAb+ IFNβ-1a treated patients.

The predominant IgG isotype of anti-IFNβ antibodies is IgG. However, Di Marco et al. (2006a) detected the IgM isotype after 3 months in one of two patients undergoing IFNβ-1a treatment, but this was gradually replaced by IgG after 6 months of treatment. The fact that IgG4 becomes more prominent as treatment continues, is not surprising, as we and others have demonstrated that repeated long-term stimulation with T cell-dependent antigens such as glatiramer acetate results in a prominent IgG4 response (Basile et al., 2006). However, IgG4 has low FcγR binding (Papadea and Check, 1989) as well as low capacity to negatively regulate B cell activation through antibody feedback, when compared to IgG1 and IgG3.

The most striking observation in this study was the extremely low levels or complete absence of

IgG3 antibodies in IFNβ-1a-treated patients. To rule out a technical problem of IFNβ-1a not binding to the microtitre plate, we tested IgG3 reactivity to both IFNβ-1a and IFNβ-1b. Results of this experiment show that sera from IFNβ-1a-treated patients had low OD values when tested against both antigens whilst sera from IFNβ-1b-treated patients exhibited high OD values against both antigens. We conclude that this strong correlation between IgG3 binding to IFNβ-1a and to IFNβ-1b, refutes the possibility that the very low ODs observed in the IFNβ-1a-treated patients could be due to a technical problem in the ELISA.

The IgG subclass study showed that among the IFNβ-1b-treated patients, there were significant differences between NAb- and NAb+ in IgG2 and IgG4 levels at month 12. This observation is in agreement with the a cross-sectional study by Deisenhammer et al. (2001a) showing that NAb+ IFNβ-1b-treated patients had a higher frequency of IgG2 and IgG4 compared to NAb- IFNβ-1b-treated-patients. However, their study also found significantly higher IgG1 and IgG4 levels in the NAb+ patients, as opposed to IgG2 and IgG4 in our hands. We similarly found that within the IFNβ-1a-treated patients, the only significant difference between the NAb+ and NAb- patients was observed in IgG2 levels at months 30 – 60.

Although the number of sera studied here was modest, they were selected according to their pattern of reactivity, and this provides evidence for the first time of distinct IgG subclass antibody responses to the different IFNβs (SC IFNβ-1a and –1b). This indicates that the intrinsic properties of the different IFNβs are important in determining the antibody response. Indeed, ample evidence in the literature supports the general notion that antigenic properties such as solubility, hydrophobicity, accessibility, mobility and the number of epitopes can influence the development of the different IgG subclasses. Thus the observed differences between IFNβ-1a and IFNβ-1b can in part be explained by their different physicochemical properties. We suspect

that the distinctive patterns of IgG subclasses could be of functional significance, and that this should be fully explored in the future to determine the relationships between IgG subclass and the antibody response to IFNβ. Furthermore, we hypothesize that the presence of high levels of IgG3 subclass antibodies in SC IFNβ-1b – treated patients and low levels in SC IFNβ-1a – treated patients contributes to the quantitative differences in antibody levels between the 2 groups of patients (Gibbs et al., 2005).

2.5 Acknowledgements

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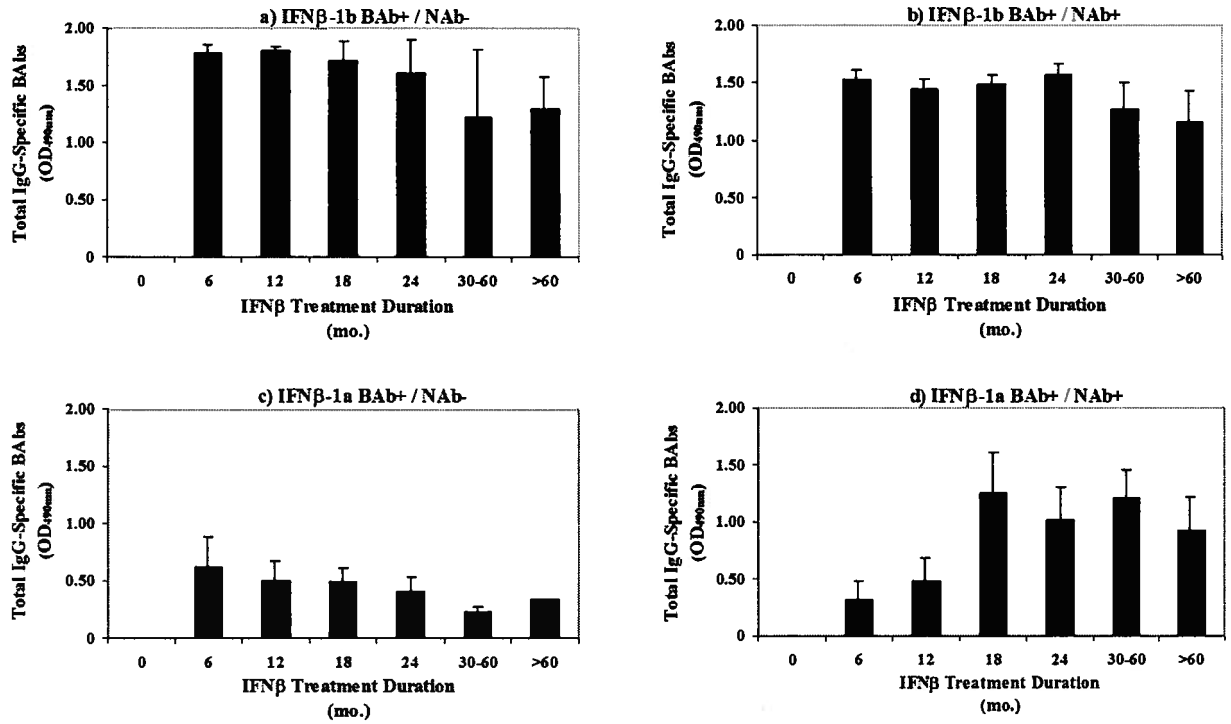

Figure 2.1. Total IgG-specific antibodies against IFNβ in the four different categories of IFNβ-treated patients at different timepoints.

(a. IFNβ-1b BAb+ / NAb- (n = 4); b. IFNβ-1b BAb+ / NAb+ (n=6); c. IFNβ-1a BAb+ / NAb- (n=5); d. IFNβ-1b BAb+ / NAb+ (n=6)).

Levels of total IgG against IFNB are expressed as the mean ± SEM for each patient category

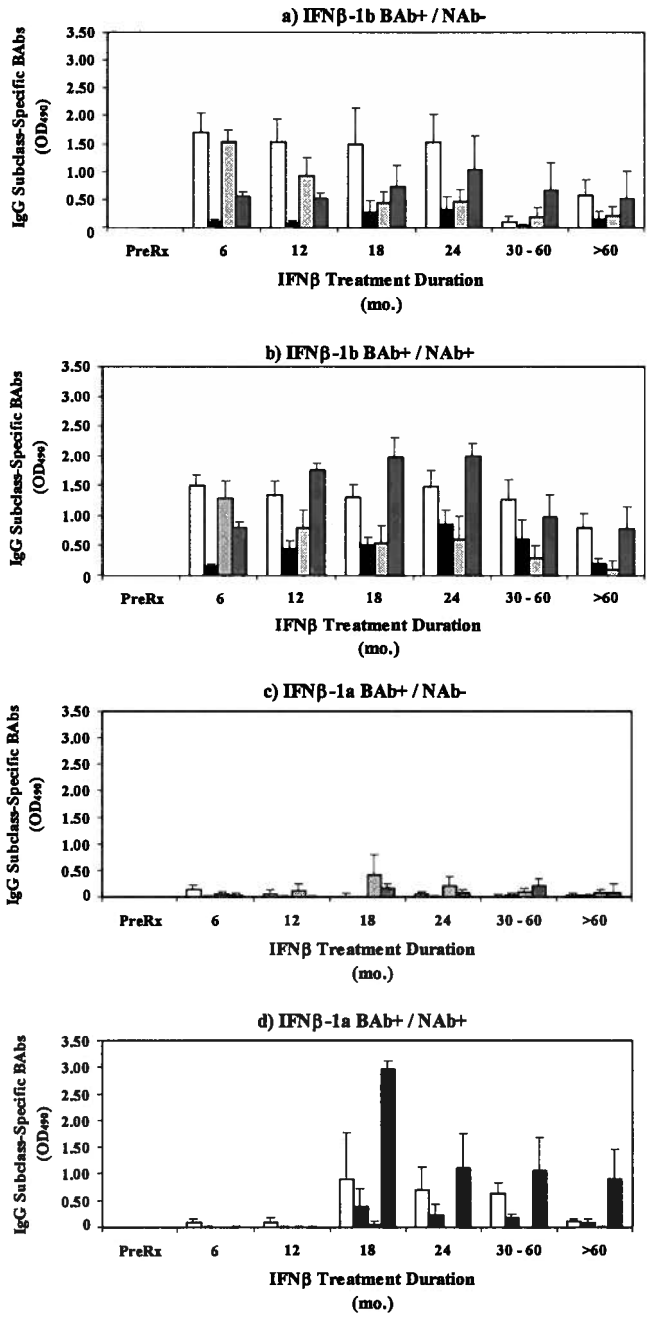

Figure 2.2. Longitudinal patterns of IgG Subclass-specific antibodies to IFNβ.

(IFNβ-1b BAb+ / NAb- (n = 4); IFNβ-1b BAb+ / NAb+ (n=6); IFNβ-1a BAb+ / NAb- (n=5); IFNβ-1b BAb+ / NAb+ (n=6)).

(IgG1□ IgG2■ IgG3▩ IgG4▤)

(Levels of IgG subclasses are expressed as the mean ± SEM for each patient category)

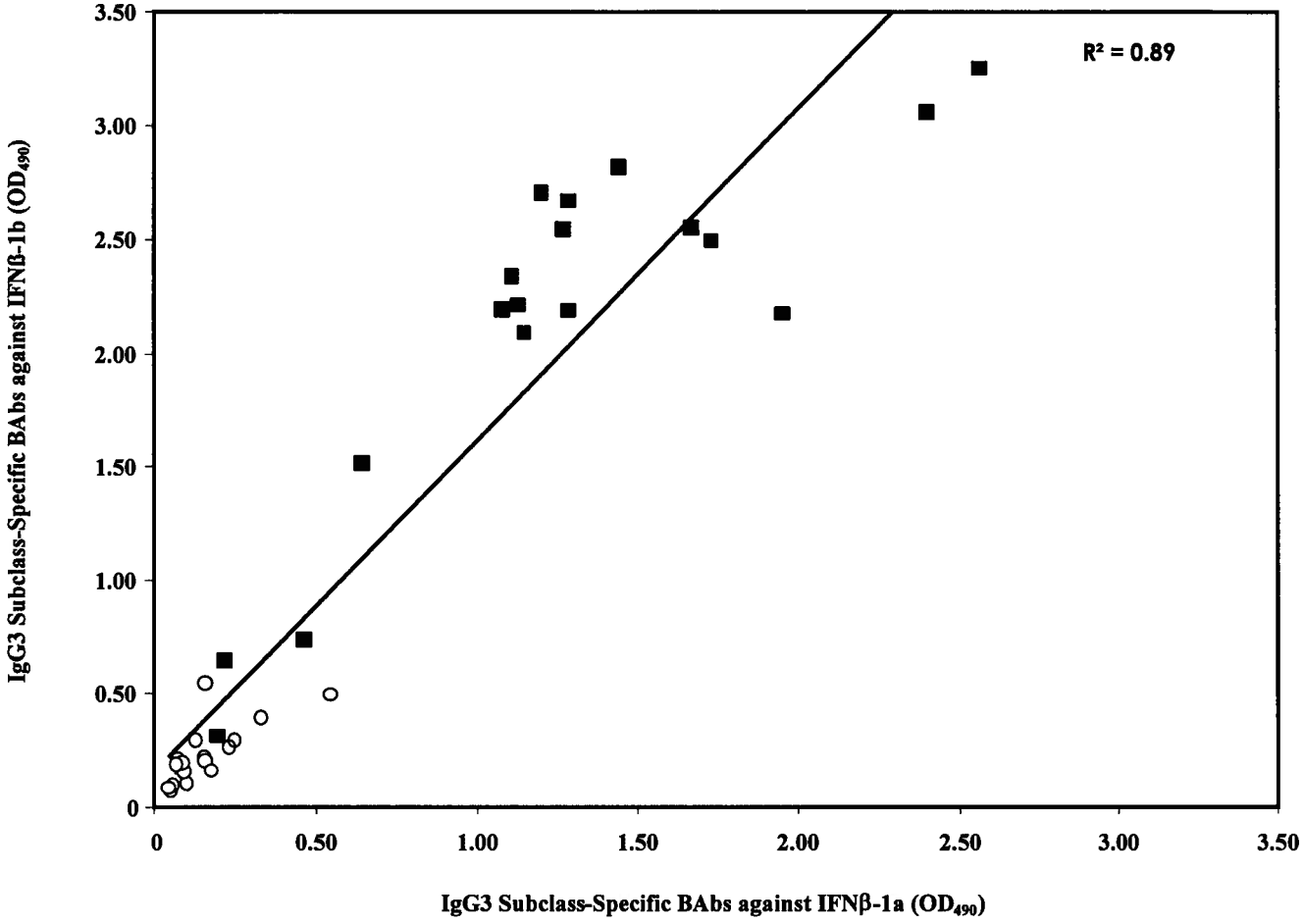

Figure 2.3. Linear regression analysis of the correlations between IgG3 subclass-specific antibodies that bind IFNβ-1a antigen and IgG3 subclass-specific antibodies that bind IFNβ-1b antigen ($R^2 = 0.89$, P = <0.0001, n = 37).

(○ IFNβ-1a -treated patients' sera (n=18) ■ IFNβ-1b -treated patients' sera (n=19))

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Chapter 3

A biosensor-based characterization of the affinity maturation of the immune response against interferon beta and correlations with neutralizing antibodies in treated multiple sclerosis patients[2]

[2] A version of this chapter has been accepted for publication. **Ebrima Gibbs**, Joël Oger. A Biosensor-based Characterization of the Affinity Maturation of the Immune Response against Interferon beta and Correlations with Neutralizing Antibodies in treated Multiple Sclerosis Patients. ***Journal of Interferon and Cytokine Research (In Press)***

3.1 Introduction

Antibodies against IFNβ represent an important aspect in MS patients undergoing treatment with IFNβ. It has now been conclusively proven that NAbs can reduce the clinical efficacy of IFNβ in a proportion of patients (Boz et al., 2007). In addition, the biological activity of IFNβ can also be abrogated by the presence of these antibodies (Vallittu et al., 2002; Bertolotto et al., 2003; Pachner et al., 2003a), the implications of which can be far reaching, as we recently discovered; an IFNβ-treated patient with NAbs persistent for over 4 years despite cessation of treatment, developed a very rare form of melanoma (Gibbs et al., 2008). This we hypothesize could be a consequence of the NAbs neutralizing not only exogenous, but the endogenously produced IFNβ, which among other functions is known to have growth inhibitory and proapoptotic effects (Chawla-Sarkar et al., 2001; Parmar and Platanias, 2003). Chronologically, in IFNb-treated patients, peak BAb levels are attained earlier than peak NAb levels, and it is hypothesized that the former, driven by the process of affinity maturation, evolve over time into NAbs (Mayr et al., 2003). It has been demonstrated that patients who develop BAbs but no NAbs (BAb+ / NAb-), tend to have lower antibody affinities when compared to BAb+ / NAb+ patients (Gneiss et al., 2006a). How this process emerges longitudinally, however, has not been ascertained.

In addition to antibody specificity and titre, the biological effectiveness of an antibody is dependent on its affinity (Steward and Lew, 1985). Affinity defines the binding strength between antigen and antibody, and affinity maturation is the overall improvement of affinity over time. As exemplified by IgG antibodies, this process is well-established and increases the effector functions of IgG antibodies during the adaptive immune response (Roost et al., 1995). Initially, during an immune response, the predominant Ig are of the IgM isotype with low antibody affinity. As the response proceeds, there is a progressive switch to the IgG isotype with a

concomitant increase in affinity. This maturation process involves B cell proliferation, germinal centre differentiation, somatic hypermutation and the competition for antigen by different clones of B cells.

Biacore™ is a state-of-the-art-technology that utilizes surface plasmon resonance (SPR) to analyze in real time, biomolecular interactions between proteins, carbohydrates, nucleic acids and peptides as they proceed (Swanson et al., 2002). An inherent advantage of this technology is that it is label-free, without the use of enzymatic, fluorescent or radioactive labelling of the interactants or secondary detector molecules. SPR is an optical resonance phenomenon occurring at the interface between a thin gold film and a liquid medium. When light with a wavelength of 760 nm is focused on the film surface of the sensor chip, SPR occurs at an angle. Analyte bound to ligand will result in an additional mass and a change in refractive index with a resultant change in SPR angle that is reported in Resonance Units (RU) (Malmqvist, 1993). A plot of RU over time produces a sensorgram, which provides real time monitoring of the binding kinetics, with 1 RU corresponding approximately to 1 pg/ml of bound analyte. Curve-fitting software, allows for the calculation of the association constant (k_{on}), the dissociation constant (k_{off}) and the equilibrium constant. Relative antibody affinity (avidity) can be estimated from the k_{off} values obtained from the dissociation phase: the higher the affinity, the slower the dissociation rate (Takacs et al., 1999).

