QUANTIFICATION AND CHARACTERIZATION OF ANTI-INTERFERON BETA ANTIBODIES IN MULTIPLE SCLEROSIS PATIENTS UNDERGOING INTERFERON BETA -1b THERAPY

by

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Experimental Pathology Program

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
OCTOBER 2001

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Date 2 OCTOBER 2001

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ABSTRACT

The increasing recognition of antibody induction in patients undergoing treatment with biologically active recombinant proteins, makes these antibodies of concern. In the case of Relapsing Remitting Multiple Sclerosis (RRMS), administration of interferon beta (IFNβ) -1b can lead to the development of anti-IFNβ antibodies in some patients. These antibodies may attenuate the effects of IFNβ, resulting in resistance to therapy or to disease relapses. On the other hand, anti-IFNβ antibodies may have carrier or stabilizing functions. The purpose of the current study was the quantification of anti-IFNβ antibodies in MS patients' sera, and to further characterize the frequency and serial profile of the antibody response. This was achieved by a sandwich enzyme-linked immunosorbent assay (ELISA) specifically developed for this study.

In the process of developing the sandwich ELISA, an anti-IFN β antibody reference pool was established by screening serum samples from 50 RRMS patients undergoing IFNβ therapy, using a qualitative direct ELISA method. Ten serum samples, identified as anti-IFNβ antibody positive, were selected for the reference pool which was arbitrarily assigned an anti-IFNβ antibody concentration of 100 Laboratory Units / mL. Serial serum samples of 20 patients on IFNβ–1β therapy were assayed using a standard curve generated from the reference pool, and each assay included a set of sera from 5 healthy individuals. The intra-assay coefficient of variation (CV) was 6-10% and inter-assay CV was 3-8%. The validity of the assay was assured through binding specificity, recovery and inhibition tests. Of 20 patients treated for 1-24 months, approximately 16 developed antibodies within the first three months. Two antibody profiles were observed: one profile consisted of a rise and gradual
decline of antibodies to pretreatment levels, and a second profile characterized by a rise which was followed by a decline to lower antibody levels that were maintained over the course of treatment. Additionally, supernatants of cultured lymphocytes were assayed, using a biotin-streptavidin amplified version of the sandwich-ELISA, and results indicate that some patients secrete anti-IFNβ antibodies in vitro.

These findings confirm that IFNβ-1b is immunogenic in MS patients and that the appearance of anti-IFNβ antibodies is an early phenomenon. The sandwich ELISA is a specific, sensitive and reproducible method for the quantification of anti-IFNβ antibodies, and can be easily adapted in any laboratory for the large-scale throughput monitoring of anti-IFNβ antibodies. The assay can also be employed as a tool to study the effects of anti-IFNβ antibodies on the therapeutic efficacy of IFNβ in MS patients, and the mode of action of IFNβ.
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<th>Description</th>
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<tr>
<td>A 490 nm</td>
<td>Absorbance at 490 nanometers.</td>
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<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity.</td>
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<td>APC</td>
<td>Antigen presenting cell.</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate.</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier.</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin.</td>
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<tr>
<td>CD</td>
<td>Cluster of designation.</td>
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<tr>
<td>CNS</td>
<td>Central nervous system.</td>
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<td>CSF</td>
<td>Cerebrospinal fluid.</td>
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<tr>
<td>EAE</td>
<td>Experimental allergic encephalomyelitis.</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay.</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum.</td>
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<tr>
<td>Gd-DTPA</td>
<td>Gadolilium Diethylene triamine penta-acetic acid.</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte colony stimulating factor</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid.</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen.</td>
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<tr>
<td>HRP</td>
<td>Horse radish peroxidase.</td>
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<tr>
<td>HSA</td>
<td>Human serum albumin.</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1.</td>
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<tr>
<td>IFN</td>
<td>Interferon.</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G subtype.</td>
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<tr>
<td>IL</td>
<td>Interleukin.</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>LFA-1</td>
<td>Leukocyte function-associated antigen-1.</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody.</td>
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<tr>
<td>MBP</td>
<td>Myelin basic protein.</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram.</td>
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<tr>
<td>mg</td>
<td>Milligram.</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex.</td>
</tr>
<tr>
<td>mIU</td>
<td>Million international units.</td>
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<tr>
<td>mL</td>
<td>Millilitre.</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase.</td>
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<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein.</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging.</td>
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<tr>
<td>MRNA</td>
<td>Messenger ribonucleic acid.</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis.</td>
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<tr>
<td>MxA</td>
<td>Myxovirus A</td>
</tr>
<tr>
<td>NAB</td>
<td>Neutralizing antibody.</td>
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<tr>
<td>ng</td>
<td>Nannogram.</td>
</tr>
<tr>
<td>NHPS</td>
<td>Normal human pooled serum.</td>
</tr>
<tr>
<td>NIAID</td>
<td>National Institute of Allergy and Infectious Diseases</td>
</tr>
<tr>
<td>OAS</td>
<td>Oligo-A synthetase.</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density.</td>
</tr>
<tr>
<td>OPD</td>
<td>Orthophenylenediamine dihydrochloride.</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell.</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline.</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PLP</td>
<td>Proteolipid protein.</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-Nitrophenyl phosphate.</td>
</tr>
<tr>
<td>PPMS</td>
<td>Primary progressive multiple sclerosis.</td>
</tr>
<tr>
<td>RRMS</td>
<td>Relapsing remitting multiple sclerosis.</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation.</td>
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<tr>
<td>SPMS</td>
<td>Secondary progressive multiple sclerosis.</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor.</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper-1 subset.</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper-2 subset.</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor.</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1.</td>
</tr>
<tr>
<td>VEP</td>
<td>Visual-evoked potential.</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4.</td>
</tr>
<tr>
<td>VH &amp; HSC</td>
<td>Vancouver Hospital and Health Sciences Center.</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia.</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization.</td>
</tr>
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Chapter 1

INTRODUCTION

1.1 Multiple Sclerosis: The disease and its manifestations

Multiple Sclerosis (MS) was first described about 160 years ago. More than 100 years has elapsed since its clinical and pathological features were characterized by Charcot, Carswell, Cruveilhier and others. MS is a chronic inflammatory disease of the central nervous system (CNS) and is the commonest cause of neurological disability in young adults of northern European origin (1). It is characterized by discrete foci of demyelinating lesions scattered throughout the CNS, with the peripheral nervous system typically being spared. Myelin is the fatty covering that insulates nerve cell fibers in the brain and spinal cord, and facilitates the smooth high-speed transmission of electrochemical signals between the CNS and the rest of the body. Loss of myelin results in the slowing or complete blockade of neurological transmission, leading to diminished or lost function. Remyelination occurs in the CNS, and contributes to clinical recovery following attacks, but it is not known if failure of this mechanism is the sole reason for permanent disability (2).

Current evidence supports the hypothesis that MS is an autoimmune disease directed at antigens in the CNS white matter, with the putative autoantigens being represented by myelin components such as myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) (3,4). The characteristic sclerotic plaques in MS consist of inflammation around axons, axonal loss, demyelination and gliosis. This is accompanied by an infiltration of macrophages and lymphocytes, typically CD4+ T-helper cells and CD8+ T-suppressor cells. It is believed that CD4+ T-
helper cells are activated outside the CNS by antigen presenting cells (APCs) which present myelin and other cross-reactive microbial antigens in the context of human leukocyte antigen (HLA) ClassII. Expression of adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 as well as their receptors is upregulated, and this allows activated T cells to adhere and extravasate across the blood-brain barrier. Matrix metalloproteinases (MMP) are also involved in the penetration of the blood brain barrier as they digest the extracellular matrix composed partly of type IV collagen (5). Once in the CNS, T cells are further activated by macrophages, microglia and astrocytes. Demyelination ensues by an inflammatory process that is consistent with a delayed-type hypersensitivity reaction: macrophages in close contact with disintegrating myelin sheath and containing intracytoplasmic, myelin breakdown products. The high numbers of myelin antigen-reactive T cells produce and express cytokines such as interferon (IFN)γ and tumor necrosis factor (TNF)α which are pro-inflammatory, and anti-inflammatory cytokines like interleukin (IL)10 and transforming growth factor (TGF)β.

The random occurrence of MS lesions results in a wide range of clinical features, including optic neuritis, sensory impairment, ataxia, tremor, nystagmus, cognitive abnormalities, fatigue, spasticity, and bowel, bladder and sexual dysfunctions. Symptoms also vary from patient to patient, and from time to time. Classification of MS is done according to the course of the disease. Typically, MS follows a relapsing-remitting course that manifests as a series of attacks followed by complete or partial remissions of symptoms. Some patients with relapsing-remitting MS (RRMS) eventually develop secondary progressive MS (SPMS) where the disease becomes chronic and progressive.
with or without occasional relief by remissions, and with a steady worsening of symptoms. In about 10% of MS patients, the disease follows a chronic progressive form from the onset: primary progressive MS (PPMS), were there are no remissions and patients never improve clinically (4,6).

MS affects about 2.0 million people world-wide. Onset of the disease is usually between the ages of 20 to 40, with women being twice as likely than men to develop the disease. Analysis of the global pattern of MS shows a north-south gradient in North America, Europe, New Zealand and Australia, with a lower prevalence in Africa, Asia and South America. This non-random pattern may provide a clue as to the etiology of the disease, whose cause is still unknown. A number of risk factors have been linked to MS, including age, race, sex, geographical location, weather, ethnicity, family status, socioeconomic status, migration, diet and HLA haplotype. It is now believed that the disease is a consequence of an interplay between environmental factors in genetically susceptible individuals (7,8).

