Neutralising Antibodies to Interferon Beta in Multiple Sclerosis

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A thesis submitted in April 2010 to University College London

for the degree of Doctor of Philosophy (PhD)

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I, Rachel Farrell confirm that the work presented in this thesis is my own. Where information has been delivered from other sources I confirm that this has been indicated in the thesis.

Abstract

Multiple Sclerosis is the most common non-traumatic cause of neurological disability in young people. Until recently few treatments existed for Multiple Sclerosis and Interferon beta was the first and remains the most commonly prescribed. As a biological product it induces antibodies to the protein which may abrogate the efficacy of the drug (neutralising antibodies - NAbs). Testing for these antibodies has been problematic as biological assays are difficult to standardise, time consuming and expensive. In the experiments described here we sought to develop a novel cell-based reporter-gene assay to reliably test for Nabs, to explore the relationship between NAbs, treatment efficacy, biological activity and correlate NAb titres with *in vivo* biomarker induction to establish guidelines to interpret results. The work presented in this thesis describes the development and validation of the luciferase assay and has shown that subjects who develop NAbs experienced increased relapse rates, (which lag behind the appearance of NAbs). Evaluating the *in vivo* biological response to IFNβ injection it was established that in subjects with NAbs there was titre dependent loss of bioactivity with reduced myxovirus resistance protein A (MxA) level in those with titres 100 – 600 NU and absent response in those with titres > 600 NU. Opinion amongst neurologists (UK, USA, Canada and Austria) regarding NAbs was evaluated and revealed uncertainty of their significance in the clinical setting, and the reluctance of some neurologists to incorporate NAb testing into routine practice. Results were similar in the countries surveyed in that 90 – 100% were aware of NAbs and thought they abrogate clinical efficacy, yet few routinely tested for them, particularly in the UK. The validated luciferase assay has since been dissemintated to 9 countries. Since it's launch in the UK in 2006 over 4,000 patients have been tested for NAbs. This work directly translates into clinical practice and provides a useful service to aid management of subjects with MS.

Acknowledgements

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Abbreviations

AD	Alternate days
AML	acute myelogenous leukaemia
ATCC	American type cell collection
ATP	Adenosine triphosphate
B O2	Monoclonal capture antibody specific for Human IFN-β
BAb	Binding antibody
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
Ca2+	Calcium
CHO	Chinese hamster ovary
CI	Confidence interval
CIS	Clinically isolated syndrome
CNS	Central nervous system
CPE	cytopathic effect assay
CSF	Cerebrospinal fluid
DMD	Disease modifying drugs
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune or allergic encephalomyelitis
EBV	Epstein Barr Virus
EDSS	Expanded disability status score
EFNS	European federation of neurological societies
ELISA	Enzyme linked immunosorbent assay
EMCV	Encephalomyocarditis virus
EOD	Every other day
FBS	Fetal bovine serum
GA	Glatiramer acetate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gd	Gadolinium
GM	Grey matter
HAT	Hybri-Max media supplement
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HERV	Human endogenous retrovirus
HEK	Human embryonic kidney
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IEF	Isoelectric focusing
IFIT-1	Interferon-induced protein with tetratricopeptide repeats 1
IFN β	Interferon β

IFNAR	Interferon receptor
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
ISRE	Interferon stimulated response element
IU	International units
IVIg	Intravenous immunoglobulin
IVMP	Intravenous methylprednisolone
LAS	Luciferse assay system
LCPS	Luminescent counts per second
LU	Laboratory unit
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MIU	million international units
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	Multiple Sclerosis
MSCRG	Multiple Sclerosis Collaborative Research Group
MxA	Myxovirus resistance protein
Na+	Sodium
NAb	Neutralising antibody
NHS	National Health Service
NIBSC	National Institute of biological standardisation
NICE	National Institute of Clinical Excellence
NIH	National Institutes for Health
NK	Natural killer cells
NU	Neutralising unit
NAWM	Normal appearing white matter
OAS	Oligoadenylate synthetase
OCB	Oligoclonal Bands
OD	Optical density
OPE	O- phenylenediamine
OW	Once weekly
SDS -PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PML	progressive multifocal leukoencephalopathy
PPMS	Primary progressive MS
PSC	Positive standard control serum
QALY	Quality adjusted life year

QC	Quality control
RIA	Radio-immunoprecipitation assay
RID	Radial immunodiffusion
RNF	Rebif new formulation
RRMS	Relapsing remitting MS
SC	Subcutaneous
SDB	Sample dilution buffer
SPMS	Secondary progressive MS
TGFβ	Transforming growth factor -β
T_h	T helper cells
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor apoptosis inducing ligand
TRU	Tenfold reduction unit
TTW	Three times weekly
TWEEN	Polyoxyethylene sorbitan monolaurate
VLA4	very late antigen 4
WHO	World Health Organisation
WM	White Matter

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Chapter 1

Multiple Sclerosis

Multiple Sclerosis

1 Introduction

Multiple Sclerosis (MS) is the most common inflammatory demyelinating disorder of the central nervous system. It is a chronic disabling disease largely affecting young people with onset between 20 – 40 years of age; onset of the disease is rare in children and those > 50 years of age. MS is typically characterised by episodes of neurological deficit followed by periods of remission. MS has an incidence of approximately seven per 100,000 per year and a lifetime risk of 1:400. Worldwide it is estimated that MS affects 2.5 million people (approximately 100,000, i.e. ~1:600 people in the UK) and its economic impact is considerable [Compston and Coles 2002; Compston and Coles 2008]. Overall life expectancy is reduced by about 7 years and living with chronic disability is the major burden of the disease [Ragonese *et al.* 2008]. Despite the strong genetic linkage and clustering of MS in some families the concordance rate between monozygotic twins is less than 30% [Willer *et al.* 2003; Mumford *et al.* 1994 ; French Research group on MS 1992 ; Ristori *et al.* 2006]. It is likely that an interaction between genetic factors and the environment causes the disease. The female to male ratio is ~2:1 worldwide, but recent evidence suggests this may be increasing [Orton *et al.* 2006]. This sex bias is conventionally attributed to MS being a putative autoimmune disorder, but may be due to hormonal and/or due to other unidentified environmental factors. The trigger or triggers have not been identified to date but attention is focused on infectious and other environmental agents [Marrie 2004].

Over the last fifteen years a number of new treatments which modulate the course of MS have emerged and treatment options continue to increase. An overview of current concepts of the aetiology, immunology and pathology of MS will be given to provide a framework for explaining the mechanisms by which these new agents modulate the immune system in MS. I will also summarise the efficacy and adverse effects of these agents and discuss how best to treat people with MS using interferon beta.

1.1.   *Epidemiology*

Epidemiological observations have consistently described an effect of geography and latitude on the risk of MS. The incidence of MS is higher in regions beyond a latitude of 42° North or South of the equator [Kurtzke 2005]. MS predominantly affects people of Northern European extraction, with lower rates in the USA, Australia and New Zealand. Countries close to the equator have a much lower incidence of MS. Migration studies have shown that children migrating before adolescence (generally < 15 years of age) assume a risk close to that of the destination country [Gale and Martyn 1995; Martyn and Gale 1997]. Several cohorts have described increased incidence of MS in the offspring of migrants to a high risk area; in Israel the frequency of MS in native born Israelis of African / Asian origin is equal to that in those of European / American origin rather than the lower rate of their forebearers [Kahana *et al.* 1994]. Similarly children born in the UK to immigrants from the Indian subcontinent acquire the risk of the native population [Elian *et al.* 1990]. Recent reviews question this, and migration prior to the age of 15 is no longer considered the crucial time, rather somepoint within the first two decades of life

[Hammond *et al.* 2000]. It is now thought that primary exposure to a particular agent after puberty or during adult life may be the trigger. There is evidence suggesting that viral infection may play an important role in pathogenesis [Gilden 2005]. The occurrence of mini-epidemics and clusters of MS, such as that in the Faroe Islands, support the hypothesis that an environmental – possibly infectious – agent plays a role in the initiation and possibly perpetuation of the disease [Kurtzke and Heltberg 2001; Kurtzke 2002]. The rising incidence of MS in some regions seems to be particularly amongst women and the rate at which this occurs indicates that genetic factors are unlikely to be solely responsible rather environmental factors are implicated and may also be influenced by increased ascertainment and altered diagnostic criteria [Noonan *et al.* 2002].

1.2.   *Aetiology*

Epidemiological evidence suggests that MS is caused by interplay between genes and the environment and thus can be considered a complex trait disorder. The genes responsible for complex diseases such as MS are not due to individual gene mutations and the production of abnormal proteins but are normal polymorphisms, each acting independently, exerting a small contributory effect on some as yet undefined structure or physiological function [Compston and Coles 2008]. Twin studies indicate a genetic susceptibility with a concordance rate of ~ 20 – 30% between monozygotic twins [French research group on MS 1992; Mumford *et al.* 1994; Willer *et al.* 2003] and increased risk of MS in those who have an affected relative [Dyment *et al.* 1997]. Susceptibility genes have been identified using population studies, candidate gene studies and whole genome linkage screens. These have elucidated susceptibility genes including HLA class II alleles (DRB1*1501, DRB5*0101 and DQB1*0602) [Olerup and Hillert 1991] and more recently specific alleles of the IL2 and IL7 receptors [Hafler *et al.* 2007] Since this time several genome wide association studies have been performed and 14 genes have been identified including CLEC16A, TNFRSF1a (CD120a), IRF8, RPL5/EVI5, CD226, TYK2, CD6, CD40 amongst others [Burton 2007, Baranzini 2009, ANZgene 2009]. The geographical distribution of people with MS, may be partially accounted for by genetic factors and homogenous populations but cannot be accounted for on the basis of genetics alone, but rather suggests a contributory role of an environmental agent in the pathogenesis of MS. It is unclear if the role of an external trigger is in initiating the disease only, or whether it also modifies the disease course. Several infectious agents have been proposed as triggers of MS, including Epstein Barr virus (EBV), human herpes virus 6 (HHV6), canine distemper virus, human

endogenous retroviruses (HERV) and Chlamydophila. However, none of the above has so far stood up to rigorous investigation [Giovannoni *et al.* 2006]. Despite inconclusive results, exposure to an infectious agent (or a sequence of agents) at a vulnerable age is currently considered an important element for MS to develop in an individual carrying specific susceptibility genes.

1.3.   *Immunology and Pathology*

The hallmarks of MS pathology are sharply defined lesions in the CNS, which are characterised by inflammation, demyelination, relative axonal preservation, and gliosis [Hohlfeld and Wekerle 2004]. These lesions (plaques) may develop in any part of the CNS, though they have a tendency to accumulate near the periventricular and outer surfaces of the brain and spinal cord. The lesions are usually round to oval, and are centered on one or several medium sized vessels [Lassmann and Wekerle, 2006]. Apart from MS plaques in the white matter (WM), studies carried out as early as 1890 have shown that plaques can also be found in the grey matter (GM) [Dawson 1962; Brownell and Hughes 1962], including the cerebral and cerebellar cortex, the basal ganglia and the spinal cord [Kutzelnigg *et al.* 2005; Kutzelnigg *et al.* 2007; Gilmore *et al.* 2006]. Usually, WM lesions in samples of MS brain can be macroscopically identified and vary in size (from less than one mm to several centimetres), shape and number. They have a pink or grey opaque appearance. Whereas lesions in the WM are pathognomonic of MS pathology, changes are also seen in the non-lesional WM, including astrocytic

proliferation, perivascular inflammation, blood brain barrier leakage, a certain degree of sclerosis of blood vessels and occasional demyelination [Allen and McKeown 1979; Albert *et al.* 2007; Miller *et al.* 2003]. Despite the relative preservation of axons and neuronal cell bodies in MS lesions, damage to both does occur, and this damage probably determines the long-term clinical manifestations of the disease [Trapp *et al.* 1998; Peterson *et al.* 2001]. Although demyelinated lesions are the most characteristic feature of MS pathology, changes also occur in non-lesional brain tissue, so-called normal appearing white matter (NAWM) [Zeis *et al.* 2008] and normal appearing grey matter (NAGM) [Albert *et al.* 2007].

1.3.1. *Lesion formation*

Current concepts of MS lesion formation are often based on findings derived from experimental allergic encephalomyelitis (EAE), an animal model that resembles certain features of MS [Mix *et al.* 2008]. Against this backdrop MS has been described as a disease that is primarily mediated by autoreactive T-cells (CD4+), which target specific epitopes in the CNS. It has been postulated that by crossing the blood brain barrier and coming into contact with target antigens, a cell mediated inflammatory reaction is initiated. Putative autoantigens include myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendroctye glycoprotein (MOG), myelin associated glycoprotein (MAG) and αB crystallin [van Sechel *et al.* 1999; Pender and Greer 2007]. Once activated, T-cells produce an array of pro-inflammatory cytokines, which stimulate other T-cells, B-cells, natural killer cells, macrophages and microglia that in turn augment and perpetuate the

inflammatory process. These cytokines promote increased permeability of the blood brain barrier, alteration of adhesion molecule expression, production of antibodies and recruitment of other cells of immune function into the CNS. The presence of macrophages and T cells (both CD4+ and CD8+) has been shown in the brain parenchyma of MS patients [Sospedra and Martin 2005] and clonotypic CD8+ T cells have also been described in the CSF [Jacobsen *et al.* 2002]. The environment in MS lesions is complex with roles for T cells (T_h1, T_h2 and Th17 CD4+ cells), cytotoxic CD8+ cells, macrophages, NK cells and microglia. Secretion of both pro and anti-inflammatory molecules; proteases, nitric oxide derivatives, reactive oxygen species, cytokines – IL-6, TNFα, IL-4 and IL-10, may occur in the same lesion and cytokines may be essential in CNS repair [Hohlfeld *et al.* 2007]. The discovery of T_h17 cells (CD4+ subset) has further advanced understanding of T cell regulation. These cells, stimulated by IL-23, secrete IL-17 along with IL-6 and TNFα, and EAE evidence suggests they play an important role in inflammation and lesion formation [Bowman *et al.* 2006].

1.3.2. *Neurodegeneration*

Whilst demyelination is the most obvious feature of the MS lesion, neurodegeneration has also been initially described 140 years ago [Charcot M 1868]. Damage to axons may be mediated directly by cytotoxic T-cells, macrophages, antibodies, loss of trophic support by oligodendrocytes and oxidative stress [Sospedra and Martin 2005; Sayre *et al.* 2008]. Axonal transection in MS lesions is well described in pathological studies, the frequency of

which appears to be related to the degree of inflammation in lesions [Trapp *et al.* 1998]. Loss of trophic support for axons and exposure to toxic metabolites leads to degeneration of axons. Redistribution of Na$^+$ channels, mitochondrial failure and Ca^{2+} mediated toxicity may all contribute to axonal degeneration in MS [Waxman 2006a; Waxman 2006b].

1.3.3.   *Lesion classification*

Several histological staging systems have been proposed to classify MS lesions in the WM. Commonly used classifications include the one that resulted from the Vienna consensus conference [Lassmann *et al.* 1998] and the staging system by van der Valk and de Groot [Van der Valk and De Groot 2000]. As a rule, demyelinated lesions in the WM are classified as active, chronic active or inactive. The relationships between remyelination (shadow plaques) [Prineas *et al.* 2001]) of formerly demyelinated lesions and lesion *activity* has yet to be clarified [Van der Valk and De Groot 2000]. Lesions in the MS grey matter have been variably classified – largely according to their location – into three, four or seven subtypes [Kidd *et al.* 1999; Peterson *et al.* 2001; Bo *et al.* 2003].

Recent pathological studies have promoted the hypothesis of lesion heterogeneity in MS with several lesion subtypes being described. The Lassmann/Luchinetti/Bruck collaboration described four different patterns of actively demyelinating lesions. Pattern 1 is characterised by a predominantly T cell and macrophage infiltrate, pattern 2 by antibody and complement deposition, pattern 3 by oligodendrocyte apoptosis and pattern 4 by non-apoptotic oligodendrocyte loss [Lucchinetti *et al.* 1996; Lucchinetti *et al.* 2000; Lucchinetti *et al.* 2004]. Whether these four patterns indeed allow distinction *between* four categories

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of patients (based on their lesion pathology), and whether the described lesion types are – as their exponents claim – unequivocally homogenous *within* a given patient, however, remains controversial [Esiri *et al.* 2009]. Another challenge to traditional views of lesion formation has emerged from the observation of oligodendrocyte apoptosis (in the relative absence of an inflammatory infiltrate), which has been interpreted as an early or initial step in lesion formation [Barnett and Prineas 2004]. According to this research oligodendrocyte apoptosis would *secondarily* lead to microglial activation and subsequent phagocytosis of dying/apoptotic oligodendrocytes. The immunoglobulin and activated complement deposited on myelin could also activate microglia, which in turn initiate a secondary immune response. Apoptotic bodies were only found in a minority of lesions and generally in those from subjects with early disease, which may reflect it being present either very early in lesion formation or a factor illustrating the heterogeneous nature of MS pathology. This hypothesis is however supported by reports of cortical demyelination in the absence of leukocyte infiltration [Peterson *et al.* 2001]. Axonal loss, which correlates more closely with disability, has been noted in the absence of focal inflammatory lesions [Lassmann *et al.* 2001]. Whether or not the axonal loss is independent of focal inflammation or due to Wallerian degeneration in response to inflammatory lesions along the axonal pathway has yet to be determined. Evidence in support of the latter hypothesis has recently been produced [Tallantyre *et al.* 2009; Frischer *et al.* 2009].

1.3.4.   *Remyelination*

Remyelination, a reparative process mediated by oligodendrocytes depends on numerous factors, including switching the balance of the inflammatory process in favour of production of immunomodulatory cytokines and growth factors such as IL-4, IL-10, Brain Derived Neurotrophic Factor (BDNF) and Transforming Growth Factor -β (TGFβ). Factors such as those mentioned above play a role in remyelination, which may, at least in part, underpin remission of symptoms and clinical signs between relapses. Research involving stem cell transplantation suggests there may be a need for a certain degree of inflammation to facilitate remyelination [Foote and Blakemore 2005]. Understanding the mechanisms underlying remyelination is key to developing treatments that will have an effect not only on the inflammatory but also on the neurodegenerative component of MS, and repair [Hartung *et al.* 2004; Hemmer *et al.* 2002; Franklin and Ffrench-Constant 2008].

1.4.   *Clinical Features*

The clinical presentation of MS reflects the impaired function at affected sites. Demyelination interferes with conduction of action potentials along axons, which results in the symptoms and neurological deficits experienced by patients. The conduction block caused by partial demyelination may be intermittent, which accounts for intermittent recrudescence of symptoms, for example in response to rises in body temperature (Uhthoff's phenomenon) [Saul *et al.* 1995]. The disease course is highly variable between subjects with 85-90% of patients experiencing a relapsing/remitting course, which over time becomes secondary progressive in the majority of patients. In a minority (10 -15%) the disease course is progressive and without "attacks" from the onset (primary progressive MS). Some patients (<5%) with an apparent primary progressive course will go on to experience superimposed relapses after a progressive onset (progressive-relapsing MS). A typical relapse frequency is between 0.8-1.2 per year, but this can be highly variable. Over time the relapse frequency declines [Compston and Coles 2008]. However, in all clinical subtypes disability eventually accrues over time. Fixed disability arises from either incomplete recovery from relapses, or insidiously in the non-relapsing primary or secondary progressive forms of the disease.
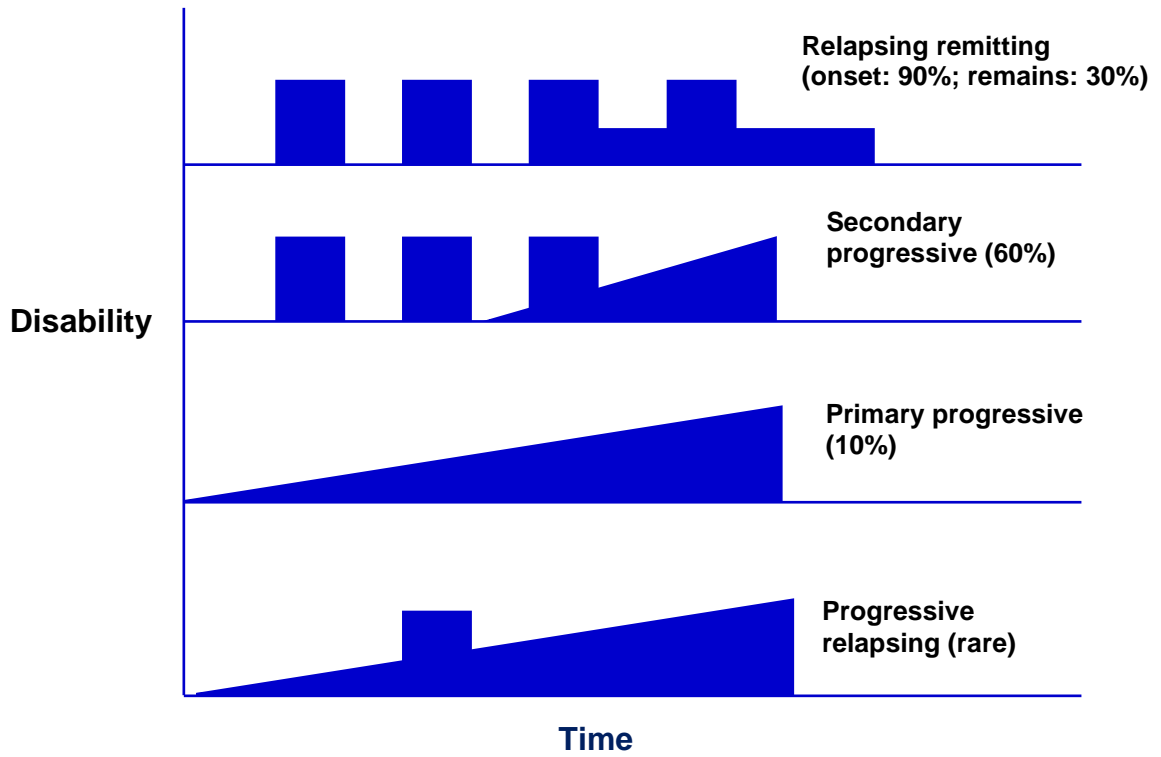
Figure 1-1 Clinical Course of MS

1.4.1.   *Diagnosis*

The diagnosis of MS requires demonstration of dissemination of the disease process in both time and space, and exclusion of other causes. The diagnosis is made clinically with the use of paraclinical tools, such as MRI [Figure 1-2], CSF analysis (showing intrathecal synthesis of oligoclonal bands or IgG) [Figure 1-3], visual, brainstem or somatosensory evoked potentials. The introduction of the McDonald criteria (2001) and subsequent revision have enabled an earlier diagnosis of MS following a single clinical event [McDonald et al. 2001; Polman et al. 2005]. Many patients initially present with an acute monosymptomatic episode of neurological dysfunction due to a single lesion, known as a clinically isolated syndrome (CIS) [Miller et al. 2008]. Most of these patients (~80%) will eventually develop MS [Fisniku et al. 2008]. The limited pathological examinations carried out on patients with CIS suggest that histological changes are similar to those in MS.
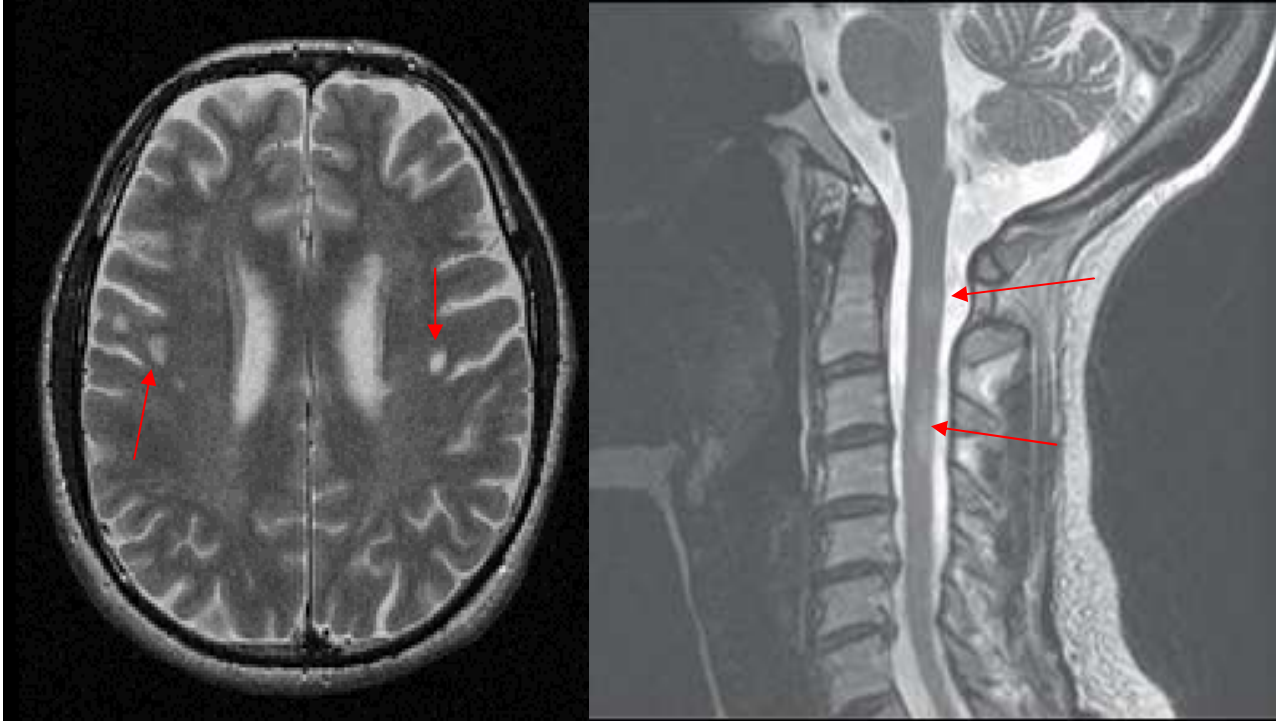
Figure 1-2 MRI is a useful tool in diagnosing MS
Hyperintense lesions in the brain and spinal cord (indicated by red arrows) are characteristic of MS
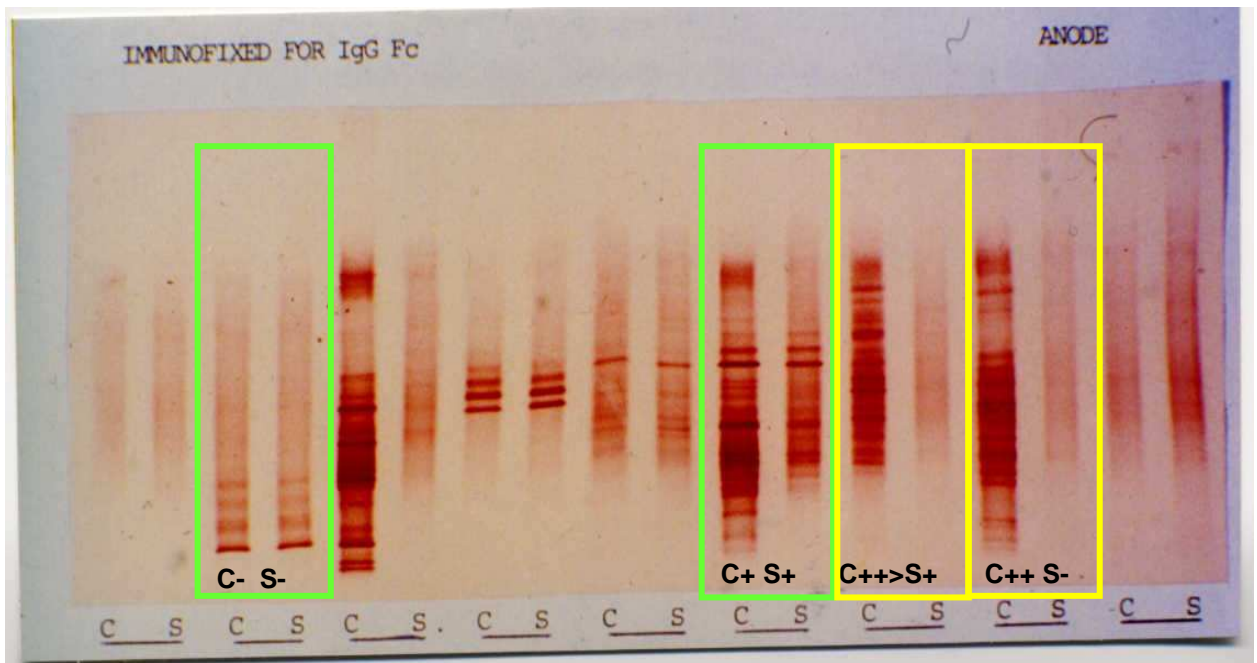
Figure 1-3 Immunoblot of oligoclonal bands
C represents CSF, S represents serum. Working from left to right blots are shown with (i) negative CSF and Serum (C- S-), (ii) matched bands in CSF and serum (C+ S+), (iii) CAF bands more numerous than serum bands (C++>S+) denoting central and systemic production and (iv) Unmatched bands in CSF not present in serum (C++ S-) which is typical of OCBs in subjects with MS
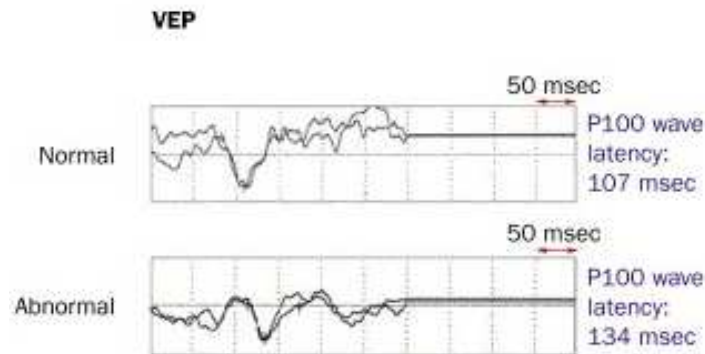
Figure 1-4 Visual evoked potentials.
Recordings of a subject with optic neuritis due to MS. The conduction is seen to be delayed in the lower panel which is consistent with demyelination

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1.5.   *Treatment*

First line disease modifying treatments (i.e. the beta interferons and glatiramer-acetate) have now been available in the UK for about 15 years. These drugs are on average partially effective in reducing the number of relapses by about one third and possibly delaying disease progression. Current opinion favours starting treatment early in the course of the disease, as neurodegeneration (for example brain atrophy) can be detected from the very first manifestations of the disease, and at least a proportion of these degenerative changes may be secondary to inflammation [Frischer *et al.* 2009a]. Hence, starting treatment early could potentially reduce the occurrence of relapses and subsequent development of disability [Jacobs *et al.* 2000; Kappos *et al.* 2007; Comi *et al.* 2001; Clerico *et al.* 2008]. Emerging treatment strategies take into account the different pathological mechanisms that have been identified in MS, in particular strategies to protect against demyelination and axonal loss. Treatments predating interferons generally and non-specifically suppressed the immune response, with sub-optimal outcomes. Although in hindsight the majority of clinical trials performed in the pre-interferon era were underpowered. Potential targets for treatment include immune dysfunction, permeability of blood brain barrier, components of the inflammatory cascade, putative autoantigens, demyelination, axonal loss (neuroprotection) and remyelination and regenerative processes (growth factors).

The complexity of decision making with respect to treatment choices in MS, continues to increase. The number of products on the market is steadily increasing. The effects of the

currently used first-line agents are modest but they have a very good safety profile that seems to have stood the test of time (IFNβ & Glatiramer Acetate). Two drugs that have more recently been licensed for use in MS are potentially more efficacious, but their use is limited by unfavourable adverse effect profiles (Natalizumab, Mitoxantrone).