In the present study, IFNβ of high purity was immobilized onto sensor chips and used in a Biacore™3000 instrument to evaluate the binding characteristics and relative dissociation rates, and thus apparent affinities, of anti-IFNβ antibodies. To address the question of how the affinity maturation of the anti-IFNβ antibodies evolves in NAb+ and NAb- patients, we examined serial samples from 18 IFNβ–1a and IFNβ–1b-treated patients, 12 of whom were NAb+ and 6 NAb-.

In addition, we report the IgG subclass specificities of these antibodies, some of which have previously been published (Gibbs and Oger, 2007).

3.2 Materials and Methods

3.2.1 Patient Sera

Serum samples were collected serially from MS patients who had been continuously treated with subcutaneous IFNβ-1a or subcutaneous IFNβ-1b. Samples were selected from the UBC MS Clinics' serum bank based on the following criteria: 1) patients were receiving one single type of IFNβ therapy, 2) completion of at least 60 months of IFNβ therapy, 3) at least 3 serum samples collected with a period of at least 6 months separating these sera, and 4) availability of results for BAbs and NAbs. We ended up with 9 IFNβ-1a and 9 IFNβ-1a-treated patients with treatment duration ranging from 66 to 198 months. These patients were stratified into BAb+ NAb- (n=6) and BAb+ NAb+ (n=12) based on detection of BAbs by ELISA (Gibbs and Oger, 2007), and NAbs by both the CPE (Grossberg et al., 1986) and Luciferase Reporter Gene Assays (Farrell et.al., 2008). A patient was considered BAb+ if the OD value of the serum sample was greater than the mean + 3 standard deviations of 5 healthy control sera included in each assay. Patients were designated as NAb+ if they tested positive (>20 Ten-fold Reduction Units (TRU)) on at least 2 consecutive samples. Otherwise, patients were considered NAb- but with IFNβ binding activity (BAb+). Control sera were obtained from patients whose BAb and NAb status had been identified by the BAb/NAb testing program at UBC, and from healthy donors.

3.2.2 Equipment and Data Analysis Software

The binding characteristics of serum antibodies to IFNβ were assessed using Biacore 3000™

instrument (Biacore AB, Uppsala, Sweden) that utilizes SPR technology. Data were analyzed using BiaEvaluation 3.1 software (Biacore AB, Uppsala, Sweden).

3.2.3 Immobilization of IFNβ Antigen

Under conditions of continuous flow of running buffer (HEPES buffered saline (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% P-20 surfactant) (HBS-EP, 0.05% P-20), pure recombinant IFNβ-1a (Avonex®, Biogen-IDEC, Cambridge, MA) was immobilized onto a CM-5 sensor chip (Biacore, Uppsala, Sweden). Using an amine coupling kit (Biacore AB), the active flow cell was first activated with a 7-minute injection of equal volumes of 0.05 M *N*-hydroxysuccinimide (NHS) and 0.2 M *N*-ethyl-*N*'- (3 diethylaminopropyl) carboiimide (EDC), according to the manufacturers instructions. The EDC/NHS mixture converts the carboxymethyl groups of the dextran matrix on the sensor chip to succinimide esters. Subsequent injection of IFNβ-1a (7.5 μg/ml in HBS-EP, 0.005% P-20, pH 7.4) over the surface results in the formation of amide bonds between NH2 groups on the IFNβ and succinimide groups of the activated surface. Finally, unreacted succinimide groups were blocked by a 7-minute injection of 1 M ethanolamine hydroxide (pH 8.5) (Figure 3.1a). The amount of IFNβ-1a immobilized on the active flow cell ranged typically from 135 – 155 RUs. On the reference flow cell, following activation by NHS/EDC mixture, the surface was immediately blocked with ethanolamine hydroxide (Figure 3.1b). All immobilization procedures were carried out at 25°C.

3.2.4 Biacore Analysis of Serial Serum Samples

A 96-well microtitre plate format was used to perform the Biacore assays, at a temperature of 37°C, under conditions of continuous running buffer flow (except otherwise stated). Serum samples were diluted 1 in 10 in HBS-EP, 0.005% P-20 containing 1 mg/ml carboxymethyl

dextran (CM-D). All samples originating from each patient were included in the same assay. Each assay also contained a positive and a negative control, and a blank control consisting of the sample diluent. Each sample was sequentially injected over the active IFNβ-1a coated surface and the reference control surface (no IFNβ-1a) at a flow-rate of 60 μl/min for 2 minutes during which time binding was assessed in the association phase. Subsequently, sample injection was stopped and antibody dissociation was allowed for 5 minutes during which time only running buffer was injected over the sensor chip surface (Figure 3.2a). The binding response was measured in RU with 1 RU typically corresponding to a surface mass change of 1pg/mm^{2}protein, and the dissociation phase was measured in seconds-^{1} (s^{-1}). After the completion of a cycle, association and dissociation, the sensor chip surface was then regenerated with a pulse of 50 mM NaOH (15 μl at 60 μl/min).

3.2.5 Determination of Binding Responses and Relative Antibody Affinities

Antibody binding to immobilized IFNβ was measured as the increase in SPR signal, in response units (RU), from 15 seconds (s) prior to sample injection (baseline) to 15 s prior to end of sample injection. The true antibody binding response was obtained by an on-line reference method that subtracts the SPR signal obtained on the reference surface from the active surface, resulting in normalized sensorgrams (Figure 3.2b). For the analysis of relative antibody affinities, the BiaEvaluation 3.1 Software (Biacore AB, Uppsala, Sweden) was used. First the normalized sensorgrams were x-transformed to start at the same sample injection startpoint, followed by y-transformations at 0.00RU and subtraction of the sample diluent. The sensorgrams were then fitted to Langmuir 1:1 binding model (single site binding) from 10 to 310 seconds post cessation of sample injection (5 minutes dissociation). The resultant dissociation rates (second^{-1}) are

reflective of the relative antibody affinity, with high affinity antibodies having a relatively slower dissociation rate and low affinity antibodies with fast dissociation rates. Due to the polyclonal nature of serum however, the relative dissociation rates and affinities should be considered as apparent estimates, representing an average of the multiple populations and interactions of the different antibody clones (Sem et al., 1999). To make comparisons between patients, the dissociation rate of pretreatment serum was further subtracted from subsequent serum samples for each patient.

3.2.6 Determination of IgG Subclass-specificities of Anti-IFNb Antibodies

The IgG subclasses of these antibodies were previously analyzed by a capture enzyme-linked-immunosorbent assay (ELISA) as part of a larger cohort of patients (Gibbs and Oger, 2007). Briefly, 96-well microtitre plates (Costar 3576, Cambridge, MA.) were coated with a capture antibody, mouse anti-human IFNb monoclonal antibody (Chemicon MAb416, Temecula, CA.), and incubated overnight at 4°C. Plates were then washed 3 times with PBS containing 0.05% Tween 20 and non-specific sites blocked with 1% Bovine Serum Albumin (BSA) at 37°C for 1 hour. After washing with PBS/Tween, diluted commercially available IFNb-1b (Berlex, Canada) or IFNb-1a (Serono, Canada) was added and plates incubated further overnight at 4°C. Following 3 washes, diluted patient sera were added and incubated at 37°C for 1 hour. Serial samples of each patient were assayed together on the same plate. After washing, horse radish peroxidase (HRP)-conjugated mouse monoclonal anti-human IgG1, Fc region-specific (clone HP6069), anti-human IgG2, heavy chain Fd region-specific (clone HP6014), anti-human IgG3, heavy chain hinge region-specific (clone HP6047) and antihuman IgG4, heavy chain Fc-region-specific (clone HP6025), all obtained from Zymed Laboratories (San Francisco, CA.), were added in the

appropriate wells for 1hr 37°C. Bound IgG antibodies were then detected by addition of *o*-phenylenediamine (OPD) substrate and optical densities measured at 490 nm wavelength in an MRX ELISA Microplate Reader (Dynex Technologies, Chantilly, VA.).

3.2.7 Statistical Analysis

Statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL.) The descriptive statistics consisted of the mean and standard error of the mean (SEM) for each group of patient and at different time-points. We determined the significance of the differences in Biacore binding responses and relative antibody dissociation rates between NAb negative and NAb positive patients using the Mann-Whitney test. The relationship between Biacore binding and NAb titres, and between relative antibody dissociation rates and NAb titres were determined by Spearman's correlation test.

3.3 Results

3.3.1 Evaluation of the Assay

The specificity of the Biacore assay to detect antibodies to IFNβ was assessed using serum samples from 43 healthy donors and 11 IFNβ-treated MS patients. Figure 3.3 shows that the 43 samples from healthy donors had no detectable binding to immobilized IFNβ-1a, whereas 5 out of 11 IFNβ-treated patients' samples had a detectable binding response above baseline (serum diluent blank). These 5 samples (M5, 8, 9, 10 and 11) also tested positive for BAbs by ELISA, and samples M10 and 11 were also NAb+ with titres of 15360 and 32250 TRU /ml, respectively. Thus these data indicate the specificity of the Biacore assay in detecting serum antibodies from treated MS patients that bind to immobilized IFNβ-1a. To address the sensitivity of the assay, 2

representative sera, an anti-IFNβ positive (70000 TRU /ml) and anti-IFNβ negative, were serially diluted (2-fold) and injected over immobilized IFNβ-1a. Even at the lowest concentration (1:160 dilution) of the positive serum sample, the binding response was still greater than the negative serum (Figure 3.4).

3.3.2 Serial Biacore Analysis of Antibody Binding Responses

Serial serum samples from 18 IFNb-treated MS patients were evaluated for binding to a low density immobilized IFNb-1a. All samples from each patient were assayed on the same 96-well microtitre plate, and a depiction of sensorgrams of serial samples of one patient is shown in Figure 3.5; the magnitude of the binding responses as well as the relative dissociation rates clearly changed as a function of the duration of IFNb treatment. The mean binding response peaked at month 18 among the NAb- patients at 28.5 ± 14.9 RU followed by a fast decline. In contrast, in NAb+ patients, a mean peak response of 54.8 ± 21.2 RU occurred later at month 36, followed by a second peak of 56.9 ±19.5 RU at month 60. The mean binding response was also higher during the first 12 months in the NAb- patients (mo.6 = 24.3 ± 8.5 RU, mo.12 = 25.4 ± 11.2 RU) compared to the NAb+ patients (mo.6 = 10.0 ± 2.9 RU, mo.12 = 15.6 ± 4.8 RU); this difference in binding response was statistically significant at mo.6 (Mann-Whitney U, 13.0, $p = 0.044$). However, with continued treatment, the antibodies generated in the NAb+ patients developed a higher binding response after month 12 compared to the NAb- patients (Figure 3.6), this being significant at mo.48 (Mann-Whitney U, 0.0, $p = 0.017$) and mo.60. (Mann-Whitney U, 0.0, $p = 0.021$).

3.3.3 Serial Determination of the IgG Subclass-Specificities of anti-IFNb Antibodies

As shown in Figure 3.7a, among the NAb- patients, overall there was no predominant expression of any particular IgG subclass, with all subclasses being expressed. As the immune response progressed, the relative levels of all subclasses decreased, such that by month 36 only low levels of IgG3 and IgG4 could be detected in the NAb- group. In contrast, NAb+ patients demonstrated a distinct pattern of IgG subclass distribution (Figure 3.7b); there was a relative predominance of IgG1 during the first 6 months of IFNb therapy, but as the immune response evolved, IgG4 levels increased markedly and became the predominant subclass, with peak levels at month 18. Relative levels of IgG2 and IgG3 were generally low and did not feature prominently in the immune response, although there was a notable peak of IgG3 at month 6, but which precipitously declined thereafter.

3.3.4 Serial Biacore Analysis of Relative Antibody Dissociation Rates

On average NAb+ patients had slower rates of antibody dissociation than NAb- patients. Over time, the mean antibody dissociation rate continued to decrease in the NAb+ patients, whereas there was only a very slight decrease and a subsequent increase in the NAb- patients (Figure 3.8). In NAb+ patients the mean antibody dissociation rates decreased from 0.00118 ± 0.00030 s^{-1} at month 6 to 0.00021 ± 0.00008 s^{-1} at month 36, and followed by a slight increase to 0.00027 ± 0.00003 s^{-1} at month 60. In NAb- patients, there was a negligible decrease in mean dissociation rate from 0.00130 ± 0.00025 s^{-1} to 0.00105 ± 0.00020 s^{-1} at mo 18, followed by an increase to 0.00244 ± 0.00099 s^{-1} at month 60. Thus NAb+ patients generally had lower antibody dissociation rates than NAb- patients, and this was significant at month 24 (Mann-Whitney U, 10.0, $p = 0.030$), month 36 (Mann-Whitney U, 0.0, $p = 0.025$) and month 60 (Mann-Whitney U,

0.0, p = 0.014).

3.3.5 Serial Measurement of NAbs and Correlations with Relative Antibody Dissociation Rates

In the NAb+ patients, mean NAb titre increased from 79 ± 26 TRU at month 6 reaching peak levels of 3800 ± 1371 TRU at month 36, followed by a gradual decline to 2007 ± 1180 RU at month 60. Figure 3.9 a) demonstrates that as IFNb treatment duration continued, there was an increase in mean NAb titres and a concomitant decrease in mean antibody dissociation rates, such that peak NAb titre coincided with the lowest rate of antibody dissociation rate at month 36. Additionally, as mean NAb titre decreased after month 36, so too did the mean antibody dissociation rate increase. Thus there was a significant inverse correlation between NAb titres and relative antibody dissociation rates (Spearman's Correlation, R2 = -0.374, $p < 0.001$). At the individual level, Figure 3.9 b) and c) also demonstrate the close relationship between the two parameters.

Similarly, Figure 3.10 demonstrates the relationship between NAb titres and Biacore binding responses in NAb+ patients. There was a strong positive relationship between the two (Spearman's Correlation, R2 = 0.537, $p < 0.001$), both parameters increasing in parallel and with their peak levels coinciding at month 36.

3.4 Discussion

In this study, the assessment of the antibody binding characteristics to IFNβ by SPR technology was effective in providing both quantitative binding responses and qualitative antibody

dissociation rates. The results were sensitive and specific enough to distinguish between anti-IFNβ antibodies in treated MS patients and the absence of anti-IFNβ antibodies in healthy donors or in anti-IFNβ antibody negative treated MS patients. However it is well documented that autoantibodies against IFNb do exist in healthy donors, albeit extremely rare (0.1%), provided antibody assays are sufficiently sensitive (Bendtzen, 2002). Using the 96-well microtitre plate format, instead of tubes, enabled the rapid analysis of serial samples of multiple patients. Initially, it was necessary to determine the appropriate concentrations of IFNβ-1a for immobilization, analyte (antibody) flow rate and regeneration buffer. To efficiently immobilize the IFNβ-1a antigen to the sensorchip surface, it first had to be electrostatically attracted towards the hydrophilic negatively charged dextran matrix surface. This process of preconcentration was achieved by imparting a net positive charge on the IFNβ-1a molecule; the isoelectric point (pI) of IFNβ is 9.8, and therefore dissolving it in HBS-EP, P-20 (pH 7.4) results in a net positive charge. We used a low surface antigen density of between 135 – 155 RUs of IFNβ-1a for immobilization after preliminary experiments showed that a high surface antigen density of 3000 RUs resulted in different serum samples having similar antibody dissociation rates. However, under the ideal conditions of limiting antigen and high analyte (antibody) flow rates, only high affinity antibody molecules would bind long enough for association and dissociation data to be collected. Low affinity antibodies are out-competed and would not bind under such conditions. The IFNβ-1a was also of high purity, and unlike the commercial available forms administered to patients, it did not contain bovine serum albumin (BSA). This was an absolute necessity, as any additives with amine groups would also bind to the sensorchip surface and cause non-specific binding of antibodies. Non-specific binding was further reduced by an on-line referencing system that subtracts the binding curve (sensorgram) obtained by flowing serum sample through the

reference surface (no IFNb-1a) from the active surface that contains immobilized IFNb-1a. Sensorgrams were further double-referenced by subtracting out the blank serum diluent curve, and for each patient the pretreatment curve was subtracted from all other subsequent curves. The latter was necessary in order to make comparisons between patients.

Our study demonstrates the dynamics of the binding responses of antibodies to IFNb, as measured by Biacore, in NAb- and NAb+ patients. A quantitative comparison of the two groups of patients shows that binding antibody responses were higher in NAb- patients, significantly so at month 6 ($p = 0.044$), than in NAb+ patients during the first year of IFNb therapy. Thereafter, the antibody binding responses became higher in NAb+ patients compared to NAb- patients, with significant differences being observed at months 48 and 60. Furthermore, whilst the binding response in NAb- patients peaked at month 18, peak antibody binding responses were attained at month 36 in the NAb+ patients. This pattern of antibody binding, as measured by Biacore, in NAb- and NAb+ patients, is reminiscent of the temporal profile of BAbs, as measured by ELISA, and NAbs, with BAbs peaking early and NAbs attaining peak levels later. [18]

We have also examined the IgG subclass distribution of anti-IFNb antibodies, and our results show a very dynamic pattern in NAb+ patients, with a predominant expression of IgG1 antibodies during the first year of IFNb therapy, followed by a relative predominance of IgG4 antibodies after month 12. In contrast, IgG subclass distribution in NAb- patients was less dynamic, as no subclass featured prominently. This suggests that there is a shift from IgG1 to IgG4 in NAb+ patients, and not so in NAb- patients.