Diagnosis of MS is primarily clinical and involves the demonstration of a minimum of two separate sites of CNS damage in a patient with at least two episodes of neurological dysfunction characteristic of MS. However, in patients where the disease is in its early course, investigational techniques of CNS structure, function and immunological status are required (6). These include visual-evoked potentials (VEP) which assesses sensory function, magnetic resonance imaging (MRI) that provides confirmatory evidence for diagnosis, and immunological abnormalities in the cerebrospinal fluid (CSF). Isoelectric focusing in CSF protein electrophoresis shows IgG oligoclonal banding in 95% of patients with clinically definite MS (9). But with the
introduction of MRI, CSF studies are less often used except in complex cases were evidence of immunological abnormality in relation to the CNS is helpful.

The working consensus that MS is an autoimmune disease mediated by immune reaction against CNS antigens, stems largely from its resemblance to experimental allergic encephalomyelitis (EAE) which is the most intensively studied experimentally induced animal model of MS. 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 TNFα, and adhesion molecules and MMPs (10,11). EAE was initially observed after repeated injections of monkeys with spinal cord material (12). Later Kabat suggested an autoimmune etiology and that the autoantigen was in the CNS white matter (13). EAE can also be induced in susceptible strains of mice, rats, rabbits and guinea pigs by immunization with whole spinal cord, MBP, PLP and MOG, or by experimental infection with neurotropic viruses such as SemLiki forest (14) and Theiler's viruses (15). Another EAE animal model involves the adoptive transfer of MBP-specific CD4+ T-helper cells to naïve recipient (16,17).

1.2 Immunoology of multiple sclerosis

The CNS has traditionally been considered as an immunologically privileged organ, excluded from immune cells and mediators, and with limited immune responses to infectious agents and tissue injury (18). Over the past decade though, experimental evidence has shown that the immunoprivileged status of the CNS is conditional: the blood brain barrier (BBB) is not an absolute barrier as immune cells are found in the CNS.
under normal conditions, albeit in very low numbers (19). Under pathological conditions such as MS, the CNS can be crossed by activated lymphocytes. It becomes an inflammatory milieu with a bidirectional communication between the invading immune and resident cells, the most abundant of which are microglial cells (20). Through a combination of chemotactic cytokines (chemokines) and cell adhesion molecules displayed on the luminal surface of brain microvascular endothelial cells, activated T-cells with their corresponding adhesion molecules bind to the BBB and extravasate into the CNS. The activated T-cells survey the CNS for their target antigens being presented by antigen presenting cells such as macrophages. T-cells further release cytokines which attract more T-cells, B-cells and macrophages, the latter being the final vectors of demyelination. Once the inflammatory process has begun, the BBB opens up and more immune cells, immunoglobulins, complement and coagulation factors gain access into the CNS. This breakdown of the BBB can be assessed in MS plaques using gadolium-diethylenetriamine penta-acetic acid (Gd-DTPA) enhanced MRI: Gd-DTPA is a large molecule that is usually excluded from the CNS by the BBB and can only enter when the BBB is disrupted (21). Despite BBB breakdown, the lymphocyte traffic in the CNS remains more selective than in other organs, as only activated lymphocytes can get through and these are a minority of the population of circulating immune cells.

1.2.1 Cerebrospinal fluid abnormalities

Since the seminal finding of Kabat and colleagues in 1948 of elevated levels of immunoglobulins in cerebrospinal fluid (CSF), it has become increasing recognized as a powerful diagnostic tool. CSF is routinely sampled by lumbar puncture and provides
supplemental information as well as clues to the pathogenesis of MS. Normally CSF contains small amounts of immunoglobulins and plasma proteins. But in 80-90% of MS patients, the chronic inflammatory process results in pleocytosis, and intrathecal IgG synthesis (22). The elevated IgG levels have restricted heterogeneity and under electrophoresis, migrate in discrete bands called oligoclonal bands (23,24). However, elevation of CSF-IgG may also arise as a result of a defective BBB that can be distinguished from intrathecal IgG synthesis by comparing CSF-IgG levels with CSF albumin levels: albumin is used as a permeability index as it can only gain access into the CSF as a result of a leaky BBB (18).

Oligoclonal bands are not specific to MS and can be detected in other immunological and infectious diseases such as subacute sclerosing panencephalitis, acute idiopathic polyneuritis, neurosyphilis, acute aseptic meningitis and rubella panencephalitis. Analysis of the bands reveals that CSF-IgG in MS patients contains anti-viral IgG, usually directed against measles, herpes zoster, cytomegalovirus and rubella (25). However, the search for the antigenic specificities of oligoclonal IgG remains largely unsuccessful, as the patterns of specificities are varied and inconsistent. Furthermore, the affinities of these virus-specific antibodies are low (26).

1.2.2 Cellular abnormalities

Autoreactive myelin-specific T cells are present in both healthy individuals and MS patients (27). Normally, the immune system has mechanisms that prevent activation of these T cells and subsequent attack of the body. However in MS, the T cells become activated by an as yet unidentified trigger. Evidence from laboratory and brain tissue studies demonstrate that CD4+ T cells are the major initiators of tissue destruction, and
this is further supported by the observation that adoptive transfer of MBP-specific T cells produces EAE in susceptible animals (11,28). T cells are characteristically classified as TH1 cells and TH2 cells which produce proinflammatory and anti-inflammatory cytokines respectively, and as TH0 that is neither pro- nor anti-inflammatory. The intracellular milieu dictates induction of the CD4+ T cell subsets, and in MS, the immune deviation is towards a TH1 proinflammatory response (3).

Another subset of T cells which may play a role in the pathogenesis of MS are the cytotoxic CD8+ T cells. These are found in pathological lesions of MS and EAE, and normally function to downregulate the inflammatory response. However, accumulated data show a weak correlation between CD4/CD8 ratios and disease (18). γ/δ cells which represent another class of T cells 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 in EAE has been shown to reduce disease activity. In vitro, these cells co-localize with oligodendrocytes and lyse them by recognizing and binding to stress-induced heat shock proteins (29,30). The MS plaque also contains B lymphocytes and plasma cells, and as demonstrated in CSF, these cells contain mainly IgG. Their implication in the pathogenesis of MS is substantiated by evidence such as demyelination of cells by culturing in serum. A potential role of B cells in MS includes the production of autoantibodies against myelin which 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 which can lyse oligodendrocytes and microglia (31).
Macrophages are some of the most abundant infiltrating cells in MS lesions and their cytoplasm contains myelin breakdown products. Further evidence for the role of macrophages in MS is that demyelination occurs mainly in the presence of macrophages, and animals devoid of macrophages do not develop EAE (11). Besides scavenging and phagocytosing myelin, macrophages are the major antigen presenting cells in MS lesions, and also secrete neurotoxins like IL-1, IL-6 and TNF-α which damage myelin (32).

1.2.3 Cytokines in MS

Cytokines are soluble factors secreted by immune cells during an antigenic challenge and are involved in immune activation, cell movement, antigenic recognition and effector functions. The role of cytokines in the pathogenesis of multiple sclerosis is becoming better appreciated with the development of novel detection systems with high sensitivities. A growing number of cytokines have been identified in active MS lesions, CSF and blood (33). During relapses, there is a preferential augmentation of pro-inflammatory cytokines like TNF-α, whilst anti-inflammatory cytokines dominate during remissions. 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 (34). 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 (35). 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 HLA class II molecules and therefore increases antigen presentation to
autoreactive T cells and promotes the inflammatory process. However, treatment of MS patients with TNF antibodies resulted in worsening of the disease (36).

Another pro-inflammatory cytokine that plays an important role in MS is interferon gamma (IFN\(\gamma\)), produced by T cells in response to myelin antigens. As with TNF\(\alpha\), IFN\(\gamma\) levels correlate with MS disease activity. IFN\(\gamma\) 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 which is a major mediator of myelin and oligodendrocyte damage, and it further stimulates phagocytosis of myelin fragments by macrophages (37,38). Paradoxically, a clinical trial to ascertain the efficiency of IFN\(\gamma\) in MS was discontinued as IFN\(\gamma\) exacerbated the disease (39).

Proinflammatory cytokines are counteracted by anti-inflammatory cytokines such as IL-4, IL-10 and TGF-\(\beta\), all of which have been shown to decrease or abrogate the symptoms of EAE. In serial studies of peripheral blood mononuclear cells derived from relapsing remitting MS patients, it was found that levels of TGF\(\beta\) and IL-10 mRNA decreased during clinical relapses.

### 1.2.4 Adhesion molecules in MS

The migration of inflammatory cells through the blood BBB into the CNS is an essential step in the pathogenesis of MS. This is dependent upon the interaction of adhesion molecules on resident endothelial cells and receptors on leukocytes. Endothelial cell molecules include intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule (E-Selectin), all of
which are upregulated by proinflammatory cytokines (47). Counter-receptors on leukocytes include very late antigen-4 (VLA-4) which binds VCAM-1, and lymphocyte functions associated antigen-1 (LFA) that binds ICAM-1. Following interactions with their ligands, cellular adhesion molecules are shed from the cell surface and secreted into body fluids. These soluble forms can competitively inhibit cellular interactions and migration. Consequently, quantitation of adhesion molecule levels have been correlated to disease activity and used as surrogate markers for disease progression (34). Compared to controls, MS patients were found to have significantly increased serum levels of VCAM-1 and L-Selectin (48). In another study, serum levels of ICAM-1 and E-Selectin were also increased in MS patients compared to controls (49).

1.2.5 Immunogenetics of MS

Several lines of investigation have shown that genetic factors influence the development of MS. Standard epidemiological studies reveal clustering in families with the risk of developing the disease being greatly increased for relatives of MS patients as compared to the general population (40). The strongest evidence for genetic predisposition comes from twin studies performed in Canada. Sadovnick et al. (41) identified clinical concordance rates of about 30% among monozygotic twins compared to about 5% in dizygotic twins. However, with long-term follow-up of monozygotic twins, only about 28% develop the disease. Despite substantiating data, no single gene has been found necessary and sufficient for disease susceptibility (42). To date, only the MHC Class II genes have been consistently linked to MS. The DR2 haplotype seems to have the strongest association, but this is not complete as approximately 40% of MS patients do
not carry this haplotype and about 20% of the normal population does (43). Moreover, systematic genomic screens in multiplex families from different countries failed to identify any genetic loci that was statistically significant (44,45,46). Thus it appears that MS susceptibility is polygenic with the epistatic interactions of several genes.