The currently licensed disease modifying treatments for MS are:

- Interferon-β 1a IM (Avonex™ – Biogen-Idec)
- Interferon-β1a SC (Rebif™ - Merck-Serono)
- Interferon-β 1b SC (Betaferon™- Bayer-Schering)
- Interferon-β 1b SC (Extavia™- Novartis)
- Glatiramer Acetate SC (Copaxone™- Teva-Aventis)
- Natalizumab IV (Tysabri™ - Biogen-Idec)
- Mitoxantrone IV (Novatrone® - Merck-Serono USA or generic product in UK)

Other agents used in MS, but not specifically licensed for this disorder, include: Corticosteroids, Cyclophosphamide (Cytoxan®), Azathioprine (Imuran®), Methotrexate and intravenous immunoglobulin (IVIg). Reducing relapse rate probably prevents the acquisition of permanent disability caused by incomplete recovery from relapses, but this does not necessarily have an effect on the insidious progression that occurs independently of attacks. IFNβ and GA have both been shown to reduce relapse rates and MRI measures of disease activity. Although they are well tolerated their effectiveness is only partial, and none has been shown to be effective in primary progressive MS [Wolinsky 2004; Leary *et al.* 2003; Montalban 2004].

1.5.1.   Interferon beta

Interferons are a family of proteins which stimulate inter- and intra- cellular responses to regulate viral infections, modulate the immune response and cell survival. Type 1 interferons were originally developed as therapeutic agents in MS because of their anti-viral activity [Borden *et al.* 2007] and although this may partially explain their effectiveness in MS, they have numerous other immunomodulatory activities including altering the T_h1/T_h2 balance [Hussien *et al.* 2001], antagonising pro-inflammatory cytokines (IFNγ, IL-12 and TNFα), down regulating MHC class II expression - affecting antigen presentation [Yong *et al.* 1998], anti-proliferative effects on T cell expansion, differentiation and increased T cell apoptosis [Sharief *et al.* 2001; Yong 2002]. There is also evidence that type 1 interferons inhibit transmigration of immune cells across the blood-brain barrier (BBB) [Leppert *et al.* 1996]. Production of IFNs is a transient response to microbes including viruses, bacteria and protozoa. [Akira *et al*. 2006]. Interferons mediate their response by binding to their high affinity cell surface receptors and initiating a signalling cascade. The type 1 IFN receptor is composed of two subunits IFNAR1 and IFNAR2 which are associated with cytoplasmic tyrosine kinases TYK2 and Janus Kinase 1 (JAK1) [Uze *et al.* 2007]. The IFN molecule usually binds to IFNAR2 which then recruits IFNAR1 and induces autophosphorylation of JAK1 and subsequently STAT (signal transducers and activators of transcription). These inturn activate nucleotide sequences or interferon stimulated response elements (ISRE) in the promoters of several genes [Platanias 2005] [Figure 1-5].
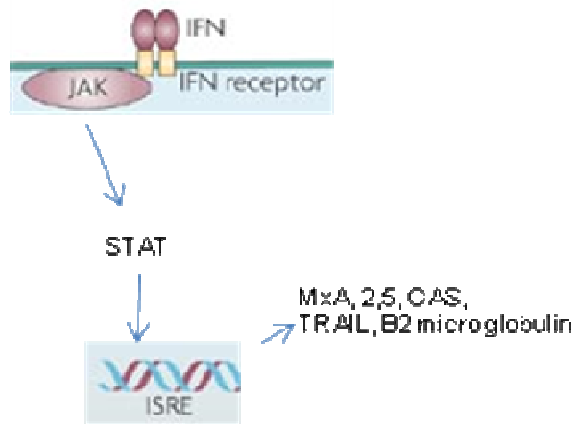
Figure 1-5 Interferon molecule interacting with IFN receptor

Two types of recombinant IFNβ products have been synthesised; IFNβ-1a is produced in Chinese hamster ovary (CHO) cells and is genetically identical to the human form of IFNβ. IFNβ-1b is produced in E. Coli, is truncated and contains one amino-acid sequence that differs from the human form, and is non-glycosylated. Several separate trials have been conducted for each agent, and more recently head-to-head trials have been published. On average all IFNβ products have been shown to reduce the annualised relapse rate by approximately one third [MS study Group 1993; Jacobs *et al.* 1996; PRISMS 1998]. MRI indices are also favourable with a 50 – 70% reduction in disease activity using conventional MRI markers of disease activity (Gd-enhancing T1 lesions and new or enlarging T2 lesions). Efficacy regarding disability measures has been variable, with some trials showing an effect, others being inconclusive, and others that did not include disability as an outcome measure [PRISMS 1998; PRISMS-4 2001; Jacobs *et al.* 1996]. The IFNβs have generally been well tolerated, and side effects included flu-like symptoms, injection site reactions, myalgia and – in a few cases – possibly depression. Various strategies have

been proposed to manage these side effects [Munschauer, III and Kinkel 1997]. Further adverse effects include abnormal liver function tests, anaemia, leukopenia and thrombocytopenia, all of which can be monitored with regular laboratory tests.

One of the most significant problems of IFNβ treatment is the production of drug specific antibodies. Up to 45% (mean ~ 25%) of patients develop neutralising antibodies (NAbs) to IFNβ products. NAbs may abrogate the clinical efficacy of IFNβs. Antibodies are more frequently induced by IFNβ-1b than 1a [Malucchi *et al.* 2004; Bertolotto *et al.* 2003; Cook *et al.* 2001; Panitch *et al.* 2002]. Of the two IFNβ-1a products the subcutaneous IFNβ-1a (Rebif®) is more immunogenic than intramuscular IFNβ-1a (Avonex®). The development of NAbs is a significant factor contributing to treatment failure, and the reduction in relapse rate in subjects who remain NAb negative may be as high as 50% [IFNbeta MSSG 1996]. In order to define the role of IFNβ in treating patients with MS, the impact of NAbs, and the response of neurologists treating MS patients with IFNβ to NAb status, it is important to be aware of the alternative therapies for MS, their efficacy and safety profiles. NAbs to IFNβ will be discussed in detail in the section following DMTs [chapter 1.6].

1.5.2.   *Glatiramer Acetate.*

Glatiramer Acetate (GA, Copaxone®), is a mixture of synthetic polypeptides that quantitatively resembles MBP, a putative autoantigen in MS. GA was found to suppress MBP-induced EAE [Teitelbaum *et al.* 2004]. Its mode of action is unclear but it has been shown to compete with MHC binding of MBP and limit activation of MBP reactive T-cells. A broad T-cell response is seen after GA injection, and it is therefore likely that GA also mimics antigens other than MBP. GA leads to a shift in the T-cell population towards a Th_2 cytokine response profile [Miller *et al.* 1998; Kim *et al.* 2004]. It has been proposed that these Th_2 cells migrate through the BBB into the brain parenchyma, where they are activated. As a result they produce immunomodulatory cytokines that counteract the pro-inflammatory Th_1 response, an effect called "bystander suppression". GA reactive T-cells have also been shown to secrete brain derived neurotrophic factor (BDNF), which has anti-inflammatory and neuroprotective functions [Stadelmann *et al.* 2002; Ziemssen *et al.* 2002]. In animal models of MS it has been shown that GA reactive T cells cross into the CNS and produce these factors [Aharoni *et al.* 2003]. Most of the described effects have been demonstrated in *in vitro* studies, and a number of questions with regard to GA's mechanism of action remain as yet unanswered. In the pivotal trial 20 mg of GA SC daily was shown to reduce relapse rates by ~ one third and also had a favourable effect on MRI outcomes; Gadolinium enhancing lesions and measures of brain atrophy however a significant effect was not seen on T2 lesions [Johnson *et al.* 1995, Ge *et al.* 2000]. Subsequent studies showed significant reduction of T2 lesions [Comi *et al.* 2001] and reduction of T1 black holes [Filippi *et al.* 2001]. Regarding adverse events injection site reactions are common with GA and lipodystrophy has been described [Drago *et al.* 1999].

The most common side effect (~ 15%) however is an idiosyncratic self limiting reaction of chest tightness, flushing, dyspnoea and palpitations which can be quite frightening for the individual but generally resolves quickly and spontaneously.

1.5.3.   *Natalizumab*

Natalizumab (TYSABRI®, Biogen-Idec/Elan) is a humanised monoclonal antibody, which targets α4-integrin, acting as a selective adhesion molecule inhibitor. The glycoprotein $\alpha_4\beta_1$ integrin is also known as very late antigen 4 (VLA4). It is expressed on the surface of lymphocytes and monocytes and plays an important role in cell adhesion and trafficking across the blood-brain and other endothelial barriers. VLA4 also acts as a regulator of activation of the immune system in areas of inflammation [Frenette and Wagner 1996a; Frenette and Wagner 1996b]. Natalizumab is given as a monthly infusion at a standard dose of 300mg. It is not clear exactly how Natalizumab exerts its clinical effect but is thought to be due to the significantly reduced migration of leukocytes into the CNS parenchyma.

Two phase III trials of Natalizumab have shown significant reduction in relapse rate and disease progression: The first trial (AFFIRM) involved treatment naive patients randomised to placebo (n=315) or 300mg Natalizumab (n=627) every 28 days for up to 28 months. The second trial (SENTINEL) involved patients who were established on IFNβ 1a 30μg IM once weekly (Avonex®) and had experienced one or more relapses in the previous year. Patients continued Avonex® therapy and were randomised to either 300mg

Natalizumab (n=589) or placebo (n=582) infusions every 28 days. In the AFFIRM study patients treated with Natalizumab had a 66% reduction in relapse rate compared with placebo, 96% of subjects receiving Natalizumab had no new Gd enhancing lesions as compared with 68% on placebo [Polman *et al.* 2006; Miller *et al.* 2007]. A reduction in new and enlarging T2 lesions was also found. In the SENTINEL study patients on both Avonex® and Natalizumab had a 54% reduction in annualised relapse rate as compared to placebo. With regards to MRI outcomes 96% of the combined treatment group had no enhancing lesions as compared with 76% of the Avonex® only group. Similarly, fewer patients developed new or enlarging T2 lesions, on combination therapy [Rudick *et al.* 2006].

However, at the end of February 2005 the use of Natalizumab was temporarily suspended after two patients receiving Natalizumab in combination with IFNβ-1a (Avonex®) developed progressive multifocal leukoencephalopathy (PML) one of whom subsequently died. A third subject with Crohn's disease whose death had been attributed to glioma was also confirmed as having PML. Since this time Natalizumab was relaunched in June 2006 and several patients have subsequently developed PML (43 as per March 2010) and many have been naïve to prior immunomodulatory treatments. Emerging evidence suggests that subjects treated for > 2 years are at highest risk (European medicines agency Jan 2010). Although many patients are now treated with Natalizumab worldwide, vigilance is required with regards to its safety profile. Other side effects include anaphylactic reactions (~0.8%) and other hypersensitivity reactions, increased risk of infection, headache, depression, arthralgia and rash. Approximately 10% of patients receiving

Natalizumab develop antibodies, which are neutralising *in vitro*, with about 6% persisting beyond 12 months of treatment [Calabresi *et al.* 2007]. Therapeutic effectiveness was reduced in those who remain persistently antibody positive and relapse rates returned to that of those on placebo in both trials. Infusion reactions were also found to be more frequent and severe in those remaining antibody positive. Consideration of the safety profile and immunogenicity of Natalizumab as another biological agent to treat MS are factors to consider in its use.

1.5.4.   *Corticosteroids.*

Corticosteroids are commonly used to treat acute relapses in patients with MS. They have potent anti-inflammatory, immunosuppressive and membrane-stabilising properties. They act by preventing disruption of the BBB, alter cell migration expression of adhesion molecules, decrease the production of pro-inflammatory cytokines and also promote apoptosis of auto-reactive T cells. All of these actions help restore the integrity of the BBB and reduce local inflammation and demyelination. A number of studies have shown that administration of high dose steroids (>500mg IVMP for at least 3 days) accelerates the recovery of disability, but does not influence the final outcome after a relapse or the risk of recurrent relapses [Brusaferri and Candelise 2000; Miller *et al.* 2000; Kapoor *et al.* 1998]. MRI studies have shown decreased number of T1 Gadolinium- enhancing lesions following steroid treatment [Barkhof *et al.* 1991; Miller *et al.* 1992]. A dose response effect has been noted, with high dose Methylprednisolone having more effect than low dose (<500mg/day) [Oliveri *et al.* 1998]. The beneficial effect of a single acute course of

corticosteroids lasts for a relatively short period of time, with MRI and other markers returning to baseline after approximately 2 months. Regular long-term administration of corticosteroids has recently shown a beneficial effect in RRMS and SPMS by reducing the accumulation of disability and MRI activity [Zivadinov *et al.* 2001; Goodkin *et al.* 1998]. High dose IVMP given at time of relapse to patients treated with IFNβ, shows sustained reduction of Gadolinium-enhancing lesions as compared to steroids alone [Gasperini *et al.* 1998].

Recently studies have explored the effect of corticosteroids in preventing the development of neutralising anti-IFNβ antibodies (NAbs) or minimising their levels. It has been shown that treatment with concomitant steroids delays the appearance of NAbs and reduces the eventual titre. Concomitant use of immunosuppressive therapies in combination with various biological products is associated with a lower titre or the reversal of NAbs. In an open labelled study of 161 MS patients, receiving IFNβ-1b (Betaferon®, 8MIU s.c. on alternate days), randomized to receive either intravenous methyl-prednisolone 1g monthly for 12 months compared to no corticosteroids the prevalence of NAbs at 15 months in the prednisone treated group was 12.1% compared to 26.8% in untreated group, a relative reduction of 54.9% [Pozzilli *et al.* 2002a]. The beneficial effect of short-term steroid administration in acute relapse justifies their use, but their modest long-term benefits are outweighed by an unacceptable side-effect profile, which includes reduced bone density, cataracts, weight gain, hypertension, diabetes, avascular bone necrosis and propensity to infection.

1.5.5. Immunosuppression

1.5.5.1. Mitoxantrone

Mitoxantrone (Novantrone®, Merck-Serono) was originally designed as a chemotherapeutic agent and is most commonly used in treating breast cancer. It is an anthracenedione, which inhibits topoisomerase-2 preventing the successful unwinding of DNA. It was shown to be effective in EAE [Lublin *et al.* 1987] and in both active RRMS and SPMS [Edan *et al.* 1997; Hartung *et al.* 2002; Le Page *et al.* 2008]. It is currently licensed in some countries (not the UK) for use in patients with aggressive relapsing MS who have failed first-line therapy. The mechanism of action of Mitoxantrone in MS may be mediated on a number of levels. It is immunosuppressive, inhibiting the proliferation of T cells, B cells and monocytes and reducing secretion of pro-inflammatory cytokines [Fidler *et al.* 1986], however it is non-specific in its immunosuppressive action. It is typically administered as IV pulses of 12mg/m^2 every 3 months for two years (Hartung protocol) or 20mg monthly for 6 months (Edan protocol). It has several serious side effects; most notably it is cardiotoxic and warrants monitoring of cardiac function [Ghalie *et al.* 2002]. In view of its cardiotoxicity the maximum recommended lifetime dose is 140mg/m^2. There have been several reports of therapy-related acute myelogenous leukaemia in patients with MS who have received Mitoxantrone [Brassat *et al.* 2002; Ghalie *et al.* 2002]. Typically therapy related leukaemia, with topoisomerease II inhibitors such as mitoxantrone develops within 2-4 years after chemotherapy has been started, and the prognosis is generally poor amongst the breast cancer patients, those with MS appear to fare better. Initially it was reported to occur in 0.07% patients receiving Mitoxantrone but recently Merck-Serono have released data describing two cases in 802 MS patients (0.25%) [Edan 2005], and more

recently other studies have described the risk as 1 – 2 % [Le Page *et al.* 2008]. The post marketing data in breast cancer has shown a higher risk in patients who have had a combination of chemotherapeutic agents. It has therefore been advised that strict adherence to blood count recommendations should be followed including complete blood count, white cell differential and platelets prior to each infusion (Prescribing information NOVANTRONE- MerckSerono). There is also a risk of premature ovarian failure or infertility, which may be permanent. Transient amenorrhoea occurs in ~12% of patients and persistent amenorrhoea in ~10% of patients. The risk of persistent amenorrhoea is higher in woman older than 35 years (14%) and lower in women less than 35 years of age (6.5%) [Edan 2003].

1.5.5.2. *Azathioprine*

Azathioprine (Imuran® FARO Pharmaceuticals, Inc.) is a purine analogue which interferes with DNA synthesis. It's exact mechanism of action is unknown but it is relatively T cell specific due to their lack of salvage pathway. It was previously widely used off-license to treat MS however, since the emergence of IFNβ and Glatiramer Acetate it is used less commonly. It is relatively T-cell specific but also suppresses both cell-mediated and humoral immunity non-specifically. Much of the data concerning its efficacy is inconclusive as many of the studies performed were on heterogeneous populations in the pre-MRI era. Meta-analyses of published studies show a trend of reduced relapse rates and slowing of disability [Yudkin *et al.* 1991; Palace and Rothwell 1997]. In general Azathioprine cannot be recommended for use in MS because of doubts about its efficacy.

The Avonex Steroids Azathioprine trial compared three treatment arms evaluating the use of add on oral methylprednisolone +/- azathioprine in patients who had failed first line treatments. Participants were followed for 3 years with a one year extension phase. An effect was found was the reduction in ARR in the triple therapy group vs dual-placebo arm (0.73 vs 1.05 p <0.05) and regarding T2 lesion volume at 2 years, but this effect was not sustained at 5 years [Hardova *et al.* 2009]. It may however have a role in combination with other therapies, for example IFNβ to treat or prevent the development of NAbs.

1.5.5.3. Cyclophosphamide

Cyclophosphamide (Cytoxan® Bristol-Myers-Squibb) is an alkylating agent and a potent cytotoxic and immunosuppressive drug which is commonly used in oncology but also in autoimmune conditions (vasculitis, lupus). Pulsed IV cyclophosphamide has been used in patients with evidence of active inflammatory MS however results have been conflicting. Early studies included a heterogenous group of MS patients and did not show significant benefit. Other trials however involving patients with very active disease showed significant improvement in relapse rate during treatment [Weiner and Cohen 2002]. Recent trials using pulsed IV Cyclophosphamide with IFNβ, showed significant clinical improvement during treatment and in the 18-month follow up period. Relapse rate declined significantly and was maintained for the follow-up period. MRI parameters also improved [Smith *et al.* 2005; Patti *et al.* 2001]. Due to its toxicity and risk of malignancy (transitional cell bladder carcinoma), it is only used as a second line agent. In general, the off-license use of cyclophosphamide for MS has been replaced by the licensed drug Mitoxantrone.

1.5.5.4. Methotrexate

Methotrexate (Rheumatrex® Lederle Pharmaceuticals) is an anti-metabolite with immunosuppressant properties. It confers its action by competitively inhibiting dihydrofolate reductase activity preventing thymidine synthesis and ultimately DNA synthesis. Its use in MS has been investigated in a number of small clinical trials and has been shown to slow disease progression in subjects with advanced chronic progressive (secondary and primary progressive) MS [Goodkin *et al.* 1995]. There remains a need for definitive placebo-controlled trials however with the advent of other oral therapies this is unlikely to occur.

1.5.5.5. Intravenous Immunoglobulin (IVIg)

IVIg is widely used in immune mediated systemic and neurological disorders and has been found to be of benefit in some aspects of MS [Achiron *et al.* 2004; Dalakas 1998]. A number of open label studies were initially performed followed by four randomised-placebo controlled trials. Most have addressed its use in relapsing remitting MS and one more recently in SPMS. The four placebo controlled trials did show a reduction in relapse rate and number of gadolinium enhancing MRI lesions [Achiron *et al.* 1998; Sorensen *et al.* 1998; Fazekas *et al.* 1997 & 2008].The European Immunoglobulin in MS group recently concluded a study involving patients with SPMS and no clinical or paraclinical benefit was found [Hommes *et al.* 2004]. IVIg was also investigated with regards to long-term visual outcome in patients with acute optic neuritis and no difference in outcome was found

[Roed *et al.* 2005]. The use of IVIG is not recommended in MS in view of the uncertainty about its impact on the more objective MRI-based outcome measures, in comparison to its apparent effect on relapse rate, and its relatively high cost and limited availability.

1.6.   *Binding and Neutralising antibodies*

The production of anti-drug antibodies has been described during treatment with a variety of biological products. This phenomenon is well described with agents such as Insulin [Fineberg *et al.* 1983], Factor VIII [Bray *et al.* 1994], erythropoietin [Casadevall *et al.* 2002], botulinum toxin [Goschel *et al.* 1997] and also with interferon alpha and beta [Antonelli *et al.* 1996; Vallbracht *et al.* 1981]. Naturally occurring proteins, which are genetically altered or synthesised in non-mammalian cells, may have subtle changes in their structure, which trigger a loss in immune tolerance. The rate at which these occur is related to the immunogenicity of the protein – defined as the ability of a protein to evoke an immune response against it. The antibodies induced can be binding or neutralising. A neutralising antibody (NAb) binds to the biological agent preventing its interaction with its receptor (or – in the case of enzymes - its active site), thus inhibiting or neutralising the biological action of the drug/protein. A binding antibody (BAb) does not significantly affect the action of the molecule and, hence, is therefore commonly called non-neutralising antibody. Non neutralising auto-antibodies are thought to possibly function as carriers, preventing the rapid elimination of cytokines from the circulation, thus increasing their bioactivity [Durelli and Ricci 2004] or in the case of some molecules increasing their elimination.

1.6.1. Anti-Interferon Beta Antibodies

As described in section 1.5.1 IFNβ is an endogenous cytokine which is secreted by a variety of cells – T cells, B cells, macrophages, endothelial cells and fibroblasts, in response to microbes (viruses and bacteria in particular). It alters the expression of numerous genes and this confers its anti-viral, anti-proliferative and immunoregulatory activities. There is some redundancy in its activities which may also be mediated by other cytokines [Borden and Wadler 1996]. Low levels of naturally-occurring antibodies directed against cytokines are found in the circulation of individuals who have never been exposed to a biological therapy and are thought to play a regulatory role in the intensity and duration of the immune response [Morgensen 1981, Fomsgaard 1989, Ross 1990, Bendtzen 1994]. As with the majority of biological products IFNβ can also induce the production of antibodies. BAbs can be detected in 50 – 80% of treated patients [Scagnolari 2002, Deisenhammer 1999, Kivisakk 2000]. The reasons that some subjects develop NAbs and others only BAbs are unclear. Those who develop NAbs – but not all cases – tend to have higher BAb levels indicating that qualitatively NAbs differ from BAbs, in that BAbs bind with lower affinity to the IFNβ molecule than NAbs [Gibbs and Oger 2008].

There has been much controversy with respect to the significance of these antibodies in patients with multiple sclerosis treated with IFNβ and how to manage them [Hartung and Munschauer, III 2004; Farrell and Giovannoni 2007]. The therapeutic efficacy of IFNβ has been shown in trials to be diminished by the presence of neutralising antibodies, which form in 2-45% of patients, depending on the product used. Neutralising antibodies may be detected as early as 3 months after commencement of therapy, but usually appear between 6 –18 months. Binding antibodies occur earlier. It has been shown in numerous trials that

patients who become antibody positive, have higher relapse rates, lesion activity on MRI and also higher rate of disease progression (this is discussed in more detail in later sections).

1.6.2. Immunological mechanisms underlying NAb development

Factors which affect the development of antibodies remain obscure but may include production and purification methods, dose and frequency of administration and potentially heterogeneity of the immune response in subjects with multiple sclerosis. IFNβ-1a preparations (Avonex® IM, Rebif® SC) are synthesized in hamster ovary and glycosylated, as is the endogenous human form; the specific glyosylation pattern, however, may differ. IFNβ-1b (Betaferon®, SC) is synthesized in *Escherichia coli* and has a different amino acid sequence to the human form with deletion of methionine at position one and a cysteine to serine mutation at position 17 and is non-glycosylated. These changes lead to it being less hydrophilic and more prone to aggregation, resulting in a higher dose required to achieve bio-activity similar to the IFNβ-1a preparations. These differences are likely to play a role in the relatively high immunogenicity of IFNβ-1b compared to IFNβ-1a. However, the above mentioned differences do not explain the difference in immunogenicity between IFNβ-1a products. Other issues such as production methods, purification, storage and dose also contribute. There is no clear evidence that the route of administration impacts on immunogenicity. Perini *et al* conducted a study, which compared IFNβ-1b 250μg (8 miu) IM once weekly vs. SC every other day. The rate of antibody production was slower in the IM group and NAb titres were lower [Perini *et al.*

2001]. However, higher frequency of dosing and cumulative weekly dose in the SC group could have affected this. Another study which compared 22µg Rebif IM once or twice weekly with 22µg Rebif SC three times weekly showed no difference in NAb frequencies [Bertolotto *et al.* 2002]. Other studies have addressed these issues but have used different products to compare IM and SC routes and thus results are difficult to interpret [Ross *et al.* 2000].

1.6.3. Frequency of NAb production

1.6.3.1. Avonex

Avonex IFNβ-1a (30µg IM once weekly) has the lowest rate of NAb production; rates are generally between 2 and 6% [Panitch *et al.* 2002; Clanet *et al.* 2002; Jacobs *et al.* 1996]. The original formulation used in the pivotal trial was more immunogenic than the commercial products that are currently on the market. It is unclear why this was the case, but it probably relates to modifications to its synthesis, purification and formulation. The rate of NAbs in the EVIDENCE trial using the freeze-dried Avonex preparation was 2% [Panitch *et al.* 2002]. Currently Avonex is available as a freeze dried vial 30µg which is reconstituted with 1mL sterile water or as a prefilled syringe 30µg in 0.5 ml. The immunogenicity of the new prefilled syringe preparation was assessed in a recent study; at 2 years 2% of patients had developed NAbs, which is similar to the data using the freeze-dried product are the same Table 1-1[Phillips *et al.* 2004].

1.6.3.2. *Rebif*

The PRISMS trial using freeze-dried Rebif IFNβ-1a (22 μg or 44 μg SC three times weekly) found that the NAb rate on high dose or 44 μg Rebif was lower than patients on 22 μg (12.5% on Rebif 44 μg vs. 23.7% Rebif 22 μg after 2 years); at the end of the 4 year extension phase 24% of patients on Rebif 22μg had developed NAbs as compared to 14% Rebif 44 μg, by their last visit [Francis *et al.* 2005j]. Subjects who had originally been treated with placebo and subsequently switched to either 22 μg or 44 μg arms had similar rates on NAb during years 3-4 (both ~ 28%) however persistent antibodies were noted in 24% of patients receiving 44 mcg tiw and in 23/83 (28%) patients receiving 22 µg tiw [Oger *et al.* 2005]. The earlier OWIMS study, published in 1999, showed a dose response in that patients receiving Rebif 44μg once weekly developed NAbs sooner and had a higher rate of positivity at the end of the trial; 5% of patients on Rebif 22μg were NAb positive as compared with 16% on Rebif 44μg once weekly [OWIMS 1999]. However this study was only 48 weeks duration and was probably too short to evaluate the natural history of NAbs or their impact on disease course. NAb frequencies vary between 15 to 25% in most studies.

Efforts have been made to reduce the immunogenicity of Rebif by altering its production methods - free from human serum albumin and produced without fetal bovine serum, called Rebif new formulation (RNF). Recently RNF and EVIDENCE studies have published NAb rates with NAbs at both 24 and 48 weeks of 2.5% (95% CI, 0.9-5.5) and 14.3% (95% CI, 10.7-18.6), respectively [Giovannoni *et al.* 2007]. In the 96 weeks results the proportion of neutralizing antibody-positive (NAb +ve) patients at week 96 was 17.4% (exact 95% confidence interval [CI]: 13.0-22.5), compared with 21.4% (95% CI: 17.2-

26.2) in the EVIDENCE study, and 27.3% (95% CI: 22.8-32.1) in the subsequent REGARD study. These results suggest that RNF had a lower immunogenic potential compared with the approved IFNβ-1a formulation assessed in the EVIDENCE and REGARD studies [Panitch *et al.* 2002; Mikol *et al.* 2008].

1.6.3.3. *Betaferon*

Betaferon has generally shown the highest rates of NAb formation (25-47%). In the IFNβ study group (1993) after three years patients on low dose IFNβ 1b (1.6 MIU) had NAb rates of 47% and those on higher dose (8 mIU) had a rate of 45% [MS study Group 1993]. Further data published in 1993 compared IM and SC routes with 8 mIU/AD and NAb rates were 35% in the SC group and 41% IM [1996]. Therefore it did not show a clear dose or route dependent effect. The more recent European trial using an MxA assay showed NAb rates of 27.8% in the 8 mIU / AD SC dose group as compared to placebo [Table 1-1].

Trials	Product/dose	Assay method	NAb positivity	% positive
[Jacobs et al 1996] (MSCRG)	30μg Avonex IM OW	Not reported	No definition given	22%. Production altered afterwards.
[Rudick *et al.* 1998a] (MSCRG)	Avonex 30μg IM OW	CPE	≥ 20 x 1 titre	6%
[Jacobs *et al* 2000] (CHAMPS)	Avonex 30μg IM OW	CPE	≥ 20 x 1 titre	2%
[Clanet *et al* 2002]	Avonex 30μg IM OW.	CPE	≥ 20 x 1 titre	2.3%
	Avonex 60μg IM OW.			5.8%
[PRISMS 1998b]	Rebif 22μg SC TTW.	CPE	Titre ≥20 at end of study.	23.8%
	Rebif 44μg SC TTW.			12.5%
[EVIDENCE 1999]	Rebif 22μg SC OW.	CPE	Titre ≥20	5.3%
	Rebif 44μg SC OW			16.3%
[PRISMS 4 2001]	Rebif 22μg SC TTW.	CPE	Titre ≥ 20 at end of trial.	
	Rebif 44μg SC TTW.			23.7%
	Placebo group			14.3%
	Rebif 22μg SC TTW from year 3			27.7%
	Rebif 44μg SC TTW from year 3			28.0%
[Interferonβ MS study group 1996]	Betaferon. 1.6MIU SC AD 8MIU SC AD	CPE	1 Titre ≥ 20	47% 45%
[Interferonβ MS study group 1996]	Betaferon 1.6MIU SC AD 8MIU SC AD 8MIU IM AD	CPE	2 consecutive titres ≥ 20.	42% 35% 41%
[European study Group 1998a]	Betaferon. 8 MIU SC AD	MxA protein	2 consecutive titres ≥ 20	27.8%

Table 1-1 Frequencies of NAbs in the pivotal and dose comparison trials

1.6.4. Comparing NAb frequencies

Frequencies of NAbs in pivotal studies are shown in [Table 1-1]. However, comparing rates of NAb development between individual studies poses several problems. The recommended weekly dose of IFNβ-1a IM (Avonex) is 6 mIU as compared to 12 mIU IFNβ-1a SC three times weekly (Rebif 44 μg - total 36 mIU weekly) and 8 mIU IFNβ-1b every other day (250 μg Betaferon – total 28 mIU weekly). There are large differences in the potency of each preparation and the amount of protein delivered in each dose. 8 miu Betaferon contains 250 μg, 6 mIU of Rebif contains 22 μg and 6 mIU Avonex 30 μg of protein. The specific activity of a protein describes the relationship between the absolute weight of the protein and its biologic activity and is measured in IU/weight. The specific activity of Betaferon is 32 mIU/mg, Rebif 273 mIU/mg and Avonex 200 mIU/mg [Deisenhammer *et al.* 2000]. The IU given by the manufacturers may not be comparable as they are measured using different IFN standards. This was illustrated by Deisenhammer *et al* who directly compared the potency of each product by quantifying each preparations in vivo activity in patients treated with IFNβ without NAbs. They found a significant increase from baseline of MxA expression with 10 IU/mL Betaferon as compared to requiring 100 IU/mL of Rebif or Avonex to achieve a similar response. However, if this was adjusted relative to IFNβ weight Rebif had the highest specific activity (called 100%), Avonex 93% of this and Betaferon 71-80% [Deisenhammer *et al.* 2000].

It is difficult to compare the rate of NAb positivity between studies as different assays and acceptance criteria have been used to define antibody positivity. It is therefore important to consider the results of comparative trials [Table 1-2].