Not surprisingly, the mean relative antibody affinities among NAb- patients were lower (faster dissociation rates) than among NAb+ patients (slower dissociation rates), confirming previous findings (Gneiss et al., 2006a). However, using serial samples, our study revealed for the first

time the temporal differences in affinity distributions of anti-IFNb antibodies in NAb- and NAb+ patients. In the same vein, our longitudinal study documented the marked shift from low to high affinity antibodies (affinity maturation) in NAb+ patients compared to minimal changes in affinity in NAb- patients. Our study was limited by the fact that as the actual concentration of anti-IFNb antibodies is unknown, absolute affinity could not be calculated. Thus we measured relative affinity based on relative dissociation rates. Dissociation rates were obtained by fitting curves in a 1:1 Langmuir binding model which is a simplistic approach for the modeling of the antigen-antibody reaction, as we know that antibodies are bidentate in nature and that in any given serum, there are subpopulations of different antibodies. Thus our analyses of dissociation rates were an average of all the different antibody clones in polyclonal serum. Our Biacore studies were performed with a common antigen, IFNb-1a, even though 50% of the patients were treated with IFNb-1b.This may have resulted in higher antibody binding responses than if a homologous antigen challenge was used, considering that NAb titres measured using IFNb-1a as challenge antigen are generally higher than titres obtained with IFNb-1b as antigen, regardless of whether the sera are from IFNb-1- or IFNb-1b-treated patients Files et al., 2007: Scagnolari et al., 2002).

Furthermore, in order to ascertain whether high NAb titres were indicative of the maturation of the IFNb immune response, or vice versa, a correlation analysis was performed between NAb titre and antibody dissociation rates. We found a significant relationship between the two (Spearman's Correlation, $R2 = -0.374$, $p < 0.001$). We reveal that there is parallelism between the the NAb titre and affinity of anti-IFNb antibodies, and that they peak at month 36, a period which coincides with notable reduction in IFNb clinical efficacy in NAb+ patients, as demonstrated by increased relapse rates and Expanded Disability Scale Scores (EDSS) (Boz et

al., 2007). A closer examination of the relationship between antibody binding response, as measured by Biacore, and NAb titres shows that the 2 are inextricably linked, and that there is a positive correlation between them (Spearman's Correlation, $R2 = 0.537$, $p < 0.001$).

A summation of the profiles of the binding responses and relative antibody dissociation rates of NAb+ and NAb- patients obtained by Biacore analysis warrants us to also conclude that during the first year of IFNb treatment, NAb- patients possess a higher level of low-affinity antibodies, as opposed to the prevalence of higher levels of high-affinity antibodies among the NAb+ patients after year one. Thus our biosensor-based approach for the characterization of the immune response against IFNb reveals a quantitative and qualitative maturation of a subset of BAbs into NAbs. Our study also demonstrates, for the first time, that the affinity maturation of anti-IFNb antibodies can be objectively monitored in IFNb-treated MS patients, and that measuring relative antibody affinity contributes to the comprehensive profiling of anti-IFNb antibodies.

3.5 Acknowledgements

We thank Regina Lam and Tariq Aziz for excellent technical assistance. This work was supported by a Studentship (E. Gibbs) from the Multiple Sclerosis Society of Canada.

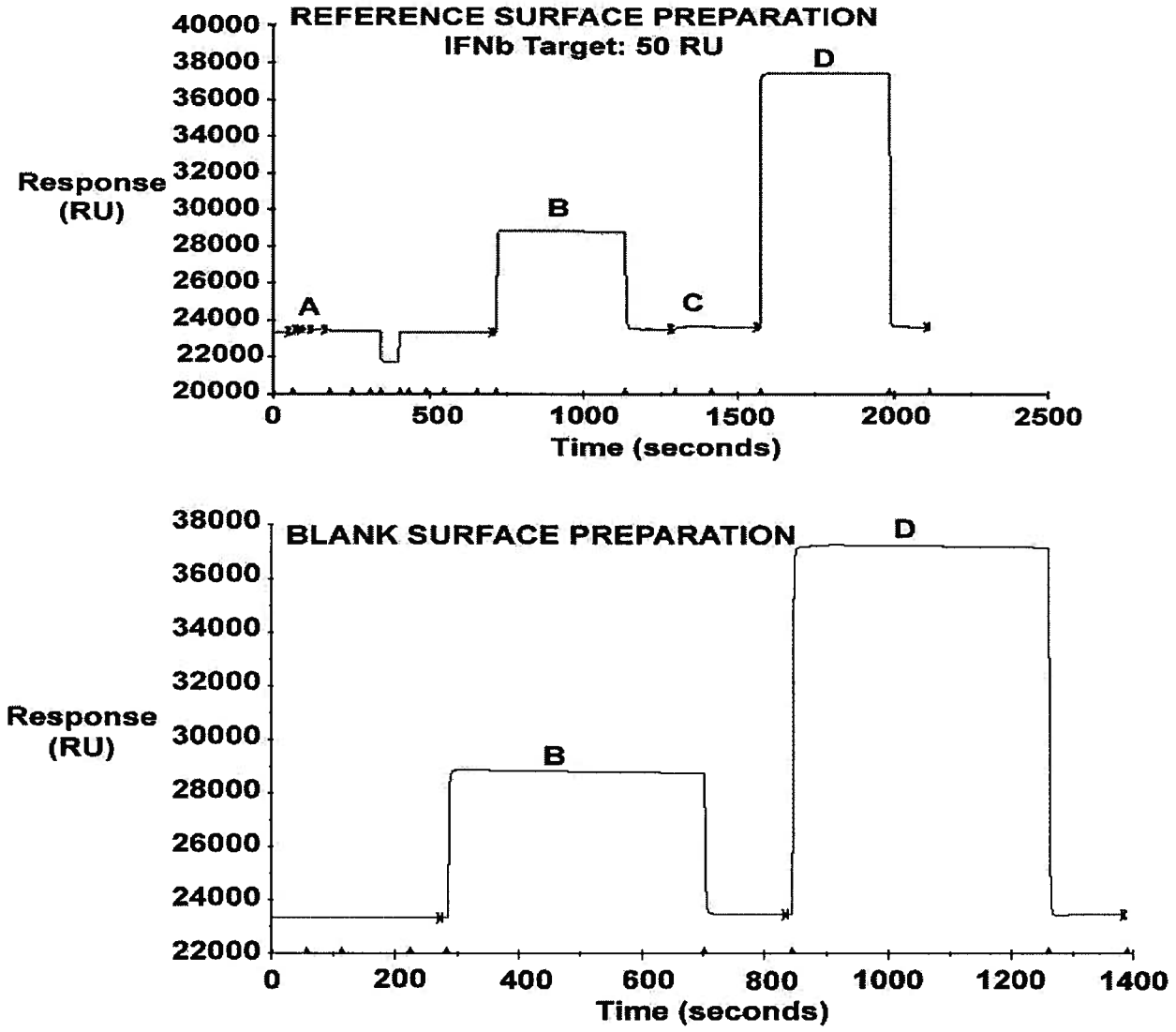


Figure 3.1. Sensorgrams showing the procedures for the preparation of the Active and Reference Surfaces. (A) Preconcentration of IFNβ-1a antigen, a process by which IFNβ-1a is electrostatically attracted to the negatively charged dextran matrix, and the target level of immobilization is tested; (B) Activation of surface with mixture of 0.2 M EDC and 0.05 M NHS (v/v) to generate succinimide groups; (C) Injection of 7.5 ug/ml of pure IFNβ-1a in HBS-EP, 0.005% P-20 (pH7.4). During this process, the amine groups of IFNβ-1a couple with reactive succinimide groups of the activated surface to form covalent amide bounds; (D) 1 M ethanolamine hydrochloride is used to block the uncoupled succinimide groups.

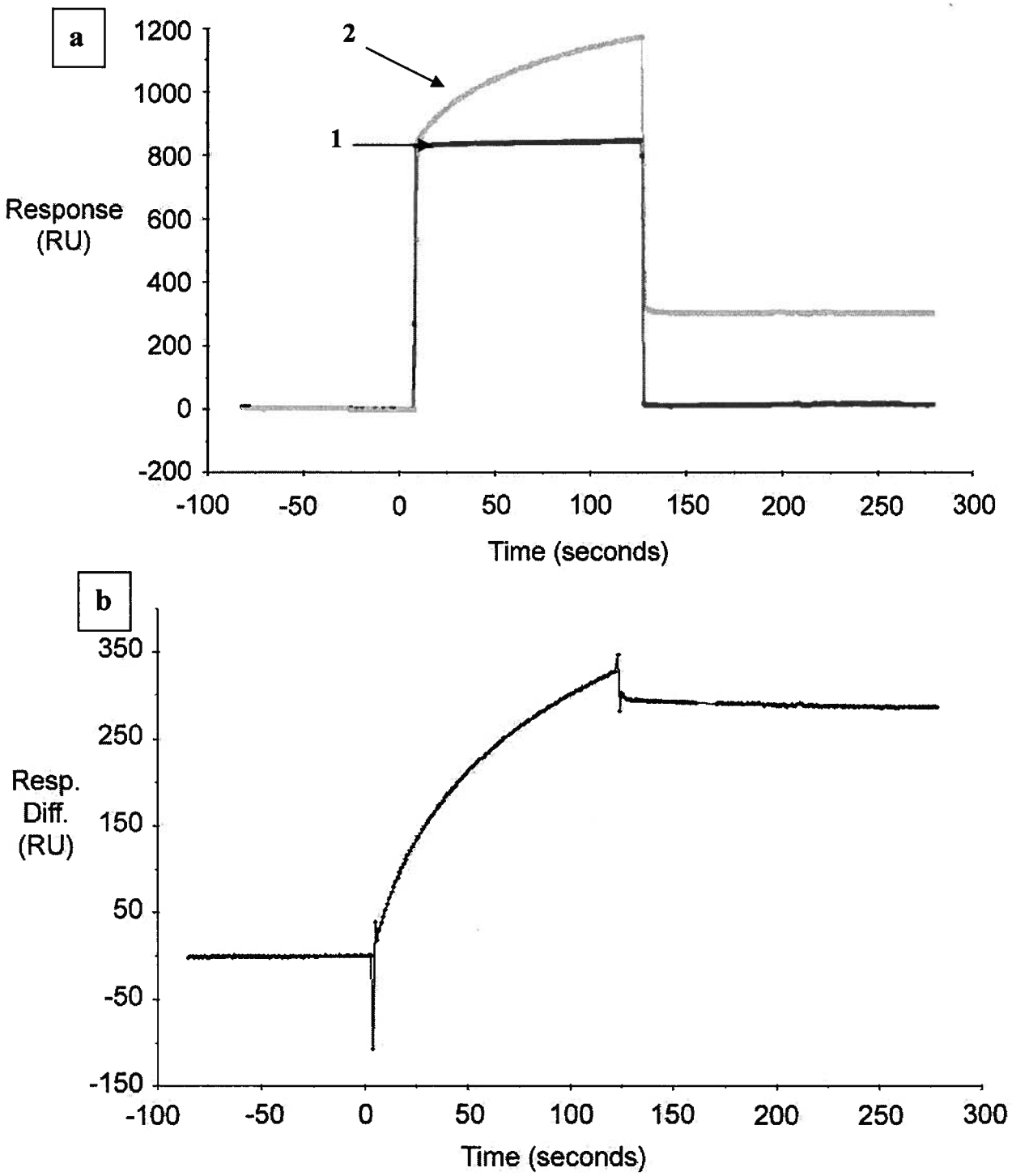


Figure 3.2. Sensorgram plots of a representative anti-IFNβ antibody positive serum sample. a) Binding to the Reference (1) and Active (2) Surfaces, b) Sensorgram obtained by subtraction of 1 from 2.

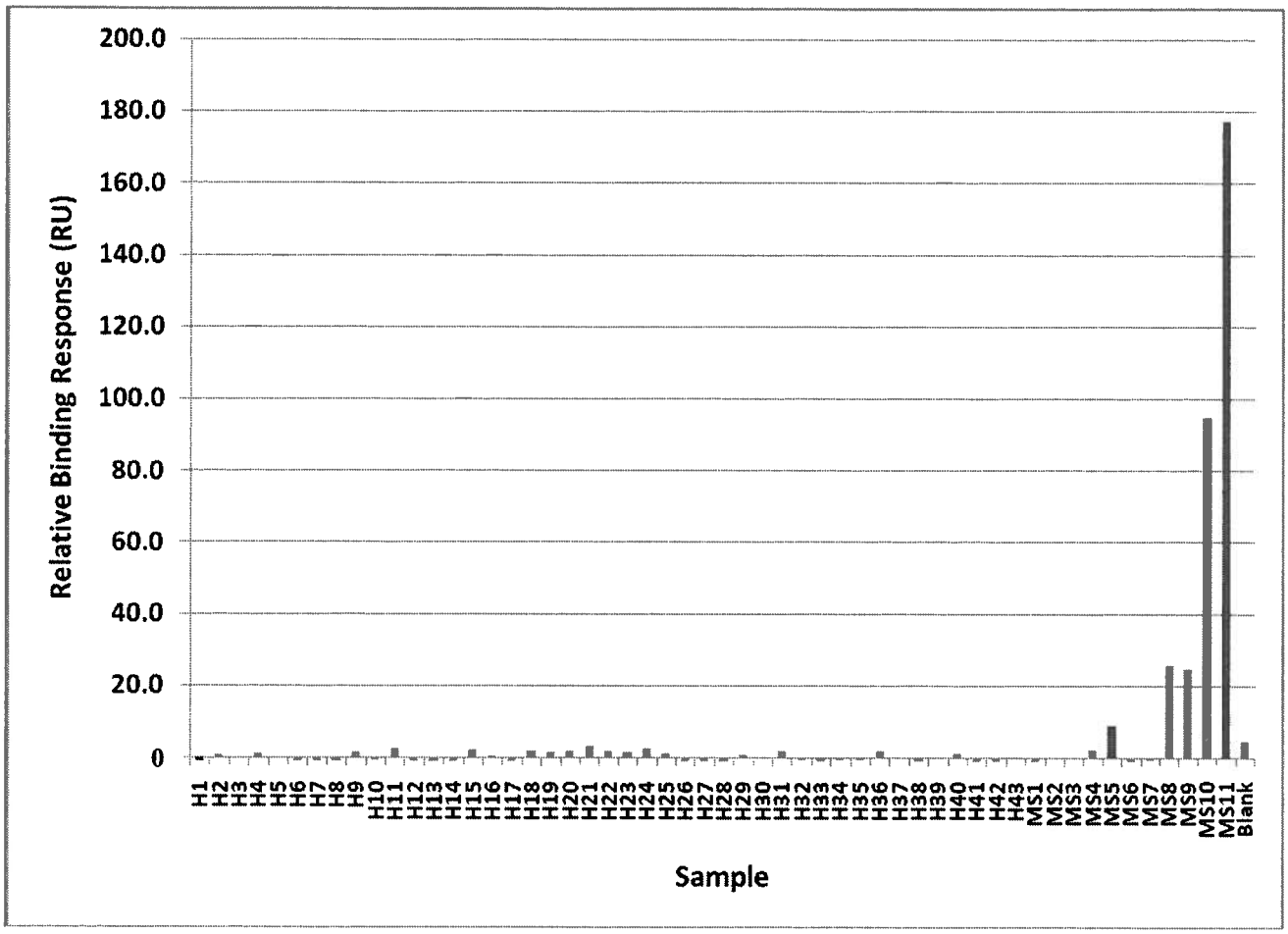


Figure 3.3. Biacore reactivity of serum samples to immobilized IFNβ-1a. Fifty-four serum samples, diluted 1:10, from healthy donors (H1 – H43) (n=43) and IFNβ-treated MS patients (MS1 – MS11) (n=11) were tested. Sera were obtained from the UBC Clinics' serum bank, and whose BAb and NAb status were identified by the BAb / NAb testing program at the UBC. MS1 to MS3 are BAb- / NAb-, MS4 to MS9 are BAb+ / NAb-, MS10 is BAb+ / NAb+ with a NAb titre of 15360 TRU / ml and MS11 is BAb+ / NAb+ with a NAb titre of 32250 TRU / ml.