1.3 Management of MS

While not medically curable, several effective strategies exist for MS therapy. These include treatment of symptoms, management of acute attacks, prevention or modulation of relapses and prevention of disease progression. The rationale behind current therapeutic approaches depends on the aetiology and pathogenesis of the disease, and on the hypothesis that MS is an organ-specific autoimmune disease.

1.3.1 Management of acute attacks

Attacks in MS consist of the development of new neurological symptoms or worsening of preexisting symptoms that last for 24 hours or longer. Mild attacks usually resolve spontaneously and are best left untreated (50). However, moderate to severe attacks do not resolve spontaneously and interfere with the patients' daily activities. The standard practice for treatment of major attacks is to use corticosteroids which include adrenocorticotropic hormone, prednisone, methylprednisolone and dexamethasone. These drugs have immunomodulatory and anti-inflammatory effects that reduce brain edema, inhibit T<sub>H</sub>1 cells and macrophages, restore BBB integrity and improve neuronal condition. They shorten the duration of the relapse and accelerate recovery, but long-term
use is not recommended as corticosteroids are associated with numerous adverse side effects (51).

1.3.2 Immunosuppressive therapy

The goal of immunosuppressants is to dampen the autoreactive mechanisms in MS, reducing the frequency and severity of relapses and preventing progression of the disease. It is ideal to treat patients during the initial stages of the disease so as to slow the start of the chronic progression and disabling phase. However, immunosuppressive therapy has not gained widespread acceptance because of considerable toxicity, limited efficacy and non-selective nature of the agents (52). Azathioprine suppresses cell-mediated and humoral immunity, and has been shown to reduce relapse rates. But it has no statistically significant effect on disease progression. Methotrexate also inhibits cell mediated and humoral immunities, and appears to be marginally effective in reducing progression of disease (51,52). Other immunosuppressive agents with marginal effects include cyclosporine A, cladribine and cyclophosphamide, an alkylating agent which has potential life-threatening side effects.

1.3.3 Immunomodulatory therapy

The mainstay of therapy in patients with relapsing-remitting multiple sclerosis 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, they have been largely replaced by
immunomodulating agents namely interferonβ and glatiramer acetate. Both classes of agents have been documented to lessen the frequency of MS attacks.

Glatiramer acetate (Copaxone™) is a mixture of random synthetic polypeptides composed of L-alanine, L-glutamate, L-lysine and L-tyrosine. It is an immunochemical mimic of the myelin basic protein and was initially shown to inhibit EAE in various species including the guinea pig, rabbit, mouse, rhesus monkey and baboon (53,54). A small trial of 50 patients which led to the approval of the agent, suggested efficacy in the treatment of RRMS. Subsequently, in a pivotal multicenter trial of 251 patients divided into treatment and placebo groups, Copaxone™ was shown to reduce annual relapse rates by 30% (54). It binds strongly to MHC II and competes with MBP for the binding sites (55). A possible mechanism of action is the preferential induction of T_{H}1 cells that secrete anti-inflammatory cytokines (56). Copaxone™ is generally well tolerated with only mild transient systemic reactions including flushing, chest tightness, palpitations, dyspnea and anxiety. Chronic administration of the drug results in the development of serum anti-Copaxone antibodies, but the presence of these antibodies does not compromise the clinical benefits (57).

IFNs were originally used as antiviral agents in the treatments of MS as the disease was thought to be viral. IFNβ is the first choice of treatment in patients with RRMS and two forms of recombinant IFNβ have been approved by the Food and Drug Administration (FDA) and European regulatory agencies. IFNβ-1a (Rebif®, Avonex®) are glycosylated recombinant products from mammalian cells, with identical amino acid sequences to natural IFNβ, while IFNβ-1b (Betaseron®) is a recombinant non-glycosylated product from E.coli cells in which serine is substituted for cysteine at
position 17 (58). All 3 recombinant products have been studied in large double-blind placebo controlled randomized clinical trials, indicating significant reductions in the frequency and severity of relapses, and decreases in active lesions as shown by MRI (59,60,61).

1.4 IFNβ therapy in MS

1.4.1 The interferons

Isaac and Lindemann first characterized interferons in 1957 when they observed that a soluble factor produced by virus-infected cell cultures could transfer protection against subsequent viral infection to other cells (62). There are three families of interferons, IFNα, IFNβ and IFNγ, identified on the basis of their antigenic, functional and molecular characteristics. IFNα and IFNβ are collectively known as type I IFNs being induced by viruses or polynucleotides and encoded by a common gene locus on chromosome 9. In contrast, IFNγ is a type II IFN, and is induced by mitogens and antigens, and encoded by a gene located on chromosome 12.

The 3 classes of IFN are produced in different cell types, IFNα in leukocytes, IFNβ in fibroblasts and epithelial cells, and IFNγ in T-lymphocytes. The IFNs also differ in their primary amino acid sequence with IFNα and β consisting of 166 amino acids and IFNγ containing only 146. In addition, IFNα exists in multiple forms, while IFNβ and IFNγ exist as unique entities. The biological effects of IFNs are mediated by IFN binding to specific receptors on the cell surface. IFNγ binds to a distinct receptor while both IFNα and IFNβ bind to a common type 1 IFN receptor which consists of at least three subunits and is distributed throughout the body. Binding initiates the Jak-Stat signal
transduction pathway and the eventual synthesis of effector proteins like protein kinase, 2'-5' Oligo-A synthetase (2'-5' OAS), 2'-5'-phosphodiesterase, myxovirus (MX) protein, β₂-microglobulin, TNF receptor, HLA antigens and various other enzymes (63).

The original rationale for IFN 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. Jacobs et al. (64) injected a natural IFNβ intrathecally in MS patients and reported a significant reduction in relapse rates which was observed in subsequent studies. However, trials were limited due to the difficulties in obtaining natural IFNs. With the advent of genetic engineering, large quantities of recombinant IFNs have become available for clinical trials.

1.4.2 Mechanisms of action of IFNβ

1.4.2.1 Antiviral effects

Like other IFNs, IFNβ has anti-viral properties against a host of RNA and DNA viruses. However the action of IFNβ on viruses is complex and there is no single mechanism responsible for impairment of viral growth. IFNβ acts directly by inducing the synthesis of proteins and metabolites which inhibit viral growth at the level of penetration, uncoating, synthesis, methylation of mRNA, protein synthesis and assembly (65). Three enzymes of particular importance are MxA, 2'-5' OAS and a protein kinase. 2'-5' OAS polymerizes ATP into a series of small 2'-5' oligoadenyates which are allosteric activators of an endogenous ribonuclease (RNaseL). RNaseL degrades viral RNA reducing its availability as a template for viral protein translation. E1F-2x protein kinase phosphorylates the smallest subunit of protein synthesis initiation factor e1F-2,
preventing it from recycling between successive rounds of translation and thereby decreasing its efficiency. Human MX proteins are homologues of the murine Mx-1 protein. They reside either in the cytoplasm or nucleus, and by direct interaction with an intact GTP-binding site, they block different steps in viral replication, possibly by inhibiting viral polymerase. Despite the overwhelming evidence for the anti-viral properties of IFNs, none of the IFNβ trials show that IFNβ–treated patients had fewer viral illness as compared to patients receiving placebo (66).

1.4.2.2 Immunomodulation

IFNβ is an immunomodulator, inhibiting certain immune responses and augmenting others. IFNβ antagonizes the stimulatory effects of IFNγ and this may contribute to its therapeutic effects. It suppresses the synthesis of MHC II proteins and subsequently downregulates antigen presentation by macrophages, B cells, astrocytes and microglia (67,68). This IFN-mediated inhibition of MHC II antigens is thought to occur by suppression of class II transactivator, a transcription factor involved in IFNγ-induced MHC II transcription (69).

1.4.2.3 Effects on T cells

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. It also inhibits IFNγ production by T cells, this being particularly significant as IFNγ is a potent inflammatory mediator and activator of disease in MS (70). IFNβ has also been shown to upregulate IL-10 secretion by CD4+ T cells. IL-10 further suppresses
production of \( T_h \) inflammatory cells and skews T cell activation towards a \( T_h2 \) response which downregulates inflammation (71,72).

1.4.2.4 Effects on blood-brain barrier

IFN\( \beta \) is known to have a significant effect in reducing gadolium-enhanced MRI lesions, which are a measure of BBB leakiness. In particular, Stone et al. (73) found that IFN\( \beta \) reduced lesion frequency in 13 of 14 patients. Indeed other investigators have found increased levels of VCAM-1 in the sera of IFN\( \beta \)-treated patients and these levels correlated with reductions in MRI lesions (74). Down-regulation of LFA-1, the ligand for VCAM-1, was concomitantly observed in the lymphocytes of these same patients. Thus a possible mechanism of IFN\( \beta \) interference with T cell adhesion to the BBB is the release of soluble adhesion molecules that bind to and block T cells (75). Another point of interference with T cell migration into the CNS is the inhibition of matrix metalloproteinases (MMP) by IFN\( \beta \). MMPs degrade the extracellular matrix of the basement membrane which surrounds the endothelium (76,77).

1.5 Anti-IFN\( \beta \) antibodies in MS patients

Over the past decades, it has been documented that natural antibodies or autoantibodies 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 (78,79). However, this occurrence is tremendously increased when self-antigens are administered at therapeutically high doses: recombinant preparations of insulin, erythropoetin, granulocyte-macrophage colony-stimulating factor
(GM-CSF). TNF-α inhibitor, factor VII and interleukin-2 (IL-2) have all been reported to induce antibody responses in a subset of patients undergoing therapy with these agents (80,81,82,83,84,85). IFNs are no exception, and Vallbracht et al. (86) in 1981 were the first to report the development of antibodies against IFN in a nasopharyngeal carcinoma patient treated with human fibroblast IFN. 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 interferons administered exogenously as therapeutic agents in several neoplastic and infectious diseases elicit antibodies in patients (87). In the past, such antibodies have been associated with reduced biological activity and loss of clinical response to IFN in such diseases as melanoma, chronic myelogenous leukemia, hairy-cell leukemia, carcinoid tumors and chronic hepatitis C. However, the frequencies of such anti-IFNβ antibodies vary considerably ranging from a total absence to as high as 95% (88, 89, 90, 91, 92).