Comparative trials	Product/dose	Assay method	NAb positivity	% positive
[Panitch et al 2002]	Avonex 30μg IM OW Rebif 44μg SC TTW	NR	Titre ≥ 20 at any time.	2% 25%
[Ross et al 2000]	Avonex 30μg IM OW Rebif 22μg SC OW or TTW Betaferon 8MIU AD	CPE. Varying amount IFN added altered sensitivity of assay		Depended on sensitivity of assay Betaferon > Rebif > Avonex.
[Bertolotto *et al.* 2002a]	Avonex 30μg IM OW Rebif 44μg SC OW or TTW Betaferon 8MIU AD	CPE	2 x titre ≥ 20	2% 15% 31%
[Kivisakk *et al.* 2000]	Avonex 30μg IM OW Betaferon 8MIU AD	CPE	Titre ≥ 20	5% 44%

Table 1-2 Frequencies of NAbs in comparative studies

1.6.5. Effect of anti-IFNβ NAbs on efficacy in MS - Clinical outcomes and MRI

There is ongoing debate about the clinical significance of Nabs amongst the MS community. Studies, which are of short duration, may not detect the negative effect NAbs have on IFNβ efficacy. However, there is now sufficient evidence in the literature to support the view that NAbs reduce or eliminate the *in vivo* bioactivity of IFNβ – termed antibody mediated decreased bioactivity. The appearance of NAbs generally occurs between 6 and 18 months after commencing IFNβ therapy. Their impact on clinical efficacy lags further behind and is generally not seen in trials of less than 2 years duration. The findings of the pivotal trials are discussed below.

Two papers were published by the MS Study group showing efficacy of IFNβ-1b in reducing relapse rates and disease activity on MRI [MS study Group 1993; Paty and Li 1993]. A follow up paper was published to discuss the impact of NAbs in more detail. In the treatment arm 35% of subjects became NAb +ve by 18 months. Those who were NAb +ve had higher annualised relapse rates from month 13 – 36 than those who remained NAb –ve (1.08 vs. 0.56 $p < 0.05$). No difference in sustained progression (using EDSS) was found. With regards to MRI parameters those who were NAb +ve had higher accumulation of new lesions than NAb –ve during the 3rd year (1.03 vs. 0.4 $p<0.05$). In this paper the authors' concluded that NAbs did reduce the clinical efficacy of IFNβ.

The more recent BECOME study compared efficacy of IFNβ-1b with Copaxone and included analysis of the effect of NAbs on MRI outcomes. The incidence of NAbs and effect of NAbs on bioactivity were consistent with previous studies. MRI outcomes in

patients with NAbs at levels high enough to abolish bioactivity relative to patients without NAbs were analysed. Those who had preserved bioactivity, the ratio of enhancing lesions per scan decreased from 7.6 in the pretreatment period to 2.6 in the post-treatment period, which was a 66% decrease. For the lost bioactivity group, the reduction was only from 8.5 to 5.8, a 32% decrease. Thus, lost bioactivity from high levels of NAbs resulted in reduced therapeutic efficacy of IFNβ-1b as manifested by diminished reductions in enhancing lesions on MRI [Pachner *et al.* 2009a].

The 4-year extension phase of the PRISMS study showed a significant reduction in relapse rate and MRI activity in the patient group on high dose Rebif. This effect was lost in those who developed NAbs [PRISMS-4 2001]. The relapse rate was 0.5 for Rx 44μg (Rebif treatment dose) patients who were NAb -ve and 0.81 in those NAb +ve (increase of 62%). There was also a significant difference in the number of T2 lesions on MRI. Median number of lesions was 0.3 in those who were NAb -ve and 1.4 in the NAb +ve group. This annual increase in the T2 burden of disease (BOD) was similar to that seen in the 2-year placebo arm of the study.

Further analysis of this data evaluated 368 of the original patients with regards to NAbs [Francis *et al.* 2005]. In this analysis the majority of NAbs developed within the first 12 months of treatment. Thirty percent of Rx 22μg group and 19% of Rx 44μg group had a positive NAb result. At 12 months relapse rates were the same at ~ 0.9, thereafter the NAb -ve cohort showed a steady linear decline in relapse rates over the next 36 months to 0.35. The NAb +ve group however, had a fluctuating course with relapse rates consistently higher than NAb -ve and similar to the placebo group. With regards to time to confirmed progression by one point on the EDSS scale, in the NAb +ve group 44% had confirmed

progression compared to 40% of the NAb -ve cohort.

Using interval positive analysis, however, a significant difference was found between the groups: NAb +ve /NAb -ve progression rate ratio 1.50, 95% CI 1.03 – 2.17 p = 0.03. A significant difference was also seen in T2 MRI lesion load between NAb -ve and NAb +ve groups. Median lesion load was 0.3 (mean 0.1) in the NAb –ve group and 1.4 (3.2) for the NAb +ve group. The median cumulative percentage change in T2 lesion burden from baseline to years 2 and 4 was -7.2% and -8.5% in the NAb –ve group receiving 44µg tiw and 12.5% and 17.6% for the NAb +ve patients.

The finding that NAb +ve subjects tend to have fewer relapses in the first 6 months has also been described in an independent longitudinal study. They describe a similar finding of reduced RR in the first 6 months in the NAb positive cohort but that after 6 months this reverses and they experience higher RR [Sorensen et al 2007]. This finding is interesting as it implies that those who develop NAbs may do better in the first year of treatment than those who remain NAb -ve in the longer term. This would suggest a difference in the immune response of subjects, who develop NAbs, to treatment. An association has been described between NAb development and a MHC class II allele (DRB1*0701) and indicates a role for T cell activation in IFN-beta immunogenicity [Barbosa *et al.* 2006], however further investigation is required. The other hypothesis is that BAbs, which precede the development of NAbs may extend the half-life of circulating IFNβ thereby increasing its bioavailability [Sorensen *et al.* 2007]

The European dose comparison trial evaluated the clinical significance of NAbs (IFNβ 1a 30µg vs. 60 µg IM OW) [Kappos *et al.* 2005]. Patients were followed from baseline and

at three monthly intervals for 48 months. Samples were evaluated for BAbs and NAbs (in BAb positive patients). They found a higher proportion of NAb +ve patients in the high dose Avonex group. NAb +ve patients had higher (39%) relapse rate from months 12 to 48. NAb positive patients EDSS score progressed by a mean of 0.89 over 4 years compared to 0.29 in the NAb negative group. With regards to MRI findings, both T1 and T2 lesion load was higher in the NAb +ve group from 24 months to the end of the study. This study is very important as it further clarifies the significance of NAbs. It also highlights the need to design long-term studies as the clinical impact of NAbs appears to become more apparent with time and lags behind the initial appearance of NAbs.

1.6.6.   *Use of Biomarkers in monitoring IFNβ activity*

IFNβ affects the expression of several hundred genes and their downstream products. Many of these can also be induced by other factors. Some, however, have been identified as being relatively specific to type 1 IFNs. MxA is a good example of this [Deisenhammer *et al.* 2000c; Pachner *et al.* 2003b,c]. Others include Oligoadenylate synthetase (OAS) [Pachner *et al.* 2003b], Neopterin [Cook *et al.* 2001] and TRAIL (tumor necrosis factor apoptosis inducing ligand) [Wandinger *et al.* 2003; Gilli *et al.* 2006], but these are not exclusively induced by IFNβ. These have been used to show that the presence of NAbs reduces the bioactivity of IFNβ *in vivo*. A significant rise in MxA mRNA and protein has been shown in response to IFNβ administration and that this response is lost in the presence of NAbs [Bertolotto *et al.* 2003; Gilli *et al.* 2005]. In the case of mRNA levels, this response has been shown to be maximal at 12 hours. Similarly the MSCRG (1998)

showed failure to induce neopterin in the NAb positive cohort [Rudick *et al.* 1998].

Other biomarkers such as β-2 microglobulin and neopterin have been measured in relation to NAbs and in the presence of NAbs failed to show any significant response to IFNβ [Francis *et al.* 2005h]. Another study measured the *in vivo* response to IFNβ by measuring pre and post dose levels of MxA mRNA, neopterin and β_2- microglobulin. Responses were compared between NAb +ve and NAb – ve cohorts. Both pre and post dose levels were lower in NAb +ve subjects and a significant inverse correlation was found between NAb titre and pre dose levels in particular. The shows that the steady state levels are significantly suppressed in NAb +ve patients and in a titre dependent fashion [Scagnolari *et al.* 2007].

TRAIL [Wandinger *et al.* 2003] and OAS [Pachner *et al.* 2003b] have also been shown to produce a reliable response to IFNβ and failure of induction has been found in subjects who are NAb +ve [Bertolotto *et al.* 2003; Deisenhammer *et al.* 1999; Malucchi *et al.* 2004; Pachner *et al.* 2003c;Vallittu *et al.* 2002]. This loss of response to IFNβ predates any clinical evidence of loss of efficacy and could thus be used as a predictor or biomarker of impending treatment failure in those patients who show persistent antibodies.

The Impact of Neutralizing Antibodies on Interferon Responsive Genes Highlights Biomarker Response (INSIGHT) study was specifically established to examine the effect of antibodies to IFNβ on in vivo response of highly specific biomarkers for IFNβ binding to IFNAR: MxA, viperin, and Interferon-induced protein with tetratricopeptide repeats 1 (IFIT-1) [Pachner *et al.* 2009b]. In this study 718 patients with MS treated with any IFNβ for at least 12 months were included - IFNβ-1a 30 µg once weekly, subcutaneous (SC) IFNβ-1a 22 µg or 44 µg 3 times weekly, or SC IFNβ-1b 250 µg every other day. Binding

antibodies were evaluated using the capture ELISA (cELISA) and NAbs were measured using the viral cytopathic effect assay. Biomarker induction was compared between those who were BAb +ve / NAb +ve (n=149) vs. BAb –ve/ NAb -ve (n = 498) vs. BAb +ve/ NAb –ve (n = 71). MxA, IFIT 1 and viperin responses were all significantly lower in the BAb +ve NAb +ve groups as compared with those in the BAb -ve / NAb –ve arm. The reduced bioactivity was also found to be titre dependent with complete suppression of biomarker response in those with titres > 100 NU (CPE assay). This study confirmed in a large cohort the findings of other groups who have correlated NAb titres with biomarker induction or IFNβ gene induction [Sominanda *et al.* 2008; Hesse *et al.* 2009].

1.6.7. Measurement of BAbs and NAbs

1.6.7.1. Binding antibody assays

Neutralising antibodies are by definition also binding antibodies and as screening for BAbs was generally considered less troublesome, it was recommended that one should screen for BAbs before testing neutralising activity. There are several ways to screen for BAbs; ELISA, western blotting, radio-immunoprecipitation (RIA) or affinity chromatography. ELISA is the most commonly used method and this can be performed in two ways, with and without a capture antibody. Recently these methods have been compared in a cohort of 325 patient samples. Capture and direct ELISAs performed equally well, however the capture ELISA had a better correlation with NAb titres [Gneiss *et al.* 2008]. These assays and findings are described in more detail in chapter 2.

1.6.7.2. Neutralising antibody assays

IFNβ has diverse actions *in vivo* altering the expression of many genes at the nuclear level which confers antiviral, anti-proliferative and immunological actions. Neutralising assays are therefore based on quantifying these functions and their inhibition in the presence of NAbs. IFNβ binds to its cell surface receptor causing it to dimerise and activate the intracellular signaling mechanism. These transcription factors translocate to the nucleus where they bind to and activate the interferon stimulated response element (ISRE), which induces the transcription of numerous genes whose end products can be measured, such as MxA, neopterin, OAS, TRAIL and β_2 microglobulin. In the presence of NAbs, it is proposed that IFNβ fails to bind to and activate its receptor and thus the downstream

effects are lost. Therefore, the antiviral effect or the IFNβ induced gene products can be used in assays to measure the bioactivity of IFNβ. Problems, however, exist with regards to reliable measurement of neutralising antibodies. At the time this work was commenced no standardised assay, which could be applied reliably in all laboratories existed. The "gold standard" assay to measure NAbs was the Cytopathic Effect Assay (CPE) recommended by the World Health Organisation [WHO expert committee on Biological Standardisation 1985]. Guidelines have been developed by the EFNS task force, which recognise the validity of a cell based assay measuring MxA mRNA or protein which may be more widely used in the future [Sorensen *et al.* 2005a].

1.6.7.3. Cytopathic Effect Assay

This assay makes use of the anti-viral effect of IFNβ. Virus sensitive cells are seeded in micro titre plates and a known amount of IFNβ is added with serial dilutions of the patient's serum. This is incubated for 12 to 24 hours, after which the virus is added. After a further 24 hours viable cells are quantified by staining with crystal violet in 20% ethanol. The ability of IFNβ to inhibit viral replication is measured. The antibody titre is calculated using the Kawade method [Kawade 1986]. The unit of measurement is the lab unit (LU), which is defined as the amount, which reduces the cytopathic effect of the virus by 50%. The dilution of patient's serum, which reduces the activity of 10LU/mL of IFNβ to 1LU/mL, is then determined. The reciprocal of this value is the neutralisation titre of the serum. A concentration value of ≥ 20 LU/mL is generally considered a positive titre although this is an arbitrary figure [Grossberg *et al.* 2001 a & b].

In an attempt to standardise this assay the WHO have recommended the use of human

lung carcinoma cells (A549) and encephalomyocarditis virus (EMCV). Use of different cells and viruses has meant that results cannot be directly compared between labs. Variation in the amount of IFNβ used in the assay also alters the sensitivity of the assay [Ross *et al.* 2000; Sorensen *et al.* 2003a]. Adding too much will reduce the sensitivity and too little will increase the sensitivity of the assay. It is therefore crucial that the amount of IFNβ added reflects the in vivo environment. It is not clear what that level should be but the WHO currently recommends 10 LU/mL. Another difficulty is that the assay is not specific. If any other factor with antiviral properties is present it may cause the reduction in the cytopathic effect of the virus instead. The CPE assay is also relatively time consuming and prone to inter assay variability.

1.6.7.4. Quantification of Biomarkers

More recently assays which quantify either MxA protein or MxA mRNA have been developed and validated in relation to the CPE assay. These assays make use of the fact that certain proteins are induced, in response to IFNβ, in a dose-dependent manner. Therefore, after stimulating the cells with a known amount of IFNβ, the amount of MxA protein or mRNA produced is measured. MxA protein can be measured by sandwich ELISA, chemoluminesence [Kob *et al.* 2003] or fluorescence activated cell sorting (FACS) [Pachner *et al.* 2003c; Vallittu *et al.* 2002]. Cells are pre-incubated with serial dilutions of patient's sera. The presence (and titre) of neutralising antibodies is quantified by measurement of the MxA produced, by ELISA or alternatively MxA mRNA is quantified by PCR. Whilst less time consuming than the CPE assay this is also subject to the problem of inter-assay variability.

Results comparing three PCR methods to measure MxA mRNA and evaluate response to IFNβ have been published. PBMCs were isolated from blood and RNA extracted and reverse transcribed to generate cDNA. Quantitative competitive PCR, real time PCR and semi-quantitative PCR were used and NAb titres compared with the CPE assay. All three methods showed high specificity for MxA analysis [Gilli *et al.* 2004]. Bertolotto et al have modified this assay mixing patient's serum with IFNβ (known amount) and adding in serial dilutions to a plate seeded with cells. This is incubated for 6 hours, cells are then lysed and MxA mRNA extracted. cDNA is produced and quantified by real time PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is also measured as a housekeeping gene at the same time to account for any cytotoxic agents, which may inhibit/lyse the cells. This new assay is less time consuming and has been validated with respect to the CPE assay and MxA protein assay [Bertolotto *et al.* 2007]. It is however expensive to perform, which could potentially limit its accessibility to clinical practice.

1.6.7.5. Titre Calculations

Most titres are calculated using a specific eponymously named method: the Kawade method. To date no consensus has been achieved to define what constitutes a clinically relevant titre. Generally the cut off is ≥ 20 neutralising units, but a variety of endpoints have been used in trials. It has also been shown that the bio-efficacy of IFNβ may be reduced with a NAb titre < 20 [Pachner *et al.* 2003b]. Guidelines regarding NAb testing, assay validation and clinical implications of a positive result have failed to form a consensus regarding a significant titre. The European group recommended in 2005 that measurements of binding and neutralising antibodies to IFNβ should only be performed in

specialised laboratories. Use of a validated CPE assay is still the gold standard assay and A549 cells should be used with a fixed amount of IFNβ which should be the same product that the patient is using. The stimulated cells can be challenged with the EMC virus or MxA production measured. Standard curves should be included using increasing amounts of IFNβ until saturation is reached. Titres above 20-60 are associated with loss of IFNβ bioactivity (using CPE assay and IFNβ-1b). In these guidelines however a recommendation is made to switch or stop treatment in subjects with persistent titre > 100 NU. It has also been acknowledged that there still remains a need for simpler assay methods such as quantifying the in vivo biological response [Sorensen *et al.* 2005a].

1.6.8.   *Not all neutralisation is due to NAbs*

A small number of patients exhibit neutralisation of IFNβ activity in the absence of antibodies to IFNβ. This has been associated with high levels of circulating soluble IFN receptors. Among 256 enrolled patients, 11 (4.3%) showed a significant inhibition of the IFNβ activity in vitro, but no measurable BAbs. As a whole, in vivo bioactivity was inhibited in 9/11 (82%) of these patients. A minority of IFNβ treated patients have a non-antibody mediated neutralising activity, which competitively inhibits the bioactivity both in vitro and in vivo. This has been attributed to the circulation of soluble IFN receptors (IFNAR) and has mostly been described in subjects treated with Rebif. If this is the case using the BAb assay as a screening tool will fail to detect a proportion of non-responders to IFN therapy and there is a strong argument to alter current guidelines to initially detect bioactivity.

1.6.9.   Reversion to NAb negativity

Over time ~ 30% of NAb positive patients will revert to negative status This occurs most commonly in patients with low to moderately positive titres and those treated with IFNβ-1b [Bellomi *et al.* 2003; Gneiss *et al.* 2004; Petkau *et al.* 2004; Rice *et al.* 1999; Sorensen *et al.* 2005b; Sorensen *et al.* 2003]. In the pivotal IFNβ-1b trial, 60% of those who had a NAb +ve test subsequently reverted to negative status [MS study Group 1993]. Rice et al performed NAb assays in the Canadian cohort, of those included 25 of 59 subjects (42.4%) developed NAbs [Rice *et al.* 1999]. They were followed for a median of 7 years and all but 3 reverted to NAb negative status in this time. Subsequently a longitudinal study was performed to analyse the development of NAbs in patients treated with IFNβ-1a and 1b. Gneiss et al followed 28 NAb positive patients for mean of 57 months after commencing IFNβ therapy, 16 patients received treatment with IFNβ-1a and 11 received IFNβ-1b. They found that patients who were likely to revert to NAb negative status reached their peak titre earlier and had lower peak titres (< 75 – 100NU) than those persistently positive [Gneiss *et al.* 2004]. 58% of patients treated with IFNβ-1b reverted to NAb –ve status whereas only 27% of those treated with IFNβ-1a were reverters. 11 of the 12 reverters had titres < 75 NU and only one of those who did not revert had titres < 75. During the NAb positive period, patients are seen to revert to placebo status with regards to induction of biomarkers.

The Danish MS group has collected data on all of their patients since IFN-β therapy was licensed. In a study using samples which were taken over 6 years, 455 patients were included receiving different IFNβ preparations. Of these 52.3% remained NAb -ve, 40.9% were definitely NAb +ve and the remainder fluctuated. Patients on Avonex had the lowest

frequency of NAbs and frequencies were similar in the Rebif and Betaferon groups, specific frequencies however were not published in this paper at this time. Of the 277 (60.8%) of patients who were NAb -ve at 18 months only 19 (6.5%) became persistently NAb +ve. At 24 months the rate of conversion was only 3%. If a patient reaches 24 months and has been persistently NAb -ve it is unlikely for them to subsequently seroconvert. Of those who developed persistent NAbs 33% subsequently became definitely NAb -ve. The chance of reverting was higher in those on Betaferon than the other products. 52% of patients who were NAb +ve treated with IFNβ 1b reverted to NAb -ve status compared with 19% of those NAb +ve treated with IFNβ-1a. In this study they were unable to differentiate between NAb titres of 200 and those much greater. Due to this they were unable to comment on titre predicting reversion to NAb- status [Sorensen *et al.* 2005b]. An earlier study investigated the persistence of NAbs in 42 patients treated with IFNβ and found that almost all subjects reverted to NAb negative status over a 6 year period. Those who remained persistently positive had high titres of Nabs [Bellomi *et al.* 2003].

1.7. Competitive Environment and Health Economics.

The high incidence and long duration of MS means that even cheap (but efficacious) treatments could have significant effects on health care provider budgets. Government funding of interferons and glatiramer acetate as treatments for MS has been controversial in many countries. The unpredictable course of MS and its potential to cause severe disability underpins the high demand for disease-modifying agents. Funding bodies, on the other hand, have highlighted the lack (as yet) of unequivocal long-term evidence of clinically effective drugs. Health economic evaluations performed to date have demonstrated poor cost-benefit ratios of these drugs [Whetten-Goldstein *et al.* 1998]. Research and development and production costs contribute to the high prices charged for the currently available DMDs. The cost of drugs may be further increased by administration costs where treatments have a short shelf-life, and require regular IM or SC administration. Patients on such treatments require regular follow-up, which add to health-care provider costs.

Medical and non-medical costs relating to treatment (direct costs) are relatively easy to measure, however to calculate the financial savings arising from delayed disease progression is less straightforward. In accounting terms, future financial benefits are usually discounted at an annual rate of up to 6% with further adjustments made for health service price inflation. However, no trial has directly investigated the potential of DMDs' to reduce the high costs of hospitalisation or nursing-home admission. Such benefits might mitigate high drug acquisition and administration costs.

The long-term effect that MS DMDs might have on salaried and non-salaried employment (indirect or production costs) has similarly not been investigated. MS is a disease that typically develops in young people, and in societies where unemployment is

low, the potential production benefits associated with delaying disability are likely to be very high [Prosser *et al.* 2004]. A further saving that might offset high drug costs are those arising from improved quality of life (intangible costs). These are very difficult to measure in financial terms. Recently pharmaco-economic studies of DMDs in MS have attempted to derive likely quality adjusted life year (QALY) benefits due to treatment, but have failed to demonstrate a convincing case for their widespread use [Gold *et al.*1996]. Though the QALY may not be the most appropriate measure in chronic, slowly progressing, diseases such as MS alternative measures of quality of life are equally or even more controversial.

In the UK in 2002, the National institute of Clinical Excellence (NICE) issued guidelines on treatment stating

"A recommendation to use these medicines cannot, presently, be justified, taking both benefits and costs into account. "

However, many patients were already receiving IFNβ treatment post licensing of IFNβ in Europe as a result of the pivotal trials. Thus NICE conceded that:

"People who are currently taking beta interferon or glatiramer acetate to treat MS could suffer loss of well being if their treatment was stopped when they did not expect it. Because of this, all NHS patients who are on therapy at the date of publication of this guidance should have the option to continue treatment until they and their consultant consider it is appropriate to stop, bearing in mind the criteria established for withdrawal from treatment in the Guidelines of the Association of

British Neurologists published in January 2001. This advice about continuing treatment also applies to all participating patients at the end of a clinical trial (regardless of whether they were receiving placebo or active drug) and to women whose therapy has been interrupted by pregnancy. "

Updated guidelines are anticipated but have not yet been issued. Future DMDs will be required to demonstrate their cost-effectiveness before approval by national licensing and funding authorities. High prices may be off-set by low administration costs (for example once monthly rather than daily dosing), clear clinical benefit in patient sub-groups in terms of reduced disability and quality of life, and data demonstrating reduction in economically relevant outcomes such as hospitalisation, nursing home admission, and employment. In view of this resolution of the issues surrounding the clinical significance of NAbs, cost effective assays and standardised reporting of titres is required.

1.8.   Rationale for the research underpinning this thesis

IFNβ is currently the most commonly prescribed first line therapy worldwide for relapsing MS. Evidence supporting the significance of NAbs in abrogating or at least reducing the clinical efficacy of IFNβ has been conflicting and many studies are inadequate for the purpose of assessing their impact. As routine NAb testing was not available in the UK at the time this work began, the purpose of the experiments and studies described here were to:

- establish a validated BAb assay in our laboratory

- develop and validate a novel cell based reporter gene (Luciferase) assay to reliably and cost effectively measure NAbs to IFNβ

- to evaluate the frequency of BAbs and NAbs in patients attending the National Hospital for Neurology and Neurosurgery (NHNN) London, using the Luciferase assay and to correlate NAb titres with clinical data – relapse rates, side effects, treatment cessation

- to develop guidelines to interpret NAb results obtained with the Luciferase NAb assay using in vivo biomarker data to set "cut-points" of significance

- to evaluate maturation of the immune reponse to IFNβ

- to evaluate current opinion among neurologists regarding NAbs to IFNβ, the uncertainty about their significance in the clinical setting, and the perspective of neurologists in the UK , Europe and the US on incorporating NAb testing into routine practice.

Chapter 2

Binding Antibodies

2 Binding antibodies

2.1.   Antibodies to IFNβ

Endogenous proteins which are synthesised and used as biological treatments are known to induce an immune response to the protein. The resulting antibodies that are produced may or may not alter the action of the drug. In the case of IFNβ those which bind to the protein but do not appear to alter the bioactivity of the drug are termed binding antibodies and those which reduce or neutralise the biological effect are called neutralising antibodies [Giovannoni and Goodman 2005]. These observations, however, are restricted to ex vivo bioactivity. The in vivo effect of BAbs in relation to the biological compound is complex; in some cases they may extend the half-life of the circulating protein and in other circumstance reduce the half-life [Durelli and Ricci 2004]. Extending the circulating half-life of interferon-beta is one of the hypotheses put forward to explain the observation that patients destined to develop NAbs tend to do better clinically in the intitial 6 months of therapy [Chapter 1.6.5].

Several factors are thought to contribute to the immunogenicity of the different commercial products including amino acid sequence, glycosylation, dose, production methods and individual patient factors. Binding antibodies have been described to occur in the majority of treated patients 50 – 80% depending on the assay used and its sensitivity [Durelli and Ricci 2004]. Many studies reporting on NAbs do not report BAb frequencies. It is also difficult to compare results due to different assays used.

There are several hypotheses to try to explain why some antibodies act as inhibitors of IFNβ function and others do not. These include immunoglobulin (Ig) class – IgM or

subtype IgG1 vs. IgG2 and IgG4 [Deisenhammer 2001, Sorenson 2007], low vs. high affinity antibodies [Gneiss 2006], epitope recognition pattern resulting in binding to epitopes which are not involved in receptor interaction [Bendtzen 1990, PRISMS 1998]. There remains significant debate as to the significance of BAbs and it is suggested that low affinity BAbs protect IFNβ from clearance or degradation. In the majority of publications the presence of BAbs in the absence of NAbs is not associated with a reduced efficacy of treatment with IFNβ or abrogation of serum biomarker responses [Francis *et al.* 2005; Rudick *et al.* 1998; Pachner *et al.* 2009 b,c; Zang *et al.* 2000].

2.2. BAb measurement

There are several ways to screen for BAbs; ELISA [Brickelmaier *et al.* 1999; Pachner 2003a], Western blotting [Gneiss *et al.* 2008], radio immunoprecipitation or affinity chromatography [Bendtzen *et al.* 2000; Lawrence *et al.* 2003].

2.2.1. Capture ELISA

This method involved coating a microtitre plate overnight with a mouse / rabbit monoclonal anti IFNβ antibody or phosphate buffered saline (PBS) alone in control wells. Plates are decanted and then blocked with milk protein. At this point IFNβ is added to the plate and incubated. A standard curve is made with doubling dilutions of a known BAb positive sample. Control and test sera are then added in duplicate. Antibodies which bind to the IFNβ molecule are detected by adding a horseradish peroxidase (HRP) conjugated anti-

human IgG and detected colorimetrically by adding developing solution (Tetramethylbenzidine TMB or o-phenylenediamine OPE). By attributing arbitrary values to the points on the standard curve titres can be generated for the samples which are tested [Brickelmaier *et al.* 1999; Pachner 2003a],.

2.2.2. Direct ELISA

The direct ELISA is similar to the cELISA but directly coats microtitre plates with 1:5000 IU solution of commercial IFNβ in 100μL PBS. Control wells were filled with PBS alone and incubated overnight. After blocking the plates test sera were added to the wells in doubling dilutions. The plate was then washed and HRP conjugated anti-human IgG added. After further washing this was detected by adding a developing solution (3, 3', 5, 5'-tetramethylbenzidine (TMB) or O-phenylenediamine (OPE)). The reaction was stopped by adding HCl and reading in a spectrophotometer. As with the cELISA a standard curve may be used to generate BAb titres [Gneiss *et al.* 2008].

2.2.3. Western Blotting

This method involves separation of IFNβ by SDS-PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis) and transfer of protein to a nitrocellulose membrane. The membrane is blocked with milk protein and dried. Strips of the membrane were incubated with test sera 1:1000 dilution, negative control sera and a mouse monoclonal anti-human IFNβ antibody as positive control. These were incubated with either anti-

human IgG or anti-mouse IgG. These were detected by adding p-nitro blue tetrazolium chloride and 5-bromo-4-chloride-3-indolyl phosphate. Samples were considered positive when the band detected at the level of the mouse anti-IFNβ, was stronger than the negative control. A disadvantage of this technique is relatively high levels of false negatives and that no relative quantification can be made merely weak or strongly positive.

2.2.4.   *Radioimmuno Assays*

Human IFNβ is radioiodinated (I-r) with purified and validated tracers which are known to be stable. Serum samples are preincubated with ^{125}I-rIFNβ and the amounts of free and IgG-bound tracer are determined by protein G Sepharose affinity chromatography. The bound IFNβ is eluted and cpm detected in the supernatant. This was used to calculate the amount of ^{125}I-IFNβ in the mixture. This assay has not been widely adopted and hence validated across multiple laboratories. In addition, it is not as simple to perform as ELISA [Lawrence *et al.* 2003].

Recent studies have compared these assays and ELISA appears to be the preferred method. The capture and direct ELISAs performed equally well, but the units obtained with the capture ELISA had a better correlation with NAb titres [Gneiss *et al* 2008]. The direct ELISA had higher rates of false positive and false negative results which have been attributed to the fact that IFNβ when bound directly to the plate undergoes conformational changes in the IFNβ molecule which results in it being presented in non-native form whereas in the cELISA IFNβ is bound to the capturing antibody in the liquid phase preventing such conformational change [Svenson *et al.* 1993; Brickelmaier *et al.* 1999].

Thus the consensus opinion is that the cELISA is the most reliable and efficient screening tool in use to detect BAbs. In view of this, for the purpose of this study, the capture ELISA was used [Pachner 2003a].

The aim of this chapter was to:

1. Validate a pre-existing BAb assay in our laboratory

2. Test the samples in the cohort of subjects attending the affiliated hospital.

2.3. BAb Capture ELISA

2.3.1. Analytical principles

The cELISA method is sandwich immuno-assay that utilises an IFNβ specific monoclonal antibody bound to the surface of a polystyrene 96-well microtitre plate to capture and immobilise the IFNβ protein. IFNβ specific IgG antibodies in the serum that bind to the anchored protein are detected using a horseradish peroxidase (HRP) labelled polyclonal donkey anti-human IgG. The amount of bound conjugate is determined colorimetrically by monitoring the oxidation of TMB which leads to production of a blue colour, the intensity of which depends on the quantity of HRP conjugate present. The reaction is stopped by adding 1M hydrochloric acid HCl (which changes the colour of the solution from blue to yellow) and the optical density is measured at 450 nm. A reading at 750 nm is also taken to correct for differences in volume of solution in each well. A standard curve is created using a highly positive reference serum sample and BAbs are quantified in arbitrary Units (U) based on the linear detection range of the standard curve. The method described here is based on that published by Pachner *et al*. 2003a.
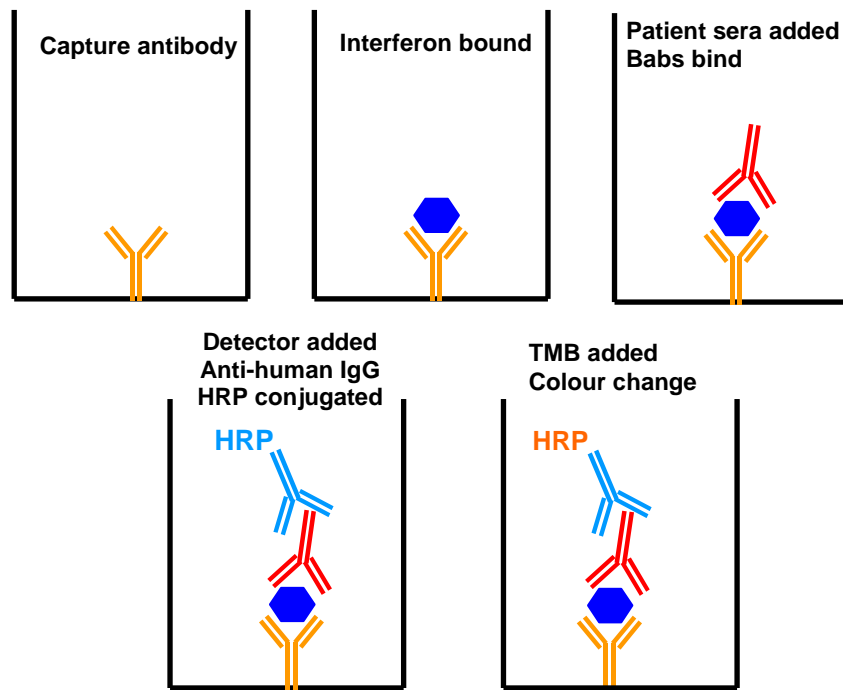
Figure 2-1 Capture ELISA
The first well shows capture antibody coating base of well, Interferon-β is added and is bound to the capture antibody. Subjects sera containing anti-Interferon antibodies (BAbs) bind to the IFNβ molecule. HRP conjugated anti human IgG is added which binds to BAbs and is detected by addition of TMB.