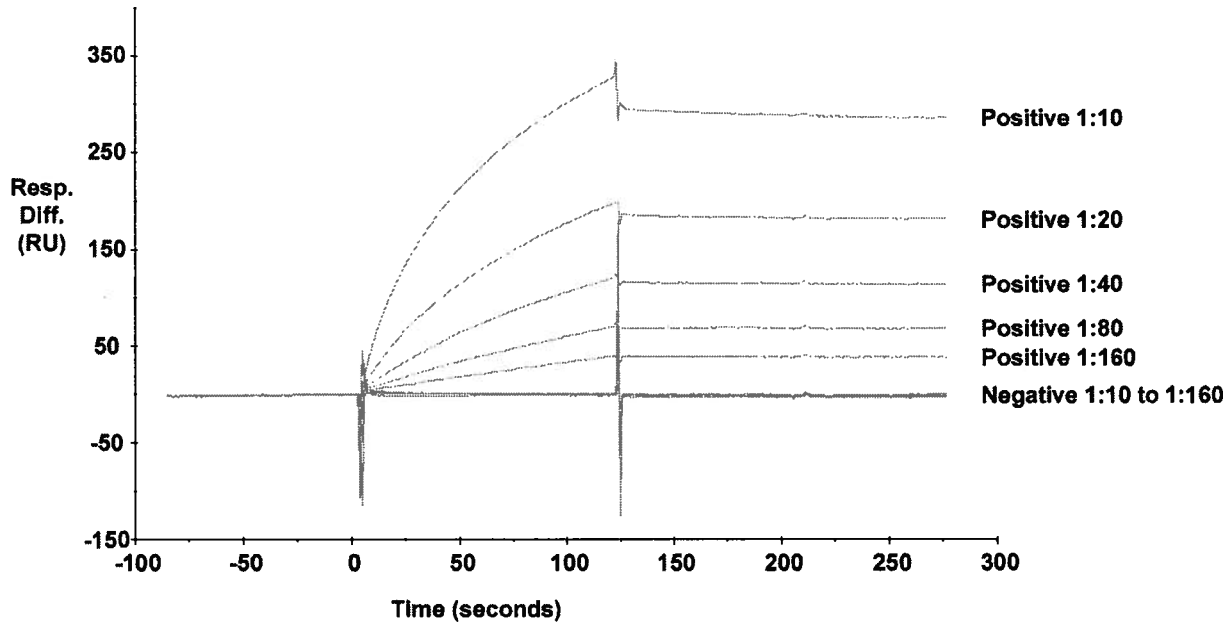


Figure 3.4. Sensorgram overlay plots of 2-fold serial dilutions of representative sera binding to immobilized IFNβ-1a . The sera consisted of an anti-IFNβ antibody positive (BAb+ / NAb+) serum with a NAb titre of 70000 TRU / ml, and BAb- / NAb- serum (< 20 TRU / ml).

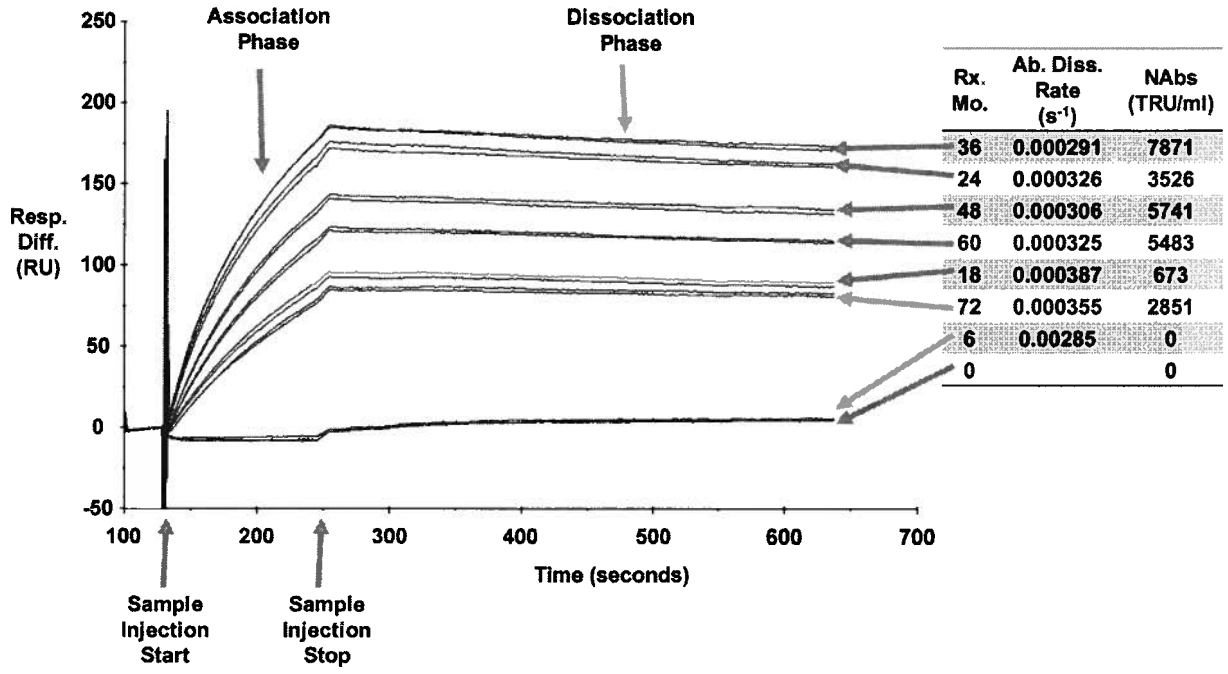


Figure 3.5. A representative plot of sensorgrams from serial samples of one patient, showing the interaction between anti-IFNβ antibodies and immobilized IFNβ-1a. Serum samples were taken at different time points during IFNβ treatment. Samples were analyzed in duplicate over the Active Surface (containing 143 RU IFNβ-1a) and the Reference Surface (no IFNβ-1a). The sensorgrams were evaluated with a BIAevaluation Software 3.1 (Biacore AB) by first X-transforming all curves to start at the same injection startpoint, followed by Y-transformations at 0.0RU and subtraction of the sample diluent. Curves were then fitted by the 1:1 Langmuir model to obtain relative dissociation rates (s^{-1}) between 255 to 655 seconds.

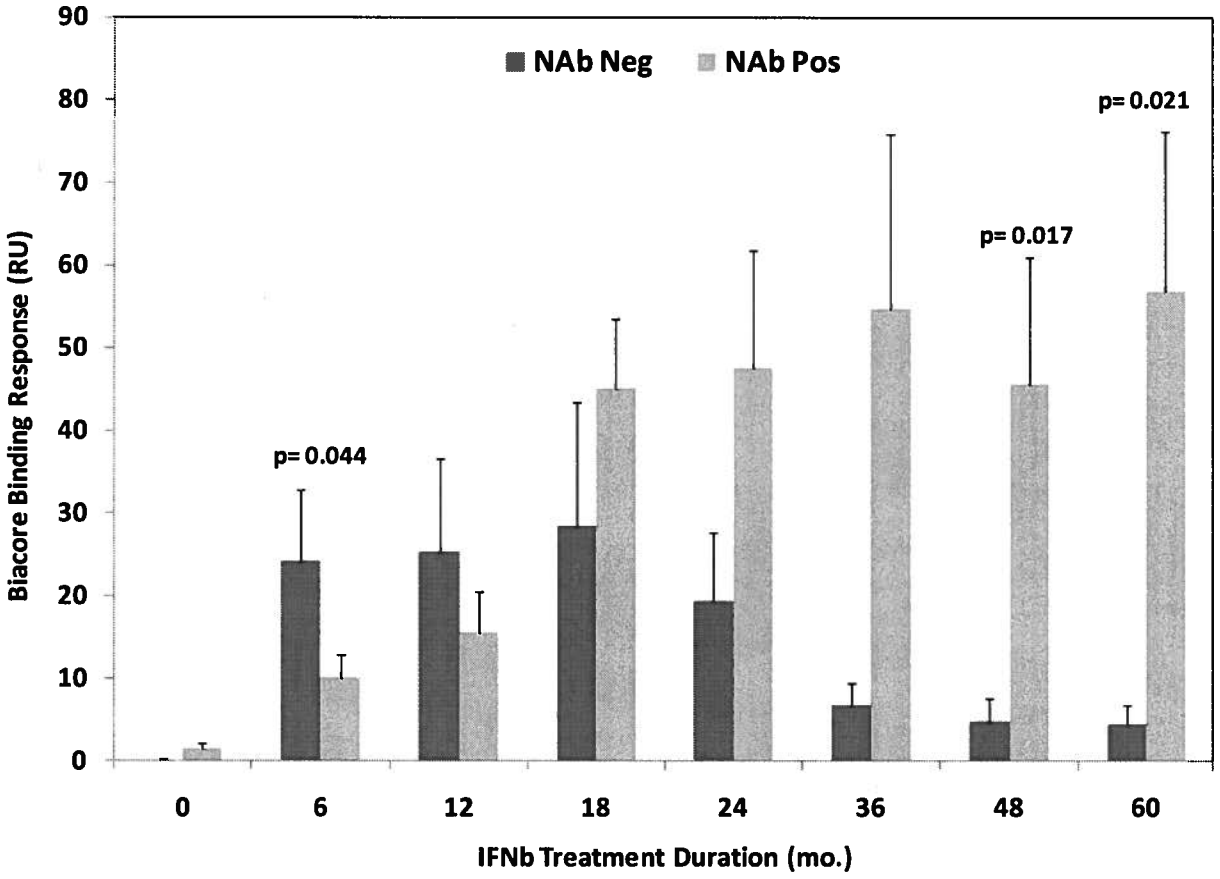
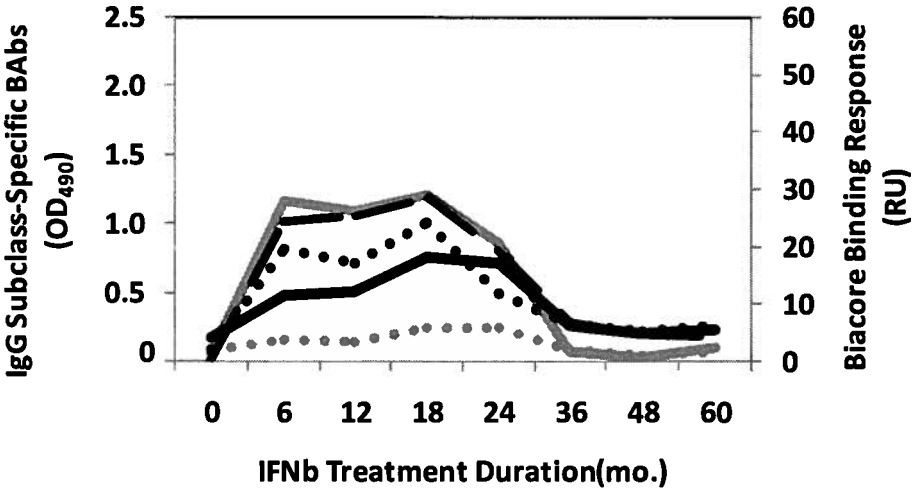


Figure 3.6. Comparison of the anti-IFNβ antibody response in NAb negative (n=6) and NAb positive (n=12) patients using Biacore. Biacore binding responses (mean values (+ SEM) are plotted over time.

a)



b)

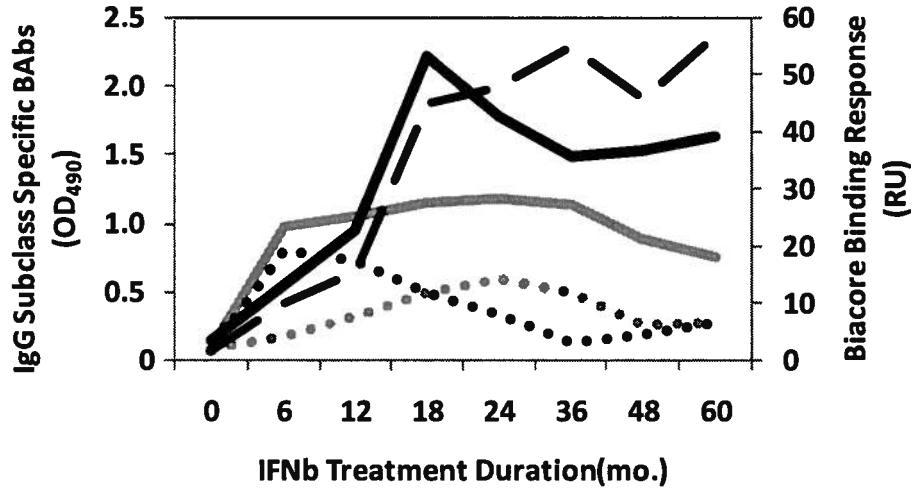


Figure 3.7.The patterns of changes in IgG subclass-specificities in relation to Biacore binding response of anti-IFNb antibodies. a) NAb negative patients (n=6), and b) NAb positive patients (n=12). Data represent mean values. IgG subclasses were measured by ELISA. (Biacore binding(▬ ▬), IgG1(▬▬▬), IgG2(▪ ▪ ▪ ▪ ▪ ▪), IgG3(▪ ▪ ▪ ▪ ▪ ▪), IgG4 (▬▬▬)).

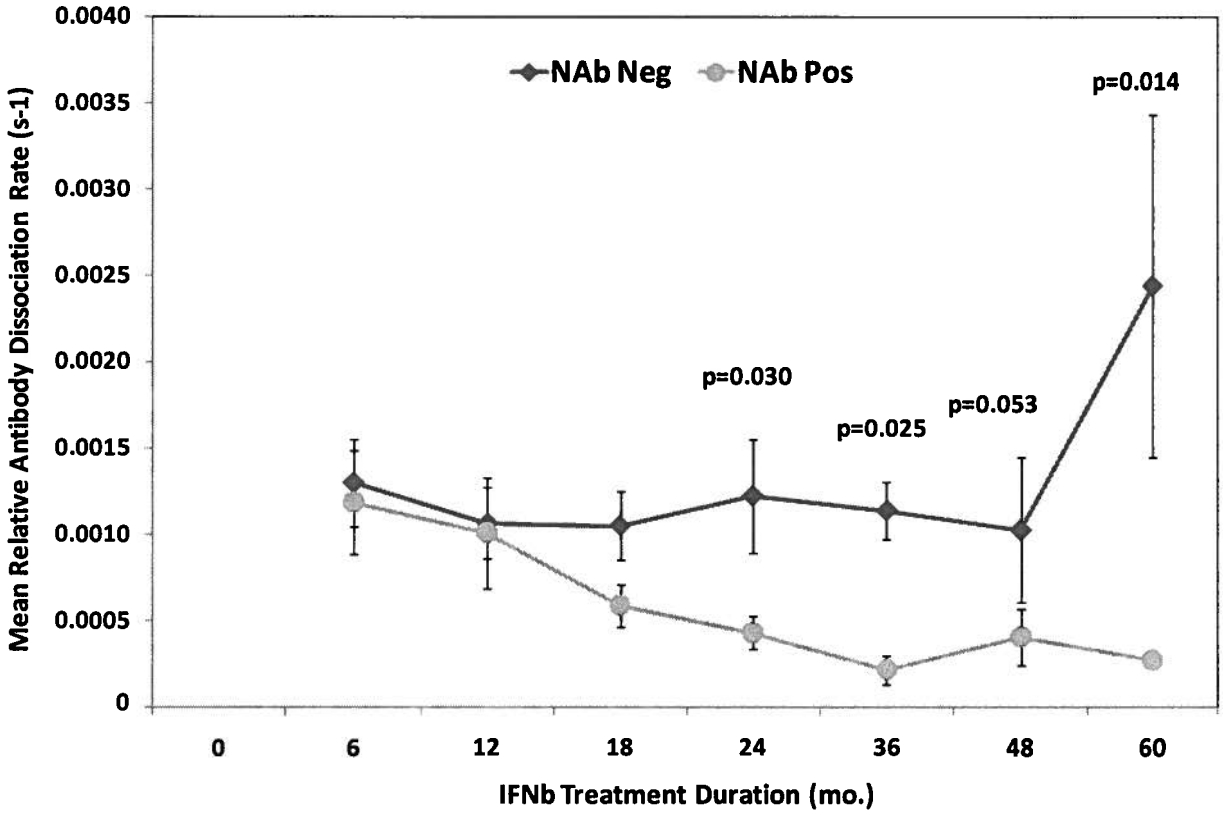


Figure 3.8. Comparison of the relative antibody dissociation rates in NAb- (n=6) and NAb+ (n=12) patients as measured by Biacore. Data represent the mean (±SEM) at different time points during treatment with IFNβ.