As seen in other patients, the prolonged clinical use of IFNβ in MS patients can result in the induction of anti-IFNβ antibodies (93, 94, 95, 96, 97, 98, 99, 100). Both neutralizing antibodies (NAB) and binding antibodies have been detected in MS patients sera, and in correlation with MRI studies, it has been demonstrated that these antibodies are associated with a failure of IFNβ therapy. In two such studies, NAB positive patients appeared to benefit less from IFNβ therapy, with higher relapse rates and greater numbers of new or enlarging MRI lesions (93,94). In addition, NABs have been shown to neutralize the biological effects of IFNβ; the presence of NABs correlates with decreasing levels of IFN-induced biological markers such as neopterin, β2-microglobulin.
and MxA (94). Deisenhammer et al. (101) measured MxA blood levels in IFNβ-1b treated patients and concluded that once NAB develops, the bioavailability of the IFNβ is completely inhibited. Perini et al. (102) have also 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. The reported frequency of patients developing anti-IFNβ antibodies is not consistent, varying among trials and among laboratories. This may be attributed in part to the differences in treatment regimens and trial designs. Other factors include the type and stage of the disease, type of IFNβ and antigenicity, route of administration and serum sampling. Most importantly, the detection of antibodies is affected by the type of assay employed. Currently, there is no standardized anti-IFNβ antibody assay. The available assays are fraught with limitations. Neutralizing antibody assays are bioassays that indirectly measure the ability of antibodies to neutralize the antiviral effects of IFNβ. At present, this is considered the “gold standard” assay. A similar assay measures the ability of antibodies to inhibit IFNβ induced MxA expression. However, besides having no biologically relevant cutoff titre and being labour intensive, these NAB assays do not directly measure anti-IFNβ antibodies as serum factors can inhibit IFN. Serum can also be directly cytotoxic to the cell lines used in these assays (103). Thus, with so many constraints on the assay systems, the precise assessment of anti-IFNβ and their clinical relevance remains a debate.

1.6 Thesis objectives

The primary objective of this study was to measure anti-IFNβ antibodies, and to further characterize these antibodies with regards to their frequencies and serial profiles
in MS patients undergoing IFNb therapy. To achieve this objective, it was imperative to develop a sandwich ELISA that was validated and proven to be reliable, readily available and relatively inexpensive.
Chapter 2

MATERIALS AND METHODS

2.1 Study subjects

All MS patients in this study were seen at the Vancouver Hospital and Health Sciences Centre / University of British Columbia (VH&HSC/UBC) MS Clinic, and were participating in the Berlex-Schering Study, with ethics approval from UBC. The patients had clinically definite relapsing-remitting MS (RRMS) and were receiving IFNβ-1b at a dosage of 8 mIU (250 μg) subcutaneously, every other day. Blood and sera were obtained from patients prior to start of IFNβ-1b therapy, and thereafter every month for up to 24 months, with samplings performed 24 hours following the last injection of IFNβ-1b. Blood for the isolation of peripheral blood mononuclear cells (PBMC) was collected by venipuncture in 15 mL heparinized Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ), and used within 24 hours of collection. Blood for the measurement of serum anti-IFNβ antibodies was collected in 6 mL Vacutainer™ tubes containing SST® gel and clot activator. Sera were then prepared by allowing blood to clot, followed by centrifugation at 450 X g for 10 minutes at 4 °C. Sera were aliquoted and stored at -20 °C until assayed.

Blood and serum samples were also obtained from healthy, individuals who had never received IFNβ-1b therapy. These samples were kindly provided by staff and students at the VH&HSC / UBC MS Clinic, with ethics approval.
2.2 Establishment of an anti-IFNβ antibody reference pool

An initial qualitative ELISA to measure anti-IFNβ antibodies was performed on serum samples of 50 RRMS patients. The qualitative ELISA consisted of coating microtitre plates with 1.1 µg/well of IFNβ-1b in carbonate-bicarbonate (pH 9.6) and incubating overnight at 4 °C. The plates were then washed 3 times with 0.5M Tris/HCl buffer (pH 8.0) containing 0.05% Tween 20 (Sigma Biosciences, St. Louis, MO) and once with dH₂O. Serum samples were then diluted 1/400 in Tris/HCl buffer (pH9.8) containing 1% normal human pooled serum (nhps) and added in triplicate to the plates, followed by incubation at room temperature for 1 hr. Plates were again washed 3 times with Tris/HCl, 0.05% Tween 20 and once with dH₂O. Goat antihuman IgG (gamma-specific)-Horse Radish Peroxidase conjugate (Sigma) diluted 1/12,000 was added and incubated at room temperature for 1 hr, followed by washing. The substrate, orthophenylenediamine dihydrochloride (OPD P8287, Sigma) diluted in citrate-phosphate buffer (pH 5.0) with 30% H₂O₂ was added and incubated until color developed (10-20 minutes) and the reaction stopped with 2N H₂SO₄. The plates were then read at 490 nm. Included in each plate were a total of 3 healthy controls. Samples with mean optical density (OD) values exceeding twice that of the mean OD of the healthy controls were considered anti-IFNβ antibody positive.

Ten serum samples qualitatively identified as antibody positive were reassayed to confirm positivity. Subsequently, 2 mL of serum were combined from each of these samples and spun at 750 X g to generate the anti-IFNβ antibody reference pool. A pool of
normal human sera (NHPS) was similarly generated with sera from 10 healthy individuals. The sera were then aliquoted into 1 mL tubes and stored at -20 °C.

2.3 IFNβ antigen preparation

IFNβ-1b (Betaseron®, Berlex, Canada) was obtained as therapy-grade vials consisting of 0.3 mg IFNβ-1b, 15 mg Human Serum Albumin (HSA) and 15 mg Dextrose. The antigenic preparation was reconstituted in 0.9% saline to produce a stock solution of 100 μg/mL, which was aliquoted and stored at -20 °C. Using the anti-IFNβ antibody reference pool and NHPS, the optimal coating concentration of IFNβ-b was determined. Ninety-six well microtitre plates (Costar 3576, Cambridge, MA), precoated with mouse monoclonal anti-IFNβ (Chemicon MAB 416, Temecula, CA) and blocked with 0.1% BSA, were then coated with three separate antigenic concentrations containing from 1 to 100 μg/mL of IFNβ-1b in 1x phosphate buffered saline (PBS), 0.5M, pH 7.4. Following overnight incubation at 4 °C, sera were diluted two-fold from 1/100 to 1/12,800 and added. The minimum IFNβ-1b concentration that could discriminate different serum dilutions ranged from 1 to 10 μg/mL. To ensure complete antigen saturation of the microtitre plates, the higher concentration of 10 μg/mL was chosen as the optimal concentration.

2.4 Secondary (detector) antibody and substrate systems

The secondary antibody, goat antihuman IgG (Fc-specific)-horse radish peroxidase conjugate was purchased from Sigma (Lot No. A-0170). The lyophilized protein conjugate was reconstituted in 1 mL of 50% glycerol and further diluted 1/10 in
1x PBS, and stored in 100 µl aliquots at -20 °C. The optimum dilution of the conjugate was 1/10,000.

The enzyme substrate consists of a solution of 0.4 mg/mL of OPD in 0.05M citrate-phosphate buffer (pH 5.0), and containing 5% freshly prepared hydrogen peroxide. The photosensitive, chromogenic substrate was prepare immediately prior to use and protected from light. The substrate reaction was stopped with a solution of 2N H₂SO₄.

2.5 Sandwich ELISA protocol

Ninety-six well microtitre ELISA plates were coated with 110 µl/well of 0.01 ng/mL of mouse monoclonal antihuman IFNβ in 0.05 M carbonate-bicarbonate buffer (pH 9.6). The monoclonal antibody (mAb) is purified and contains no preservatives (Chemicon International Product Info. Sheet). It recognizes amino acids 30 to 47 of IFNβ and has no crossreactivity with human IFNα or IFNγ (104). Plates were then incubated overnight at 4 °C. Following manual decanting, the plates are washed four times with 1x PBS, 0.05% Tween 20 (Sigma), and blocked with 300 µl/well of 1x PBS, 0.1% BSA for 1 hr at room temperature. Wells were washed with 1x PBS, 0.05% Tween , and 100 µl/well of IFNβ-1b at a concentration of 10 µg/mL in 1x PBS was added. Following overnight incubation, plates were again washed and 100 µl test sera diluted 1/100 in 1x PBS, 0.1% NHPS was added in triplicate, according to the plate template.

Included in each plate was a standard curve generated by serially diluting the anti-IFNβ antibody reference pool (2 Lab.Units/mL to 0.0004 Lab.Units/mL). A set of five normal control sera, diluted 1/100 in 1x PBS, 0.1% NHPS was also included. Sera were incubated at room temperature for 1 hr, followed by washing. Bound anti-IFNβ
antibodies were detected using goat antihuman IgG (Fc-specific)-horse radish peroxidase (Sigma A-0176,) diluted 1/10,000 in 1x PBS (pH 7.4). The conjugate was incubated at room temperature for 1 hr, washed and plates developed by the addition of 100 µl of OPD substrate in 0.05M citrate-phosphate buffer (pH 5.0). Just prior to adding to plates, 5 µl of 30% H₂O₂ solution was added to the substrate. The development reaction was carried out for exactly 20 minutes at room temperature after which it was stopped with 50 µl of 2N H₂SO₄. Plates were read with an MRX ELISA Microplate Reader (Dynex Technologies, Chantilly, VA) at 490nm and results expressed as mean absorbance values of triplicate wells. Mean absorbance values were automatically converted to Lab.Units/mL of anti-IFNβ antibodies using the anti-IFNβ standard curve.