2.3.2. Materials

Monoclonal capture antibody specific for Human IFNβ Clone #B-02 Yamasa SAM-260871-1, IFNβ-a (Avonex) Biogen Idec, Donkey anti-human IgG -HRP conjugate Jackson Immuno-research #709-035-1, TMB Microwell Substrate Kirkegaard & Perry Labs 50-76-06, 96-well Microtitre Plates ICN 76-381-04, Microtitre Plate Covers Corning-Costar 3095, Micro Pipette Tips VWR, Reagent Reservoirs Labcor 730-001, Sodium Chloride S3014, Potassium Chloride P9541, Sodium Diphosphate S7907, Postassium Monophosphate P0662, Sodium Carbonate S1641, Sodium Bicarbonate S8875,

Polyoxyethylene sorbitan monolaurate (Tween 20) P1379: all from Sigma, Instant Non-Fat Dry Milk Pathmark, Sulphuric Acid CEM SX1244-3

2.3.3. Preparation of solutions

All solutions were prepared prior to the day of the assay except the blocking buffer and the sample dilution buffer which were prepared on the day of the assay.

2.3.3.1. Carbonate buffer

A 0.1M solution was made with 4.2g Sodium Bicarbonate added to 500mls distilled water. This was poured into a large beaker. The 0.1M sodium bicarbonate solution was made with 3.18g Sodium bicarbonate added to 300mls distilled water. This was slowly added to the Sodium Bicarbonate until pH reached 9.6. The buffer was stored at room temperature for up to 1 month.

2.3.3.2. 10 X PBS Solution

80g NaCl, 2g KCl, 11.5g NaH_2PO_4 (dibasic), 2g KH_2PO_4 (monobasic) were brought to 1L with distilled water.

2.3.3.3. Blocking buffer

0.25g non-fat, dried, milk powder was added to 5mls of 10XPBS and 50 mls distilled water. This was prepared fresh each day the assay was being performed.

2.3.3.4. Wash buffer

0.05% Tween 20 in PBS. 5 ml Tween was added to 200ml 10xPBS and volume brought to 2L.

2.3.3.5. Sample dilution buffer

0.05% Tween 20 & 0.5% milk in PBS. 1.25g non-fat dry milk powder was added to 250ml wash buffer. This was prepared on the day of the assay.

2.4. Methods

2.4.1. Day 1: cELISA

The appropriate number of microtitre plate wells were coated with 55µL/well of BO2 capture Ab at 2 µg/ml: To prepare 1 plate: 28 µL BO2 stock was diluted in 5.5 ml carbonate buffer. Plates were incubated overnight at 4°C.

2.4.2. Day 2: cELISA

Blocking buffer and sample dilution buffer (SDB) were prepared as described above (2.3.3.5). The coated microtitre plates were taken from the refrigerator and washed 3 times with PBS-TWEEN wash buffer by filling the wells to the brim and then decanting the entire volume and blotting on paper towel. Using a multi-channel pipette, the plates were

blocked with 300 µL/well blocking buffer and placed on a shaker for 1 hour at ambient temperature. The IFNβ solutions were prepared using sample dilution buffer: 20 µL stock IFNβ (Avonex®) + 4 ml PBS-TWEEN-20/Milk = 0.15 ug/mL (volume for 1 plate). The blocking buffer was decanted and plate inverted and tapped sharply on paper towels to remove excess buffer. Using a multi-channel pipette, 50 µL/well of IFNβ or diluent (for neg. control wells) was added to the appropriate wells [Figure 2-2] and this was further incubated on the shaker for 1 hour at ambient temperature.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent	
B	BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent	
C	BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent	
D	BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent	
E	BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent	
F	BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent	
G	BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent	
H	BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent		BO_2 + IFN		BO_2 + diluent	

Figure 2-2 Coating of plate with IFNβ or diluents (sham well).

2.4.3. *Preparation of Calibrators, controls and samples*

The standard curve was prepared using a known positive serum sample which was diluted 1:400 in doubling dilutions to 1:12,800. Several samples were evaluated for use as the standard which had low background reactivity and had a high level of BAbs. Arbitrary units were assigned to each point on the standard curve – see table 2-1. A negative control sample and samples for testing were prepared by diluting 1:100 with SDB 5 µL of sample

added to 500 µL of sample diluent buffer. The plate was emptied, blotted on paper towels

and washed 4 times. All standards, controls (buffer and negative) and samples were added

in duplicate 50 µL / well. This was incubated on the shaker for 2 hours at room temperature

[Figure 2-3].

Dilution	Serum or diluted serum	Vol required	Vol SDB	Dilution Factor	Assigned units
1	Positive standard serum	5 µL	2000 µL	1:400	100 U
2	1:400	1000 µL	1000 µL	1:800	50 U
3	1:800	1000 µL	1000 µL	1:1600	25 U
4	1:1600	1000 µL	1000 µL	1:3200	12.5 U
5	1:3200	1000 µL	1000 µL	1:6400	6.25 U
6	1:6400	1000 µL	1000 µL	1:12,800	3.125 U

Table 2-1 Preparation of standards

	1	2	3	4	5	6	7	8	9	10	11	12
A	buffer control		buffer control		test sample #1		test sample #1		test sample # 9		test sample # 9	
B	negative control		negative control		test sample #2		test sample #2		test sample#10		test sample#10	
C	PSC dilution 6		PSC dilution 6		test sample#3		test sample#3		test sample#11		test sample#11	
D	PSC dilution 5		PSC dilution 5		test sample #4		test sample #4		test sample#12		test sample#12	
E	PSC dilution 4		PSC dilution 4		test sample #5		test sample #5		test sample#13		test sample#13	
F	PSC dilution 3		PSC dilution 3		test sample #6		test sample #6		test sample#14		test sample#14	
G	PSC dilution 2		PSC dilution 2		test sample #7		test sample #7		test sample#15		test sample#15	
H	PSC dilution 1		PSC dilution 1		test sample #8		test sample #8		test sample#16		test sample#16	

Figure 2-3 BAb ELISA - Application of samples to the plate.

The HRP conjugated detector antibody was prepared by adding 0.5µL donkey anti-human IgG HRP-conjugated antibody and 8 µL goat anti-human IgG and adding to 11.65 mls SDB (per plate). The samples were decanted from the plate and washed 4 times, plates were blotted after each wash on paper towel. 100 µL of detector antibody solution was added to each well and incubated for 1 hour on the shaker at room temperature.

This was decanted and washed 4 times with wash buffer and 100 µL of TMB added to each well. This was incubated at room temperature in the dark until the colour of the highest concentration standard was sufficiently developed to the naked eye (~ 5 – 10 minutes). The reaction was stopped by adding 50 µL of 1M HCl to each well. The plate was then read at 450 nm and 750 nm using the Wallac Victor2 1420 plate reader [Figure 2-4].
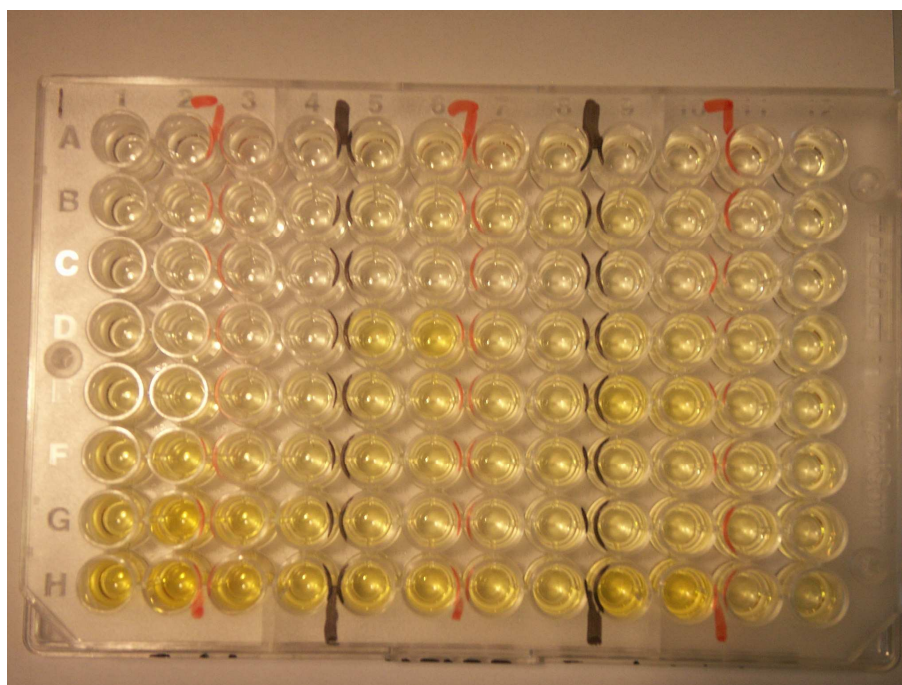
Figure 2-4 Photograph of BAb cELISA plate
Layout of standards and samples is as described in Figure 2-3

2.4.4. Calculation of results

The OD 750 nm readings were subtracted from the OD 450 nm readings; this corrected for subtle variations in volume. The mean of each duplicate was obtained and the CVs of each duplicate were calculated to ensure variation was < 10%. The mean readings without IFNβ were subtracted from those with IFNβ to correct for background levels. To create a standard curve, Units were plotted on the x-axis vs. corrected A-450 values of the five doubling dilutions of the positive control sera on the y-axis (both log 10 scale). A linear regression analysis was performed on the x-y points of the curve to obtain slope and y-intercept. Using these values and the background corrected A-450 values of the test samples as y values, the x value (Units) was calculated for each test sample using the following formula:

Test Sample Units = [(test sample background-adjusted A-450) – y-intercept] / slope

2.4.5. Reference ranges

Reference ranges were established previously by measuring the OD and thus mean units in subjects (n=10) who were treatment naïve and thus BAb negative. The mean OD + 3 SD was used as the cut off for negative / low positive and results were categorised as follows:

Units	Interpretation	
< 3.5 U	Low	Negative
3.5-8 U	Moderate	Positive
8.0-55 U	High	Positive
> 55 U	Very High	Positive

Table 2-2 Result interpretation.

2.5.   *Testing patient samples*

Several NAb+ve samples were selected and used at varying dilutions to generate a standard curve. Those with background < 0.15 were stored for use. The plate below shows the typical OD values obtained. Columns highlighted in red are wells coated with IFNβ and those highlighted in green without (control for background) Figure 2-5 and Figure 2-6.

	1	2	3	4	5	6	7	8	9	10	11	12
A	buffer control		buffer control		test sample #1		test sample #1		test sample # 9		test sample # 9	
B	negative control		negative control		test sample #2		test sample #2		test sample#10		test sample#10	
C	PSC dilution 6		PSC dilution 6		test sample#3		test sample#3		test sample#11		test sample#11	
D	PSC dilution 5		PSC dilution 5		test sample #4		test sample #4		test sample#12		test sample#12	
E	PSC dilution 4		PSC dilution 4		test sample #5		test sample #5		test sample#13		test sample#13	
F	PSC dilution 3		PSC dilution 3		test sample #6		test sample #6		test sample#14		test sample#14	
G	PSC dilution 2		PSC dilution 2		test sample #7		test sample #7		test sample#15		test sample#15	
H	PSC dilution 1		PSC dilution 1		test sample #8		test sample #8		test sample#16		test sample#16	

Figure 2-5 BAb cELISA Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.162	0.143	0.135	0.155	0.504	0.518	0.556	0.536	0.391	0.366	0.377	0.404
B	0.437	0.414	0.392	0.407	0.436	0.440	0.363	0.343	0.456	0.445	0.477	0.476
C	0.285	0.262	0.142	0.162	0.781	0.786	0.840	0.813	1.278	1.310	0.357	0.394
D	0.272	0.256	0.107	0.115	0.385	0.365	0.426	0.410	0.364	0.380	0.368	0.381
E	0.331	0.306	0.093	0.094	0.607	0.648	0.389	0.380	0.325	0.316	0.396	0.358
F	0.471	0.470	0.092	0.090	0.595	0.625	0.308	0.282	0.405	0.469	0.365	0.303
G	0.677	0.704	0.128	0.134	0.728	0.719	0.943	0.863	0.468	0.436	0.301	0.329
H	1.135	1.124	0.162	0.152	0.374	0.394	0.493	0.460	0.165	0.161	0.189	0.197

Figure 2-6 OD from positive standard curve and patient samples.

2.5.1.   *Standard Curve*

The mean of each dilution of the positive standard, negative control and diluents in wells with or without IFNβ was calculated. These values were subtracted to give the corrected value [Table 2-3]. Dilutions 1 – 6 of the PSC were assigned unit value, which were plotted against corrected absorbance at 450nm to generate the standard curve [Figure 2-7].

	Mean IFNβ	Mean no IFNβ	corrected	units	SD IFNβ	CV %	SD no IFNβ	CV %
dil	0.152	0.145	0.008		0.0137	9.0	0.0143	9.9
c neg	0.426	0.400	0.026		0.0159	3.7	0.0106	2.7
6	0.273	0.152	0.121	3.125	0.0164	6.0	0.0143	9.4
5	0.264	0.111	0.153	6.25	0.0112	4.2	0.0052	4.7
4	0.318	0.094	0.225	12.5	0.0182	5.7	0.0010	1.1
3	0.470	0.091	0.379	25	0.0007	0.1	0.0012	1.3
2	0.690	0.131	0.559	50	0.0191	2.8	0.0047	3.6
1	1.129	0.157	0.972	100	0.0077	0.7	0.0077	4.9

Table 2-3 Data analysis to derive standard curve

Units of each dilution plotted against corrected absorbance @ 450nm

$$y = 0.0087x + 0.1162$$
$$R^2 = 0.994$$
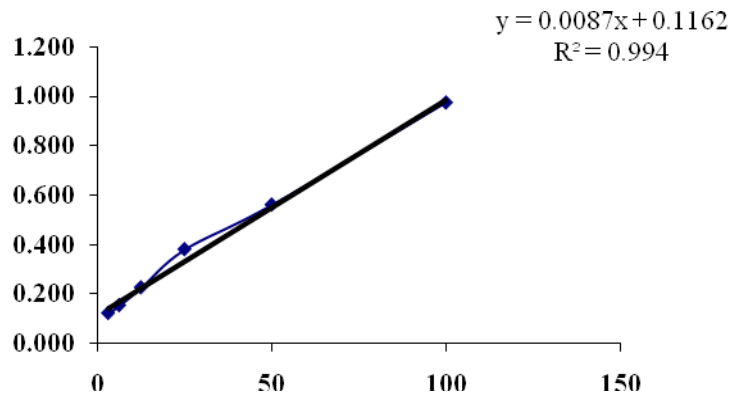
Figure 2-7 BAb standard curve

To calculate the titre of the unknown samples the OD was corrected and inserted as the y value in the equation of the standard curve. Thus x (sample titre) was calculated. In the data provided below the background values of sample 3 and 7 were > 0.6 therefore these results were not accepted and required retesting. Samples 5, 6 and 11 were positive and sample 15 was a low positive, all others were regarded as negative

Sample	IFNβ	no IFNβ	Corrected	Result	SD IFNβ	CV	SD no IFNβ	CV
1	0.511	0.546	-0.035	-20.4	0.0098	1.9	0.014	2.6
2	0.438	0.353	0.085	-4.4	0.0026	0.6	0.014	3.9
3	0.784	0.827	-0.043	-21.5	0.0035	0.4	0.019	2.3
4	0.375	0.418	-0.044	-21.6	0.0138	3.7	0.012	2.8
5	0.627	0.384	0.243	16.6	0.0288	4.6	0.007	1.7
6	0.610	0.295	0.315	26.2	0.0212	3.5	0.018	6.2
7	0.723	0.903	-0.180	-39.8	0.0063	0.9	0.057	6.3
8	0.384	0.477	-0.093	-28.1	0.0143	3.7	0.023	4.9
9	0.378	0.391	-0.012	-17.4	0.0183	4.8	0.020	5.0
10	0.451	0.477	-0.026	-19.3	0.0079	1.7	0.001	0.2
11	1.294	0.375	0.918	106.6	0.0226	1.7	0.026	6.9
12	0.372	0.374	-0.002	-16.1	0.0108	2.9	0.009	2.5
13	0.320	0.377	-0.056	-23.3	0.0068	2.1	0.027	7.1
14	0.377	0.334	0.043	-2.0	0.0452	8.3	0.044	7.1
15	0.452	0.315	0.137	2.5	0.0224	5.0	0.020	6.4
16	0.163	0.193	-0.030	-19.7	0.0028	1.7	0.006	2.9

Table 2-4 Typical BAb results with BAb capture ELISA

2.5.2. *Evaluation of protocol performance*

To evaluate performance of the assay each plate has a standard curve of dilutions of a known positive sample. Each plate also contains control wells (containing buffer alone) and control negative serum. After identification of known BAb +ve sample and untreated BAb –ve sample these were aliquoted in small volumes and frozen at –80°C for use in each assay. A sample was considered negative when the A-450 values were <0.4 prior to background correction and less than 0.15 after correction. The positive control serum was serially diluted as previously described, each dilution should have A-450 values curve ranged from 0.2 to 1.0 after correction for background (background levels < 0.4 were accepted). The dilutions were plotted on a log scale and the adjusted R value for the linear regression of the standard curve should be >0.95 to accept the plate. Serial standard curves were monitored for performance and plates which did not fulfil criteria were discarded and repeated. Figure 2-6 shows the plot of ten sequential "accepted" standard curves with 95% confidence intervals of each dilution point. Using repeated measures ANOVA no difference was found between the OD at each point (p=0.093).

Standard curve (mean with 95% CI)
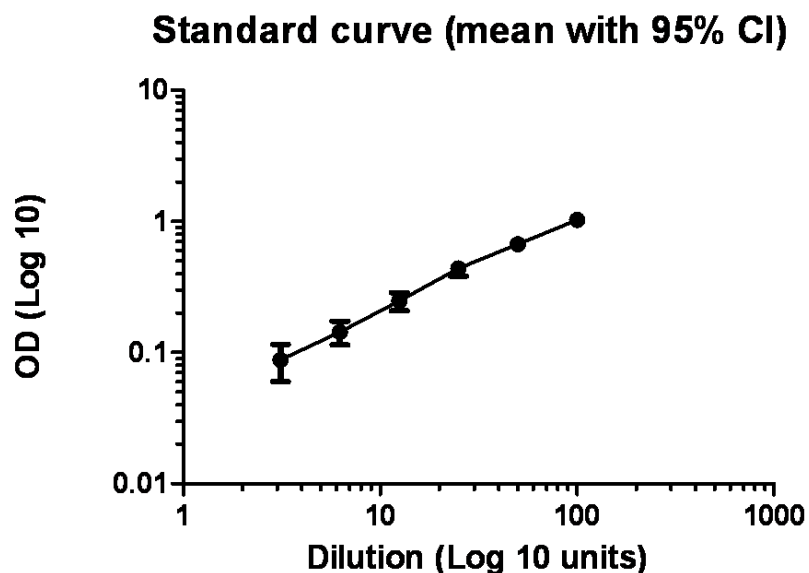
Figure 2-8 Plot of BAb standard curve.
Mean OD of ten sequential standard curves with 95% CI.

2.6.   Limitations

The sensitivity of the assay was 3.5 U. Samples with background corrected A450 values out of the linear range of the standard curve (i.e. above the top value or below the lowest value of the curve) could not be quantified accurately. To obtain a more accurate Unit value samples were re-tested in the next assay using a higher dilution if above top end of curve (e.g. 1:500 or 1:1000) or lower dilution when below bottom value of curve (e.g. 1:25 or 1:50). Samples with background levels >0.6 A-450 were also retested. Accession of samples less than 6 hours after IFN-β injection can theoretically result in falsely low values due to quenching, by IFNβ, of the binding to antibodies in the circulation.

2.7.   Conclusions

The cELISA is accepted as the optimal screening assay for BAbs thus we sought to establish it as the BAb assay in our laboratory and to use this as a screening tool to evaluate subjects treated with IFNβ in the affiliated hospital prior to NAb testing. We found this assay simple to establish in the laboratory but many samples required retesting due to high backgrounds. In this way ~700 samples were tested with respect to BAb status (details of these results are given in chapter 4). In this way we could identify samples which should be NAb -ve and also those potentially with neutralising capacity. These samples could be used in developing and validating a novel NAb assay (chapter 3). The cELISA was simple and relatively time efficient to perform. Difficulties were experienced related to the sourcing and expense of the capture antibody which required shipping from Japan. Each 96-well plate had capacity to test only 16 sera and thus high throughput screening would be very labour intensive in the absence of a robot to apply samples or a plate washer. This may make the assay less accessible for some laboratories / countries.

Chapter 3

Development and validation of a novel cell based anti-Interferon beta neutralising antibody assay

3 Development and validation of a novel cell based anti-IFNβ neutralising antibody assay.

3.1. Introduction

The development of NAbs has an important impact on the use of IFNβ in the treatment of people with MS. The objective of this chapter was to develop a novel bioassay to accurately detect neutralising antibodies to IFNβ, which was easy to perform, reliable and utilised commercially available products and to validate it against existing assays in other European Laboratories.

The firefly - Lampyridae of the beetle order Coleoptera, also called lightning bugs or gloworms are winged beetles which are capable of producing a cold light. The enzyme luciferase acts on luciferin, in the presence of magnesium ions, ATP (adenosene triphosphate), and oxygen to produce light [Figure 3-1]. Luciferase genes can be synthesised and inserted into organisms or transfected into cells. Mice, silkworms, and potatoes are amongst the organisms that have already been engineered to produce the protein. The gene has been incorporated into many cell systems to provide a sensitive, rapid means to assay transcriptional activity of regulated activation sequences of DNA when fused to the protein coding sequence of the luciferase gene. In biological research, luciferase commonly is used as a reporter to assess the transcriptional activity in cells that are transfected with a genetic construct containing the luciferase gene under the control of a promoter of interest [Fan and Wood 2007].
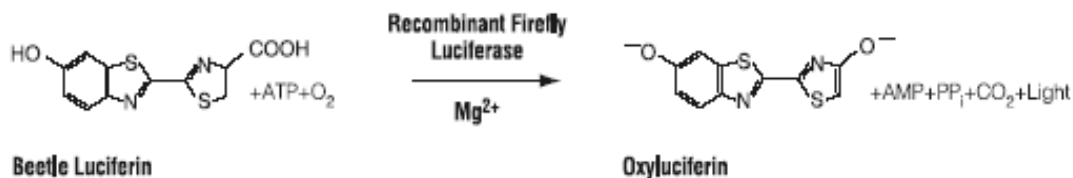
Figure 3-1 Diagram of firefly reaction with substrate - beetle luciferin to yield light.

The premise to this work was to use a human cell line which was stably transfected with the firefly luciferase gene under transcriptional control of the ISRE. There are a number of cell lines available using the luciferase reporter gene system, thus we sought to evaluate two cell lines – a fibrosarcoma cell line (ATCC HT 1080 - HL 116 clone) developed by Gilles Uze, University of Montpellier France [Uze *et al.* 1994] and also a human embryonic kidney cell line - HEK293 (ATCC CRL-1573™). The HL 116 clone was isolated in after co-transfection of the human HT 1080 [Pellegrini *et al.* 1989] cell line with p6-16 luciferase and pBB3 which contains the Eco GPT gene (glutamic-pyruvate-transaminase) [Bourachot *et al.* 1982]. When interferon binds to its receptor, the reporter cassette is activated by the JAK/STAT intracellular signalling mechanism and luciferase is expressed within a few hours [results 3.7.1]. In the presence of Nabs this response is inhibited [Figure 3-1] (courtesy of Dr Susan Goelz – Biogen Idec). The HEK293 (ATCC CRL-1573™) cell line was donated by Biovation Ltd. who had transfected the cell line with the luciferase gene. The signaling reporter vector pISRE–TA-luc containing the firefly Luciferase gene was under the control of the ISRE. This cassette was transferred to the mammalian episomal expression vector pREP4 (Invitrogen) to create pREP-ISRE

which was subsequently transfected into HEK293 cells. Stable transfectants were selected with hygromycin creating a stable HEK293-ISRE cell line [personal correspondence from Biovation].

Reporter Gene System for detection of Neutralizing Antibodies
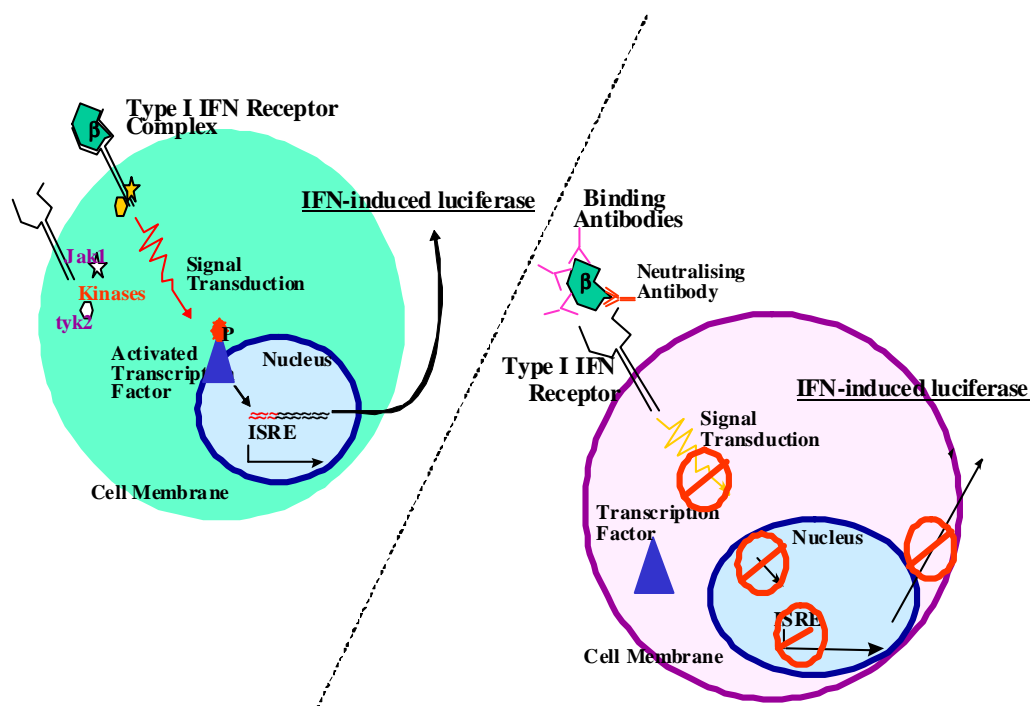
Figure 3-2 Mechanism of action of IFNβ
Diagram of interaction of INFβ molecule with its cell bound receptor. activating JAK/STAT cell signalling mechanisms which in turn cause activation of the ISRE and transcription of downstream nuclear genes including the transfected luciferase gene. In the presence of NAbs this effect is lost.

3.2.   *Principles of assay development and validation*

The process by which a specific bioanalytical method is developed, validated and transferred to routine use divides into three main stages (i) reference standard preparation, (ii) bioanalytical method development and establishment of assay procedure and (iii) application of validated method to routine sample analysis and acceptance criteria (www.fda.gov/cder/guidance). A reference standard is a substrate of known identity and purity and should be used to prepare solutions of known concentrations, which would be used in the standard curve. For our purposes we utilised readily available IFNβ standards (NIBSC 3rd International Standard Interferon Beta 00/572) and therapeutic products (Avonex IFNβ-1a (Biogen Idec Ltd), Rebif IFNβ-1a (Merck-Serono) and Betaferon IFNβ-1b (Schering)), and NIH antiserum to IFNβ (NIH anti-IFNβ antibody standard #G038-501-572). The cell line used was an established line in the public domain and all other materials were obtained from verified suppliers. In developing the method fundamental parameters needed to be addressed including: accuracy, precision, calibration curve, robustness and system suitability. Accuracy is the closeness of agreement between the measured value and that which is accepted as the true value. This requires a "gold standard" or method to which the new method may be compared. Accuracy is determined by replicate analysis of the same samples using > 5 determinations at a variety of titres. Precision describes the degree of agreement among individual measures when a single sample is repeatedly measured. This should also be performed using at least 5 determinations of >3 samples at a range of concentrations. Acceptance criteria of coefficients of variation (CV) < 25 % should be applied to a biological assay. The standard curve is the relationship of known amounts of standard and the cells – a predictable dose response curve. A sufficient number of standard

samples should be used to adequately define this relationship and it should be reproducible. Anchoring points beyond the established upper and lower limits of quantification may be employed. The robustness of a procedure is the capacity of the method to remain unaffected by small but deliberate variations in the method parameters and provides an indication of its reliability in normal use. In a cell based assay this includes: cell bank (beginning, middle or end of freeze), cell passage level, seeding density, stock density (days in culture), incubation time, different plates and varying reagent sources. If the assay method meets these validation criteria it can then be used to test routine samples.