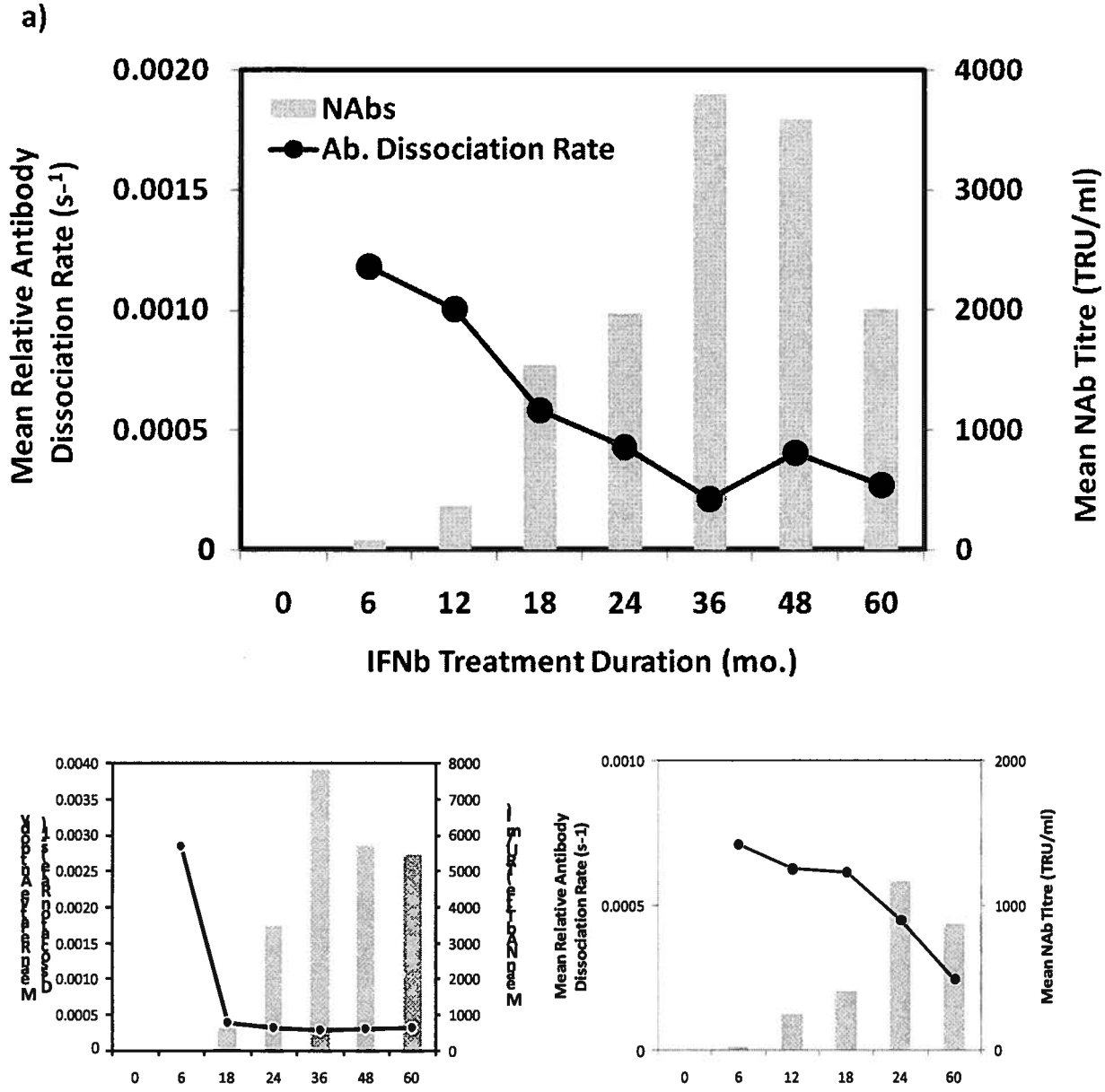


Figure 3.9. Relationship between changes in NAb titres and changes in relative antibody dissociation rates during the anti-IFNβ immune response. There is a strong correlation between the two parameters (Spearman's correlation, $R^2 = -0.374$, $p < .001$) in 12 NAb+ patients (a). Examples of 2 individual patients (b, c).

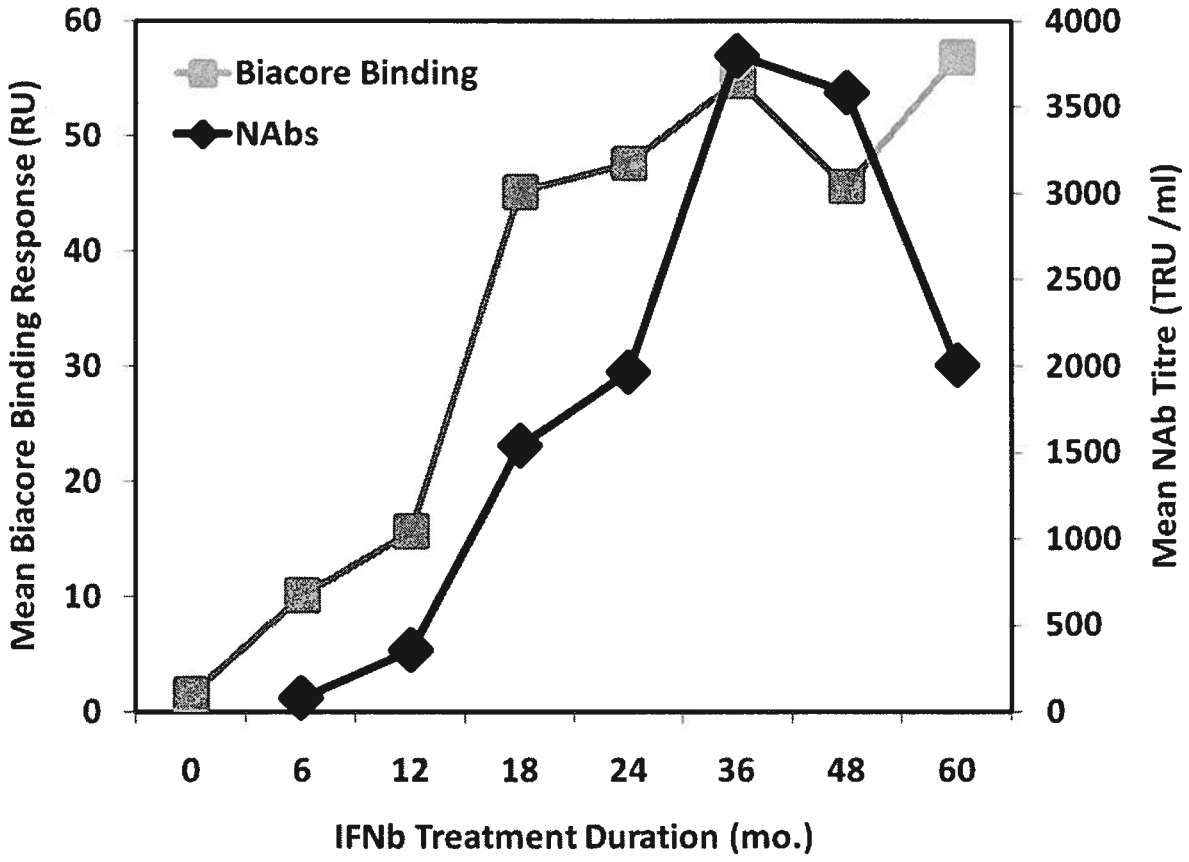


Figure 3.10. Profile of Binding antibodies as measured by Biacore (Biacore Binding Response) and Neutralizing antibodies as measured by bioassay, showing the significant positive relationship between the two (Spearman's Correlation, $R^2 = 0.537$, $p ,< 0.001$). Data represent the mean (±SEM) at different time points during treatment with IFNb, in NAb+ patients (n=12).

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Chapter 4

Reduced effectiveness of long-term interferon-b treatment on relapses in neutralizing antibody- positive multiple sclerosis patients: a Canadian multiple sclerosis clinic-based study[3]

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4.1 Introduction

Interferon-beta products (IFNβ) are the mainstay of MS therapy and have proven to be efficacious in reducing relapse rates and MRI activity. Their effect on disease progression is less evident. During treatment, both binding antibodies (up to 80% positivity) and neutralizing antibodies (up to 47%) have been reported from early clinical trials (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996; The PRISMS Study Group and the University of British Columbia, 2001). The appearance of anti-IFNβ antibodies occurs sooner in treated MS patients than in cancer patients (Larocca et al., 1989). Both *in vivo* and *in vitro* studies have demonstrated a heightened antibody response to various natural antigens by MS patients (Oger et al., 1983). Among the three preparations used, IFNβ-1b (Betaseron®) has been shown to be most immunogenic and IFNβ-1a subcutaneous (Rebif®) less so, while IFN β-1a intramuscular (Avonex®) is the least immunogenic (Antonelli et al., 1998; Ross et al., 2000). The technique used to detect anti-IFNβ antibodies determines their classification as binding antibodies (BAb) or neutralizing antibodies (NAb). BAbs most likely represent all the antibodies that are elicited by, and that bind to IFNβ, whilst NAbs block the interaction with the IFNβ-receptor (Redlich et al., 1991; Runkel et al., 2001) either because they bind to the IFN receptor binding site or because they induce conformational changes in the molecule. Although IFN bioavailability is reduced in the presence of NAbs (Deisenhammer et al., 1999; Bertolotto et al., 2003) , the effect of NAbs on clinical efficacy is less well delineated (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996; Rudick et al., 1998; Bertolotto et al., 2003; Sorensen et al., 2003; Malucchi et al., 2004). Furthermore, it is not clear if NAbs have the same effect on all 3 preparations. Persistently NAb+ patients were demonstrated to have reduced MxA induction both at the protein (Deisenhammer et al., 1999; Vallittu et al., 2002) and

at the mRNA levels (Bertolotto et al., 2003; Pachner et al., 2003a). Previous studies have demonstrated that three out of four IFNβ treated MS patients develop BAb within 6 months of therapy and that these BAbs may disappear over time. Approximately 50% of BAb+ patients develop NAbs during IFNβ-therapy (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996; Rudick et al., 1998; Pachner et al., 2003c; Sorensen et al., 2003; Perini et al., 2004).

Initially the short pivotal studies did not support an effect of NAbs on IFNβ clinical efficacy (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1995) but extension of these studies showed a negative influence on the limited clinical effect of interferons based on clinical and MRI findings (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996). Two recently published studies have further supported the detrimental clinical effect of Nabs (Sorensen et al., 2003; Francis et al., 2005). However, the clinical significance of BAbs and NAbs is still debated and there is no consensus about decision-making surrounding treatment when NAbs appear. Our study aimed to correlate NAb status and clinical effects of NAbs in a cohort of patients treated at the University of British Columbia Multiple Sclerosis Clinic. Patients were prospectively evaluated clinically on a yearly basis in a program specifically created for systematic, population-based follow-up. A cross-sectional study of their IFN-β antibody status was correlated with their clinical course retrospectively. Our findings confirm that NAbs reduce the effectiveness of therapy on relapse rate with a greater effect on subcutaneous IFNβ-1a than on subcutaneous IFNβ-1b.

4.2 Materials and Methods

4.2.1 Patients

British Columbia is one of the 10 provinces of Canada and has an independent decision making program of reimbursement for medications. To be reimbursed for the cost of IFNβ medications patients have to fulfill conditions put forward by the British Columbia Pharmacare Program (only a very rare patient-less than 1%- would be able to afford the cost of Interferon without this support): at least 2 clinical relapses in the last 2 years; ability to walk; and age >18 to 65. Until July 2005, the privilege of prescribing the Disease Modifying Drugs (Interferons and Glatiramer Acetate) for MS was limited to neurologists working in the MS clinics affiliated with UBC. To renew their prescription, each patient had to be re-evaluated yearly in the MS clinic by history and by examination with recording of the number of relapses and by examination generating an EDSS value. Each patient was followed by a single treating neurologist who ordered antibody testing. Five neurologists of the UBC MS clinic systematically tested their patients at the time of yearly clinical review for renewal of the IFNβ prescription.

4.2.2 Criteria for inclusion

Data were generated systematically through the Pharmacare program by which patients were reviewed on a yearly basis. This study is based on patients who had been treated and followed clinically up to 3 years or more before July 2004, who had received one single interferon, had not been on clinical trials, had not stopped IFN for more than 1 month, had antibodies tested between July 1st, 2003 and June 30th, 2004 and had been treated for more than 3 years at the time their blood was drawn. This cohort includes 340 patients. Maximizing the number of such long-term-treated patients in the study may have introduced bias towards persistent positivity since

some patients may revert to sero-negativity before 3 years, especially those patients with low antibody titers (Rice et al., 1999). Our criteria also excluded patients who discontinued treatment early. These patients have been studied in another report (Tremlett and Oger, 2003). Charts were reviewed by a single neurologist (C.B.), blinded to the laboratory results, who verified eligibility and recorded total numbers of relapses in the 2 years before treatment, EDSS scores at onset, number of relapse for each year during treatment, and EDSS score in each year during treatment. The following outcome measures were evaluated: yearly relapse rate, percentage of relapse free patients and disability progression. Disability progression was defined as time to a sustained increase of $\geq$1 point on the EDSS scale persisting for at least 6 month for subjects with baseline EDSS scores $\leq$5, or 0.5 point increase for subjects with a baseline EDSS score >5. In our clinic, patients who suspect they may have a relapse contact our MS nurse. All our patients have been trained by our clinic nurses to recognize relapses and report them to our nurse practitioner who then communicates with the neurologist. If there is any question about the relapse, the patient is seen acutely in the clinic. Otherwise the relapse is confirmed historically at the next visit. At the time of the annual review, the neurologist reviews the documentation and records the number of bonafide relapses: such as appearance of new symptoms or worsening of old symptoms lasting more than 24 hours in the absence of fever.

4.2.3 Binding antibodies

We used a sandwich ELISA as a screening assay. Briefly, 96 well microtitre plates (Costar 3576, Cambridge, MA.) were coated with mouse monoclonal anti-IFNβ antibody (Chemicon MAb 416, Temecula, CA.) and incubated overnight at 4 °C. Plates were then washed 3 times and blocked for 1 hour with 0.1% BSA. After 3 consecutive washes, IFNβ-1b (Berlex, California) or

IFNβ-1a antigen (Serono, Canada) was added at a concentration of 10 μg/ml and plates incubated at 4 °C overnight. Patient's sera were added to the plate coated with the corresponding IFNβ, and bound antibody was detected using goat anti-human IgG (Fc-specific)-horse radish peroxidase conjugate (Sigma A-0176, Sigma Chemical Corporation, St. Louis, MO.) and with O-phenylenediamine substrate (Sigma). Color development was stopped after exactly 20 minutes with 2 M H_2SO_4. Absorbances were then immediately read at 490 nm wavelength with an MRX ELISA Microplate Reader (Dynex Technologies, Chantilly, Va USA.). Included in each plate was a set of 5 normal healthy control sera and 3 positive controls from IFNβ-treated patients (positive high, medium, low). Sera with BAb levels above the cut-off point determined as mean of the 5 healthy controls + 3 standard deviations (SD) were considered BAb positive. We have established the sensitivity of our binding assay on 675 samples as being 97.8% compared to neutralizing antibodies done by CPE. The coefficient of variation for inter-assays was 6-10% and for intra-assays 3-6%.

4.2.4 Neutralizing antibodies

BAb-positive samples were sent to the Medical College of Wisconsin for NAb assay (by Dr. Sidney E. Grossberg). Neutralizing antibodies were measured by the constant IFN method in an objective, cytopathic effect (CPE), naphthol blue black dye-uptake procedure, utilizing A549 human lung carcinoma cells and the encephalomyocarditis virus (Grossberg et al., 1986). The data were processed by a computer program created by Leslie D. Grossberg that provides the dose- response curves, statistical analyses of different parameters, calculation and final adjustment of titers according to the formula $t = f(n-1) / 9$, reportable as Tenfold Reduction Units (TRU)/ml (Grossberg et al., 2001a; Grossberg et al., 2001b).

Samples were considered positive if they had titers greater than 20 TRU/ml. They were considered negative if they had negative ELISA titers or, if BAb+, when they were below 20 TRU/ml in the CPE bioassay, considered to be the gold standard (Sorensen et al., 2005a). Although quantitative NAb data were available, the cut-off of >20 TRU/ml was taken as positive indication of anti-IFN antibody levels since that has been essentially the universally accepted level of significance. There is currently no agreement on what constitutes "high" or "low" NAb titers, and further stratification of patients by any such arbitrary assignments would have introduced unnecessary complexity; further, it would have reduced the power of the statistical comparisons of IFN-β1a with IFN-β1b inasmuch as these two interferons have very different frequency histograms when titers are reported (Gibbs and Oger, 2006).

4.2.5 Statistical Analysis

Since antibody testing had been done on a cross-sectional basis (July 2003 to June 2004) the patients were stratified by the above cut-offs, thus extending their status retrospectively to the date of onset of the follow-up. Clinical data of NAb+ and NAb– patients were compared to assess the clinical effects of NAbs. Pre-treatment annual relapse rate was calculated from the number of relapses documented during the 2 years before therapy. Since the number of patients who had been treated more than 6 years was relatively low, the values for their relapse rates and EDSS changes for years 6 to years 10 were pooled.

Age at onset of disease, sex distribution, duration of disease, duration of treatment, relapse rates, proportion of relapse free patients and EDSS scores were analyzed. The effect of IFNβ on relapse rate during the treatment period was analyzed using the repeated measures ANOVA test. After the normal distributions of variables were evaluated, all relevant data were descriptively

compared between NAb+ and NAb– groups using the chi-square test for categorical variables and Mann Whitney U (relapse rates) or Student-t test for continuous variables (age, age at onset, duration of disease, duration of therapy, and time to sampling).

Time to sustained disability progression one point or more on EDSS were evaluated in NAb+ and NAb– patients using Kaplan Meier curve-fitting, and significance was tested with the log-rank test. Statistical analyses were done using SPSS program (version 10.0, Chicago, IL). Ethics approval had been granted by the University of British Columbia Institutional Review Board.

4.3 Results

4.3.1 Subjects

Figure 4.1 illustrates patient flow at the UBC MS clinic. Of 560 patients who were seen for Interferon renewal and tested for antibody, 340 had been on a single drug for 3 years or more. Any charts lacking the required data were eliminated, reducing the number by 78 to obtain a study population of 262 patients.