2.6 Specificity assays

2.6.1 Analysis of dilution curves

A W.H.O. Reference Antiserum (G-038-501-578) containing antibodies against human natural interferon-β was obtained from the National Institute of Allergy and Infectious Diseases (NIAID, Bethesda, MD). The reference serum was obtained originally from a patient who had been receiving natural IFNβ therapy. The lyophilized product (3.96 mg) was reconstituted in dH₂O as recommended by the NIAID to give a stock concentration of 1 mg/mL. Two-fold serial dilutions in 1x PBS, 0.1% NHPS, were made each for the W.H.O. reference antiserum (1/10 to 1/1280) and for the anti-IFNβ antibody reference pool (1/133 to 1/17066). The sandwich-ELISA was then performed to generate dilution curves of the two sera.
2.6.2 Binding assay

Sandwich-ELISA was performed using various antigenic preparations: IFNβ-1b (10 μg/mL), HSA (500 μg/mL), BSA (0.1%) or no antigen. They were diluted in 1x PBS (pH 7.4) and added to ELISA plates, that had been precoated with mouse antihuman IFNβ monoclonal antibody, blocked, and incubated overnight at 4 °C. Diluted sera (1/100, 1/200, 1/400 and 1/800) were added and incubated at room temperature for 1 hr. After washing, goat antihuman IgG (Fc)-HRP at a dilution of 1/10,000 was added. Plates were developed with a 0.4 mg/mL solution of OPD and after 20 minutes, the reaction stopped and OD values obtained.

2.6.3 Inhibition assay

Anti-IFNβ antibody reference pool (100 Lab.Units/mL) was diluted two-fold from 1/100 to 1/12,800, and preincubated with increasing concentrations of IFNβ-1b antigen (0.50, 1.00, 10.00 μg/mL) in a test tube overnight at 4 °C. The tubes were spun at 15,000g for 20 minutes at 4 °C and supernatant collected and assayed for anti-IFNβ antibodies. The results are expressed as percent inhibition of binding calculated as \((A_{490\text{Non-Incubated Serum}} - A_{490\text{Incubated Serum}} / A_{490\text{Non-Incubated Serum}}) \times 100\).

2.7 Validation assays

2.7.1 Reproducibility

Assay reproducibility was assessed by calculating the coefficient of variation (CV) within and between assays. The intra-assay variability, expressed as % CV, was
calculated from the mean and standard deviation (SD) of 10 replicate analyses of four different serum samples. Inter-assay variability, also expressed as % CV, was obtained from the mean and SD of four other different serum samples, each assayed independently and on four separate occasions (assays).

2.7.2 Recovery

The recovery of anti-IFNβ antibodies was obtained by measuring the concentration of antibodies in four sera, before and after the addition of 100 Lab.Units/mL of anti-IFNβ antibodies. Percentage Recovery was calculated as \( \frac{\text{Measured [Anti-IFNβ Ab]} - \text{Initial [Anti-IFNβ Ab]}}{\text{Initial [Anti-IFNβ Ab]} + \text{Added [Anti-IFNβ Ab]}} \times 100 \).

2.8 Amplified sandwich ELISA for detection of anti-IFNβ antibodies in cell culture supernatants

Heparinized whole blood was layered onto Ficoll-Paque® (Pharmacia Biotech, Uppsala, Sweden) and spun by density gradient centrifugation at 450 X g for 30 minutes at 20 °C. Peripheral blood mononuclear cells (PBMC) were isolated from the gradient interface, washed three times in Hank's Balanced Salt Solution (Gibco BRL, Grand Island, NY) and once in RPMI medium 1640 (Gibco BRL, Grand Island, NY). Cells were resuspended in 1 mL of RPMI 1640 and counted in a hemocytometer using methylene blue dye. PBMCs were then cultured in 1 mL aliquots of RPMI 1640 at a concentration of \( 1 \times 10^6 \) cells/mL in 100 mL Falcon culture flasks, in a humidified incubator with 5% CO₂ at 37 °C. After 7 to 10 days, cell cultures were centrifuged at 750 X g for 15 minutes and supernatants harvested.
Microtitre plates precoated with captured IFNβ-1b and blocked, were incubated overnight at 4 °C with 100 µl of undiluted cell culture supernatant. After washing, 100 µl of mouse monoclonal antihuman IgG-biotin conjugate (Sigma B-3773) was added at a 1/100 dilution and incubated for 1 hr at room temperature. Plates were again washed and 100 µl of streptavidin-alkaline phosphatase conjugate (Sigma B-2890) diluted 1/50 in 0.5M Tris/HCl (pH 8.0) was added and plates incubated for 3 hr at room temperature in the dark. p-Nitrophenyl phosphate (pNPP) substrate (Sigma), was then added at a concentration of 1 mg/mL in 0.1M diethanolamine ((HOCH₂CH₂)₂ NH) buffer (pH 9.8), and incubated for 3 hr at room temperature, in the dark. Plates were read at 405 nm with the Dynex ELISA plate reader and results were expressed as mean absorbances.
Chapter 3

RESULTS

3.1 Anti-IFNβ antibody reference pool

Serial serum samples from 50 MS patients on IFNβ-1b therapy, and sera from healthy donors were initially tested for the presence of anti-IFNβ antibodies using a direct qualitative ELISA. Briefly, 96-well microtitre plates were coated with 1.1 µg/well of IFNβ-1b and incubated with 1/400 dilutions of test sera. Sera were identified as anti-IFNβ antibody positive, using a cutoff of 2x mean Optical Density (OD) of 3 healthy controls. A total of 10 sera, from 10 different patients demonstrating high OD values, were selected to establish the anti-IFNβ antibody reference pool, as shown in Table 1. These sera were reassayed together with sera from normal individuals. Fig.1 confirms that the individual sera comprising the positive reference pool were antibody positive and had greater OD values than sera from normal individuals. Following the pooling of the antibody positive sera to generate the anti-IFNβ antibody reference pool, and normal sera for normal human pooled sera (NHPS), the pooled sera were analysed by serially diluting them. Fig. 2 shows that the antibody reference pool was more reactive and had higher OD values than NHPS. It can also be seen that across serum dilutions, NHPS demonstrated little difference in OD values and thus does not appear to contain anti-IFNβ antibodies.
Establishment of anti-IFNβ antibody reference pool

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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</thead>
<tbody>
<tr>
<td>Mean absorbance (OD&lt;sub&gt;490&lt;/sub&gt;)</td>
<td>1.604</td>
<td>1.749</td>
<td>2.112</td>
<td>1.544</td>
<td>1.705</td>
<td>1.484</td>
<td>0.804</td>
<td>1.226</td>
<td>1.004</td>
<td>1.532</td>
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<tr>
<td>2X Mean OD (3 healthy controls)</td>
<td>0.720</td>
<td>0.646</td>
<td>0.617</td>
<td>0.453</td>
<td>0.787</td>
<td>0.646</td>
<td>0.442</td>
<td>0.847</td>
<td>0.646</td>
<td>0.811</td>
</tr>
<tr>
<td>Anti-IFNβ antibody status</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1: Serum samples from a total of 50 RRMS receiving IFNβ-1b therapy were initially assayed for anti-IFNβ antibodies using a qualitative ELISA. 10 patient sera designated as antibody positive (>2x Mean OD of 3 healthy controls) were chosen to establish the antibody reference pool.

Qualitative reassay of MS patients' and healthy control sera

Figure 1: 10 serum samples previously identified as antibody positive, were reassayed to confirm anti-IFNβ antibody status. Sera from 10 normal individuals were similarly assayed and combined to generate normal human pooled serum (NHPS). Anti-IFNβ antibody binding was expressed as absorbance and each point represents the mean of triplicate samples.
Dilutional analysis of anti-IFNβ antibodies in the anti-IFNβ antibody reference pool and in normal human pooled sera

Fig. 2: The antibody reference pool (n=10) and normal human pooled sera (n=10) were diluted serially between 1/50 and 1/102,400, and assayed for the presence of anti-IFNβ antibodies. Data are expressed as the mean absorbance of triplicate determinations.
3.2 Primary antibody coating

Mouse monoclonal antibody (mAb) to human IFNβ was immobilized to microtitre plates to capture IFNβ-1b antigen. The optimal coating concentration of the monoclonal antibody was assessed using pretreatment serum and serum obtained during IFNβ-1b treatment from the same patient. Fig. 3a shows that when 10 and 1 ng/mL of mAb was used for coating, and sera diluted 1/20,000, there was no difference in OD readings between pretreatment and treated samples. Decreasing the mAb concentration to 0.1 and 0.01 ng/mL resulted in similar low OD values for both sera. However, at a serum dilution of 1/200, OD values for the treated serum sample remained high across the mAb coating concentrations, while OD values for pretreatment serum decreased sharply when 0.1 and 0.01 ng/mL of mAb was used. Thus the lowest concentration of mAb (0.01 ng/mL) was chosen as the optimal concentration, as it could effectively discriminate between antibody negative (pretreatment) and antibody positive (treated) sera.

3.3 Comparison of blocking agents

The efficacy of two blocking agents were studied to determine their ability to block unoccupied sites on the microtitre wells and reduce background activity. Microtitre wells pre-coated with mouse monoclonal anti- IFNβ antibody, were incubated with 0.1 and 1% of bovine serum albumin (BSA) and fetal calf serum (FCS), diluted in 1x PBS, and incubated for 1 hr at room temperature. As shown in Table 2, both blocking agents were able to reduce binding as demonstrated by the observation that antibody negative serum had lower absorbance readings in comparison to antibody positive serum at all
serum dilutions. Both concentrations of blocking agents were also shown to effectively block background binding. However, the data show BSA to be more effective in blocking, with lower absorbance values for normal human pooled sera. As there was no difference between 1.0 and 0.1% BSA, the latter was selected for all subsequent assays.