In the case of NAb assays a specific technique is used to calculate the titre to correct for changes in the bioactivity of the IFNβ protein used in the assay [Grossberg *et al.* 2001 a & b]. Rather than employing the international unit assigned by the IFNβ producer a Lab Unit (LU) is assigned by the laboratory performing the assay and this denotes activity as measured by any given IFN bioassay method in that lab, without reference to a standard. The LU is expressed per unit volume (LU/mL) as the IFNβ dose response is concentration dependent. In a bioassay the dose response forms a sigmoidal curve with upper and lower asymptotes and the endpoint of any assay is taken as the midpoint of the straight line portion of the dose response curve [Fig 3.3]. In the luciferase assay, the lower limit was set as the minimum amount of light the cells without any IFNβ stimulus emit under the established conditions of the assay. The upper limit was taken as the maximum amount of light emitted by cells maximally stimulated by a concentration of IFNβ that is considered to be saturating the transfected cell line.
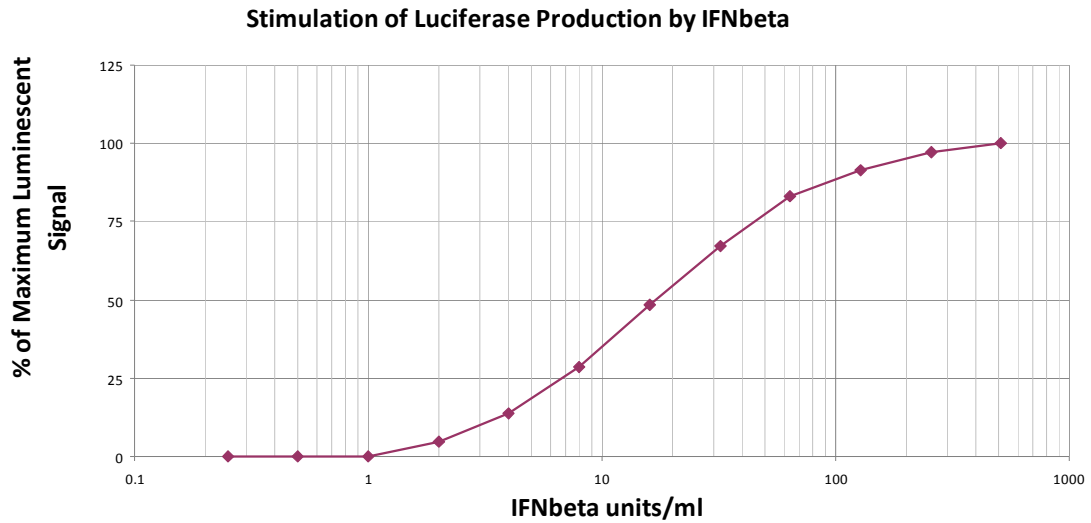
Figure 3-3 Graph of luminescence in response to stimulating cells with IFNβ

The Kawade technique defines the neutralisation potency of serum as the titre that reduces IFNβ bioactivity by ten-fold, i.e. the serum titre that reduces the activity of IFNβ from 10 LU/mL to 1 LU/mL. The concentration of IFNβ at the 50% endpoint is defined as being 1 LU/mL and is the point at which 50% of the biological effect of IFN-beta is lost. To calculate the ten-fold Reduction Units (TRU)/ml, the formula $t = f (n-1) / 9$ is used, where t equals the titre, n the number of LU/ml of IFNβ applied to the samples actually measured in the assay on this occasion (originally assumed to be 10), and f the dilution of antibody at the 50% endpoint. One LU/ml is subtracted from n because that is the endpoint, not zero (there is no IFN to measure at zero), and therefore 9 is used as the divisor (difference between 10 and 1). This calculation method will be described in subsequent passages detailing calculation of *n* and the titre [Chapter 3.4.9].

3.3.   Materials and Methods

3.3.1.   Materials

Flasks 80 cm^2, sterile, with filter cap, Nunc (VWR, 734-2131). 96-well TC treated microtitre plate, black with clear bottom, sterile (Greiner Bio-One Ltd, 655097). Microplate lid, sterile (Greiner Bio-One Ltd, 656170). 10mL Stripette (Sigma, CSL 4488). Eppendorf combitips (2.5 mL) (VWR, 613-3531). 50 mL centrifuge tubes (Fisher, CFT-900-011L). 15 mL centrifuge tubes (Fisher, FB55950). 5 mL syringes (VWR, 7008169). Sterile syringe filters 0.2µm (VWR, 514-4011).

ATCC HT -1080 HL-116 clone (Gilles Uze, University of Montpellier, France,), bank of cells stored in liquid nitrogen. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Ltd. 11960-044) stored at 4°C. Fetal bovine serum, heat-inactivated (FBS) (Invitrogen Ltd. 10108-165). L-Glutamine solution 200 mM, sterile filtered (Sigma, G7513). HAT Media Supplement (50x), Hybri-Max, γ-irradiated (Sigma. H-0262). Antibiotic-Antimycotic αM (Sigma, A5955). Hank's Balanced Salt Solution (HBSS) (Sigma, H-6648). Dimethyl sulfoxide, (DMSO) Biotechnology Performance Certified, sterile-filtered (Sigma, D2438). Trypsin-EDTA Solution, 10x concentration (Sigma, T-4174). Trypan Blue (Sigma). Steady-Glo Luciferase Assay System (Promega, E2550).

Avonex IFNβ-1a (Biogen Idec Ltd). NIBSC 3rd International Standard Interferon Beta 00/572. Sheep anti human IFN-beta antibody (Serotec AHP294Z). NIH anti-IFNβ antibody standard #G038-501-572 .

Tissue culture facilities: 37°C incubator with 5% CO_2, laminar flow hood water bath,

set at 37°C refrigerated bench centrifuge (set at 2000rpm, 5min, 4°C). Microscope cell counting chamber and counter liquid nitrogen cell storage facility Pipettes: 10 µL, 200 µL, 1000 µL, 30-300 µL multichannel adjustable volume pipettes - Eppendorf Research$^®$. Aluminium foil, ELISA plate shaker, ELISA plate reader (Wallac Victor^2 1420 Multilabel Counter Plate Reader), set to read luminescence.

3.3.2.   Solutions and Media

3.3.2.1. Complete culture medium

Culture medium without αM	Volume
DMEM	500 mL
FBS	57 mL
Glutamine	6 mL
Sterile 7.5 % w/v NaHCO$_3$ solution	6 mL
HAT	1 vial

Culture medium with αM	Volume
(DMEM-αM as prepared above)	100 mL
1% Antibiotic-Antimycotic	1 mL

Table 3-1 Preparation of culture media

3.3.2.2. Calibration solutions

Preparation of stock solution – 10 LU/mL IFNβ-1a (Avonex) [Table 3-2]. Ten LU/mL of IFNβ-1a was generally equivalent to about 15 IU/mL of Avonex (10 – 20 IU/mL). Stock solutions were made on this basis. The actual concentration of IFNβ-1a in LU/mL used to stimulate the cells was calculated and adjusted on each plate in line with the Kawade technique. A vial of Avonex (IFNβ-1a) was dissolved in fresh DMEM-αM to give a final volume of 6.0 mL and a concentration of 1 million IU/mL. This was equivalent to approximately 500,000 LU/mL. The solution was diluted with DMEM-αM, to give a final IFNβ-1a concentration of 500 LU/mL (ie take 1mL of the 500,000LU/mL solution and add to 9 mLs of DMEM, then take 1mL of this solution (50,000 LU/mL) and add to 9 mLs DMEM to create the 5,000 LU/mL solution, finally take 8mLs of the 5,000 LU/mL solution and add to 72mLs of DMEM to create the working solution of 500 LU/mL [Table 3-2].

IFNβ-1a – 500 LU/mL   Stock solution.

[IFN] LU/mL	IFN (mL)	DMEM-αM (mL)	Final [IFN] LU/mL
500,000	1	9	50,000
50,000	1	9	5000
5000	8	72	500

Table 3-2 Preparation of IFNβ stock solution.

3.3.2.3. *Promega Steady-Glo Luciferase Assay System*

Substrate was reconstituted as described in the manufacturers' protocol and aliquots (5.3 mL) were prepared in foil covered tubes and frozen at -20°C ready for use. Each aliquot contained sufficient substrate for one complete 96 well plate.

3.4. Cell Culture

The HL-116 cells were stored in complete DMEM in 1.5 mL aliquots at a density of 4 million cells / mL in liquid nitrogen and thawed for use as required.

Complete DMEM+αM (12 mL) was added to an 80 cm^2 culture flask and incubated at 37°C, with 5% CO_2 for ~15 minutes prior to the cells being thawed. A vial of cells was removed from liquid nitrogen storage and thawed rapidly at 37°C in a water-bath. The thawed cells were added to culture flask. Cells are cultured for ~ 4 hours to allow the viable cells to adhere to the flask and the medium changed to remove cell debris and DMSO. The culture medium was changed every 2-3 days (depending on the cell density) and replaced with fresh pre-warmed complete DMEM +αM (10-12 mL).

3.4.1. Sub-culturing cells

Cells were sub-cultured (or 'split') when ~ 85% confluent. This was approximated by examining the flask using a light microscope. Culture medium was aspirated and the cells washed twice with pre-warmed HBSS (4 mL). Warmed trypsin/EDTA (4 mL) was added to the flask and gently washed over cells. The flask was returned to incubator until

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all of the cells had detached (~ 2 min). FBS (0.4 mL or approx 10 % of trypsin volume) was added to inhibit the trypsin. The detached cells were aspirated and added to a 50 mL centrifuge tube. A cell pellet was prepared by centrifuging the cell suspension for 5 min at 2000 rpm, 4°C. The supernatant was discarded and the cell pellet resuspended in DMEM +αM (10 mL) for sub-culturing, freezing or counting.

3.4.2. Counting cells

A cell pellet was prepared as described above and resuspended in DMEM +αM (10 mL). HBSS (30 µL) and Trypan blue 0.04 % in HBSS (40 µL) were added to a 1.5 mL tube. Cell suspension (10 µL) was added and 10µL of this suspension was placed under the cover slip of each side of the counting chamber using a pipette. The cells were counted in 8 of the 4x4 squares. This has a volume equivalent to 0.1 µL. The cell count for 8 squares (*n*) x 10,000 = cells/mL.

3.4.3. Seeding of cells into microtitre plates

Cells were counted as described and resuspended at predetermined concentrations (assay dependent) in DMEM-αM. Using an Eppendorf repeater pipette, 100 µL of the cell suspension was added to each well of a sterile Greiner 96 well plate. The gaps around the wells at the edge of the plate were filled with 75 µL of sterile H_2O to prevent dehydration of the external wells. A sterile lid was placed on top and the plate transferred to the incubator overnight.

3.4.4. *Freezing cells in liquid nitrogen for long term storage*

A sterile solution of 5 % (v/v) DMSO in DMEM+αM was prepared. The cell pellet was prepared as described above and resuspended in 5 % (v/v) DMSO DMEM+αM (10 mL). These were counted and diluted to a density of 1 x 10^6 cells/mL and frozen in 1 mL aliquots in cryotubes labelled with cell type, passage number, initials and date. Cryotubes were frozen, at the rate of 1 ^{o}C/min (using a 'freezing pot' filled with isopropyl alcohol) in a – 80 ^{o}C freezer for a minimum of 16 h. The following day the cryotubes were transferred to a liquid nitrogen cryo-conservation vessel.

3.4.5. Development of a standard protocol to measure anti-Interferon β neutralising antibodies

3.4.6. Assessment of cell response to IFN β: Dose response

Cells were cultured in DMEM+αM at 37 °C and 5 % CO_2. Cells were counted and seeded into microtitre plates at a density of 40,000 cells per well in DMEM-αM. The plates were incubated overnight at 37 °C and 5 % CO_2. Standard interferon solutions were prepared with NIBSC human IFNβ 00/572 (reference standard), IFNβ-1a (Avonex, Biogen-Idec) and IFNβ-1b (Betaferon, Bayer-Schering). Doubling dilutions were used to prepare standards of concentration 1000 IU/mL to ~0.08 IU/mL and these were added to the plates in duplicate. The plates were incubated for 5 hours at 37 °C and 5 % CO_2. The 'Promega Steady-Glo Luciferase Assay System' substrate was warmed to room temperature, protected from light and mixed by gentle inversion prior to use. The plate was allowed to equilibrate at room temperature for 10 min. The 'Promega Steady-Glo' substrate was added to each well (50 μL/well). The plate was covered with aluminium foil (to protect it from the light) and placed on a plate shaker for 10 minutes. Luminescence was measured on a Wallac Victor[2] 1420 plate reader. A curve was constructed by plotting Log10 IFN dilution (x-axis) vs. Log10 LCPS (y-axis) to illustrate the dose-response of the cells to stimulation with IFNβ. From these curves the maximal and basal responses were determined to be used subsequently as anchor points. Both HL 116 and HEK cell lines were evaluated. HL 116 cells were found to be more sensitive to IFNβ stimulation and had lower levels of baseline leakage and thus HL 116 cells were used to establish the assay [Figure 3-4].
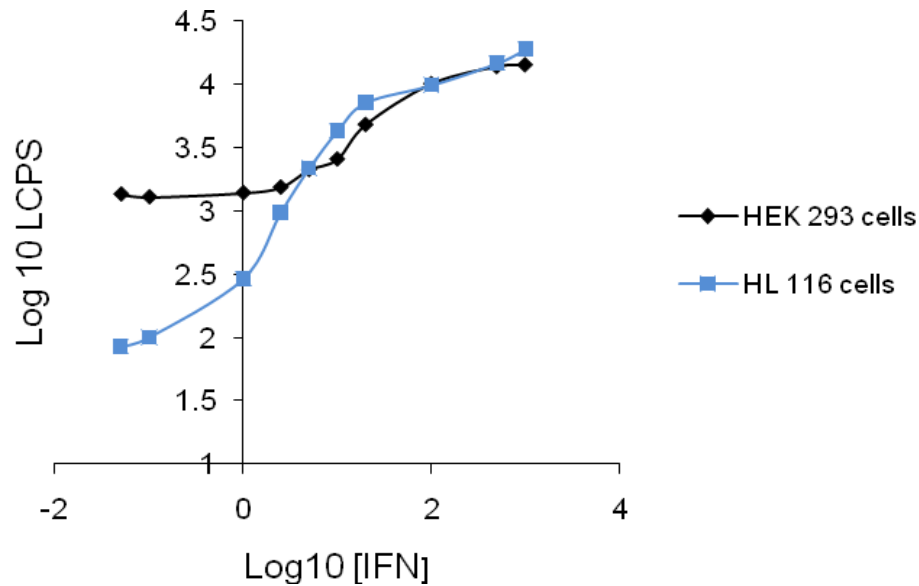
Figure 3-4 Response of HL 116 and HEK 293 cells to IFNβ

3.4.7. *Inhibition of luciferase induction by commercial Anti-IFNβ neutralising antibodies*

Commercial sheep anti-human IFNβ antibody (Serotec AHP294Z) has a known neutralising activity of 1 NU/mL neutralising the bioactivity of 1 IU/mL of standard IFNβ. This was pre-incubated in known concentrations of 0, 0.1, 1, 10, 100 and 1000 NU/mL with IFNβ preparations from (0.5 to 100 IU/mL). These samples were added in duplicate to the microtitre plate and incubated for 5 hours. Promega substrate was added, luminescence measured and inhibition of activity calculated.

3.4.8.   *Uniformity Experiments*

This experiment was run to determine whether the position of the sample on the plate affects the final results. IFNβ standard (10 LU/mL) was added to each well (100 µL/well) and the plate incubated for 5 hours [Figure 3-5]. A second plate [Figure 3-6] was prepared with the standard curve in the centre wells and at the edges and the curves and values compared. A total of 3 of each uniformity plate were run on 3 different days. The data from these experiments was subjected to statistical analysis (ANOVA) to determine putative plate effects that may impact on data interpretation.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10	10	10	10	10	10	10	10	10	10	10	10
B	10	10	10	10	10	10	10	10	10	10	10	10
C	10	10	10	10	10	10	10	10	10	10	10	10
D	10	10	10	10	10	10	10	10	10	10	10	10
E	10	10	10	10	10	10	10	10	10	10	10	10
F	10	10	10	10	10	10	10	10	10	10	10	10
G	10	10	10	10	10	10	10	10	10	10	10	10
H	10	10	10	10	10	10	10	10	10	10	10	10

Figure 3-5 Uniformity plate layout 1. All wells contain 10 LU IFNβ.

	1*	2*	3**	4**	5*	6*	7**	8**	9*	10*	11**	12**
A	10	10	QC+	QC+	10	10	QC+	QC+	10	10	QC+	QC+
B	5	5	100	0	5	5	100	0	5	5	100	0
C	2.5	2.5	100	0	2.5	2.5	100	0	2.5	2.5	100	0
D	1.25	1.25	100	0	1.25	1.25	100	0	1.25	1.25	100	0
E	0.63	0.63	100	0	0.63	0.63	100	0	0.63	0.63	100	0
F	0.32	0.32	100	0	0.32	0.32	100	0	0.32	0.32	100	0
G	0.16	0.16	100	0	0.16	0.16	100	0	0.16	0.16	100	0
H	0.08	0.08	QC-	QC-	0.08	0.08	QC-	QC-	0.08	0.08	QC-	QC-

Figure 3-6 Uniformity plate layout 2.
*Columns 1, 2, 5, 6, 9 & 10 contain IFNβ standard curve from 10 LU/mL to ~0.08 LU/mL
** Columns 3, 4, 7, 8, 11&12 contain QCs and the "maximum" and "minimum" response to IFNβ

3.4.8.1. Evaluation of 96 well plates

Three types of sterile, tissue culture plates were tested for use in the luciferase assay: 96 well black plates with a transparent bottom (Greiner), black plates with a black bottom (Greiner) and all white plates (Greiner). The three plates were evaluated simultaneously with the same calibration curve and samples in each plate. Absolute LCPS, CVs and 50% endpoints were compared between plates to determine which was optimal for the assay.

3.4.9. Determination of the 50 % endpoint and Kawade correction factor

The Kawade technique as previously described [3.2], defines the neutralisation potency of serum as the titre that reduces IFNβ bioactivity by ten-fold, i.e. the serum titre that reduces the activity of IFNβ from 10 LU/mL to 1 LU/mL. The concentration of IFNβ at the 50% endpoint is defined as being 1 LU/mL and is the point at which 50% of the biological effect of IFN-beta is lost. The maximal and basal responses (anchor points) were identified from the extended dilution curve and the 50 % endpoint calculated.

50 % endpoint = Average (Log$_{10}$ Max CPS: Log$_{10}$ Min CPS)

The undiluted stock of commercial IFNβ-1a (Avonex) is assigned a concentration of 6 million IU/mL (freeze dried preparation). The dilution factor was calculated for each IFNβ-1a standard used. The Log mean LCPS (x-axis) vs. Log IFN Dilution (y-axis) was

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plotted as described above. The "linear" appearing part of the curve was expanded and a trend-line fitted with equation and R^2 value. This equation was used to determine the Log IFN dilution at 1LU/mL (i.e. at the 50% endpoint). The IFNβ dilution was then calculated (**IFNβ dilution = $10^{\wedge\text{LogIFNDil}}$**) and used to calculate the LU/mL used to stimulate the cells on the day.

n = (IFNβ Dilution at 1 LU/mL) / (IFNβ Dilution used on cells)

This value (n) was used as a correction factor in the Kawade formula.

t = f (n-1) / 9 (expressed as TRU/mL)

Where: t = NAbs titre (corrected), f = serum dilution achieving the endpoint, TRU = Ten-fold Reduction Unit.

3.4.10.   Screening IFNβ treated patients' sera for neutralising activity

Cells were seeded at density of 40,000 per well and incubated overnight as previously described. The patients' sera samples to be tested were thawed at 4 °C. Stock IFNβ was

diluted to give a ~20 IU/mL concentration. Samples were screened for presence of any neutralising activity by mixing equal volumes of serum at 1:10 dilution with 20 LU/mL IFN beta 1a (Avonex) for 1 hour at 37ºC. A calibration curve of IFN-beta of concentrations from 10 LU/mL to 0.08 LU/mL was prepared and a solution of 100 LU/mL IFNβ was prepared to detect the maximal saturated response of luciferase induction. Known negative and positive patient sera were included in each plate at 1:20 dilution. Overnight media was decanted (by inversion and gentle tapping) and the IFNβ dilutions and serum/IFNβ preparations added in duplicate to the microtitre plate [Figure 3-7]. Plates were incubated at 37 °C in a 5 % CO_2 atmosphere for 5 hours. Luciferase substrate 50µL was added to the plate and left for 10 minutes before reading in a luminometer. The LCPS obtained by the maximum (100 LU) concentration of IFNβ and minimum (no IFNβ) were calculated and the 50 % endpoint identified. The mean LCPS of each sample was calculated and those with values below the endpoint were considered positive. Positive samples were analysed further to determine the Nab titre.

	1 *	2 *	3 **	4 **	5 ***	6***	7***	8***	9***	10***	11***	12***
A	10	10	QC+	QC+	S1	S1	S9	S9	S17	S17	S25	S25
B	5	5	100	0	S2	S2	S10	S10	S18	S18	S26	S26
C	2.5	2.5	100	0	S3	S3	S11	S11	S19	S19	S27	S27
D	1.25	1.25	100	0	S4	S4	S12	S12	S20	S20	S28	S28
E	0.63	0.63	100	0	S5	S5	S13	S13	S21	S21	S29	S29
F	0.32	0.32	100	0	S6	S6	S14	S14	S22	S22	S30	S30
G	0.16	0.16	100	0	S7	S7	S15	S15	S23	S23	S31	S31
H	0.08	0.08	QC-	QC-	S8	S8	S16	S16	S24	S24	S32	S32

Figure 3-7 Screening plate Layout.
*Columns 1 & 2 IFNβ standard curve from 10 LU/mL to ~0.08 LU/mL
** Columns 3 & 4 contain QCs and the "maximum" and "minimum" response to IFNβ
*** Columns 5 to 12 contain screening samples at 1:20 dilution preincubated with 10 LU of IFNβ

3.4.11.   Nab titre calculation

IFNβ calibration curve, 20 LU/mL and 100 LU/mL IFNβ solutions and QCs were prepared as described above. Serial dilutions of NAb positive samples were prepared, from 1:20 to 1:2560 (final dilution after IFNβ added) [Table 3-3]. IFNβ solution (125 µL) was discarded from the final dilution tube, so that the final volume in all of the tubes was 125µL.

	DMEM-αM Pre-warm (37°C)	Serum (or QC)	IFNβ-1a (20 LU/mL)	Final [IFN] (LU/mL)	Final serum dilution
1	225	25	125	10	1:20
2	125	125	125	10	1:40
3	125	125	125	10	1:80
4	125	125	125	10	1:160
5	125	125	125	10	1:320
6	125	125	125	10	1:640
7	125	125	125	10	1:1280
8	125	125	125	10	1:2560

Table 3-3 Serum sample dilutions – NAb assay

IFNβ solution 20 LU/mL (125 µL) was added to each serum dilution (125 µL) to give a final dilution of sera of 1:20 and an IFNβ concentration of 10 LU/mL. The tubes were incubated at 37 ºC for 1 hour. Overnight media was decanted from the plates and the IFNβ calibration curve, QCs, maximum / minimum controls and samples added in duplicate [Figure 3-7].

	1*	2*	3**	4**	5***	6***	7***	8***	9***	10***	11***	12***
A	10	10	QC+	QC+	S1	20	S2	20	S3	20	S4	20
B	5	5	100	0	S1	40	S2	40	S3	40	S4	40
C	2.5	2.5	100	0	S1	80	S2	80	S3	80	S4	80
D	1.25	1.25	100	0	S1	160	S2	160	S3	160	S4	160
E	0.63	0.63	100	0	S1	320	S2	320	S3	320	S4	320
F	0.32	0.32	100	0	S1	640	S2	640	S3	640	S4	640
G	0.16	0.16	100	0	S1	1280	S2	1280	S3	1280	S4	1280
H	0.08	0.08	QC-	QC-	S1	2560	S2	2560	S3	2560	S4	2560

Figure 3-8   Dilution Plate layout.
*Columns 1 & 2 IFNβ standard curve from 10 LU/mL to ~0.08 LU/mL
** Columns 3 & 4 contain QCs and the "maximum" and "minimum" response to IFNβ
*** Columns 5 to 12 contain diluted samples at 1:20 to 1:2560 dilutions preincubated with 10 LU of IFNβ

Plates were incubated for 5 hours at 37°C, equilibrated at room temperature for at least 5 minutes, 50 μL luciferase substrate was then added and the plate was incubated for 10 minutes on a plate shaker before LCPS was measured. The actual LU of IFNβ used to stimulate the cells was calculated using the Kawade technique. The mean LCPS at each dilution of sample, log10 LCPS and CVs were calculated and Log10 LCPS plotted vs. Log10 serum dilution to generate a standard curve. The dilution at which the curve crosses the 50 % endpoint (1 LU) was determined and the reciprocal of this denotes the unadjusted Nab titre.

3.4.12. Optimisation of other parameters

3.4.12.1. Use of Promega Steady-Glo Luciferase Assay System

The commercial product protocol advised addition of an equal volume of Steady-Glo Luciferase assay substrate to the culture medium (i.e. 100µL). This should be incubated for 10 minutes to ensure complete cell lysis. To optimise the efficiency of the assay, performance of 25, 50 and 100 µL of luciferase assay substrate was investigated by measuring luminescence counts per second in standard curves of IFNβ- 1a and IFNβ- 1b [Figure 3-9, results 3.7.7]. Following addition of substrate, plates were shaken for the duration of the incubation period with the substrate to ensure that solutions were fully mixed and cells were lysed. Absolute LCPS data were compared.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1		
B	0	0	1	1	1	1	1	1	1	1		
C			2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5		
D			5	5	5	5	5	5	5	5		
E			10	10	10	10	10	10	10	10		
F			20	20	20	20	20	20	20	20		
G			100	100	100	100	100	100	100	100		
H			Units of Avonex /ml				Units of Betaferon / ml					

Figure 3-9 Plate layout assessing volume of Luciferase assay substrate required
Columns in green contain media only, columns in yellow (3 – 6) contain standard curves using Avonex, columns in blue (7 to 10) contain standard curves using Betaferon.

3.4.12.2. *Evaluation of Luminometer parameters*

To ensure optimal reading of the plates, the effects of changing the acquisition time settings of the luminometer were investigated. The luminometer was set to read for 0.1, 0.5, 1 and 2 seconds per well and the raw data compared with respect to absolute values, range and CVs of duplicates. The shortest acquisition time giving optimal results was 1 second and thus was employed in all subsequent work [Figure 3-10].
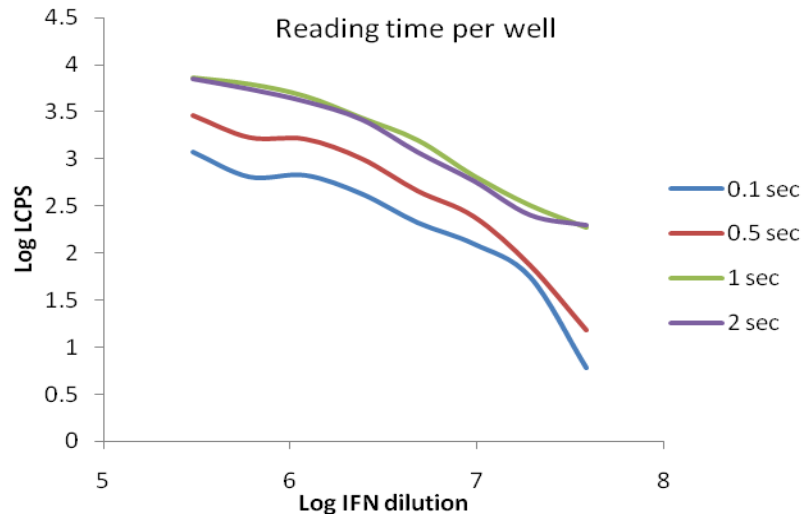
Figure 3-10 Standard curves with varied aquistion time

3.5.   *Validation of Assay*

The accuracy and precision of the assay was examined by response to NIBSC standard IFNβ preparation #00/572, this is the WHO reference standard which acts as a calibrant to measure the potency of glycosylated IFNβ derived either from human fibroblasts or from CHO cells (commercial IFNβ 1a products). Repeated testing of NIH anti-human IFNβ antibody reference standard #G038-501-572 (pooled human NAbs generated against IFNβ1a) was also performed to ensure accuracy of titres obtained using the standard operating procedure. Additional samples with known NAb titres, determined either by a validated MxA protein ELISA or cytopathic effect (CPE) assay were obtained from other accredited European laboratories – Regional reference laboratory, Orbassano, Italy (CPE & MxA ELISA); Karolinska Institute, Stockholm Sweden (MxA ELISA); Biogen Idec, Cambridge US (CPE & MxA ELISA); University of Innsbruck, Innsbruck, Austria (MxA ELISA). These were tested on 10 occasions to determine assay accuracy and precision. To test robustness, sera were tested repeatedly (i) on the same day by two operators and (ii) on different days, with different cell passage number, varying pre-incubation / incubation times and cell densities. External validation was performed by blind exchange of samples with other European Laboratories using the MxA protein ELISA [Files *et al.* 1998] and cytopathic effect assay [WHO expert committee on Biological Standardisation1985]. Samples encompassing a wide range of NAb titres were included. Results were unblinded only after testing had taken place.

3.6.   *Statistical analysis*

Statistical analysis and graphs were prepared using Microsoft Office Excel (2003) and STATA software (version 9, 2006, Statacorp, Texas, USA). To test for plate effects Levene's test for equality of variances and students T-Test were used. To assess accuracy and precision the coefficients of variation for repeated measures were evaluated. To compare titres obtained with the Luciferase reporter gene assay and the "gold standard" methods, paired combinations and mean-difference plots were assessed using Bland-Altman method [Bland and Altman 1999]. This method is favoured when comparing results measured using a new test with an established one. If the two readings (old test and new test) were identical all results would fall upon the mean difference line (which would be zero). By plotting the data this gives a visual assessment as to the degree of agreement between the two measures and whether this is skewed. The Pearson correlation coefficient was used to test association between assay methods. Percentage agreement was calculated and inter-rater kappa coefficient used as measure of agreement corrected for chance. A value of 0 reflects no agreement and 1 denotes perfect agreement [Kundel and Polansky 2003; Chan 2003]

NAb Assay - Validation procedure summary

Dose response of cells to IFNβ

Inhibition of response with commercial NAbs

Uniformity experiments

Evaluate performance of 96 well plates

Application of Kawade technique to determine activity of IFNβ on the day (n)

Screening of patient sera

Dilutions of sera to determine NAb titre

Optimisation of Assay parameters

Luciferase substrate

Luminometer parameters

Cell density in wells

Internal Validation

Accuracy – ten known positive patient samples tested on five occasions

Precision - NIH anti-human IFNβ antibody reference standard antibody titres

Repeatibility – NAb titres obtained with repeated testing

Robustmess – effect of varying assay parameters

External Validation

Blinded sample exchange with 4 other laboratories

NAb assay step by step

Make solutions and Interferon Calibration standards

Count cells and seed in 96 well plate 100μL (40,000 cells)

Incubate overnight

Prepare serum samples to be tested and controls

Preincubate sera with IFNβ (10 LU/mL) for 1 hour

Prepare IFNβ standard curve

Decant DMEM from 96 well plate

Add IFNβ standards to 96 well plate in duplicates

Add sera and controls to 96 well plate in duplicate

Incubate for 5 hours

Add Luciferase substrate to each well

Measure luminescent counts per second in Luminometer

Calculate value for n and NAb titres as per Kawade method

3.7.   Results

3.7.1.   Response of cells to IFNβ

Plates were prepared in accordance with operating procedure. Stock IFNβ was diluted uniformly to prepare aliquots of IFNβ with concentrations ranging from 1000 IU/ml (dilution factor 6000) to ~0.12 IU/ml (dilution factor 49152000). 100µl of each dilution point was added in duplicate to the plate and incubated for 5 hours. Luminescence (as measured by luminescent counts per second) was used to detect the presence of the luciferase enzyme. The response was equivalent with NIBSC standard IFNβ1a or 1b and commercial products Avonex and Betaferon. Plotting the Log_{10} IFN dilution vs. Log_{10} LCPS produced a sigmoidal curve [Figure 3-11] shows the Log_{10} IFN dilution plotted on the x-axis vs. Log_{10} LCPS on the y-axis (ANOVA, p= 0,53).
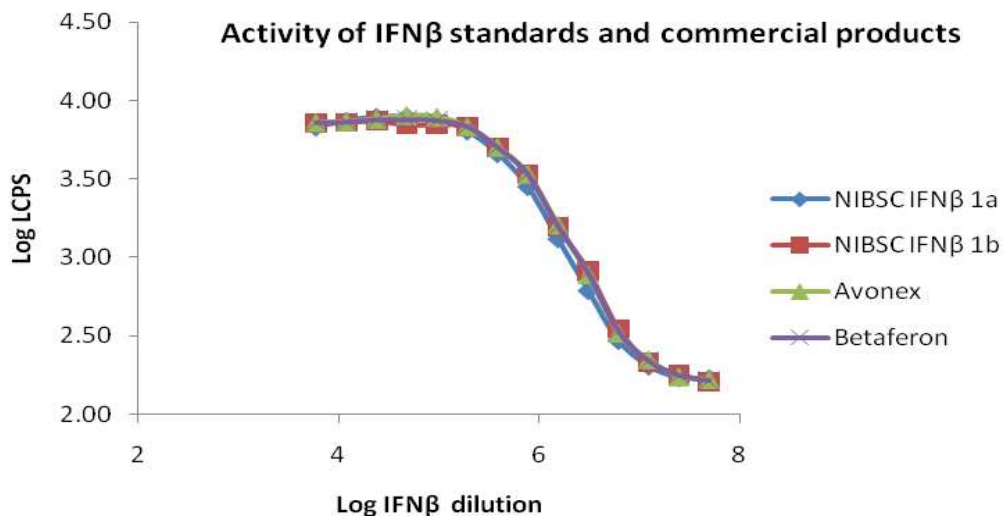
Figure 3-11 Response of cells to IFNβ
Log IFNβ dilution vs. Log luminescent counts per second measured when
luciferase substrate added to the cells

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3.7.2. Inhibition of Luciferase induction by sheep anti human IFN-beta antibody

Figure 3-12 shows the inhibition of IFNβ by commercial sheep anti-human IFNβ antibody. The log IFNβ concentration is plotted on the x-axis and log LCPS on the y-axis. Each line represents the concentration of NAb used to inhibit the response of the cells to IFNβ. The samples incubated with no NAb, 0.1 NU/mL and 1NU/mL showed a good response with all concentrations of IFNβ, the samples with 10 NU/mL of NAb added shows some inhibition of response at low IFNβ concentrations but this inhibition is overcome at higher IFNβ concentrations, however using 100 NU/mL and 1000 NU/mL of NAb the inhibition of response was complete at all IFNβ concentrations. This confirms the dose response effect of Nabs.
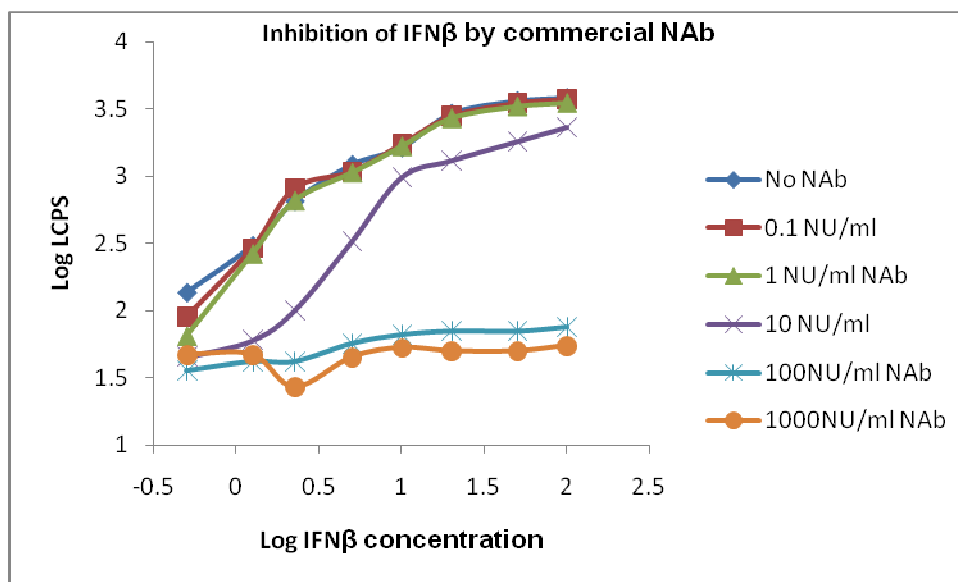
Figure 3-12 Inhibition of IFNβ (Avonex) by commercial sheep anti-human IFNβ NAb
IFNβ concentrations range from 0.5 IU/ml to 100 IU/ml. The bioactivity of IFNβ is inhibited by NAb as the NU/ml increases.