4.3.2 Demographic and Clinical Characteristics

Demographic and clinical characteristics of the group of 262 patients fulfilling the entry criteria are shown in Table 4.1. Age, age at disease onset, sex, mean duration of disease before therapy, mean duration of therapy, mean time from treatment initiation to time of sampling, pre-treatment relapse rate and pre-treatment EDSS scores did not differ between Betaseron® and Rebif® subgroups. The demographics of the 78 patients with incomplete charts did not differ from the study group of 262: (age: 46.9±9.1, female/male ratio: 53/25, age at onset: 32.9±7.9, EDSS at onset: 2.7 ±1.1, pre-treatment annual relapse rate: 1.16±0.6).

4.3.3 Efficacy of IFNß Therapy

Although not a primary aim of the study, the beneficial effect of IFNβ therapy was demonstrated on relapse rate in IFNβ-treated MS patients. Irrespective of antibody status, the mean annual relapse rate decreased significantly from a pre-treatment value of 1.18 relapse/year to a mean of 0.56 in year 1, 0.49 in year 2, 0.35 in year 3, 0.30 in year 4, 0. 34 in year 5 and 0.28 in year 6-10 (repeated-measures ANOVA ($p<0.001$).

4.3.4 Percentage of BAb and NAb Positivity

Numbers and percentages of BAb+ and NAb+ patients who had been followed over time are shown in Figure 4.2. One hundred and fifteen patients (43.9%) were BAb+, 34 patients (13%) were NAb+ out of a total 262 patients who had been followed for 3 years. The number of patients treated decreased over time after year 3. However the proportion of NAb+ patients did not differ significantly throughout subsequent follow-up visits ($p=0.77$) The percentages of BAb+ and NAb+ patients in Betaseron® and Rebif® group are shown in Figure 4.3 and 4.4. The incidence of BAb positivity was significantly higher in Betaseron® treated patients (72/119, 60.5%) than in Rebif® treated patients (40/131, 30.8%) ($P<0.001$) in year 3. However, the incidence of NAb positivity did not differ significantly between Betaseron® (18/119, 15.1%) and Rebif® (16/131 12.2%) treated patients ($p=0.31$). Three (25%) out of 12 Avonex® treated patient were BAb+, none of them were NAb+.

4.3.5 Clinical Effects of BAbs

Demographic features of the patients, pre- and post-treatment relapse and disability values did

not differ between BAb+ and BAb – patients (data not shown).

4.3.6 Patient Demographics and Baseline Clinical Characteristics

Age, age at onset, sex, mean duration of disease before therapy, mean duration of therapy, mean time from treatment initiation to time of sampling, pre-treatment relapse rate and pre-treatment EDSS scores did not differ between NAb+ and NAb– groups, either when considering all treatment groups or when comparing Betaseron® to Rebif®-treated patients (Table 4.2).

4.3.7 Effect of NAbs on Clinical Efficacy

Relapse rates in NAb+ and NAb– patients are shown in Table 4.2 and illustrated in Figure 4.5. Relapse rate were not statistically different between NAb+ and NAb– patients in year 1, and 2 and some tendency to better response was, again in this study, found in the patients destined to become NAb+. In Years 3 and 4, however, NAb+ patients had significantly more relapses than NAb– patients ($p=0.015$ and $p=0.027$ respectively). Similarly the proportion of patients with relapses did not differ in year 1 (84/228 in NAb– versus 9/34 NAb+ $P=0.22$) and year 2 (75/228 in NAb– versus 9/34 in NAb+, $P=0.51$), but was higher in NAb+ patients than NAb– patients in year 3 (59/228 in NAb– versus 16/34 in NAb+, $p=0.007$) and year 4 (35/127 in NAb– versus 10/25 in NAb+, $p=0.044$). In year 5 and in years 6-10, average relapse rate did not differ between NAb+ and NAb– groups, but the smaller numbers reduced the power of the analysis.

The relapse rates in NAb+ and NAb– patients in Betaseron® and Rebif® subgroups were analyzed separately. Relapse rates in NAb+ and NAb– Betaseron® treated patients are shown in Table 4.2 and illustrated in Figure 4.6. Although the differences did not reach statistical significance, NAb+ Betaseron® patients tended to have more relapses in year 3 than NAb–

Betaseron® treated patients (p=0.056). Concomitantly, during year 3, more NAb– patients were relapse-free compared to NAb+ patients, with the difference tending toward significance (p=0.065). Relapse rates were comparable for NAb + and NAb – Betaseron® groups in year 4, year 5 and year 6-10.

Relapse rates in NAb+ and NAb– Rebif® treated patients are shown in Table 4.2 and illustrated in Figure 4.7. In the Rebif®- treated group, NAb+ patients had more relapses than NAb– groups in year 3, (with a trend toward significance (p=0.074) and year 4 when it was highly significant (p=0.009). Again, a greater proportion of NAb– patients were relapse-free than NAb+ patients during year 3 (P=0.033, Fischer χ^2) and year 4 (with a trend toward significance, p= 0.062 Fischer χ^2).

As shown in Table 4.2, other statistics related to relapses did not differ between NAb+ and NAb– patients either in the Rebif® or Betaseron® groups.

4.3.8 Sustained Disability Progression

Pre-treatment EDSS scores were comparable in NAb+ (median of 2.4) and NAb– patients (median of 2.6). Kaplan-Meier analyses showed comparable time to sustained progression of one point or more on EDSS scores in NAb+ and NAb– patients (log rank, 0.67, p=0.4) (Figure 4.8)

4.4 Discussion

Our study population is not different from the overall population of patients attended in our UBC MS. In British Columbia only physicians working in an MS clinic are permitted to prescribe disease-modifying drugs (e.g. interferons and glatiramer acetate), and patients who receive disease-modifying drugs must be seen yearly for prescription renewal. This set of circumstances

provides a unique situation to follow treated patients and the effect of NAbs on their treatment response. In this study, we confirmed earlier results that IFNβ-1a IM (Avonex®) appeared the least immunogenic, with none of 12 patients developing NAbs (too small a number to allow firm conclusions). NAb incidence was 15.7% in SC IFNβ-1b (Betaseron®) and 13.9% of SC IFNβ-1a (Rebif®). Pooled data from patients receiving any one of the three medications alone showed that NAb reduced efficacy of IFNβ in MS patients. NAb+ patients had more relapses than NAb– patients in years 3 and 4. Additionally, the number of relapse-free patients was higher in NAb– patients in years 3 and 4. When the clinical effects of NAbs were analyzed based on treatment subgroups, NAb+ Betaseron®-treated individuals tended to have more relapses in year 3 than NAb– patients, although not statistically significant. There was no statistically significant effect on year 4. In the Rebif® group, NAb+ patients had significantly more relapses in year 3, with a significantly higher number of relapses in year 4 than in NAb– patients. A similar trend was noted in the European secondary progressive MS trial of Interferon Beta-1b (Polman et al., 2003) where NAbs induced a reduction in effect, which was not significant. In contrast, in PRISMS (Francis et al., 2005) using IFNβ-1a, the reduction in clinical effect is clear and significant at both years 3 and 4. Inasmuch as IFNβ-1a is less immunogenic than IFNβ-1b, and antibodies to IFNβ-1b disappear faster than antibodies to IFNβ-1a, we hypothesize that those IFNβ-1b treated patients whose titers became undetectable render the IFNβ-1b group less homogeneous in terms of NAb levels than the IFNβ-1a group, who may be on a slope of increasing titers.

As explained in the Methods section, stratification based on a quantitative ranking of NAbs titers was purposely not used, and instead a grouping of patients as NAb-positive and NAb-negative at the accepted cut-off titer of 20. A number of researchers have reported that even at a neutralizaing titer of 20, the bioavailability of interferon is already compromised (Bertolotto et

al., 2003). Further, we have presented preliminary evidence that the frequency histograms of the titers are greatly different between the 2 types of interferons used in this study: IFNβ-1b treated patients tend to have a high frequency of positive NAbs but with lower titers than IFNβ-1a treated patients (Gibbs and Oger, 2006).

We have also combined the results of the 2 doses of IFNβ-1a treated patients (22 mcg and 44 mcg subcutaneously TIW), because we found no differences between the titers of the two groups and because separating the groups would have reduced the power of the statistical analysis.

Finally, we have eliminated patients who had switched or discontinued drugs, or had been treated less than 2 years in order to concentrate on the longer-term efficacy of treatment and how antibodies would affect it. It should be stressed that these criteria of selection of our population may have resulted in a bias selecting long term sustained NAb-positive patients. Such patients with sustained positivity are known, to have higher titers, and it is this group in which it may be most important to describe the effect of NAbs on the clinical course. For these reasons despite a possible biased selection, we think our population is as representative as can be of MS patients followed for long term (more than 3 years) interferon treatment, and thus brings up results never reported in clinical trials.

Although initial reports (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1995; The PRISMS Study Group and the University of British Columbia, 1998; The PRISMS Study Group and the University of British Columbia, 2001; Vallittu et al., 2002) failed to show negative effects of NAbs on the beneficial effects of IFNβ, strong evidences of the negative impact of NAbs on clinical efficacy of IFNβ has been observed. Pivotal phase III trial of Betaseron® (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996) and the 2-year extension of Rebif® pivotal phase III trial showed negative effect of NAb

on relapse rate and MRI activity. In the phase III trial of Betaseron® (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996), the mean relapse rate during months 18-36 was greater among NAb+ patients in comparison to NAb– patients. Similarly, the 2-year extension study of Rebif® showed significantly higher relapse rate and higher MRI activity in year 3 and year 4 in NAb+ patients compared to NAb– patients. Further, two recent studies have also demonstrated diminished clinical efficacy of IFNβ in NAb+ patients (Sorensen et al., 2003; Malucchi et al., 2004). In the Danish study (Sorensen et al., 2003), where patients were stratified by NAb titer, a ceiling effect was noted for titers above a maximum (which might be equivalent to about 100 TRU/ml), lending additional basis for our avoiding a NAb stratification beyond our indicated cut-off.

The effect of NAb status on disability progression is controversial, probably because the effect of IFNβ on disability is still controversial and at best quite limited. In line with our results, no effect of NAb status on disability progression has been found in previous studies (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996; The PRISMS Study Group and the University of British Columbia, 2001). Conversely, Malucchi et al. (Malucchi et al., 2004) reported a higher proportion of NAb+ patients worsening over a 3-year period when compared to NAb- patients. Sorensen et al. (2003) found significant difference in EDSS scores between NAb+ and NAb– patients only at 42 months and at 48 months from treatment start. Although some decreases in NAb titers have occurred over time, the percentage of NAb+ patients was not significantly reduced between year 4, year 5 and years 6-10. These data may support findings by Sorensen, showing that the majority of patients, who had become NAb+ in year 3, remained NAb+ for years. These findings do not contradict our previous results of long term switch from NAb+ to NAb– in individuals followed sequentially (Rice et al., 1999), as in this study the NAb

status was not followed prospectively in individual patients. Previous reports show that NAbs appear 12 to 18 months following the initiation of IFNβ therapy. A recent study showed that patients who have remained NAb– during the first 18-24 months of IFNβ therapy would only rarely develop Nabs (Sorensen et al., 2003; Perini et al., 2004; Sorensen et al., 2005b). The authors suggested that the risk of becoming NAb+ is negligible in patients who have remained NAb– for 24 months. In the light of previous studies, we suspect that our NAb+ patients developed NAbs in the first 2 year of treatment and that they thus continued to receive interferons in the presence of circulating NAbs.

In contrast to earlier suggestions that NAbs are more likely to occur in patients who are either older (Polman et al., 2003), or have higher disease activity (Antonelli et al., 1998) prior to IFNβ therapy, we could not find any clinical features predictive of the development of NAbs. In agreement with our results, Sorensen et al. (2003) showed no correlation between age or disease activity and patients who develop NAbs. In an earlier preliminary report (Oger et al., 1997) we showed that high in-vitro T-cell dependent B cell secretion of IgG was a marker for developing NAb, supporting the possibility that development of NAbs may depend on genetic factors. Although relapse rates in the NAb+ patients at year 3 and 4 were significantly higher than in NAb–, this finding does not exclude a possible residual clinical effect of IFNβs. Since we did not have a placebo group, we cannot evaluate in which proportion the presence of NAbs had abolished the clinical effects of IFNβ. In year 5 and later, we could not find any significant differences in the relapse rate between NAb+ and NAb– groups. However, this observation may be attributable to the small number of patients followed during this time or that the clinical efficacy of IFNβ decreases with time. The clearly decreasing pattern of relapse rates over six years in both IFNβ and placebo groups in previous reports and the natural course of MS (Petkau

et al., 2004) should be taken into account in interpreting our results at year 5 and later. The knowledge that IFNβ reduces the absolute risk of relapse by approximately 30% is based on studies with a duration of 2 to 4 years. Since NAbs do not have any appreciable clinical effect in the first 2 years, lack of efficacy during this interval is probably not related to NAb development, whereas their effects are greater in years 3 and 4, and more evidently so in IFNβ-1a (S/C. 22 or 44 mcg TIW) than in IFNβ-1b (S/C 250 mcg BID) treated MS patients.

In conclusion, our findings further support the negative clinical impact of NAbs at years 3 and 4 in MS patients treated with IFNβ. The rationale for incorporating NAb testing into clinical practice is compelling. How best to integrate this information in clinical practice must await further therapeutic trials.

4.5 Acknowledgement

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	All	Betaseron® group	Rebif® group	Avonex® group	P* value Betaseron® vs Rebif® group
Number of patients	262	119	131	12	
Male/female	72/190	39/80	32/99	1/11	0.93
Age (Year)	46.2±9.3	47.9±8.6	44.9±9.6	42±10.4	0.061
Age at onset of MS	32.7±9.9	34.1± 9.7	31.8±9.8	30±12.3	0.07
Pre-treatment disease duration (years)	8.8±7.3	8.8±6.94	8.7±7.4	8.6±9.8	0.8
Total number of previous relapses	5.3±3.1	5.25±3.24	5.45±3.21	4.3±1.1	0.43
Pre-treatment annual relapse rate	1.18±0.7	1.16±0.73	1.19±0.67	1.37±0.8	0.63
Pre-treatment EDSS	2.94±1.63	3.11±1.57	2.85±1.65	2.3±1.9	0.21
Duration of IFN-β	4.9±1.9	5±1.47	4.91±2.34	3.36±0.6	0.71
Time to sampling (median)	54.8	61.3	49.9	43.6	0.9
(range)	24-174	26-174	24-118	30-62	

Table 4.1. Demographic and clinical characteristic of all patients as a group and of patients sub-grouped according to treatment. (*P values are from χ^2 for sex comparison; Mann Whitney U test for total number of relapse and pre-treatment relapse rate; Student-t test for age, age at onset, duration of disease, duration of IFNβ and pre-treatment EDSS scores).

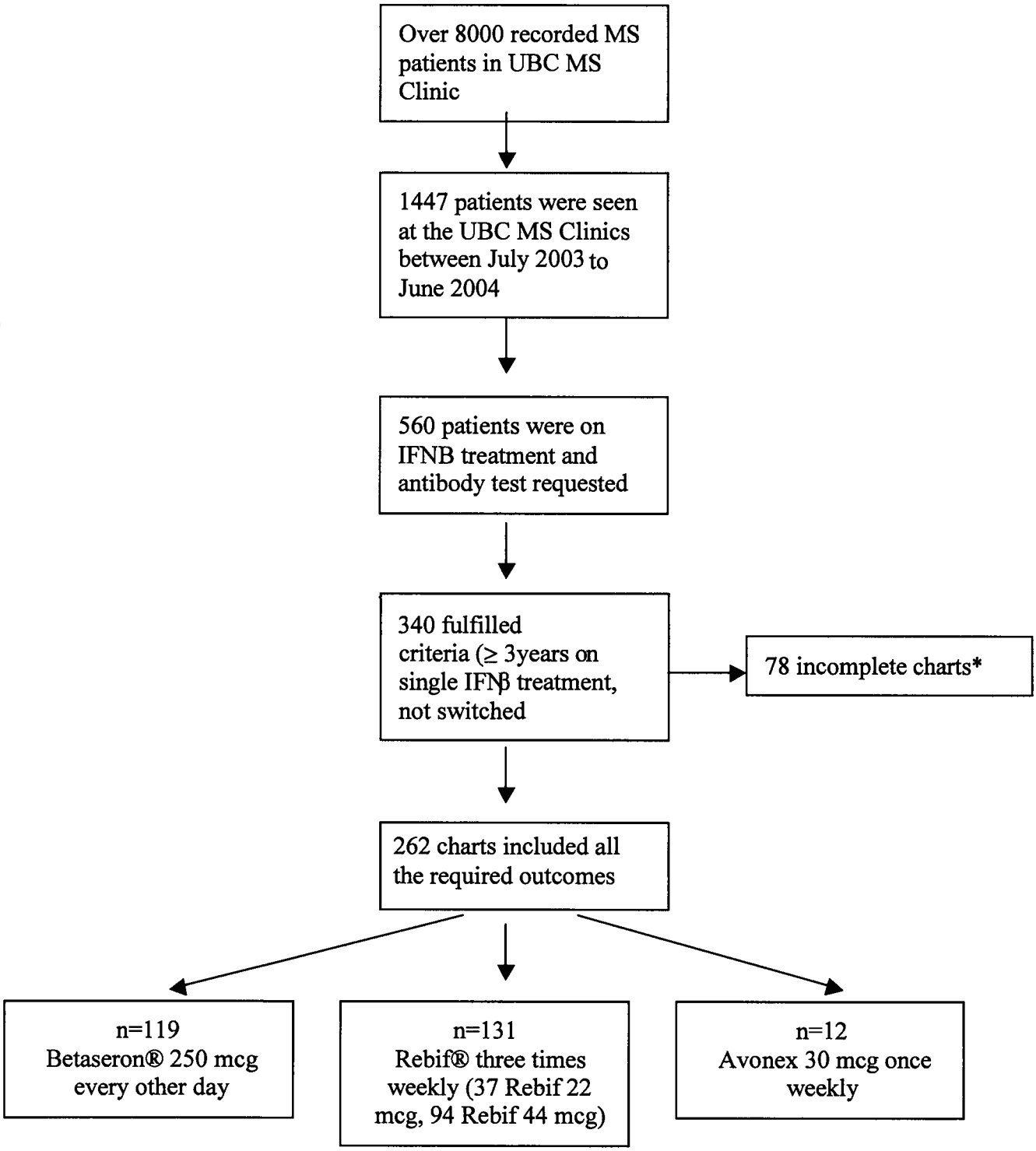
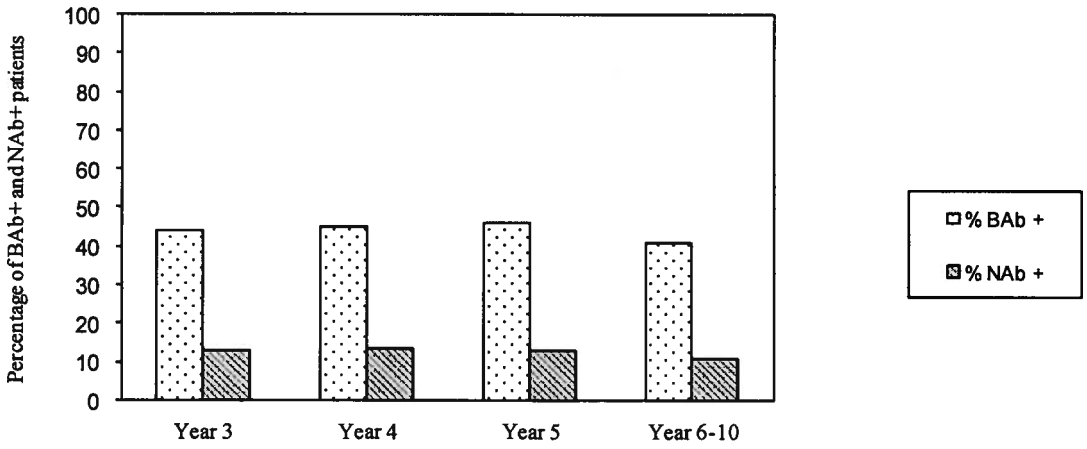