3.4 IFNβ antigen coating

The optimal condition with regard to IFNβ–1b antigen coating was ascertained using varying concentrations. IFNβ–1b ranging from 1 to 100 μg/mL was added to microtitre plates that had been precoated with mouse anti-human IFNβ (0.01 ng/mL) and blocked with 0.1% BSA in 1xPBS. The anti-IFNβ antibody reference pool and normal human pooled sera were diluted two-fold from 1/100 to 1/12,800 and added. As shown in Fig. 4, the anti-IFNβ antibody reference pool bound IFNβ–1b at all antigenic concentrations, with increasing absorbance at greater antigen concentrations.

Absorbance was also a function of the concentration of anti-IFNβ antibodies: the more diluted the sera, the lesser the OD values. However, there was no binding with normal human pooled serum, and no difference in absorbance between the different antigenic concentrations. Thus all 3 antigenic concentrations could discriminate antibody positive and negative sera, and differing concentrations of antibody. Subsequently, all assays were performed using 10 μg/mL of IFNβ–1b antigen.
Determination of optimal coating concentration of IgG antibody
(Mouse antihuman IFNβ monoclonal antibody)

a) Sera diluted 1/20,000

b) Sera diluted 1/200

Fig. 3: The optimal concentration for coating with the mouse MAbs was assessed by diluting it ten-fold from 10 to 0.01 ng/mL. The IFNβ-1b was added at 10.0 ng/mL. Test sera were obtained from an MS patient prior to receiving IFNβ-1b and during treatment when the patient became antibody positive. Antibody binding was measured by absorbance. Bars are means of four measurements.
Performance comparison of blocking agents

<table>
<thead>
<tr>
<th>Serum Dilution</th>
<th>1% BSA</th>
<th>0.1% BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibody Ref. Pool</td>
<td>NHPS</td>
</tr>
<tr>
<td>1/200</td>
<td>2.797</td>
<td>0.676</td>
</tr>
<tr>
<td>1/800</td>
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<td>1/3200</td>
<td>0.966</td>
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<td>1/12800</td>
<td>0.710</td>
<td>0.609</td>
</tr>
</tbody>
</table>

a) Bovine serum albumin (BSA)

<table>
<thead>
<tr>
<th>Serum Dilution</th>
<th>1% FCS</th>
<th>0.1% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibody Ref. Pool</td>
<td>NHPS</td>
</tr>
<tr>
<td>1/200</td>
<td>2.868</td>
<td>1.005</td>
</tr>
<tr>
<td>1/800</td>
<td>1.721</td>
<td>0.908</td>
</tr>
<tr>
<td>1/3200</td>
<td>1.172</td>
<td>0.868</td>
</tr>
<tr>
<td>1/12800</td>
<td>1.030</td>
<td>0.879</td>
</tr>
</tbody>
</table>

b) Fetal calf serum (FCS)

Table 2: BSA and FCS were diluted 0.10 and 1.0% in 1xPBS, and added to ELISA plates pre-coated with IFNβ-1b. Serum samples diluted in 1xPBS, 0.1% NHPS were then added. Anti-IFNβ antibody binding was measured by absorbance and results shown are means of triplicate measurements.
Determination of optimal coating concentration of IFNβ-1b (Betaseron®) antigen

Figure 4: Plates pre-coated with mouse monoclonal anti-human IFNβ and blocked with 0.1% BSA were incubated with varying concentrations of IFNβ-1b. Diluted sera were added to determine optimal binding of antibodies to IFNβ-1b antigen. Results are expressed as mean of triplicates.
3.5 Reactivity of goat antihuman IgG(Fc)-horse radish peroxidase conjugate

To assess the reactivity of the secondary (detector) antibody, sera diluted 1/400 and 1/800, were added to antigen-coated plates and incubated overnight at 4 °C. Plates were then incubated with the peroxidase conjugate at dilutions of 1/5000 to 1/20,000, for 1 hr. at room temperature. Figure 5 indicates that the antibody reference pool bound with the conjugate, and normal human pooled serum did not. The conjugate dilution of 1/10,000 was employed in all subsequent experiments, as it proved sufficient to effectively distinguish between positive and negative sera.

3.6 Standard curve

Figure 6 shows the standard dose-response curve for the quantitation of anti-IFNβ antibodies. The curve was established from the antibody reference pool which was arbitrarily assigned an anti-IFNβ antibody concentration of 100 Laboratory Units/mL. The reference pool was diluted two-fold from 1/50 to 1/102,400, and the resulting standard curve reveals a characteristic sigmoidal shape with a linear working range between 0.0016 and 2.0 Lab.Units/mL. The anti-IFNβ antibody concentrations in serum samples are determined by multiplying the values read from the standard curve by the dilution factors.

To determine the range of concentrations measurable from the established standard curve, a total of 18 sera from MS-treated patients and healthy controls were diluted 1/100. The antibody concentrations extrapolated from the curve show that it can detect a wide range of antibody levels as shown in Figure 7.
Reactivity of goat antihuman IgG (Fc-specific) - horse radish peroxidase conjugate

Figure 5: Reactivity of the peroxidase-conjugated goat anti-human IgG (Fc-Specific) detector antibody, diluted 1/5000 to 1/20,000. Antibody reference pool and normal human pooled serum (NHPS) were diluted 1/400 and 1/800 and used to determine the optimal concentration of the conjugate. Antibody binding was expressed as the mean of triplicate measurements.
Figure 6: The standard curve for the quantification of anti-IFNβ antibodies reveals a characteristic sigmoidal shape. The anti-IFNβ antibody pool (100 Lab.Units/mL) was diluted two-fold from 1/50 to 1/102,400. The useful working range is between 0.0016 and 2.000 Lab.Units/mL. Each point represents mean of triplicate measurements.
Concentration of anti-IFNβ antibodies in MS-treated patients and normal controls

MS-treated patients (n=12) Normal controls (n=6)

Figure 7: Reactivity of anti-IFNβ antibodies in serum samples of normal controls (n=6) compared to MS-treated patient's (n=12). Sera were assayed in triplicate and each point represents mean concentration of antibodies. Antibody concentrations range from 1.22 to 146.38 Lab.Units/mL.
3.7 Assay specificity

3.7.1 Parallelism of dilution curves

The specificity of the ELISA was verified by testing for parallelism between dilution curves generated from the W.H.O. Reference Antiserum and from the anti-IFN\(\beta\) antibody reference pool. Figure 8 shows that the two curves are parallel over a wide range of dilutions, thus confirming that both sera contain an identical analyte (anti-IFN\(\beta\) antibodies), and that the ELISA is specific for anti-IFN\(\beta\) antibodies.

3.7.2 Binding specificity

The IFN\(\beta\) antigen used in the ELISA is an impure source containing additional stabilizers such as HSA. Thus it might be perceived that the antibodies are binding to HSA or BSA which is used as a blocking agent. To examine this possibility and to establish the specificity of the ELISA in detecting anti-IFN\(\beta\) antibodies, different antigenic preparations were used. Plates were coated with either IFN\(\beta\) (10 \(\mu\)g/mL), HSA (500 \(\mu\)g/mL), BSA (0.1%) or no antigen, followed by incubation with varying dilutions of the antibody reference pool or NHPS. As shown in Figure 9, there was no difference in absorbance readings of NHPS between the 4 different antigen coating conditions. However, it is evident from Figure 10 that the antibody reference pool bound with high specificity only to IFN\(\beta\)-1b, and that this binding was a function of the anti-IFN\(\beta\) antibody concentration: increasing serum dilutions correspond to decreasing absorbance levels.
Parallelism of dilution curves

- Anti-IFNβ Antibody Reference Pool  ▲ W.H.O. Reference Antiserum(G038-501-572)

**Figure 8**: Specificity of the ELISA was verified by dilutional analysis of W.H.O. reference antiserum (1/10 to 1/1280) and the anti-IFNβ antibody reference pool (1/133 to 1/17066). The resultant curves show parallelism across a range of dilutions. Each plotted point represents the mean of triplicate measurements.
Figure 9: Normal Human Pooled Serum was diluted from 1/100 to 1/800 and incubated on plates pre-incubated with different antigens. Binding is expressed as mean absorbance of triplicates. No binding was observed with any antigen.
Figure 10: The anti-IFNβ antibody reference pool bound specifically only to IFNβ, and not to BSA or HSA. Binding was directly proportional to the concentration of antibodies.
3.7.3 Inhibition of binding

The specificity of the ELISA was also ascertained by a direct inhibition test. Test serum with known concentrations of anti-IFNβ antibody was serially diluted and pre-incubated with increasing concentrations of IFNβ-1b antigen in a test tube at 4 °C, and the supernatant assayed. Figure 11 shows a dose dependent increase in inhibition of antibody binding with increasing concentrations of IFNβ-1b antigen. Prior incubation with 0.50 µg/mL of IFNβ-1b resulted in a maximum of 59% inhibition. Similar pre-incubation with 1 µg/mL and 10 µg/mL yielded maximum inhibition of 78 and 95%, respectively. This trend was also reflected in an antibody dose-dependent manner, with decreasing antibody concentrations yielding higher inhibition rates.

3.8 Assay validation

3.8.1 Reproducibility

Reproducibility of the ELISA was assessed by precision tests, and the mean, standard deviation (SD) and coefficient of variation (CV) calculated. Intra-assay variability was determined by repetitive measurements (10 times) of four different serum samples, and the CV calculated to be 10.1, 9.0, 6.3 and 7.8%, respectively (Table 3a). Interassay variability was determined by comparing four other different samples each assayed on four independent assays. In this instance, the CV was 8.5, 3.0, 5.2 and 7.4%, respectively (Table 3b).