3.7.3. Application of Kawade method

3.7.3.1. Calculation of n

An extended standard IFNβ-1a curve (using doubling dilutions between 100 and 0.02LU/mL, approximate concentrations) was prepared and incubated [3.4.9]. In the example below [Figure 3-13 & Table 3-4] the LCPS at maximum (100LU/mL) was 9574 (ln 3.98) and at minimum (no IFNβ) was 381 (ln 2.58), thus the $\log_{10}$ LCPS at the 50% endpoint was 3.98-2.58 = 3.28. The 50% endpoint reflects 1 LU/mL and the $\log_{10}$ IFN dilution at this point was 6.43. Thus the defined interferon concentration of 1LU/mL (50% endpoint) would be equivalent to a dilution of 1: 2710834 of the original stock (inverse Log 6.43). Each sample to be tested was incubated with ~10LU/mL which was a 1:300,000 dilution of stock IFNβ. Thus from the standard curve the *actual* LU value of IFN β corresponding to the *estimated* 10LU/mL could be determined. Thus the **n** of the Kawade equation in the assay on this occasion = 2710834 / 300000 = 9.04. A further example of calculation of **n** is given on page 149 (worksheet from laboratory).
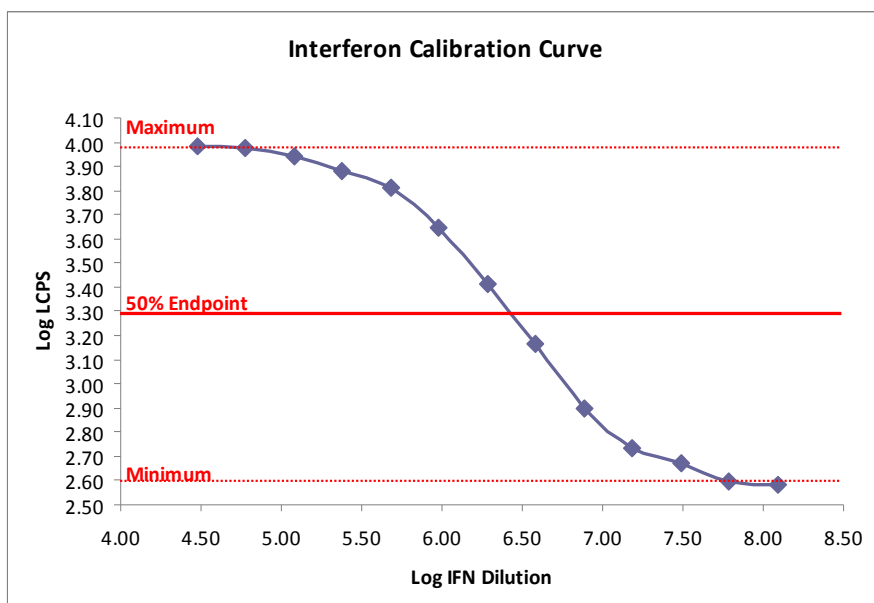
Figure 3-13 Maximum and minimum asymptotes with 50% endpoint

Estimated LU/mL	IFN dil	CPS	Log CPS	Log Dil	IFN	Log CPS
100.00	30000	9574	3.98	4.48		3.98
50.00	60000	9520	3.98	4.78		3.98
25.00	120000	8779	3.94	5.08		3.94
12.50	240000	7606	3.88	5.38		3.88
6.25	480000	6518	3.81	5.68		3.81
3.13	960000	4415	3.64	5.98		3.64
1.56	1920000	2573	3.41	6.28		3.41
0.78	3840000	1475	3.17	6.58		3.17
0.39	7680000	786	2.90	6.89		2.90
0.20	15360000	545	2.74	7.19		2.74
0.10	30720000	468	2.67	7.49		2.67
0.05	61440000	393	2.59	7.79		2.59
0.02	12288000	381	2.58	8.09		2.58
0.00		414	2.62			

Table 3-4 IFNβ standard curve in NAb assay (raw data)

3.7.3.2. Titre calculation

To determine whether a test sample was NAb -ve or +ve the mean LCPS from the duplicate readings was calculated [3.4.10]. This was converted to 'Log $_{10}$ CPS.' If this value was greater than the 50% endpoint the sample was negative for Nabs. If this value was less than the 50% endpoint the sample was positive for NAbs and was therefore serially diluted to determine the titre at which the endpoint was reached as decribed in 3.4.11. The Log $_{10}$ LCPS at each serum dilution were plotted on the x-axis vs. the serum dilution on the y-axis. A trendline was fitted (using points either side of the 50% endpoint) and the trendline equation was used to calculate the serum dilution achieving the 50% endpoint. This was the value (f) in the Kawade formula. Using the Kawade formula above the corrected NAbs titre was estimated. A further example of titre calculation is given on pages 149 -150 (pages from laboratory worksheet).

Serum Dilution Curve

$$y = 0.236x^{10.104}$$

$$R^2 = 0.9999$$
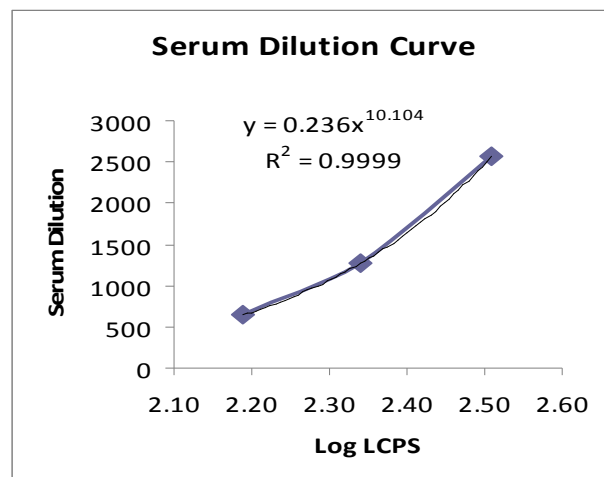
Figure 3-14 Sample dilution curve

3.7.4.   *Incubation time*

The graph below [Figure 3-15] shows the log10 IFNβ dilution (x-axis) vs. log10 LCPS (y-axis). Each line represents the IFNβ standard curve obtained with a specific incubation time. Longer incubation times resulted in higher absolute LCPS but did not affect the slope of the curve. Saturation was dependent on the IFNβ concentration used. Repeated testing of 4 samples with different incubation times did not show any significant variation in titre with CVs <10.4% [Table 3-5].
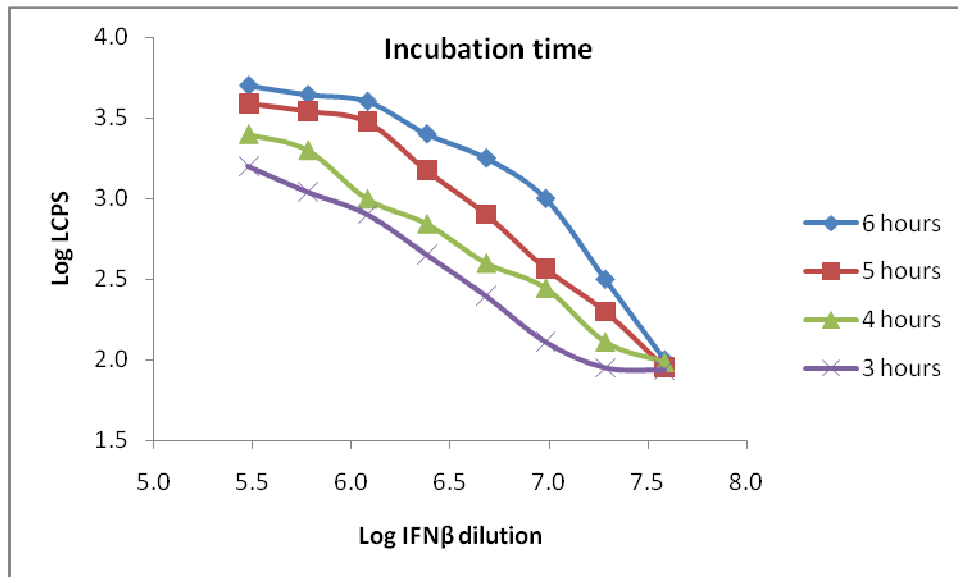
Figure 3-15 The effect of incubation time on LCPS measured

Sample	3 hours	4 hours	5 hours	6 hours	mean	CV
1	< 20	< 20	< 20	< 20	< 20	NA
2	232	252	293	256	258	9.8
3	782	843	702	898	806	10.4
4	> 1280	> 1280	> 1280	> 1280	> 1280	NA

Table 3-5 Nab titres obtained testing four samples using different incubation times

3.7.5. Uniformity Experiments

The LCPS detected in each well when cells were stimulated with 10 LU/mL of standard IFNβ were analysed using repeated measures ANOVA, no significant plate effect was detected (p = 0.13). Similarly no significant difference was found relating to position of standard curve in the plate (p = 0.21).

3.7.6. Evaluation of 96 well plate performance

The LCPS, performance of standards and titres obtained were not significantly different when using black clear bottomed or solid plates. The luminescent counts obtained with white plates were higher than those in the black plates; white plates mean IFNβ minimum LCPS 234 (range 165-321) black plates minimum LCPS 156 (132 – 176) t-test p=0.0021, white plates maximum (100LU IFNβ) LCPS 21354 (range 17634 – 23187) black plates mean maximum (100LU IFNβ) 10342 (85679-12753) p < 0.0001. However, NAb titres of known positive samples remained unchanged. Thus either black or white, clear bottomed or

solid plates are suitable for use in this assay. Black clear bottomed plates were selected for use in the standard operating procedure as the cross talk was reduced between wells and the cells could be easily visualised.

3.7.7. *Optimisation of Promega Steady-Glo Luciferase Assay System*

The manufacturers recommend use of 100 µL of substrate in each well for use in the Luciferase assay system. The performance of 25, 50 and 100 µL of LAS was evaluated and LCPS compared using MANOVA. No significant difference was found in LCPS of standard curves using 50 or 100 µL ($p = 0.53$) but there was a difference when 25 µ µL was used $p = 0.003$. In the interest of cost effectiveness we reduced this volume to 50 µL [Figure 3-16]
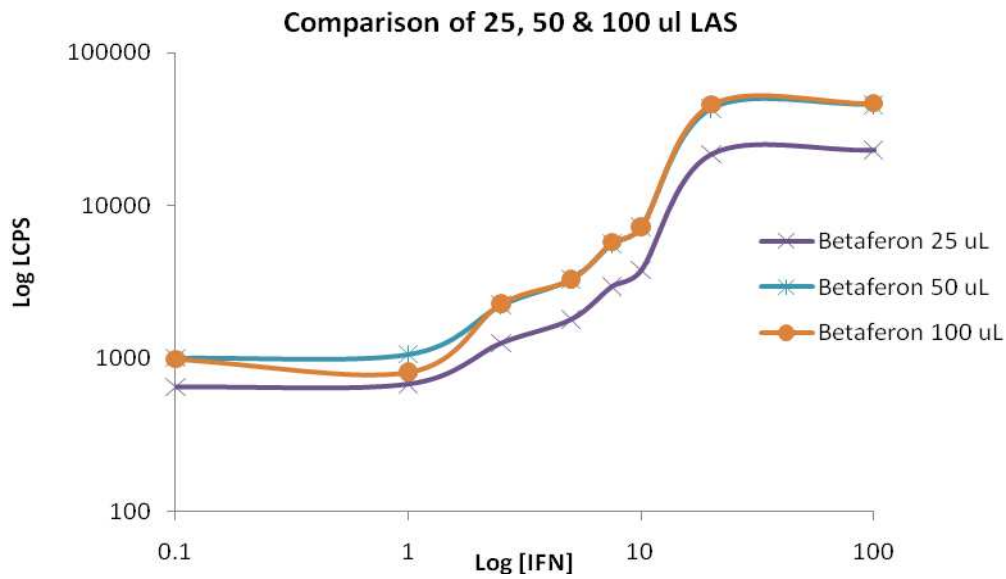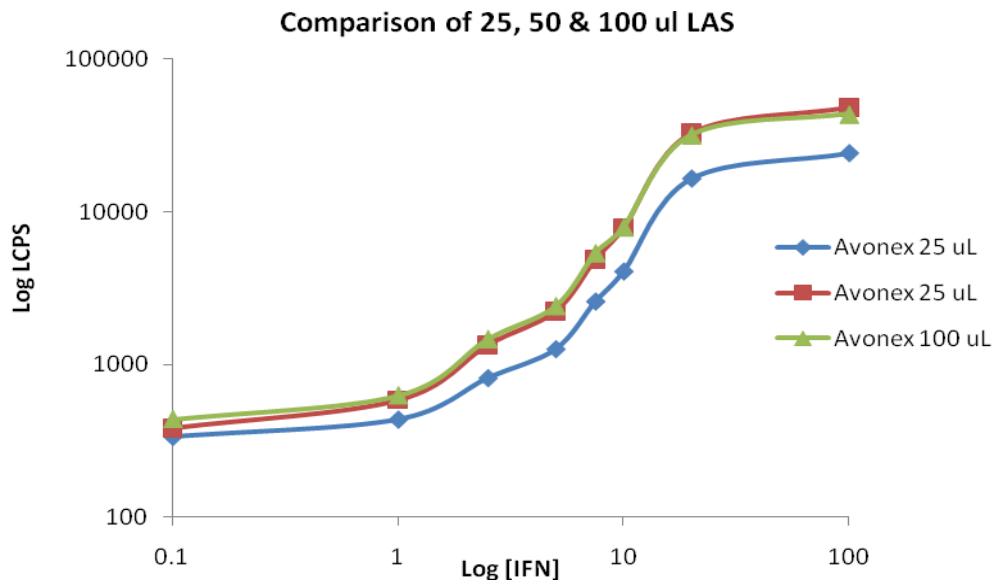
Figure 3-16   Standard curves of IFNβ-1a Avonex
IFNβ-1a (Avonex) and IFNβ-1b (Betaferon) detected using 25, 50 or 100μL of Luciferase assay substrate

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3.7.8.   Evaluation of Luminometer parameters

A single plate was read on 4 occasions altering the duration of measurement of LCPS from each well. Readings at 0.1 s/well, 0.5 s/well, 1s/well and 2s/well were obtained. The effect on maximum LCPS measured increased significantly from 0.1 to 1 s/well and began to plateau after this point [Table 3-6]. Reading times of 0.5 – 2 seconds per well did not significantly affect the endpoint and using 1 s/well ensures the total reading time of the plate is < 2 minutes which is more convenient when analysing several plates.

s/well	Max LCPS	Min LCPS	50% endpoint	Actual LU
0.1	1601	24	2.29	17.5
0.5	4687	15	2.35	15
1	8834	159	3.07	14.61
2	9534	177	3.10	14.07

Table 3-6 Luminometer settings
Maximum and minimum LCPS obtained varying the duration of measurement from each individual well.

3.7.9.   Effect of cell density

Plates were prepared with cell densities ranging from 20,000 to 100,000 cells per well. Six samples were tested in each plate to ascertain the effect of cell density on titre obtained. No significant difference in titre was found (repeated measures ANOVA p = 0.079), coefficients of variation range 0-21% [Table 3-7].

	100,000/well	80,000/well	60,000/well	40,000/well	20,000/well	CV
S1	<20	<20	<20	<20	<20	NA
S2	69	62	64	67	63	4
S3	167	180	160	155	200	10
S4	83	80	75	72	79	6
S5	600	702	653	590	612	7
S6	>1280	>1280	>1280	>1280	>1280	NA

Table 3-7 Cell density effect.

3.7.10. *Precision, Accuracy, Repeatability and Robustness*

The precision of the assay was assessed by measuring NIH anti-human IFNβ antibody reference standard antibody titres. The reference antibody was reconstituted as per NIH recommendations. This has an attributed titre of 1:1700 using a CPE assay and titre calculated using the Kawade method. This was measured on 10 separate occasions to assess accuracy of the Luciferase assay. Figure 3-17 shows the results obtained; mean titre 1716, CV 8.8 %, [range 1492 – 1989].
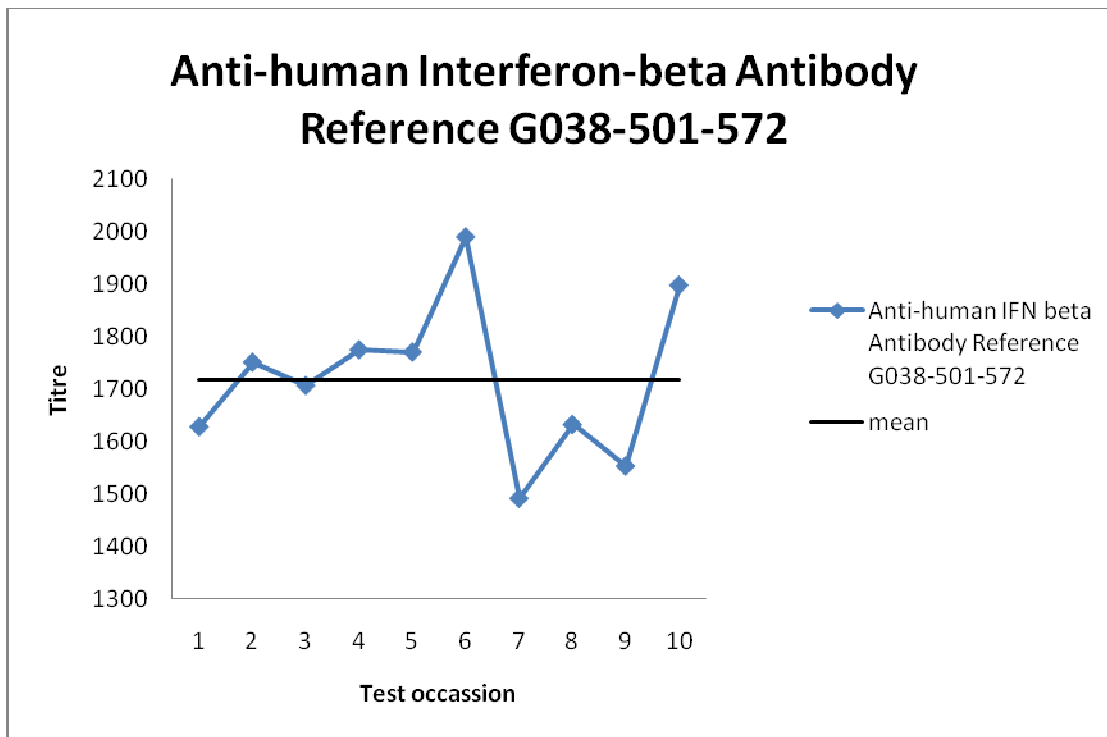
Figure 3-17 Repeated testing of NIH NAb reference standard on 10 occassions against IFNβ 1a (Avonex).

To evaluate accuracy ten known positive patient samples, encompassing a range of titres were tested on five occasions on five different days, using cells of different passage number. CVs ranged from 8 -23% depending on titre [Table 3-8] Samples with low titres were prone to higher % variability although in absolute terms the differences were small.

Sample	1	2	3	4	5	mean	SD	CV %
1	> 1280	> 1280	> 1280	> 1280	> 1280	> 1280	0	NA
2	647	754	598	671	605	655	80	12
3	258	238	292	301	275	273	29	11
4	171	160	183	154	163	166	13	8
5	93	83	90	75	80	84	8	10
6	56	58	62	66	49	58	4	8
7	35	33	34	27	40	34	4	11
8	36	25	31	22	20	27	6	23
9	22	12	18	20	25	19	4	22
10	<20	<20	<20	<20	<20	<20	<20	NA

Table 3-8 Repeated testing of 10 known NAb samples

3.7.11. External Validation of Luciferase assay

Blinded exchange of 200 samples took place between the Neuroimmunology laboratory in London and laboratories in Turin, Innsbruck, Stockholm and Biogen Idec (USA). Samples were tested using the current Luciferase assay (IoN) and the MxA protein ELISA in Turin, Innsbruck and Stockholm. A further 24 samples were also tested using the cytopathic effect assay in Turin. Samples were diluted to a factor of either 1:1280 or 1:2560 depending on the laboratory. For the purpose of calculations, titre values > 1280 or > 2560 were considered equal.

3.7.11.1. Turin

Samples (n=49) were exchanged between Dr. A Bertolotto's laboratory and IoN. Overall agreement – positive vs. negative outcome = 83.7% (41/49) of observed agreements. Inter-rater Kappa =0.67. Pearson correlation = 0.83. Of these samples 24 were also tested using the CPE. Overall agreement was 87.5% [Table 3-9]. The Kappa inter-rater agreement was=0.75 and Pearson correlation = 0.96. Bland Altman plots are shown below and shows overall good agreement between the titres obtained as the mean difference line sits on the zero line [Figure 3-18].

A.
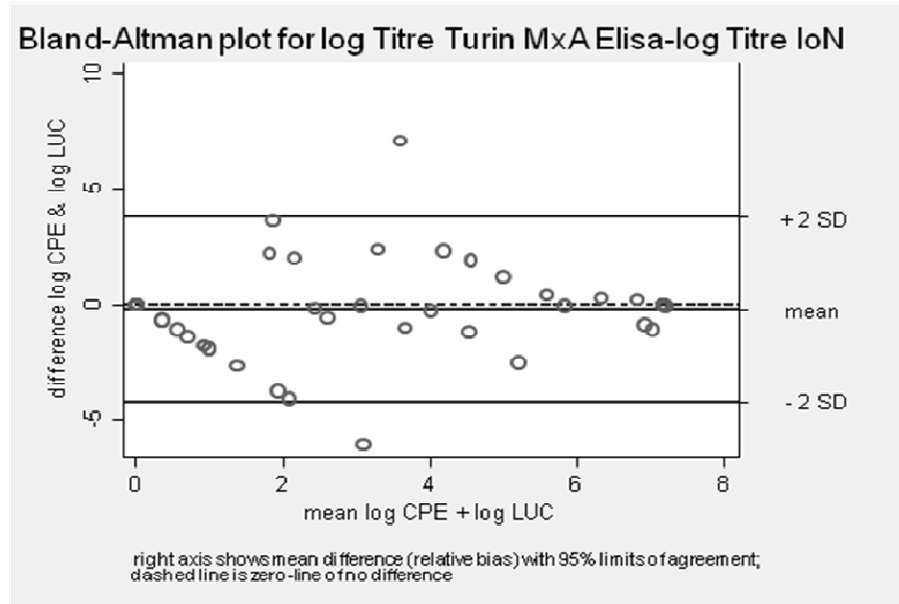
B.
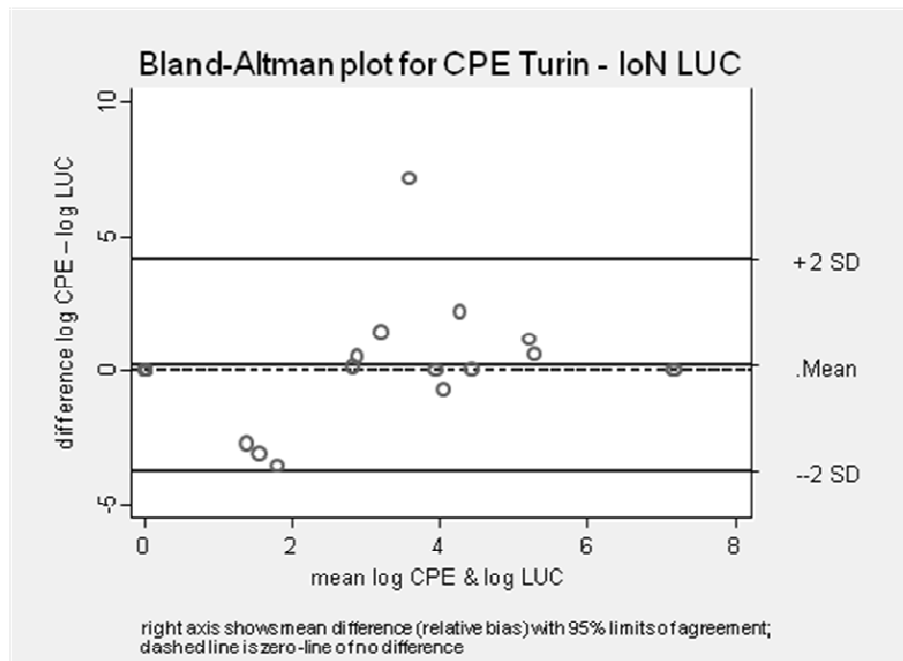
Figure 3-18 Bland Altman Mean difference plot - Italy
Figure A shows the agreement between Nab titres obtained using the MxA ELISA in Turin and the LUC assay at IoN, UK. Figure B shows the agreement between CPE assay in Turin and LUC assay UK

3.7.11.2. Innsbruck

Samples (n=57) were exchanged. All samples were tested using IFNβ-1b as this is always used for Nab assays in Innsbruck irrespective of subject treatment. Overall agreement – positive vs. negative outcome = 94.7% (54/57) of observed agreements [Table 3-9]. The inter-rater Kappa =0.87, Pearson correlation coefficient = 0.90. The Bland Altman plot showed that the titres obtained using the Luciferase assay tended to be higher than those obtained using the MxA ELISA although when testing for differences these were not significant (t test p = 0.067).
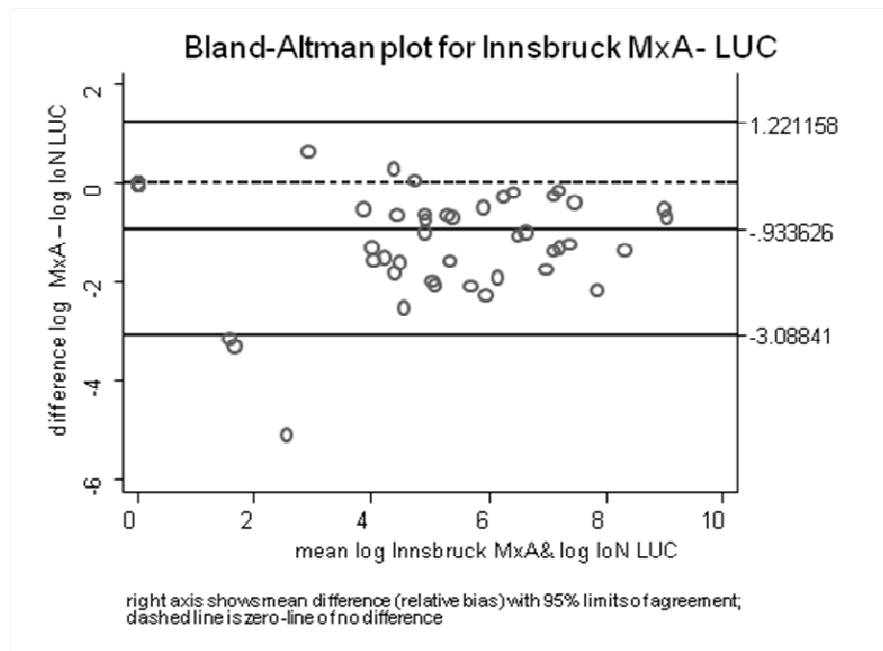
Figure 3-19 Bland Altman mean difference plot – Austria.
The figure shows the agreement between the MxA ELISA performed in Innsbruck Austria and the luciferase assay in the UK. Samples tend to have higher titres with the Luciferase assay as compared with the MxA ELISA as many sit below the zero line.

3.7.11.3. Stockholm

Samples were exchanged on 2 occasions. In total 86 samples were exchanged. Overall agreement was 84%, kappa inter-rater agreement was 0.56 and Pearson correlation coefficient was 0.83 [Table 3-9]. The Bland Altman plot shows that there is a tendency for the titres obtained with the Luciferase assay to be higher than those with the MxA ELISA in Stockholm as the mean difference line is at -0.4. This was noted to be related to some samples which were negative with the ELISA and low positive using the Luciferase assay.
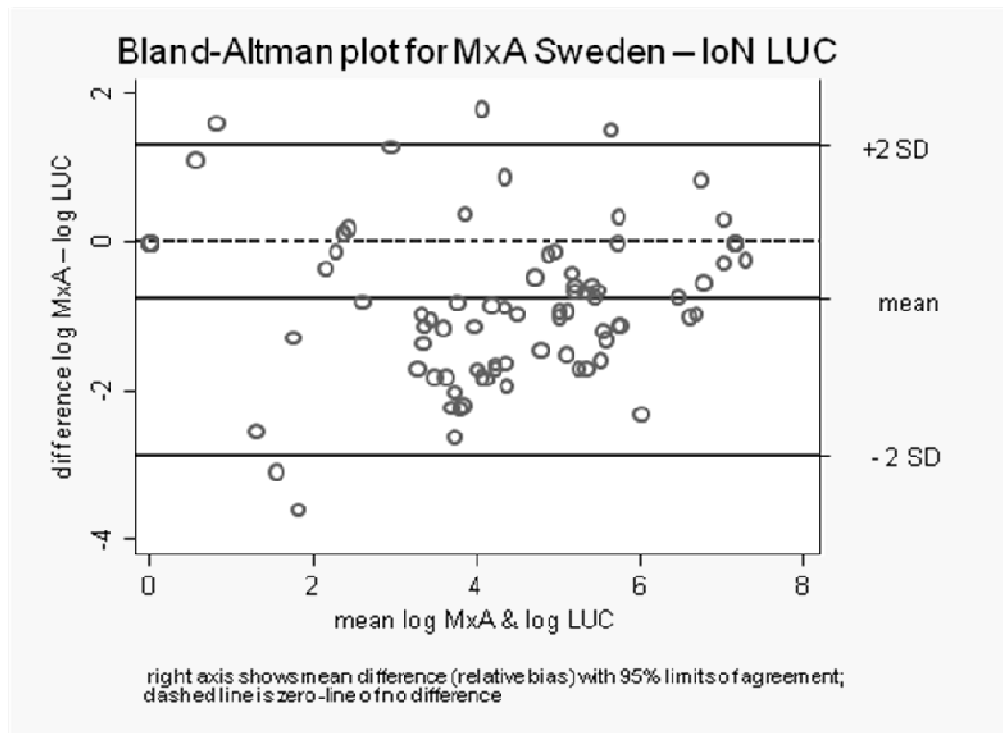
Figure 3-20 Bland Altman mean difference plot - Sweden
The figure shows the agreement between the titres obtained between the MxA ELISA in Sweden and the LUC assay UK.