Figure 4.1. Selection of patients from the UBC MS Clinic. *Demographic features of the 78 incomplete charts that were excluded did not differ from those of our study group.



	Year 3	Year 4	Year 5	Year 6-10
BAb+	115	84	58	33
NAb+	34	25	16	9
Total	262	187	125	80

Figure 4.2. Percentage of BAb+ and NAb+ patients in whole group during treatment.

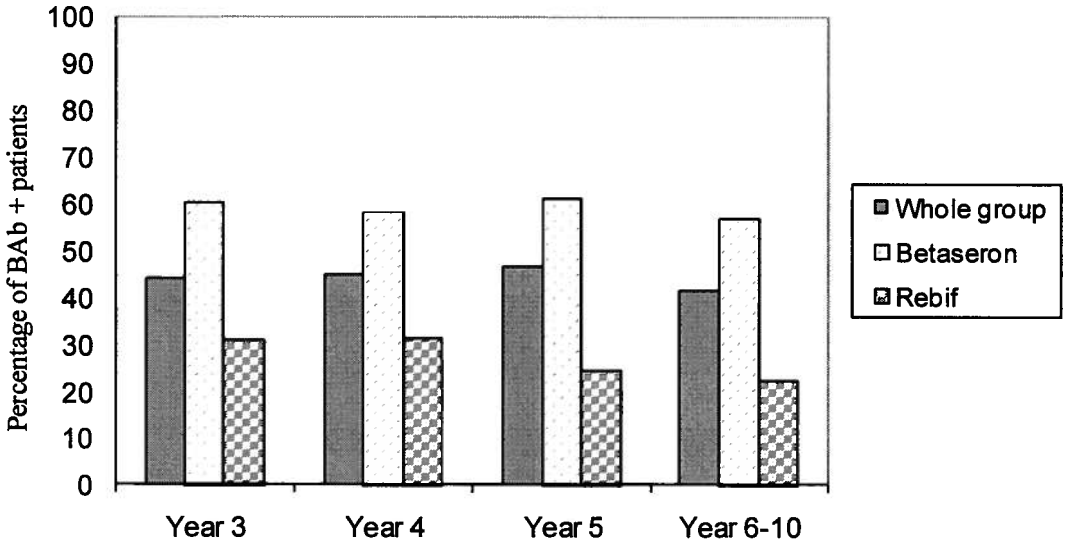


Figure 4.3. Percentage of BAb + patients in whole group, Betaseron® and Rebif® groups.

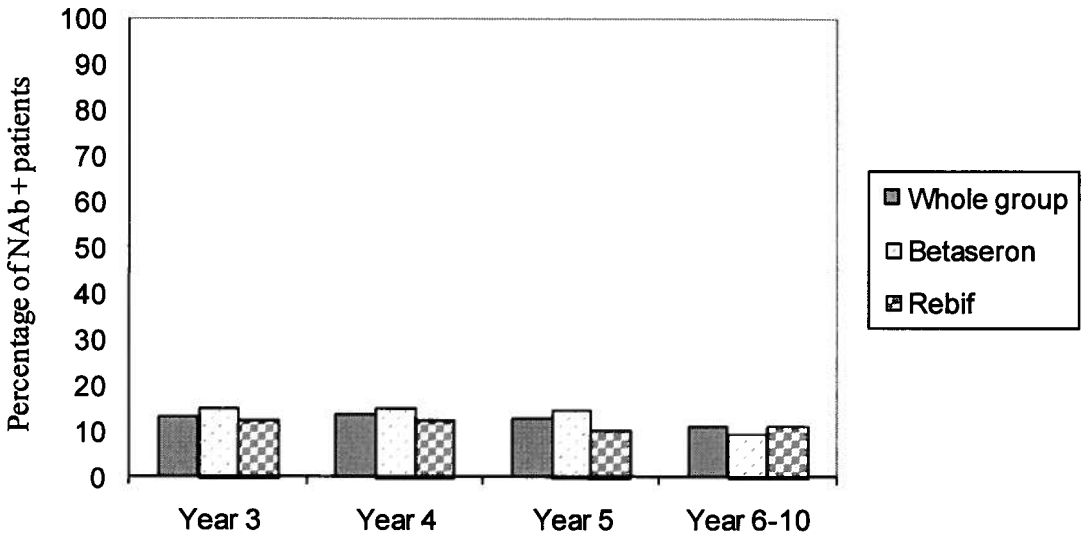


Figure 4.4. Percentage of NAb + patients in all group, Betaseron® and Rebif® groups.

	All group			Betaseron®			Rebif®		
	NAb–	NAb+	P	NAb–	NAb+	P	NAb–	NAb+	P
Male/female	63/165	9/25	0.53	34/67	5/13	0.42	28/87	4/12	0.58
Age	46.1±9.2	46.2±9.9	0.93	47.4±8.9	50.5±5.9	0.16	45.5±9.2	42±11.6	0.14
Age at onset of MS	32.7±9.7	33±11.2	0.81	33.8±9.7	35.8±9.2	0.41	32±9.4	29.8±12.5	0.38
Disease duration	8.8±7.3	8.5±7	0.89	8.6±6.7	9.9±7.9	0.48	8.9±7.6	7.1±5.8	0.33
Duration of drug	4.90±1.97	4.81±1.9	0.78	5.1±1.5	4.7±1.3	0.35	4.9 1±2.33	4.9±2.45	0.9
Time from onset of treatment to sampled	61.7±25.5	57.5±21.2	0.34	62.9±20	57.3±15.9	0.26	62.5±29.9	57.6±28.2	0.54
Pre-treatment EDSS	3±1.7	2.6±1	0.14	3.17±1.62	2.75±1.22	0.32	2.9±1.7	2.4±0.8	0.19
Pre-treatment relapse rate	1.18±0.70	1.19±0.74	0.860	1.16± 0.69	1.17±0.91	0.763	1.18±0.69	1.22±0.52	0.845
Year 1 relapse rate	0.50±0.80	0.41±0.86	0.277	0.43±0.82	0.50±1.04	0.893	0.56±0.78	0.31±0.60	0.196
Year 2 relapse rate	0.52±0.90	0.33±0.60	0.414	0.52±0.91	0.35±0.61	0.622	0.50±0.90	0.31±0.60	0.538
Year 3 relapse rate	0.33±0.63	0.52±0.57	0.015	0.25±0.57	0.47±0.62	0.056	0.38±0.67	0.56±0.51	0.074
Year 4 relapse rate	0.27±0.57	0.56±0.77	0.027	0.26±0.61	0.36±0.63	0.430	0.24±0.49	0.82±0.87	0.009
Year 5 relapse rate	0.37±0.68	0.19±0.54	0.209	0.36±0.70	0.27±0.65	0.567	0.39±0.65	0.00±0.00	0.165
Year 6-10 average relapse rate	0.27±0.48	0.33±0.37	0.263	0.26±0.48	0.29±0.34	0.594	0.29±0.48	0.36±0.44	0.472

Table 4.2. Comparison of demographics and clinical characteristics between NAb+ and NAb– patients in all groups and in the different treatment subgroups.

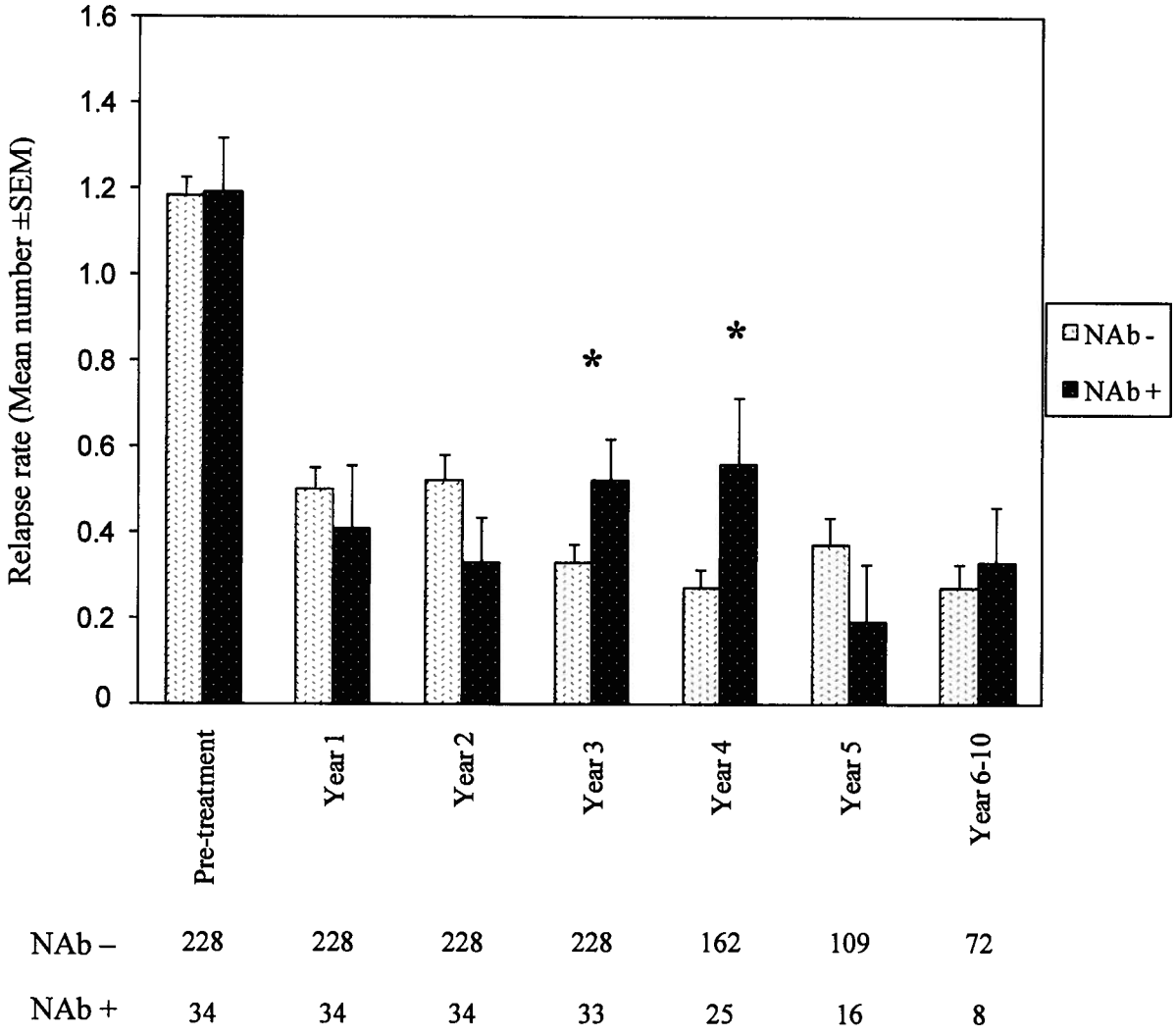


Figure 4.5. Relapse rates (mean ±SEM) over time in NAb + and NAb– groups.

* indicate statistically significant difference between NAb+ and NAb– groups.

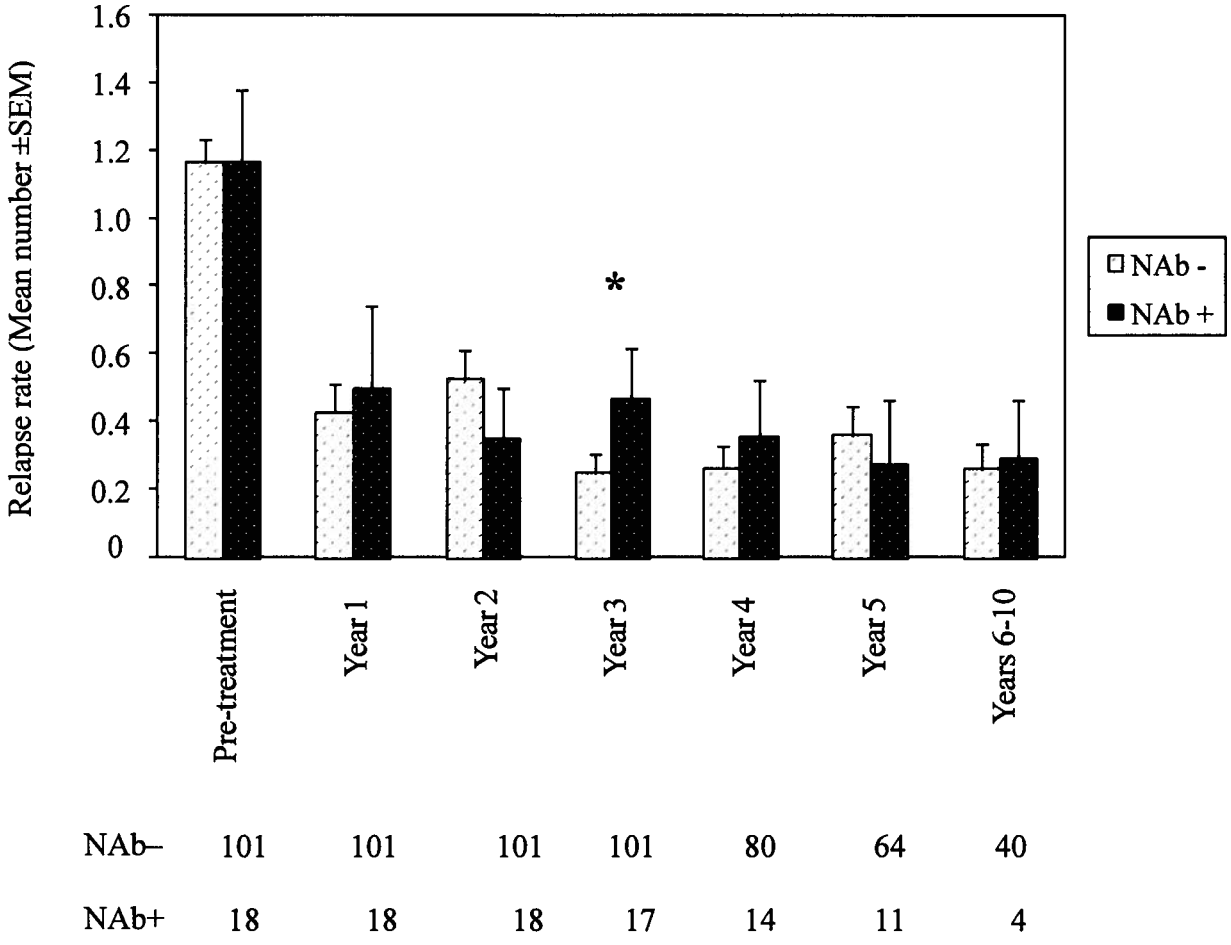


Figure 4.6. Relapse rates (Mean+SEM) in NAb + and NAb– Betaseron® patients.