3.8.2 Recovery test

Using four distinct samples supplemented with 100 Lab.Units/mL of anti-IFNβ antibodies, the recoveries of the added antibody to the serum samples were investigated.
As shown in Table 4, the recovery rates ranged from 66 to 104%. The rate was dependent on the initial antibody concentration of the test sera, as sample 1 with the lowest initial concentration showed the highest recovery rate, and sample 4 with the highest antibody concentration having the lowest rate.
Inhibition of Anti-IFNβ Antibody Binding

Fig 11: Pre-incubation of serum containing various concentrations of anti-IFNβ antibodies, with increasing concentrations of IFNβ-1b antigen in test tubes overnight. The resulting supernatants were assayed for antibodies. Percent inhibition was calculated as (A₄₉₀ non-preincubated serum - A₄₉₀ preincubated serum / A₄₉₀ non-preincubated serum) X 100
Reproducibility of ELISA

a) Intra-assay variability

<table>
<thead>
<tr>
<th>Sample</th>
<th>Replicates</th>
<th>Mean</th>
<th>SD</th>
<th>%CV</th>
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<tr>
<td>1</td>
<td>10</td>
<td>2.70</td>
<td>0.27</td>
<td>10.09</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
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</tr>
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<td>3</td>
<td>10</td>
<td>115.50</td>
<td>7.29</td>
<td>6.31</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>6.45</td>
<td>0.50</td>
<td>7.79</td>
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</table>

b) Inter-assay variability

<table>
<thead>
<tr>
<th>Sample</th>
<th>Replicates</th>
<th>Mean</th>
<th>SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
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<td>4</td>
<td>35.48</td>
<td>3.03</td>
<td>8.45</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>47.95</td>
<td>1.45</td>
<td>3.02</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>78.80</td>
<td>4.09</td>
<td>5.19</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>124.50</td>
<td>9.26</td>
<td>7.43</td>
</tr>
</tbody>
</table>

Table 3: Precision of the ELISA was determined by 10 repetitive measurements of four serum samples (intra-assay variability), and four independent assays of four other different patients (inter-assay variability).
Recovery test for the determination of anti-IFNβ antibodies by the sandwich ELISA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial (Lab.Units/mL)</th>
<th>Added (Lab.Units/mL)</th>
<th>Initial+Added (Lab.Units/mL)</th>
<th>Measured (Lab.Units/mL)</th>
<th>Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.731</td>
<td>100.00</td>
<td>100.731</td>
<td>105.167</td>
<td>104.404</td>
</tr>
<tr>
<td>2</td>
<td>5.753</td>
<td>100.00</td>
<td>105.753</td>
<td>88.446</td>
<td>83.635</td>
</tr>
<tr>
<td>3</td>
<td>10.150</td>
<td>100.00</td>
<td>110.150</td>
<td>88.188</td>
<td>80.062</td>
</tr>
<tr>
<td>4</td>
<td>124.609</td>
<td>100.00</td>
<td>224.609</td>
<td>148.213</td>
<td>65.987</td>
</tr>
</tbody>
</table>

Table 4: The recovery test was performed by measuring the anti-IFNβ antibody concentration in four distinct samples that had been spiked with 100 Lab.Units/mL of anti-IFNβ antibodies. Percentage Recovery was calculated as (Measured / (Initial+Added)) X 100.
3.9 Anti-IFNβ antibody levels: Frequency and serial profiles

Figure 12 shows the monthly anti-IFNβ antibody levels of 20 MS patients who received 8 mIU (250 μg) of IFNβ-1b subcutaneously every second day. Serial serum samples were diluted 1/100 and incubated in triplicate on microtitre plates. The sera of each patient were assayed in a single plate which also contained a standard curve generated from the antibody reference pool, and five normal controls. None of the patients had a detectable antibody level at the start of the IFNβ-1b therapy as ascertained by the mean +/- 3SD of 5 normal controls. During the first month of therapy, only 3/20 patients developed anti-IFNβ antibodies and thereafter, 16/20 developed antibodies after 3 months of therapy. Among this group of patients, the highest level of anti-IFNβ antibody level attained was 212 Lab.Units/mL, as was observed with patient 1 at month 5. Patients 1 through 13 had significant levels of antibody in comparison to patients 14, 15 and 16 whose antibody levels were barely detectable. 4/20 of the patients did not develop antibodies, and this group is represented by patients 17, 18, 19 and 20.

In 12/16 of antibody positive patients, there was a characteristic antibody profile consisting of a steady rise in antibody levels to peak levels, followed by a decrease approximating pre-treatment levels in some patients. 4/16 of antibody positive patients maintained a steady level of antibodies, as exemplified by plateaus in patients 5, 6, 9 and 11. Figure 13 shows the cumulative monthly antibody levels of all the patients. The highest antibody average antibody level was observed at month 6 (62 Lab.Units/mL), but as in individual antibody profiles, this gradually decreased to 27 Lab.Units/mL at month 18.
Figure 12: Serial monthly anti-IFNβ antibody levels of 20 MS treated patients. Serum samples were assayed in triplicate and each plotted point represents the mean anti-IFNβ antibody concentration.
Cumulative Monthly Averages of Anti-IFNβ Antibodies

Fig. 13: Average monthly levels of anti-IFNβ antibodies in all patients (n=20). Results are reported as mean ± SEM.
3.10 Secretion of anti-IFNβ antibodies in cell culture by serum positive patients

Lymphocytes isolated from MS-treated patients were grown in culture for 7 days, after which the supernatant was harvested and assayed for the presence of anti-IFNβ antibodies. The assay used was a modified version of the Sandwich ELISA containing a biotin-streptavidin amplification step. This assay was qualitative and expressed anti-IFNβ antibody levels in mean absorbance values. Figure 14 shows the anti-IFNβ antibody levels in serum and supernatant of 1 patient. Serum antibody levels steadily increased reaching peak levels at month 5, followed by a steady decline. Supernatant antibody levels peaked at month 4 by sharply declined at month 5, and thereafter followed slight increases and decreases.
Detection of Anti-IFNβ Antibodies in Cell Culture Supernatants of An IFNβ-1b Treated MS Patient

Figure 14: Peripheral blood mononuclear cells isolated from a serum anti-IFNβ positive patient were grown in culture for 7 to 10 days. Following harvesting, supernatants were incubated on IFNβ-coated plates overnight, and the presence of anti-IFNβ assayed using an amplified version of the sandwich ELISA. Both serum and supernatant antibodies are expressed as mean of triplicate measurements.
Chapter 4

DISCUSSION

At present, there is a perception among some clinicians caring for MS patients that anti-IFNβ antibodies reduce therapeutic efficacy in IFNβ-treated patients. Indeed, studies demonstrated a serious reduction of IFNβ beneficial effects in antibody positive patients (93). As a consequence, the relapse rates of patients who developed antibodies reverted to placebo levels. Furthermore, these patients experienced an increase in MRI lesional activity, as compared to antibody negative patients. Thus, given the clinical relevance of monitoring anti-IFNβ antibodies in correlation to therapeutic responsiveness, it is imperative to have a universal and quantitative assay for measuring these antibodies.

Currently, anti-IFNβ antibodies are measured mostly by using their neutralizing capacities. The anti-viral assay tests the ability of anti-IFNβ antibodies to interfere with the anti-viral effects of IFNβ. Known amounts of IFNβ are incubated with serum samples, and thereafter the serum-treated IFNβ is dispensed to a virus-sensitive cell line which is then challenged with a virus. Inhibition of viral replication by the serum-treated IFNβ, as characterized by the cytopathic effects on the cell line, is compared to inhibition by serum-free IFNβ. The titre of the serum is calculated as the serum dilution that reduces 10 Lab.Units/mL of IFNβ to 1 Lab.Units/mL. Another neutralization assay is the MxA induction assay. It is based on IFNβ-specific induction of the MxA gene in human cell lines. MxA protein levels are increased in the blood during IFNβ therapy, and in tissue cultures anti-IFNβ antibodies have been shown to reduce the levels of MxA.
induction. Briefly, cells are allowed to grow on microtitre plates for 24 hours after which serum-treated IFNβ is added. Cells are lysed after 2 hours, and the MxA protein in the lysate is measured by a chemiluminometric assay. The amount of MxA protein induced is directly proportional to IFNβ activity which is expressed as Lab.Units/mL. The anti-IFNβ antibodies in the serum are hence expressed as titres (reducing 10 Lab.Units/mL to 1 Lab.Units/mL of IFNβ). The anti-proliferative assay is another neutralizing assay in which the serum antibodies neutralize the anti-proliferative effects of IFNβ, whose activity is also expressed in Lab.Units/mL.

However, these assays will only detect antibodies if they interfere with a function of IFNβ. And since IFNβ anti-viral activity may be independent of its immunomodulatory effects, the same serum may have different anti-IFNβ antibody titres, depending on the type of assay employed. Furthermore, these assays will only detect antibodies of moderate to high affinity; in the IFNβ-1b pivotal trial, antibody negative patients had similar levels of induced MxA protein as patients with low antibody titres. And in patients with high antibody titres MxA levels were low, thus indicating incomplete neutralization by anti-IFNβ antibodies. Another limitation to these assays is that a variety of cell lines, like human lung fibroblasts, human amniotic cells, and bovine kidney cells, have been employed as target cells. As these cells have varying sensitivities to IFNβ, the assays also have different sensitivities in measuring anti-IFNβ activity. Other confounding factors include non-antibody serum factors such as platelet derived growth factor that may have anti-viral activity and decrease the sensitivity of target cell lines to IFNβ. Thus, antiviral neutralization assays may not directly be measuring anti-IFNβ antibodies. Only neutralizing antibodies are detected, and these recognize a short
linear epitope spanning residues 39-48 of the 166 amino acid molecule of IFNβ-1b (104). Non-neutralizing binding antibodies in contrast are less specific, and bind to epitopes spanning the entire amino terminal of the IFNβ-1b molecule. The binding antibodies are likely to affect IFNβ bioavailability, and will form immune complexes that are cleared from the circulation by the reticulo-endothelial system, thus decreasing serum IFNβ levels. The possibility exists also that they may stabilize IFNβ-1b and increase its half-life. The bioassays are also expensive, difficult to perform, and time consuming. And they are not appropriate in situations when large numbers of clinical samples have to be analyzed for anti-IFNβ antibodies. Most importantly, little or no information has been published regarding the reproducibility, specificity and sensitivity of these assays.