3.7.11.4. Biogen Idec

Biogen Idec supplied 8 samples which were tested. Overall agreement 100%, Inter-rater kappa 0.86 and Pearson correlation coefficient 0.93 [Table 3-9].

LUC assay	Turin MxA		Innsbruck MxA		Stockholm MxA		Biogen Idec MxA		Total
	pos	*neg*	*pos*	*neg*	*pos*	*neg*	*pos*	*neg*	
Agree	23	18	39	15	55	16	6	2	**174/ 200** *87%*
Disagree	4	4	2	1	1	14	0	0	**26/200** *13%*

Table 3-9 Agreement of NAb results with Luciferase assay vs other laboratory.
The titres obtained using the Luciferase NAb assay at IoN were compared with the "known" results obtained using the MxA protein ELISA assay in other accredited European Laboratories.

The overall agreement for the 200 samples exchanged was 87% between MxA ELISA and Luciferase assay (n=200) [Table 3-9]. There were a significant number of disagreements in the samples exchanged with Stockholm. These were samples which were called negative if titre < 20 NU. Of the discordant results the titres measured in Stockholm were between 10 – 19 TRU in 9 of 14, but > 20 NU (20 -42 range) when tested using the Luciferase assay. Whether difference in low titres make a clinical difference is a moot point and will discussed in detail in chapter 5.

3.7.12. Quality Control

Pooled serum samples were stored in small aliquots at -80° C. A negative and a positive control were included on each plate. On dilution plates a moderately positive control was diluted to obtain a titre. All samples, QCs and standards were tested in duplicate, if samples did not perform as expected the plate was rejected and the analysis repeated. IFNβ-1a standards: if the CV for any of the standard points was $> 10\%$, the graph showing a plot of the raw data was examined. If any one of the plotted points, relating to these high CVs, was obviously an outlier it was excluded from the calculations relating to the standard curve. A maximum of two outliers were removed in this manner. If more than two were present the assay was repeated.

Patient samples: If CVs of any dilution points were above 10%, it was evaluated as to whether any of the individual measurements changed the status of the sample, from either positive to negative or negative to positive or led to a significant change in titre. If the status was changed the sample was re-analysed.

NAb assay worksheet 13/07/2009

	1	2	3	4	5	6	7	8	9	10	11	12
A	3528	3050	118	90	111	110	97	104	106	81	2860	2996
B	2514	2417	4323	67	205	240	97	119	118	100	1378	1738
C	1659	1488	4574	80	601	708	121	132	179	237	2567	2580
D	882	875	4519	58	1623	1812	446	422	876	976	1071	1527
E	357	395	4287	69	2357	2290	1160	1321	1857	1826	2714	2854
F	182	197	4289	70	2831	2737	2196	2101	2639	2780	2926	3048
G	90	113	4561	68	2981	2999	3142	3023	3343	3180	2985	3029
H	83	50	2067	1736	3035	3203	3252	3174	3607	3372	3290	3266
	1	2	3	4	5	6	7	8	9	10	11	12

LU/mL	IFN Diln	CPS	Log CPS	LogIFNDil
10.00	300000	3289	3.52	5.48
5.00	600000	2466	3.39	5.78
2.50	1200000	1574	3.20	6.08
1.25	2400000	879	2.94	6.38
0.63	4800000	376	2.58	6.68
0.31	9600000	190	2.28	6.98
0.16	19200000	102	2.01	7.28
0.08	38400000	67	1.82	7.58

Log IFN Dil (x) vs Log LCPS (y)

Log IFN Dil (x) vs Log LCPS (y) Linear section

$y = -1.0385x + 9.5308$

$R^2 = 0.9955$
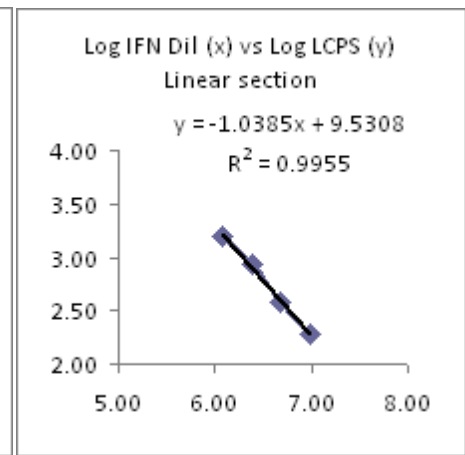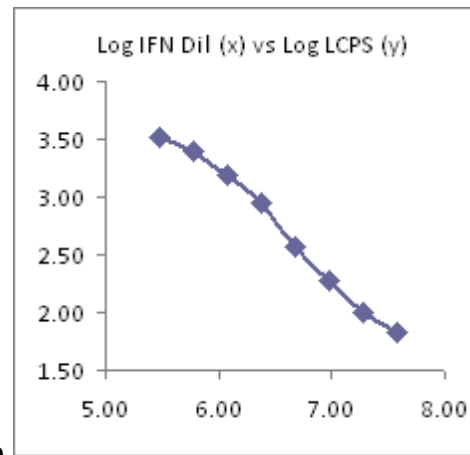
	CPS	Log CPS	LU/mL	Log IFN Dil	IFN Diln
Maximum	4426	3.65			
Minimum	69	1.84			
50% Endpoint		2.74	1.0	6.54	3447562

LU used to stimulate cells

n= **11.49**

300000

(n-1)/9= **1.17**

QA			
	Av CPS	SD	CV
Maximum	4426	140	3
Minimum	69	7	10

149

Samples	LCPS 1	LCPS 2	Average	SD	CV	Log CPS	Diln	Serum Dil (f)	NAb titre Report	Result	Sample ID	Report P SQ
10.00	3528	3050	3289	338	10	3.52						
5.00	2514	2417	2466	69	3	3.39						
2.50	1659	1488	1574	121	8	3.20						
1.25	882	875	879	5	1	2.94						
0.63	357	395	376	27	7	2.58						
0.31	182	197	190	11	6	2.28						
0.16	90	113	102	16	16	2.01						
0.08	83	50	67	23	35	1.82						
Positive QC	118	90	104	20	19	2.02	20			Positive	QC Pool	Pass
Negative QC	2067	1736	1902	234	12	3.28	20			Negative	QC Pool	Pass
								Sample 1				
Sample 1	111	110	111	1	1	2.04	20	76	88	Low positive		
	205	240	223	25	11	2.35	40		corrected			
	601	708	655	76	12	2.82	80					
	1623	1812	1718	134	8	3.23	160					
	2357	2290	2324	47	2	3.37	320					
	2831	2737	2784	66	2	3.44	640					
	2981	2999	2990	13	0	3.48	1280					
	3035	3203	3119	119	4	3.49	2560					
								Sample 2				
Sample 2	97	104	101	5	5	2.00	20	192	224	Positive		
	97	119	108	16	14	2.03	40		corrected			
	121	132	127	8	6	2.10	80					
	446	422	434	17	4	2.64	160					
	1160	1321	1241	114	9	3.09	320					
	2196	2101	2149	67	3	3.33	640					
	3142	3023	3083	84	3	3.49	1280			;		
	3252	3174	3213	55	2	3.51	2560					

3.8.   Conclusions

The Luciferase assay fulfils the international need of a reliable cost and time efficient NAb assay which can be incorporated easily into routine use. Reporter gene assays are frequently used in bioassay systems as markers of biological activity of a substrate. In this context the luciferase gene was linked downstream to the ISRE and reliably expressed when IFNβ binds to the cell surface receptor and initiates the cell signalling pathway which results in activation of ISRE transcription of the luciferase gene. To validate the assay parameters specific to assay conditions, accuracy, reliability and precision were examined and samples were exchanged with four other validated laboratories to compare titres obtained with the Luciferase assay and existing assays. The assay was found to be highly robust in relation to incubation times and cell density. High throughput screening is possible as 32 samples can be evaluated on a single plate and dilutions are only required for those which reach the 50% endpoint. Unlike existing assays it is easily completed in less than 24 hours and does not require expensive or patented materials. The cells employed were already in the public domain and with the permission of Dr Gilles Uze could be distributed to independent validated laboratories for use. As part of the NABINMS consortium the Luciferase assay was distributed to other consortium members and established in their laboratories. Results relating to distribution of the assay will be reported in chapter 7.

Chapter 4

Evaluation of the frequency and clinical effects of NAbs

in subjects receiving IFNβ

4 Evaluation of the frequency and clinical effects of Neutralising Anti-Interferon beta antibodies in subjects receiving IFNβ

4.1. Background

As described in chapter 1 IFNβ is a biological therapy which is known to be immunogenic, inducing anti-IFNβ neutralising antibodies in a proportion of subjects. The frequency of NAbs varies depending on IFNβ product and the NAb assay used. The objective in this chapter was to assess the frequency of NAbs using the newly established luciferase reporter gene assay, to evaluate their association with relapses, occurrence of side effects and to compare the results in our cohort with that of published data.

4.2. Materials and methods

BAbs were measured using the cELISA as described in chapter 2 and NAbs were measured using the Luciferase assay described in chapter 3. Testing was performed in accordance with the standard operating procedures described in earlier chapters.

4.2.1.   Subject acquisition

All subjects attending the NHNN receiving interferon beta who were being followed in the outpatient department on a regular basis were eligible for inclusion in the study. Since 1996 most subjects had serum samples stored for future NAb analysis. These samples were coded with a β-IFN number and recorded in an electronic database. This was accessed to identify those who had received treatment for $\geq$ 2 years as of 31st January 2006. Those on treatment for a shorter period were excluded as were those who stopped therapy or switched to an alternative (non IFNβ) treatment, such as Copaxone or Mitoxantrone, during this period. Those who stopped or switched treatment after 2 years of IFNβ therapy were included.

Case notes of each subject included in the study were requested from medical records in the hospital. Those which were located were reviewed for clinical information: date of birth, sex, MS subtype at time treatment commenced, IFNβ product chosen, number of relapses in 24 months prior to treatment, relapse rate on treatment, side effects experienced, treatment cessation or switching to alternative product.

4.2.2.   Samples

As NAbs generally appear between 6 – 18 months time points [Sorensen *et al.* 2005] 12 and 24 months were selected for testing. Those who had been on treatment for a further year had an additional "follow up" sample included. These samples were tested for the presence of binding antibodies to IFNβ by capture ELISA and also for the presence of

neutralising antibodies using the Luciferase assay as previously described in Chapter 3.

4.2.3.   Statistical Analysis

The percentage of subjects who developed NAbs at each time point was calculated to determine the incidence in each treatment group. All analyses were performed using Stata™ Version 9.2, 2006 (StataCorp Texas USA). Difference between groups was analysed using Chi-squared test. Measure of association between NAb status, relapse rate, side effects, treatment cessation were analysed using Pearson Chi-squared test or Fisher's exact test as appropriate. All reported p values were based on two tailed statistical tests and a significance level of 0.05.

4.3. Results

After reviewing the database of samples 348 subjects were identified who had commenced therapy prior to January 2004. A further 21 subjects were excluded due to early treatment cessation or switching to non IFNβ product, leaving 327 subjects included in the analysis. The female: male ratio was 2.56**:**1, 71.9% being female $n = 238$ and 28.1% being male $n = 93$. The mean age at time of study was 41.8 years (range 19-68), the mean time from disease diagnosis to treatment commencement was 5.56 years (median 4 years, range < 12 months – 28 years). The mean relapse rate prior to treatment was 1.53 / year. Eighty eight percent of subjects had RRMS at the time treatment was commenced; the remaining 12% had SPMS.

4.3.1. Product breakdown

Of the 327 subjects included 119 subjects (37.3%) were treated with IFNβ-1a IM (Avonex®), 55 subjects (16.2%) with IFNβ-1b SC (Betaferon®) and 149 subjects (46.5%) with IFNβ-1a SC (Rebif®) either the 22 or 44 μg dose. In total 649 samples were available for testing; 260 at 12 months, 200 at 24 months and 189 subjects had samples available for follow up testing. 238 subjects had ≥2 samples available for testing, 23% had only one sample available and with regards to data analysis these were included in the "anytime positive" analysis and NAb rates at a particular time point but were excluded from the longitudinal analysis regarding Nab persistence.

4.3.2. BAb and NAb analysis per sample.

All samples were screened for BAbs and subsequently tested for NAbs, 331 samples were BAb +ve (51% of samples tested), 317 (49%) samples were BAb -ve and NAb –ve, 174 (27%) BAb +ve NAb –ve and 156 were BAb +ve NAb +ve. There were two samples which tested positive for NAbs but were negative for BAbs. Both of these had low NAb titres (24 and 36). The percentage of BAb +ve samples by product was: 24% Avonex, 50% Betaferon, 47.6% Rebif. The total number of NAb positive samples was 156 (24 % of all samples tested). The percentages of NAb positive samples at each time point tested are illustrated below [Table 4-1].

Product	1 year		2 years		> 3 years		total
	positive	negative	positive	negative	positive	negative	
Avonex	8 (8.84%)	87 (91.6%)	6 (7.7%)	72 (92.3%)	4 (5.8%)	65 (94.2%)	242
Betaferon	15 (38.5%)	24 (61.5%)	11 (30.6%)	26 (69.4%)	11 (28.2%)	28 (71.8%)	115
Rebif	45 (35.7%)	81 (64.3%)	32 (37.6%)	53 (62.4%)	24 (29.6%)	57 (70.4%)	292
total	68	192	49	151	39	150	649

Table 4-1 Percentage of samples tested which were NAb positive at 12 and 24 months and in the follow up period

Table 4-2 depicts NAb status and titre *per product* of all subjects and reflects the samples that were available for testing. Samples were not available for all subjects

at each time-point. Titres were highest in the NAb positive samples of subjects treated with

Rebif. Of those testing positive, 50% of those on Rebif had titres > 320 NU as compared to

only 31% of those treated with Betaferon and 11% of those on Avonex.

Titre of positive samples	Avonex n=242	Betaferon n=115	Rebif n=292
< 20 NU (negative)	92.6% (224)	69.55% (80)	65.4% (191)
Total > 20 NU (positive)	7.4% (18)	30.45% (35)	34.6% (101)
20 – 99 NU	4.4 % (10)	13.9% (16)	11.0% (32)
100-320 NU	2.6% (6)	7.0% (8)	6.5% (19)
> 320 NU	0.9% (2)	9.6% (11)	17.1% (50)

Table 4-2 NAb status and titres per product

4.3.3. NAb analysis by subject

Three hundred and twenty seven subjects were included in the study. Of these, 130 subjects (40%) were BAb positive and 89 (27%) subjects were NAb positive at anytime. The risk of being NAb +ve at 12 months was: 8% IFNβ-1a IM (Avonex), 39% IFNβ-1b (Betaferon) and 33% IFNβ-1a SC (Rebif), $p<10^{-5}$. The risk at 24 months was 8% (Avonex), 31% (Betaferon) and 27% (Rebif) respectively, p=0.002 [Figure 4-1]. Only 4 subjects (2.1%) who were NAb –ve at 24 months subsequently became NAb +ve in the follow up period. There was no difference in NAb rates seen in women (27% NAb +ve) or men (31% NAb +ve) p=0.377.
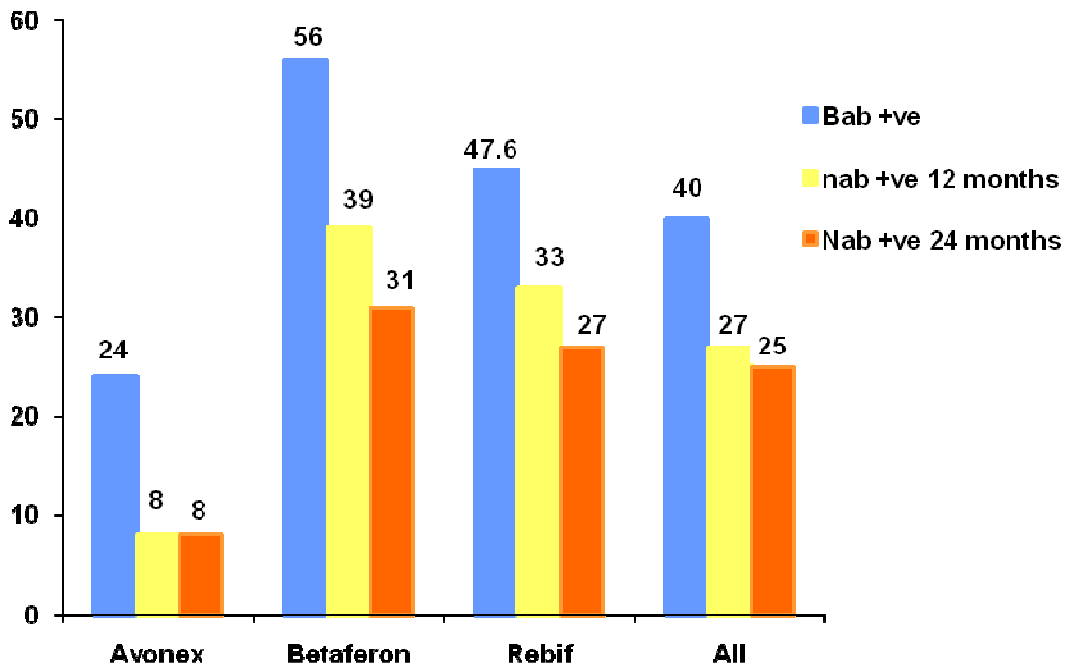
Figure 4-1 Percentage of subjects who are BAb or NAb positive

4.3.4. NAb persistence.

As 23% of subjects had only one sample available for testing they were excluded from the analysis of NAb persistence. 251 subjects had a 12 or 24 month sample and a follow up sample (greater than 3 years). Of these, 84 subjects were NAb +ve at either 12 or 24 months 61% remained NAb +ve at follow up (mean 4.3 years; 2-8 years), 39% reverted to NAb –ve status. Of those receiving Betaferon 37.5% (9/24) reverted, Avonex 50% (5/10) and 38% of those receiving Rebif (19/50) [Table 4-3]. Most subjects who reverted had titres < 100 NU and all were < 320 NU. The mean titre of reverters was 85 (SD 79) vs 374 (SD 167), p < 0.0001. These titres are illustrated in Figure 4-2.

	Avonex	Betaferon	Rebif
Persistent Neg	88.5%	44.5%	53.7%
Persistent Pos	5.8%	34.1%	28.7%
Reverters			
% all subjects,	5.8%	20.5%	17.6%
(% NAb +ve subjects)	(50%)	(37.5%)	(38%)

Table 4-3 Percentage of subjects who reverted to NAb negative status during follow up period

NAb titres by product of reverters and persistent NAb positive
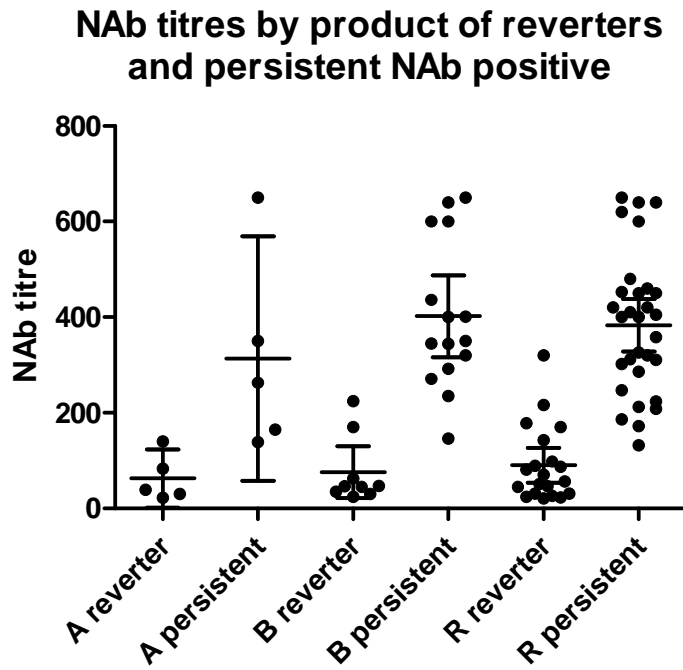
Figure 4-2 Nab titres by product in reverters vs non-reverters
Mean and 95% CI of mean shown.
A = Avonex, B = Betaferon and R = Rebif

4.3.5. Effect of NAbs on relapse rate

Medical notes were requested on all 327 subjects, of which 288 were available for retrospective review. Reliable data regarding relapse rates was available on 228 subjects, 32 % of whom had no documented relapses since treatment commenced. Pre treatment annualised relapse rate (ARR) was 1.53/year (range 1 – 12). The post treatment ARR was 0.55/year (range 0 – 5.9) for all subjects. The ARR in NAb –ve subjects was 0.50 and was 0.67 in the NAb +ve cohort (chi-squared p=0.04). There was a higher total number of relapses in subjects who were persistently NAb +ve in the follow up period as opposed to those who tested positive on only one occasion and reverted to negative status (0.27 vs. 1.58 relapses, p < 0.0005). Of those who were documented as relapse free (75 subjects), 61 (81.3%) were NAb –ve throughout, 11 (14.7%) were NAb +ve at any time and 3 (4%) were persistently NAb +ve, all having Nab titres < 100 NU.
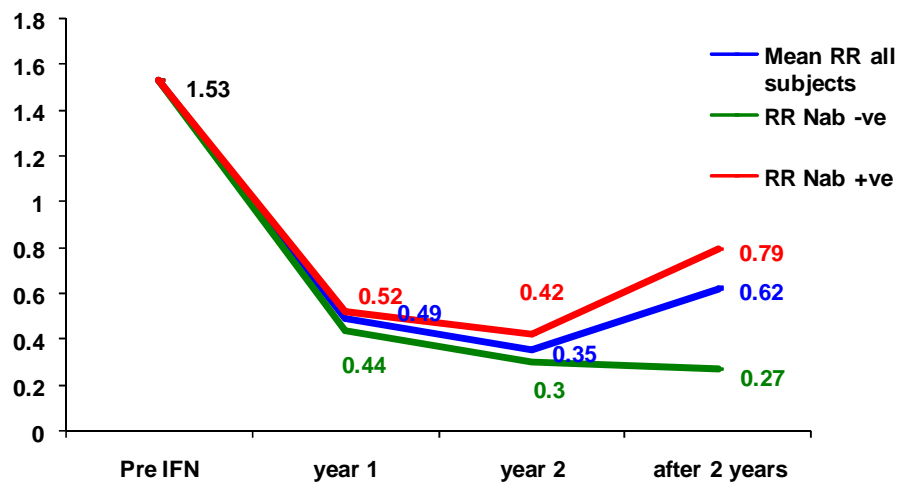
Figure 4-3 Relapse rates in subjects depending on NAb status.

The mean relapse rate during the first year of therapy was 0.49 (range 0-3) and 0.35 (range 0-2) in the second year. The mean number of relapses in the follow up period (i.e. > 2 years) was 0.62 range (0 – 6). NAb status at 12 months was not a predictor of relapses in the following 12 months, relative risk 1.13 (95% CI 0.9 – 1.4, chi2 p=0.45), however NAb status at 12 months was found to predict relapses in the period 2 years after treatment commenced, relative risk 1.55 (95% CI 1.05 – 2.42, p=0.012). Similarly there was a strong association between NAb status at 24 months and subsequent relapses (occurring after 24 month) relative risk 1.3 (95% CI 1.01 – 1.56 p=0.002). Thus the effect of NAbs on relapse rate lags behind their initial appearance.

The frequency of relapse was calculated with regards to the actual NAb titre measured. Titres were divided into NAb –ve, titre 0 – 19 NU, low positive 20-99 NU, moderate positive 100 – 319 and high positive titre > 320 NU. The NAb status at 24 months correlated with relapses. 27% of subjects whom were NAb –ve at 24 months had a subsequent relapse during follow up as compared with 52% of those with low positive, 62% of those with moderate positive titres and 65% of those with titres > 320 [Figure 4-4]. This showed that subjects with higher titres (> 100NU) were at greater risk of relapse. Higher relapse rates were also found in subjects who were persistently NAb +ve as opposed to those who tested positive on only one occasion and reverted to negative status, 0.27 vs. 1.58 (ANOVA, p<0.005).
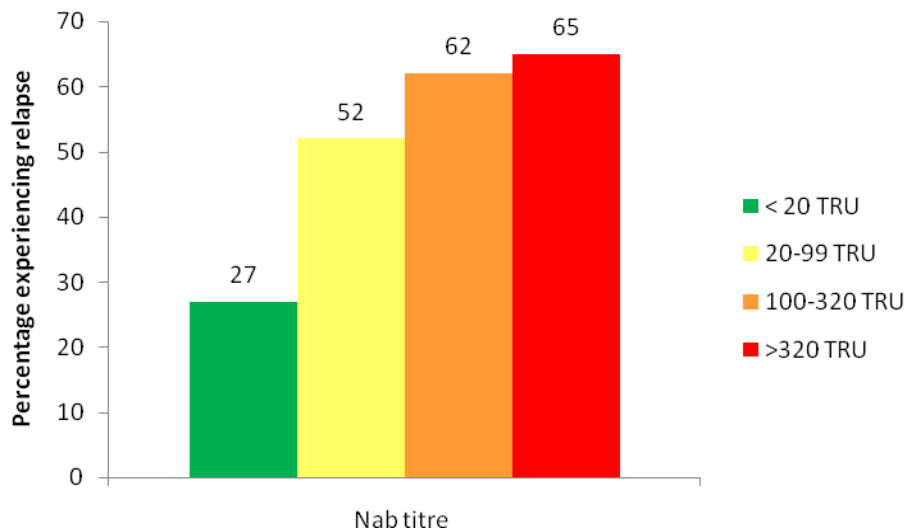
Figure 4-4 Percentage of subjects experiencing a relapse depending on NAb titre

4.3.6.   Side Effects

Charts were reviewed retrospectively for the presence or absence of side effects related to IFNβ therapy – including flu-like symptoms, skin reactions, and headache. Of the 238 NAb –ve subjects 135 had a record regarding side effects with 108 (46%) reporting ongoing troublesome side effects. In the NAb +ve cohort (89 subjects) a record was found in 56 subjects with 32 (36%) reporting troublesome side effects. Analysing those who reported troublesome side effects (140) 113 (81%) were NAb negative and 27 (19%) NAb positive. Reporting side effects was found to be a predictor of NAb -ve status (Pearson chi2 p= 0.046). Of the 51 subjects who reported an absence of side effects 24 were NAb +ve

and 27 NAb –ve. Evaluating side effects as a predictor of NAb status revealed that a positive report has a specificity of 81% in identifying those who remained NAb negative, but sensitivity of only 47%.

4.3.7.   Treatment Cessation

Medical notes and the IFNβ treatment database were reviewed to ascertain subjects' treatment status at the time the review was conducted. Data was available for 310 cases. Of these 196 were still on treatment, 91 had stopped treatment, 17 were lost to follow up and 6 had stopped in order to conceive. The 91 cases that stopped treatment included those who switched to Copaxone® or Mitoxantrone due to IFNβ treatment failure, persistent relapses and those who met other criteria for treatment cessation (EDSS > 6.5). Of those who stopped treatment 42% were NAb +ve as compared to 33% of those continuing treatment, however this difference was not significant p=0.565. Similarly correlating NAb status at 24 months with treatment cessation, no significant difference in NAb status was found between those who continued treatment and those who had stopped (22% NAb -ve vs. 29% NAb +ve, p=.057).

4.4.   Conclusions

In this cohort of 327 subjects, 649 time points were tested for BAbs and NAbs. The rate of NAb positivity in our cohort reflects that in the published literature and further supports the validity of the Luciferase reporter gene NAb assay. Rates quoted in other studies for Avonex, Rebif and Betaferon are reported as ~ 2-6%, 15-44%, 25-47% respectively (chapter 1) and depends on the sensitivity of the assay used [Rudick *et al* 1998, Jacobs *et al* 2000, PRISMS 1998, Interferon β MS study group 1996, Francis *et al* 2005, Bertolotto *et al* 2002]. As samples were not available for all subjects at each time-point analysis per sample shows NAb frequencies at each time point that was dependent on the individuals who had samples available. This accounts for the difference in the NAb frequencies reported *per sample* as compared to *per subject*. Analysis of the data *by subject* the risk of being NAb +ve at 12 months was 8%, 33%, 39% (Avonex, Rebif, Betaferon) and at 24 months was 8%, 27%, 31%. These values are similar to those published, showing that Avonex is less immunogenic than the two high frequency, higher dose preparations. Betaferon appears to have the highest frequency of NAbs but the difference between Betaferon and Rebif is small. Absolute NAb titres were highest in those subjects treated with Rebif, 50% of whom had titres >320 NU as compared to only 33% of those on Betaferon and 11% of those treated with Avonex. Many studies consider the two doses; 22 µg and 44µg of Rebif independently and have reported higher NAb frequencies in the Rebif 22 µg dose [Francis *et al.* 2005]. In this cohort it was difficult to ascertain from retrospective case note review which subjects remained on 22 µg and if and when subjects were switched to the higher dose. In view of this the two doses were analysed together. Only two subjects were BAb –ve and NAb +ve (both treated with Rebif and NAbs < 100

NU). This would suggest that in view of the luciferase assay being both cost and time efficient BAb screening is not essential prior to Nab testing and that simple screening for neutralising activity should be the first step.

Clinical information with regards to side effects was only available in 191 subjects. In those where a record was made it was more likely that troublesome side effects were reported (positive bias), this may account for the low sensitivity. The predictive value of side effects reflecting NAb negativity and the significant association found between NAbs and side effects are useful when following a subject in the clinical setting but NAb testing is still required to confirm this.

The pre-treatment relapse rate of 1.53/year was high compared to the relapse rates quoted in most clinical trials. This was due to local guidelines determining eligibility for disease modifying therapy. Subjects in the UK are only deemed eligible for treatment with a DMD if they have experienced $\geq$ 2 relapses in the previous 2 years. Due to this all relapsing remitting subjects (88%) in this study had a baseline annualised relapse rate $\geq$ 1/year. The apparent relapse rate reduction (0.55/year overall) in relation to treatment may over estimate the treatment effect as it is likely not all relapses were documented or required medical review after therapy commenced. The natural history of MS is for relapses to reduce overtime so regression to the mean is also a factor to account for reduction in relapse rates of the entire cohort. Subjects who continued to relapse or progress rapidly after starting IFNβ may have been switched to either Copaxone or Mitoxantrone during the first 2 years of treatment and were therefore excluded from the analysis, biasing the cohort in favour of responders or those with less severe disease. A difference was found in the annualised relapse rates of those who were persistently NAb –

ve and those who were NAb +ve at any time (0.5 vs. 0.67 p = 0.04). NAb +ve status at 12 and 24 months was significantly associated with relapses after 24 months of treatment showing the effect of NAbs on efficacy of IFNβ lags behind the appearance of NAbs. This has been described previously and may reflect the length of time it takes for the biological efficacy of Interferon-beta to be lost [Kappos *et al.* 2005; Francis *et al.* 2005]. A longitudinal MRI study, following 2 subjects who discontinued treatment, has shown that disease activity begins to increase 6 months after treatment has stopped and returns to baseline ~ 10 months after treatment cessation [Richert *et al.* 2000]. We have also shown that higher NAb titres are associated with increased risk of relapse.