* indicate that differences between NAb + and NAb- groups show a trend toward significance, p=0.056.

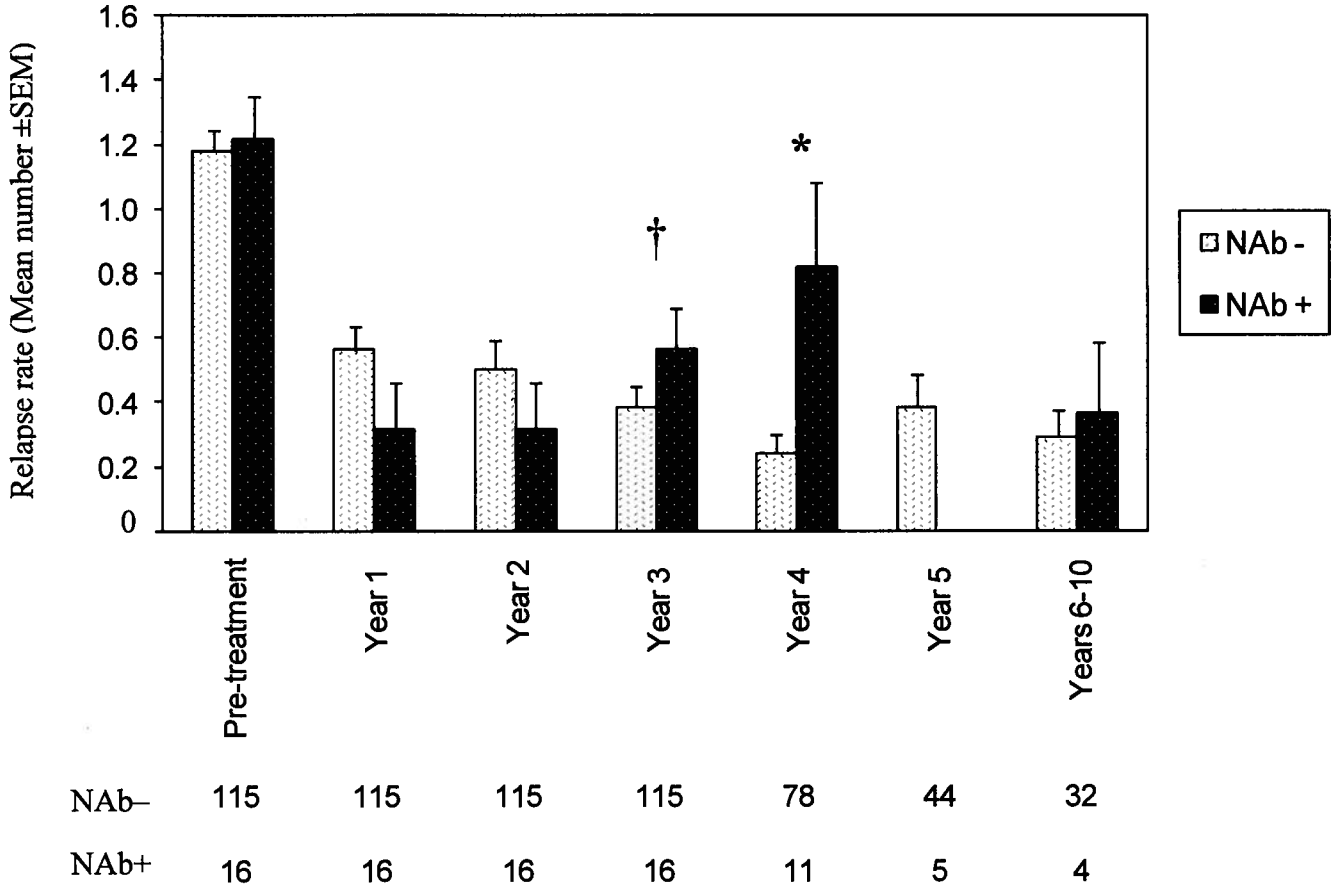


Figure 4.7. Relapse rates (Mean+SEM) in NAb+ and NAb– Rebif® treated patients.

† indicate that differences between NAb+ and NAb– groups show a trend toward significance, p=0.074

* indicate statistically significant difference between NAb+ and NAb– groups.

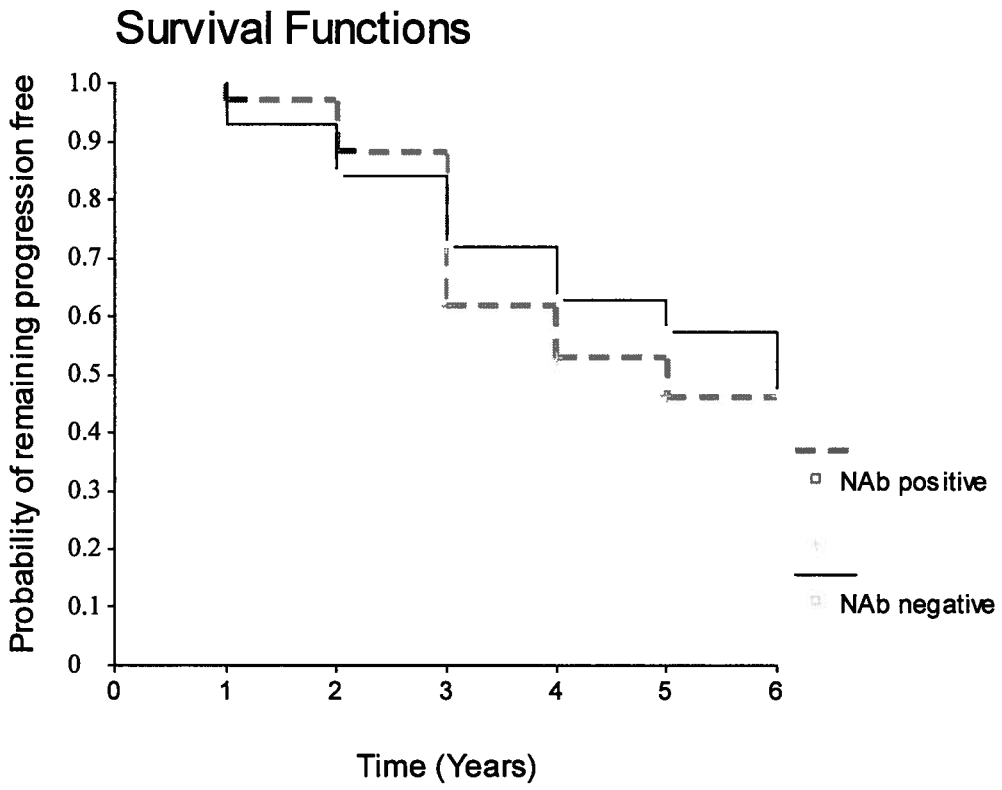


Figure 4.8. Kaplan-Meier analyses of time to sustained progression in NAb + and NAb– patients. No significant differences are noted between NAb+ and NAb– patients.

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Chapter 5

General Discussions and Conclusions

5.1 Discussion

Presently, no single encompassing method exists that can simultaneously detect and characterize the anti-IFNβ antibodies in treated MS patients. BAbs can be detected by immunoassays and further subtyped into IgG subclasses by a modified ELISA. However, in order to detect and quantify those antibodies that can neutralize IFNβ (NAbs), cell-based bioassays such as the CPE, MxA inhibition and luciferase reporter gene assays are employed. These assays, albeit very informative, cannot be routinely used for the subclassification of anti-IFNβ antibodies as this would require the separation of individual serum samples into their representative IgG subclasses and then assaying each IgG fraction for NAbs. This is not practical as it would be intensely laborious and cumbersome.

Another method for the characterization of these antibodies is to measure their effect on IFNβ bioactivity, by way of IFNβ-inducible genes. MxA induction, using both *in vitro* and *ex vivo* methods is reportedly the most specific and sensitive known biomarker for IFNβ (von Wussow et al., 1990; Deisenhammer et.al., 2000; Bertollotto et.al., 2003; Pachner et. al., 2005; Capra et.al., 2007). However, the surrogacy of MxA is not complete as its effect on IFNβ treatment efficacy and on MS disease pathogenesis is unknown. In the same vein, the effect of IFNβ on MS disease is also not clear even though it has been repeatedly demonstrated that IFNβ is effective in reducing relapse rates in about 30% of treated MS patients (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1995; Jacobs et al., 1996; The PRISMS Study Group and the University of British Columbia, 1998). Another confounding factor is the heterogeneity of the disease course of MS as well as inherent genetic differences between individual patients. Taken together, a meaningful strategy to analyze anti-IFNβ antibodies should include a whole battery of different methods.

Our study contributes to the ongoing characterization of anti-IFNβ antibodies by providing an insight into how IgG subclass-specificities of BAbs change over time and how the affinity of the immune response evolves (affinity maturation), using serial serum samples of IFNβ-treated MS patients. We also evaluated the clinical effects of these antibodies by a cross-sectional analysis of relapse rates in a larger population of patients.

In Chapter 2, our results showed that the IgG subclass-specificities of BAbs do not remain static but change over time. This pattern is clearly manifested in SC IFNβ-1b-treated patients were IgG1 and IgG3 are the predominant IgG subclasses during the initial phase of the immune response but whose levels gradually decline. Following 18 to 24 months of IFNβ treatment, IgG4 subclass-specific BAbs are increasingly expressed and become the prominent subclass in the latter phase of the immune response. One could speculate that the prevailing cytokine milieu influences the differential production of these IgG subclass-specific BAbs, as pro-inflammatory cytokines such as TNFα and IFNγ are known to induce IgG1 and IgG3 production, whilst anti-inflammatory cytokines like IL-4 and IL-10 induce IgG4 production. (Snapper and Mond, 1993; Sutherland et al., 1993; Kawano et al., 1995; Abbas et al., 1996). Interestingly, IFNβ is potently anti-inflammatory as it inhibits production of pro-inflammatory cytokines and promotes the production of anti-inflammatory cytokines. Thus by following the course of the IgG subclasses of BAbs, it can be deduced that proximally proinflammatory cytokines are the dominant cytokines but distally anti-inflammatory cytokines are prominently expressed, as IFNβ treatment continues. This shift from pro-inflammatory Th1 to anti-inflammatory Th-2 mirrors the shift from IgG1 and IgG3 to IgG4 subclass-specific BAbs.

Our results also demonstrate that the pattern of the IgG subclass specificities of BAbs is a

function of the type of IFNβ administered to MS patients. In contrast to SC IFNβ-1b, patients treated with SC IFNβ-1a expressed minimal levels of IgG3. IgG3 has a very high affinity for the Fcγ RII receptor which is very potent in downregulating antibody production by the phenomenon of Fc-mediated negative feedback inhibition. Coligation of the activating B cell receptor with the Fcγ RII receptor by antigen-antibody immune complex, results in the phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) on the cytoplasmic domain of Fcγ RII. Phosphorylated ITIM becomes a docking site for the inositol 5-phosphatase SHIP which hydrolyses B cell membrane-produced 3,4,5-triphosphate (PIP3). Normally, PIP3 interacts with the pleckstrin homology (PH)- containing domains of phospholipase C (PLC) and B cell tyrosine kinase (Btk) both of which are required for B cell receptor-mediated sinalling. Thus engagement of Fcγ RII abrogates B cell signaling and ultimately antibody production (Leibson, 2004). We reason that this feedback inhibition could be the underlying mechanism for the gradual disappearance of anti-IFNβ antibodies in SC IFNβ-1b-treated patients, and the lack of this inhibition leads to the persistence of antibodies in SC IFNβ-1a-treated patients, consistent with previous studies (Sorensen et al., 2005b).

Following the antibody dissociation rates of serial samples reveals that the relative antibody affinities differ between patients who are NAb- and those who are NAb+ are different. Generally, for both group of patients, the relative affinities where similar during the first 18 months, but diverged thereafter. The relative antibody affinity of NAb- patients did not change after month 18, whilst it increased in NAb+ patients. Antibody quality thus improved only among the NAb+ patients during the course of the immune response, and though not dramatic, this maturation of affinity distinguishes NAb+ from NAb- patients. As IFNβ is a

protein antigen, B cells require help from CD4 Th cells to differentiate into antibody secreting cells, plasmablasts and plasma cells which initially produce low affinity germ line antibody of the IgM and IgG isotype. The low affinity of these antibodies is ascribed mainly to VDJ gene segment rearrangements and to heavy / light chain pairings. As the immune response proceeds and matures, antibodies of higher affinity are produced by the process of somatic hypermutation (Song et al., 1998). Thus it is probable that in NAb- patients, B cells do not undergo somatic hypermutation and the affinity maturation is blocked. Conversely, affinity maturation in NAb+ patients indicates the presence of somatically hypermutated B cells.

We also observed a parallelism between NAb levels and relative antibody affinity, as both parameters tended to increase as the immune response proceeded. Thus NAbs are temporally, if not functionally linked to affinity maturation. Interestingly, whereas relative antibody affinity stabilized, the NAb levels began to decline after 5 years of IFNβ treatment. We postulate that this is due to a decline in anti-IFNβ-specific plasma cells and a maintenance of memory B cells, as the immune response evolves. Even though we observed a maturation of the immune response in NAb+ patients, the antibody dissociation rates were generally low (10E-2 to 10E-4) when compared to the typical dissociation rates of antigen-antibody reactions of 10E-9 to 10E-11. Thus our study is in strong agreement with the constant proportion hypothesis of antibody neutralization, which states that NAbs as measured by the Kawade formula, reduce the ratio of added IFN/residual active IFN molecules and are of low affinity (Grossberg et al., 2001a; Grossberg et al., 2001b). A possible explanation for this could be a ceiling effect for affinity maturation in which dissociation rates do not exceed 10E-4; antibody molecules with dissociation rates slower than 10E-2 would not be selected

by the immune system.

Our studies on IgG subclasses and on affinity maturation did not include any clinical correlates, the sole reason being that as the clinical benefits of IFNβ are only marginal, a larger population of patients is required to demonstrate the impact of these antibodies. Hence in Chapter 4, we used a cohort of 262 patients that are representative of the British Columbia MS population to correlate the effects of NAbs on IFNβ clinical efficacy, namely reduction in relapse rates. Previously, clinical trials have shown that NAb+ patients generally have higher or significantly higher relapse rates than NAb- patients between months 18 to 36 in SC IFNβ-1b-treated (The IFNb Multiple Sclerosis Group and the University of British Columbia, 1996), and between month 36 to 48 among SC IFNβ-1a-treated patients (The PRISMS Study Group and the University of British Columbia, 2001). Our study is the only post-marketing study in North America and confirms these previous findings. We show that among SC IFNβ-1b-treated patients, NAb+ patients had higher relapse rates than NAb- patients at year 3. Similarly relapse rates were higher among the SC IFNβ-1a-treated NAb- patients compared to NAb- patients. However whereas there was no difference in relapse rates at year 4 among SC IFNβ-1b-treated patients, NAb+ had significantly higher relapse rates than NAb- patients during this time among the SC IFNβ1a-treated patients. Thus the effects of NAbs were observable for a shorter period of time among SC IFNβ-1b treated patients compared to a longer period among SC IFNβ-1a-treated patients. This may be attributable to the faster disappearance of NAbs among SC IFNβ-1b- treated patients than among SC IFNβ-1a-treated patients. As aforementioned, the difference in NAb persistence between the 2 groups of patients may be due to IgG3 antibody feedback inhibition occurring in SC IFNβ-1b-treated patients and the absence of such a mechanism in SC IFNβ-1a-treated

patients. Another possibility could be that anti-IFNβ-specific plasma cells persist more in IFNβ-1a-treated patients than in IFNβ-1b-treated patients, as the half-life of serum antibodies is less than 3 weeks and a continuous production and secretion of anti-IFNβ antibodies would be required to maintain their levels (Manz et al., 2005). Furthermore, the long-lived plasma cells which reside in the bone marrow require survival signals to protect them from apoptosis. Stromal cells in the bone marrow provide such signals, which include the cytokines IL-6, TNFa, APRIL and Blys/BAFF, the chemokine CXCL12, the transcription factor XBP1 and the transcriptional repressor PRDM1 (BLIMP-1) (Schiemann et al., 2001; O'Connor et al., 2004; Ravetch and Nussenzweig, 2007).

5.2 Conclusions

In summary, our studies demonstrate the dynamic temporal profile of IgG subclass-specific BAbs, with a notable expression of IgG4 during the later phase of the immune response. These antibodies mature over time into NAbs that are correlated with a decrease in the therapeutic efficacy of IFNβ. Future investigations are needed to ascertain the predictive value of IgG subclass-specific BAbs for the subsequent development of NAbs, and for the practicing neurologist and his or her patient, the clinically relevant levels of NAbs. It would also be imperative to analyze the underlying mechanism behind a faster seroreversion from NAb+ to NAb- in IFNβ-1b treated patients as compared to IFNβ-1a-treated patients. We reason this would also provide an insight on why there are time differences in the effects of NAbs on relapse rates between the 2 IFNβ products as observed in our studies. Our work highlights the need for a comprehensive strategy that would include various methods to fully characterize anti-IFNβ antibodies, and to elucidate the exact role of these characteristics

within the totality of the immune response in IFNβ-treated MS patients.

5.3 References

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