This study describes the development of a sandwich-ELISA to quantify and characterize anti-IFNβ antibodies in the serum of MS patients undergoing IFNβ-1b therapy. The assay is based on a solid phase-bound capture MAb (mouse monoclonal anti-human IFNβ), which binds specifically to IFNβ. After blocking for non-specific binding with 0.1% BSA, the IFNβ-1b antigen was added followed by incubation of diluted sera. A second labeled detector antibody (goat antihuman IgG-HRP conjugate) is then dispensed into the microtitre plate wells, and binding of anti-IFNβ antibodies is visualized by the addition of OPD substrate. The absorbance of the reaction mixture is determined using an ELISA plate reader and the OD values are translated into Laboratory units/mL of anti-IFNβ antibodies.
As there is no internationally recognized standard for anti-IFNβ antibodies, an 'in house' standard was prepared. This anti-IFNβ antibody reference pool was generated from 10 anti-IFNβ antibody positive sera with high OD values. These sera were initially assayed by an in house indirect qualitative ELISA. As this assay exhibits substantial day to day variations (inter-assay variability), the selected serum samples were reassayed to confirm for the confirmation of the presence of antibodies. As expected, individual sera of the anti-IFNβ antibody reference pool demonstrated significant higher OD values in comparison to a group of 10 healthy control sera that comprise the normal human pooled sera (NHPS). Both pooled sera were subjected to dilutional analysis (from 1/50 to 1/102,400), and OD values remained essentially the same across NHPS dilutions while there was a clear relationship between anti-IFNβ antibody reference pool dilutions and OD values.

While developing the sandwich-ELISA, optimization studies were performed for each component of the assay. Mouse monoclonal anti-human IFNβ was used as capture antibody to immobilize the IFNβ-1b antigen and to increase the sensitivity of the assay. It is generally recognized in ELISAs that direct coating of the antigen onto ELISA microtitre plates results in the non-native presentation of antigen, in contrast to captured antigen that is presented in the native state (105). Thus precoating of the microtitre plate with Mab was included to remedy this effect. In the process of developing the sandwich-ELISA, it was necessary to block unoccupied sites on the MAb-coated plates which may provide the opportunity for adherence of other proteins. Various protocols using BSA and FCS were assessed for their blocking capacities. Ultimately, 0.1% BSA in 1×PBS was selected as the blocking agent as it resulted in lower background as demonstrated by the
low OD values of the NHPS. Inclusion of Tween20, a nonionic detergent, in the washing buffer (1x PBS, 0.05% Tween 20) provided additional blocking.

Taking into consideration the cost of pure IFNβ-1b and the number of patient serum samples assayed, it was more feasible to utilize the therapy grade formulation. This antigen preparation is an impure source containing 50 times more HSA than IFNβ-1b. HSA is added as a stabilizing constituent because of its high solubility and thermal stability. HSA also provides additional blocking, as it has the ability to prevent surface adsorption of active proteins, and thus no blocking step was included following IFNβ-1b antigen coating. In an attempt to determine the optimal coating concentration of the IFNβ-1b antigen, binding of the anti-IFNβ antibody reference pool was compared to that of NHPS using increasing IFNβ-1b concentrations. NHPS had very low OD values and did not show any difference in binding to 1, 10 or 100 μg/mL of IFNβ-1b antigen. Additionally, the various dilutions of NHPS exhibited similar binding. In contrast, the OD values of the anti-IFNβ antibody reference pool were noticeably high, with increases in antigen concentration resulting in concomittant increases in binding. This binding of antibodies to IFNβ-1b antigen was decreased as the anti-IFNβ reference pool was diluted. The concentration of 10 μg/mL was chosen as the optimal coating condition for IFNβ-1b as this was deemed sufficient to effectively discriminate between anti-IFNβ antibody binding and background absorbance as exhibited by NHPS.

During the development of the assay, the reactivity of the detector antibody, goat antihuman IgG (Fc)-HRP conjugate, was studied. There was no difference in OD values for NHPS across the conjugate dilutions, while the anti-IFNβ reference pool showed increasing OD values with increases in conjugate concentration. The conjugate dilution
of 1/10,000 was established as optimal for the assay. The chromogenic substrate was used at a concentration of 0.40 mg/mL, as was recommended by the manufacturer.

Subsequent to the optimization of the assay reagents the anti-IFNβ antibody reference pool was arbitrarily assigned 100 Lab.Units/mL of anti-IFNβ antibodies, and a standard curve generated. The curve is typically sigmoidal, signifying that there is no 1:1 relationship between antigen and antibody. It has a working range of between 0.0625 to 1.00 Lab.Units/mL. Thus the sensitivity of the assay is approximately 0.10 Lab.Units/mL. Using a set of 18 sera anti-IFNβ antibodies could be detected from a range of 1.22 Lab.Units/mL in a normal individual to 146.38 in an IFNβ-1b treat MS patient.

The specificity of the assay was examined through dilutional analysis of the anti-IFNβ antibody reference pool and the W.H.O. Reference antiserum. Curves generated from the two sera showed parallelism, and the anti-IFNβ antibody reference pool was exceedingly more potent than the W.H.O. reference antiserum, exhibiting higher OD values at similar serum dilutions. The parallelism of the two curves demonstrates that antibodies in the two sera recognize a similar antigen, IFNβ-1b. However, the W.H.O. serum, obtained from a human carcinoma patient repeatedly treated with human IFNβ, is not recommended by the W.H.O. to calibrate assays but to establish the function of assays.

Assay specificity was also verified by binding and inhibition studies. The HSA in the formulated therapy grade IFNβ-1b preparation could itself potentially act as an antigen against which antibodies can be directed. Another potential antigen which can bind serum antibodies include the BSA component of the blocking buffer. These possibilities were examined and the results conclusively demonstrate that anti-IFNβ
antibodies do not bind to HSA or to BSA, and that these antibodies are specifically
directed against IFNβ-1b. The assay was further validated with precision and recovery
tests. The sandwich ELISA proved reproducible with both inter- and intra-assay
variabilities of less than 10%. Recovery rates were also good and ranged from 66 to
104%.

This study provided a serial and quantitative analysis of anti-IFNβ antibodies, and
this is important as the onset and magnitude of antibody induction was variable. This can
be attributed to the immunogenicity of the IFNβ-1b antigen and the immunological status
of the patients. The results of 20 RRMS patients treated with IFNβ-1b for up to 24
months are presented here. A total of 443 serum samples, with an average of 21 sera per
patient, were assayed. Sixteen patients developed anti-IFNβ antibodies. The development
of anti-IFNβ antibodies was an early event, with patients seroconverting by month five of
IFNβ-1b therapy. In 12/16 of the antibody positive patients, the appearance of antibodies
was transitory peaking on average around month six and gradually declining. However,
in 4/16 of patients, the antibody levels were maintained constantly over time.
Furthermore, serial serum samples of 5 MS patients on placebo, were assayed and no
anti-IFNb antibodies were detected in any of these samples. This study further attempted
to measure anti-IFNβ antibodies in the culture supernatants of patients who had serum
antibodies. The amplified sandwich ELISA, modified with an extra biotin-streptavidin
step, could detect antibodies in some patients. And supernatant antibody profiles closely
matched serum antibodies, rising to a peak at around month 6 and thereafter declining.
CONCLUSION

IFNβ has been established as an effective treatment for MS, and is indeed the most prescribed drug for relapsing-remitting MS. However, IFNβ therapy is associated with the development of anti-IFNβ antibodies, whose precise biological effects have not been determined. It remains unclear how these antibodies affect the bioavailability of IFNβ and its therapeutic efficacy. The delay in determining the effects of anti-IFNβ antibodies is primarily due to the absence of a standard anti-IFNβ antibody assay.

The validated sandwich-ELISA used in this study confirms the immunogenicity of IFNβ-1b. The study demonstrates that a large number of MS patients (16/20) respond to IFNβ-1b therapy by producing anti-IFNβ antibodies, and that antibody development occurs early. No antibodies were detected prior to treatment. Among the group of patients who developed antibodies, antibody levels peaked within the first 6 months and thereafter declined despite continued administration of IFNβ-1b. However, in a subset of antibody positive patients (4/16) the antibodies declined to a plateau which was maintained during the entire course of treatment. The data also clearly delineate two groups of antibody positive patients; those with high antibody levels and those with medium to low levels. And in an adaptation of the sandwich-ELISA, using an amplification step, cultured lymphocytes from some antibody positive patients show detectable levels of secreted anti-IFNβ antibodies. The serial profile of the latter approximated that of serum antibodies.

IFNβ-1b is structurally different from the two other IFNβ-1a preparations IFNβ-1a (Rebif®, Avonex®). And as demonstrated in other families of molecules, slight
modifications generally result in changes in effect of compounds. Thus in the future, the sandwich-ELISA could be used in a side-by-side comparison of the different IFNβ preparations. This will provide meaningful and reliable insight into their relative antigenicities, and ascertain cross-reactivities of the anti-IFNβ antibodies. Future experiments should involve improvement and quantitation of anti-IFNβ antibodies in culture, and the isolation of those B cells that produce the antibodies. This will potentially be a useful tool in the identification of the mechanisms of action of IFNβ-1b. It will also be necessary in the future to determine what proportion of these anti-IFNβ antibodies have neutralizing capabilities, and how they affect the bioavailability and pharmacokinetics of IFNβ.

Finally anti-IFNβ antibody data should be correlated to clinical data of MS patients, such as MRI scans, disability scores and relapse rates. Most importantly, the question of whether patients without antibodies are clinically more stable than antibody positive patients should be addressed. Similarly, the disease states of patients prior to IFNβ therapy, as well as their genotypes should be determined. These results should provide information as to whether anti-IFNβ antibody development is due to the magnitude of the MS disease or to an immunological predisposition.
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