Over 100 subjects had stopped treatment during the follow up period, many of whom stopped due to disease progression. Those who had persistently active disease on IFNβ were considered for Copaxone or Mitoxantrone therapy. As the medical notes did not specify the reason in many cases all were considered together. This may contribute to the lack of association seen between NAb positivity and treatment cessation/failure. In addition, patients who are destined to become NAb positive have a greater reduction in relapse rate than those who remain NAb negative in the first 6 months of starting treatment [PRISMS 2001; Francis *et al.* 2005; Kappos *et al.* 2005, Sorensen *et al*. 2007] this biological effect reduces the impact of NAbs on relapse rate after 6 months of treatment [Giovannoni and Goodman 2005]. The results in this study would support implementation of guidelines which recommend routine Nab testing with the view that persistent NAb positivity is a herald of treatment failure and increased relapse rates.

Chapter 5

Validation of a novel anti-Interferon beta antibody assay:

Correlation with induction of peripheral biomarkers

5 Validation of a novel anti-Interferon beta antibody assay: Correlation with induction of peripheral biomarkers

5.1. Background

As previously discussed [chapter 1] IFNβ alters the expression of several hundred genes at the nuclear level and the downstream production of mRNA and proteins. These confer the anti-viral, anti-proliferative and immunomodulatory actions of IFNβ. Many of these can also be induced by other factors and are thus not specific markers of IFN activity. Some however, have been identified as being relatively specific to type 1 IFNs. Myxovirus resistance protein A (MxA) is a good example of this [Deisenhammer *et al.* 2000; Pachner *at al.* 2003 b]. Other useful biomarkers include 2-5 oligoadenylate synthetase [Pachner *at al.* 2003 b], neopterin [Cook *et al.* 2001] β2-microglobulin [Francis *et al.* 2005] and TRAIL (tumor necrosis factor apoptosis inducing ligand) [Wandinger *et al.* 2003;Gilli *et al.* 2006], but these are not exclusively induced by interferons. These have been used to show that the presence of NAbs reduces the bioactivity of IFNβ. Bertolotto et al have shown that there is a significant rise in MxA mRNA and protein in response to IFNβ administration and that this response is lost in the presence of NAbs [Bertolotto *et al.* 2003; Gilli *et al.* 2005, Hesse *et al.* 2009]. In the case of MxA mRNA levels, this response has been shown to be maximal at 12 hours. Similarly the MSCRG (1998) showed failure to induce neopterin in the NAb positive cohort [Rudick *et al.* 1998]. This finding has also been replicated in several other papers. Neutralising antibodies were measured in relation

to induction of β-2 microglobulin and neopterin. In the presence of Nabs failure to show any significant response to IFNβ was shown [Francis *et al.* 2005]. The second study measured the *in vivo* response to IFNβ by measuring pre and post dose levels of MxA mRNA, neopterin and β_{2-} microglobulin [Scagnolari *et al.* 2007]. Responses were compared between NAb +ve and NAb –ve cohorts. Both pre and post dose levels were lower in NAb +ve subjects and a significant inverse correlation was found between Nab titre and pre dose levels in particular. They showed that the steady state levels were significantly suppressed in NAb +ve patients and in a titre dependent fashion.

Some Neurologists argue that some bioactivity may be preserved in patients with NAbs despite lack of induction of MxA in response to IFNβ injection. The Danish group have measured gene expression by gene chip analysis in 12 NAb -ve and 12 NAb +ve patients to test that hypothesis. They were not able to detect differential expression of any of the interferon (IFN) beta-regulated genes identified in NAb -ve patients in the NAb +ve cohort, thus suggesting no residual bioactivity [Hesse *el al.* 2009]. Other studies have shown attenuation of the induction of TRAIL in response to IFNβ administration [Wandinger *et al.* 2003; Gilli *et al.* 2006].

Until the development of the Luciferase NAb assay [Farrell *et al.* 2008; Farrell and Lam *et al.* 2008] there has not been a readily available assay in the UK to measure NAbs. To further validate this assay we sought to measure biomarker induction (MxA) in a cohort of subjects with MS who were treated with IFNβ and use this to establish a "cut-off" or NAb titre, determined by the Luciferase assay, at which no detectable in vivo bioactivity of IFNβ is found. This would help develop better guidelines for interpretation of NAb results

using the luciferase assay and thus subsequent patient management. We anticipated that ~ 25% of subjects recruited would have detectable NAbs, but in only half of these the titre would be significant, i.e. at a level that inhibits the induction of the biomarkers. Patients on Avonex were excluded because the vast majority (> 95%) would be NAb negative. By measuring MxA before and after the administration of IFNβ we would be able to quantify the relative increase in each subject in response to the injection. In this way we correlated the NAb titre with relative attenuation of biomarker response and thus defined a threshold at which no IFNβ bioactivity is found.

5.2.   Hypothesis

Peripheral biomarker induction after IFNβ administration would be attenuated in a predictable fashion in the presence of NAbs. By quantifying this we would determine the titre (as measured by Luciferase reporter gene assay) at which partial and complete loss of INFβ bioactivity is seen and thus generate improved guidelines for clinical practice.

5.3. Materials

5.3.1. Sample collection

Plastic venous blood tubes 8mL EDTA Hemogard® closure, 8.5mL Serum tube with clot activator and gel for serum separation BD Vacutainer®, venepuncture kit, centrifuge, sterile 1.5 mL microtubes Sarsdedt®.

5.3.2. Processing and storage

Centrifuge, sterile 1.5 mL microtubes Sarsdedt®, sterile pipettes and tips, -80°C freezer

5.3.3. RNA preparation and quantification

PAXgene Blood RNA Tubes (cat. no. 762165), Ethanol (96–100%) for molecular biology Sigma Aldrich®, Pipettes: 2μL, 10 µL, 200 µL, 1000 µL, adjustable volume pipettes - Eppendorf Research®, Sterile, aerosol-barrier, RNase-free pipette tips, 0.1 - 10μL, 200 µL, 1000 µL (extralong) STARLAB® TipOne, Graduated cylinder, Centrifuge, Vortex mixer, micro-centrifuge, Incubator, Crushed ice, Nanodrop™ 3.1 Thermo Scientific.

5.3.4. Reverse transcription

High capacity cDNA reverse transcriptase kit (with RNase inhibitor) Applied Biosystems™ cat # 4374966, Nuclease free water, 0.2ml 8 Strip PCR Tubes, Individual attached domed caps STARLAB®, thermal cycler, microcentrifuge, pipettes and tips as before.

5.3.5.   PCR

TaqMan Universal master mix Applied Biosystems™ cat #4304437, ABI gene expression kit MxA and GAPDH, MicroAmp® Optical 96-Well Reaction Plate Applied Biosystems™ #4316813, Optical adhesive cover Applied Biosystems™ #4311971, Assays-on-demand™ Gene expression products; Mx1 assay id Hs00182073_m1, GAPDH assay id Hs99999905_m1 Applied Biosystems™, Nuclease free water, Taqman 7500 Real-Time PCR system Applied Biosystems™.

5.4. Methods

5.4.1. Subjects

Previous evaluation of subjects attending the NHNN revealed that ~ 25% of subjects treated with Rebif or Betaferon were NAb positive. NAb frequency in subjects treated with Avonex are < 8 % using the Luciferase assay, thus we elected to exclude those treated with Avonex to enrich the capture of NAb +ve subjects. Of those who were NAb +ve, the majority had low positive titres < 100 NU (~ 50%) and another ~ 40% had high positive titres > 320 NU. Other groups have also shown that ~ 10% of subjects have some neutralising activity but do not reach the 20 NU cut-off. It was estimated that by recruiting 150 subjects we would have ~ 50 subjects who would be NAb positive, with sufficient numbers of low to moderate titres to establish a cut-off level. Ethical approval to conduct the study was awarded in all sites prior to recruitment and subjects with MS treated with either Rebif or Betaferon, who regularly attended the one of several sites including the National Hospital for Neurology and Neurosurgery (NHNN), Barts and the London NHS Trust, Basildon hospital, Southend hospital and St Thomas' hospital were recruited. Recruitment began in July 2008 and was stopped in January 2010 when 144 subjects had completed the study. Recruitment was stopped before 150 subjects had been identified due to time and financial constraints related to completing the project.

5.4.2.   Samples

Blood samples were obtained on the day that IFNβ would be administered and again 12 hours after injection. Each day 4 PAXgene® tubes, 1 x 8.5 mL serum tube and 1 x 8 ml EDTA tube were taken. PAXgene® tubes were used to ensure the stability of mRNA for storage and future use. These have been commercially validated and shown to protect RNA molecules from degradation by RNases and also minimises further gene expression (www.preanalytix.com). Each PAXgene tube allows collection of 2.5 mLs of blood and should yield approximately 3μg RNA (eluted in 80μL). The PAXgene tubes were kept at room temperature for a minimum of 2 hours and were subsequently frozen at minus 80°C until use. A whole blood sample was aliqouted from the EDTA tube, the serum and remaining EDTA tubes were centrifuged for 10 minutes at 3000 rpm and serum and plasma aliquots were taken and frozen at -80°C until use. All of the samples were processed and stored at the Blizard Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry.

5.4.3.   Neutralising Antibody Quantification

Pre and post injection samples were tested for NAbs using the Luciferase NAb assay in the neuroimmunology laboratory as described in chapter 3. Titres were expressed in Neutralising Units (NU).

5.4.4.   MxA quantification

5.4.4.1. RNA extraction and purification

Two Paxgene® tubes taken prior to IFNβ injection and two taken 12 hours post IFNβ injection were used to extract and purify mRNA from each participant. Extraction was performed as using the PreAnalytix PAXgene Blood RNA system and following the manual extraction protocol. In brief the PAXgene Blood RNA Tubes were centrifuged for 10 minutes at 3000 x g. The supernatant was decanted and 2 mLs RNase-free water was added to each tube. The two PAXgene tubes from the same time point were combined at this stage creating a 4mL volume in one tube. The tube was closed using a fresh secondary BD Haemogard closure. The sample was vortexed until the pellet was visibly dissolved, and was centrifuged for another 10 minutes at 3000x g. The supernatant was decanted and 350 μL of buffer (BR1) was added. This was vortexed until the pellet was visibly dissolved and the sample was pipetted into a 1.5 ml microcentrifuge tube.

To this 300 μL of buffer (BR2) and 40 μL of proteinase K were added. This was mixed and incubated for 10 minutes at 55°C. The lysate was pipetted directly into the PAXgene shredder spin column, placed in a 2 ml processing tube, and centrifuged for 3 minutes. The entire supernatant of the flow-through was transferred into a fresh tube and 350 μL of ethanol (96–100%) was added. This was mixed and added in aliquots to the PAXgene RNA spin column which was centrifuged for 1 minute at 8000–20,000 x g. The flow through was discarded and the column placed in a new 2 ml processing tube.

At this stage the mRNA was bound to the column and the following steps are to purify and elute the mRNA. 350 μL of buffer (BR3) was added into the PAXgene RNA

spin column and centrifuged for 1 minute. The spin column was placed in a new 2 ml processing tube. For each spin column 10 µL of DNase stock solution was added to 70 µL buffer RDD and mixed before adding 80 µL to each column. This was incubated at room temperature (20–30°C) for 15 minutes. A further 350 µL of buffer (BR3) was added into the spin column, and centrifuged for 1 minute at 8000–20,000 x g, the old processing tube and flow-through was discarded. 500 µL of buffer (BR4 – containing ethanol) was then added and the spin column centrifuged for 1 minute. Another 500 µL of buffer (BR4) was added and centrifuged for 3 minutes.

Finally the mRNA was eluted by pipetting 40 µL of elution buffer BR5 directly onto the PAXgene RNA spin column membrane. Again this was centrifuged for 1 minute at 8000–20,000 x g and the step repeated with another 40 µL of buffer BR5 to generate an elution volume of 80 µL. The eluate was incubated at 65°C for 5 minutes and samples immediately chilled on ice.

5.4.4.2. Reverse transcription (cDNA)

RNA was quantified using the Nanodrop™ 3.1. The quality of the RNA material was monitored by recording the OD ratio 230:260 and 260:280. Samples with values 1.9 – 2.1 were consided of good quality however all samples were used to generate cDNA. Most extracts contained > 30 ng/µL of RNA and thus all samples were diluted in nuclease free water to a standard concentration of 30µg/mL. Master mix was prepared as described in Table 5-1. The master mix (6.8 µL) was added to the 0.2 µL microtube and 13.2 µL of template RNA was added to each individual tube. The master mix / RNA template

mixture (20 µL per reaction) was incubated using the thermal cycling conditions described in Table 5-2. The cDNA was stored at -80°C until use.

Component	Volume / Reaction
10 x RT Buffer	2 µL
25 x dNTP Mix (100mM)	0.8 µL
10 x RT Random Primers	2 µL
Multiscribe Reverse Transcriptase	1 µL
Rnase Inhibitor	1 µL
Total Volume	6.8 µL

Table 5-1 Components of Master Mix for reverse transcription

	Step 1	Step 2	Step 3	Step 4
Temperature °C	25°C	37°C	85 °C	4°C
Time	10 min	120 min	5 sec	∞

Table 5-2 Thermal cycling conditions for reverse transcription

5.4.5. *Standard Curve*

To generate a standard curve of MxA and GAPDH for use in the PCR, HL-116 (ISRE-Luc) cells were stimulated overnight with IFNβ 1a (Avonex), 1000 IU/mL which was added to DMEM in which cells were cultured. The cells were harvested and pelleted and resuspended into 2 mLs HBSS. This was added to a PAXGene tube for extraction of RNA

and cDNA generation as described above.

5.4.5.1. PCR

Real time PCR was used to quantify MxA [Bertolotto *et al.* 2007] gene expression in response to administration of IFNβ. Real time PCR reactions are characterised by the point in time at which amplification of a target is first detected rather than the total amount accumulated at the end of the PCR run. Five serial dilutions of cDNA generated from IFNβ stimulated HL-116 cells were prepared to generate both MxA and GAPDH standard curves. Values were attributed to each standard in accordance with the amount of RNA which was used to generate cDNA (100, 10, 1, 0.1 and 0.01 ng). Fresh master mix for (i) MxA and (ii) GAPDH reactions were prepared using 10μL Taqman Universal Master Mix, 8 μL molecular grade water and 1μL of ABI gene expression mix (either MxA or GAPDH), 19 μL of master mix added to each well in the reaction plate. Half of the wells contained master mix to detect MxA and the other half for GAPDH. To each well 1 μL of template cDNA was added. The plate was loaded into the Taqman® 7500 system and the PCR was run with the following conditions;

2 minutes at 50°C + 10 minutes at 95°C + [15 sec @ 95°C + 60 sec @ 60°C] x 40 cycles

After the run was completed samples were analysed using the SDS programme (Taqman® 7500).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S 1	S 1	S 2	S 2	S 3	S 3	S 4	S 4	S 5	S 5	NTC	NTC
B	U	U	U	U	U	U	U	U	U	U	U	U
C	U	U	U	U	U	U	U	U	U	U	U	U
D	U	U	U	U	U	U	U	U	U	U	U	U
E	U	U	U	U	U	U	U	U	U	U	U	U
F	U	U	U	U	U	U	U	U	U	U	U	U
G	U	U	U	U	U	U	U	U	U	U	U	U
H	S 1	S 1	S 2	S 2	S 3	S 3	S 4	S 4	S 5	S 5	NTC	NTC

Figure 5-1 PCR plate layout.
Wells A-D (Green) MxA , wells E-H (Red) GAPDH
S – standard, NCT – negative control, U –unknown sample

5.4.6.   *Analysis of PCR results*

The assay was validated by standardising the standard curve obtained for MxA. The position of the threshold line was set at the point where the highest MxA standard (S1 100 ng RNA) crossed the vertical line denoting the 25th cycle. The MxA standard curve was automatically generated where the slope was optimised at -3.3 (-3.0 to -3.6) with the y intercept at 24.5 to 25.0. Similarly the GAPDH standard curve was generated where the slope was optimised at -3.3 (-3.0 to -3.6) with the y intercept at 15.5 to 16.0. Relative quantification of the expression of MxA and GAPDH was thus calculated. The relative amount of MxA induction in each individual was calculated by dividing the amount of MxA detected by the amount of GAPDH in the same sample. Variation in RNA extraction and efficiency of reverse transcription were compensated for in this was by normalising all MxA values to GAPDH and then calculating the difference between the amount of MxA in

pre IFNβ administration sample and the post dose sample.

MxA pre/GAPDH pre = corrected MxA level pre IFNβ administration

MxA post /GAPDH post = corrected MxA level post IFNβ administration

⇓

MxA corr post – MxA corr pre = MxA induction in response to IFNβ administration

5.5. *Statistical Analysis*

The Student T test was used to determine differences between baseline demographics of participants. ANOVA was used to compare means of NAb groups and non-parametric tests i.e., Mann-Whitney U test, Kruskal-Wallis test with post Dunn correction for multiple comparison and Spearman correlation were performed where appropriate using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

5.6. Results

5.6.1. Demographics

144 subjects were recruited into the study. Of these 91 were female (64%) and 53 male (36%). Of those included 19 were treated with Rebif® 22μg TTW, 89 were treated with Rebif® 44 μg TTW and 36 with Betaferon® 250 μg EOD. Of the 144 sets of samples, 132 had complete and valid NAb and MxA / GAPDH results. The average timing of the post IFNβ administration sample was 12.6 hours (range 10 – 15 hours). Baseline characteristics of participants are given below [Table 5-3]. There were significantly more females recruited than males, in keeping with the sex distribution of MS, and the age at recruitment, duration of treatment and post treatment annualised relapse rates were higher in the female cohort. Females were also more likely to report side effects.

	Female	Male	
Sex			
	n = 91 (64%)	n = 53 (36%)	
Age (at recruitment)	45.6 (27 – 65)	41.6 (19 – 61)	p< 0.001
Duration of disease (years	12.4 (1 – 35)	8.9 (1 – 23)	p< 0.001
Years on treatment	6.68 (0.6 – 22)	5.7 (1 – 15)	p< 0.001
Annualised relapse rate post treatment	0.32 (0 – 3)	0.44 (0 – 1.67)	p< 0.001
Side effects reported	76%	68%	p< 0.001
NAb negative	66 (72%)	30 (57%)	
Neutralising activity (> 5 NU)	25 (28%)	23 (43%)	

Table 5-3 Demographics of participants

5.6.2. NAb frequency per product

Of the 144 subjects who took part 138 had valid NAb results at the time of submission. As predicted approximately one in every four participants were NAb positive (titre > 20 NU, 24.8 %) and a further 8 % (n=11) had evidence of sub-threshold neutralising activity (titres 5 – 19 NU). The number of NAb positive subjects was different depending on which product they were treated with [Table 5-4]. Subjects treated with Betaferon had the highest frequency of NAbs (42%) as compared with Rebif 22μg (32%) and Rebif 44 μg (25%). There was no significant difference in NAb titre obtained when testing the pre or post IFNβ administration samples (t test p = 0.643).

	Nab negative < 20	No neutralising activity	Neutralising activity > 5 NU	Titre 5 - 19	Titre 20 -99	Titre 100 - 600	Titre > 600
Rebif 22 n=19	13 (68%)	13 (68%)	6 (32%)	0 (0%)	4 (21%)	1 (5%)	1 (5%)
Rebif 44 n=84	67 (79%)	59 (70%)	24 (30%)	8 (10%)	5 (6%)	4 (5%)	7 (8%)
Betaferon n=35	22 (63%)	18 (51%)	17 (49%)	4 (11%)	3 (9%)	10 (29%)	0 (0%)
Total n=138	102 (73%)	90 (65%)	47 (35%)	12 (8.7%)	12 (9%)	15 (11%)	8 (6%)

Table 5-4 Nab status depending on product.

5.6.3.   *MxA induction*

MxA was measured by generating a standard curve from IFNβ stimulated HL-116 cells. Arbitrary values of 100, 10, 1, 0.1 and 0.01 were assigned to each point on the curve. The MxA level for each sample was thus calculated with reference to the standard curve and normalised to GAPDH. The MxA level pre and post IFNβ administration and the difference (MxA induction) were calculated. Data is illustrated in Figure 5-2.

Baseline MxA: Mean baseline levels of MxA expression pre IFNβ administration in NAb titre group were; NAb negative 778.4 (95% CI 643-913), NAb 5 – 19 NU 624 (95% CI 442 – 805), NAb 20-99 NU 555 (95% CI 242 – 868), NAb 100 – 600 NU 390 (95% CI 20 – 659) and NAb >600 NU 44.75 (95% CI 12 – 77). The MxA value at baseline in the NAb > 600 NU group was significantly different to all other groups (ANOVA p < 0.001).

MxA post dose: Mean MxA level post IFNβ administration was also calculated; NAb negative 2330 (95% CI 1940 – 2719), NAb 5 – 19 NU 1883 (95% CI 1335 – 2431), NAb 20 – 99 NU 1533 (95% CI 741 – 2324), NAb 100 – 600 NU 832 (186 – 1478) and NAb > 600 NU 101 (95% CI 0 – 224). The MxA value 12 hours post IFNβ administration in the NAb 100 – 600 NU and > 600 NU group were significantly different to all other groups (ANOVA p = 0.0014).

MxA induction: Mean induction of MxA in each group was; NAb negative 1762 (95% CI 1411 – 2113), NAb 5 – 19 NU 1259 (95% CI 764 – 1754), NAb 20 – 99 NU 1168 (569 – 1768), NAb 100 – 600 NU 442 (95% CI 0 – 885), NAb > 600 NU 46 (95% CI 0 – 128). MxA induction was lower in the NAb 100- 600 NU and NAb > 600 NU groups as

compared with the lower NAb + and NAb negative groups (p=0.0012, p < 0.0001). [Table 5-5, Figure 5-2].

Mean MxA	Nab negative	Titre 5 - 19	Titre 20 - 99	Titre 100 - 600	Titre > 600
Pre dose	778	624	555	390	45
Post dose (12hrs)	2330	1883	1533	832	101
MxA induction	1762	1259	1168	442	46

Table 5-5 Mean values of MxA pre and post IFNβ administration and MxA induction
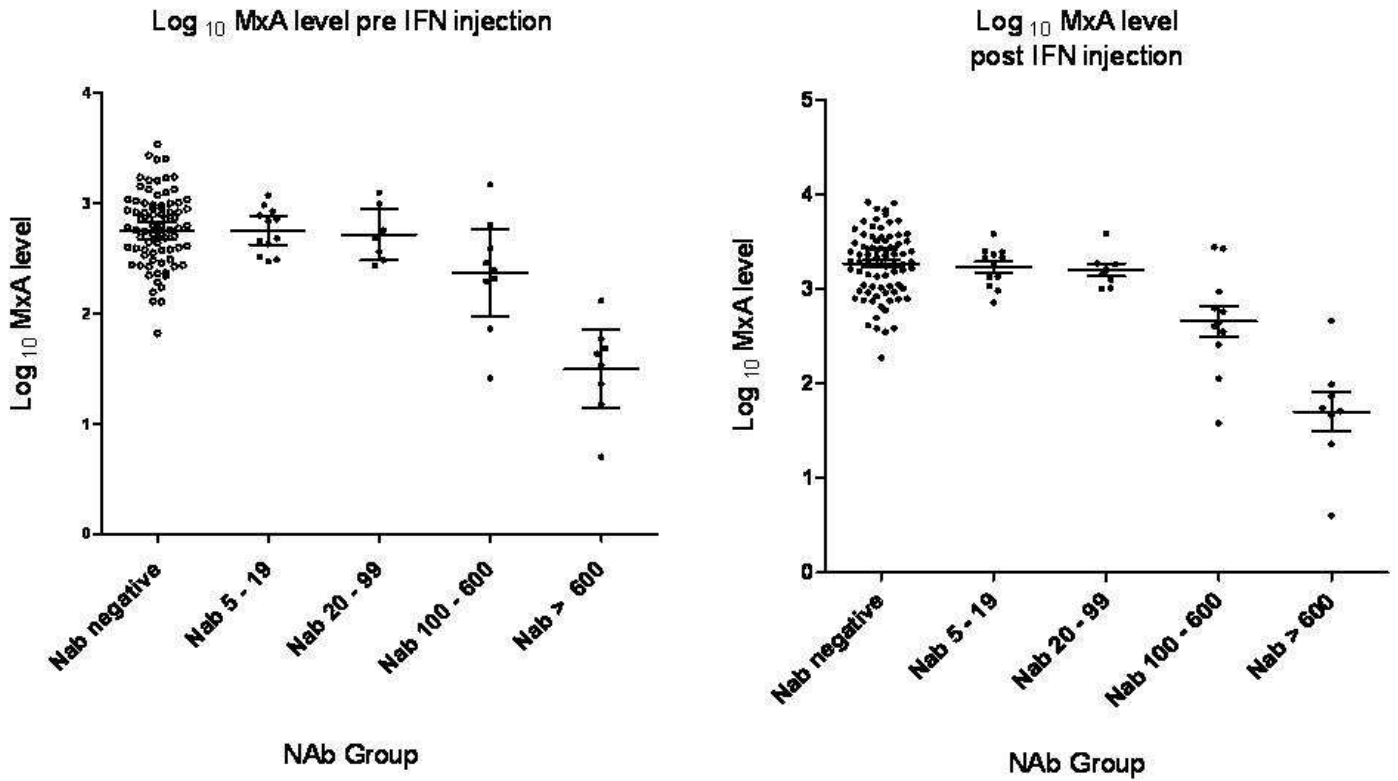
Figure 5-2 Scatterplot of Log $_{10}$ MxA level dependent on NAb group
Mean and 95% CI shown. A. MxA level in each subject before IFNβ administration. B. MxA level post IFNβ administration. MxA levels at baseline andpost injection value were significantly different in the NAbs > 600 NU group as compared with the other groups.

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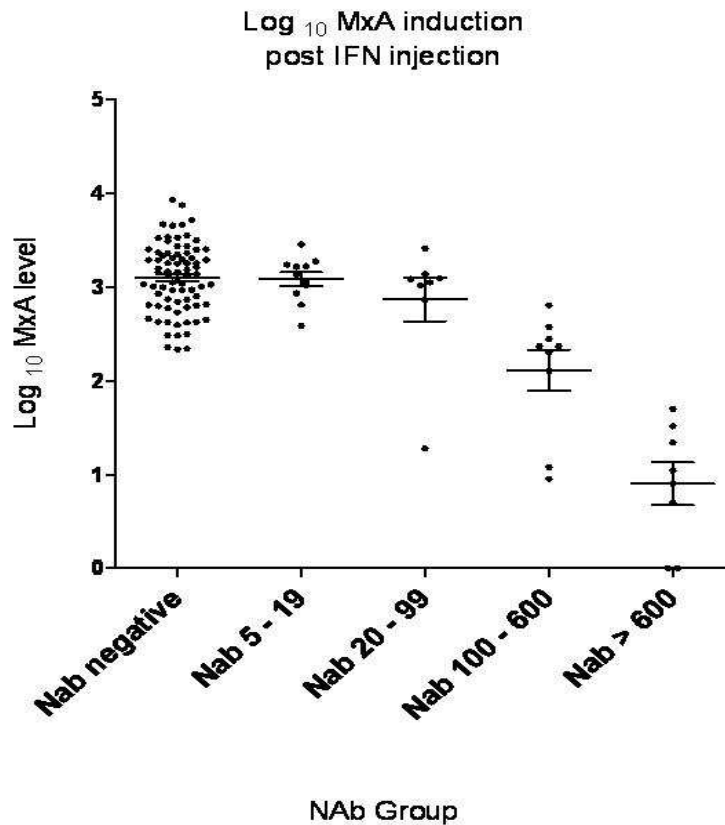
Figure 5-3 Scatterplot of Log $_{10}$ MxA induction in each subject depending on NAb titre. Mean and 95% CI shown. MxA induction levels were significantly different in the NAbs 100 – 600 NU and > 600 NU group as compared with the other groups.

5.6.3.1.   MxA induction post IFNβ administration.

To assess whether there was a difference in MxA response and inhibition by NAbs in all treatment groups, samples were divided into groups by product – Rebif 22μg, Rebif 44 μg and Betaferon. The diffence in MxA level after IFNβ injection is illustrated in Figure 5-3. Regarding the post injection sample significant differences were found in MxA level between the NAb negative and NAb positive (>20 NU) cohorts (Mann Whitney test) in each product group.
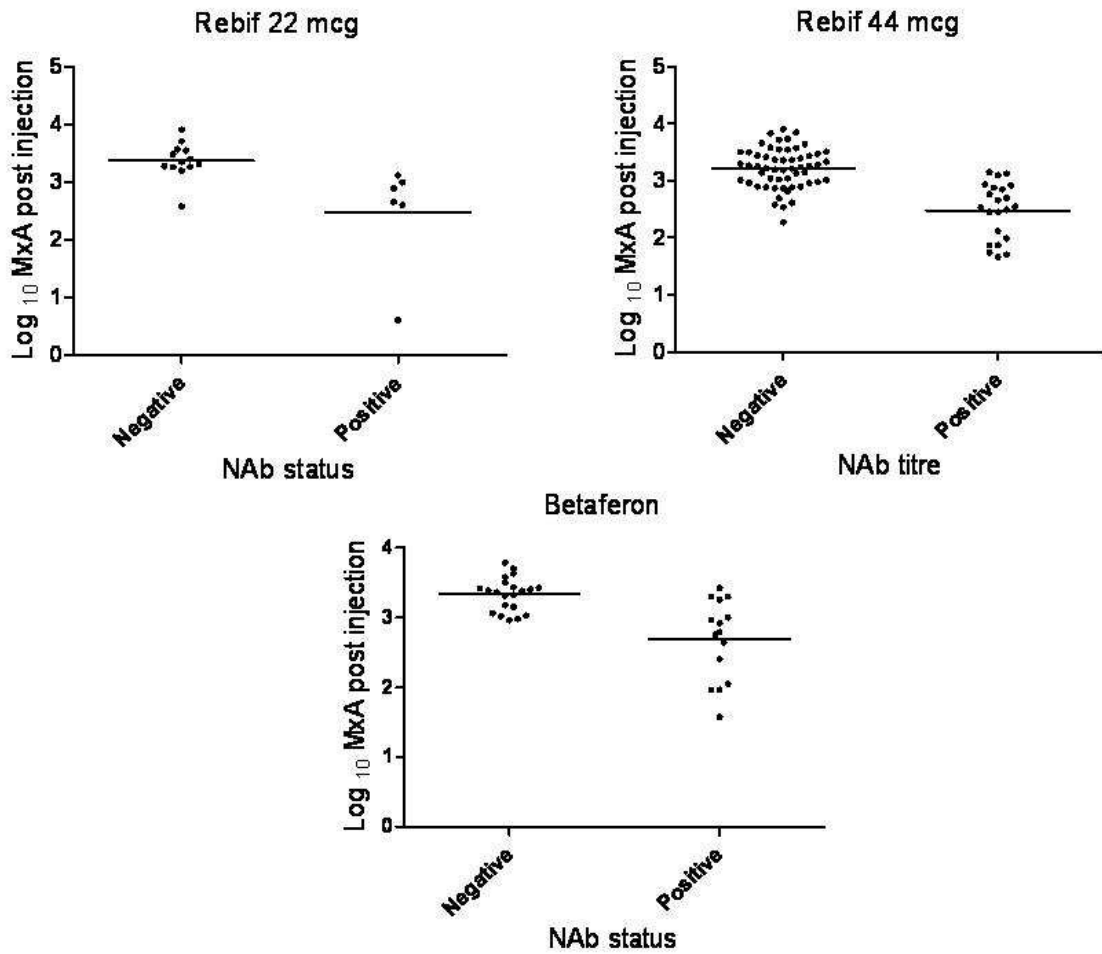
Figure 5-4 Scatterplot of Log$_{10}$ MxA level post IFNβ administration.
Mean and 95% CI depicted, significant differences were found between NAb positive and NAb negative subjects in each treatment group.

5.6.4. *MxA expression in relation to NAb titre*

In subjects with any neutralising activity (i.e. titre > 5 NU, n = 49) NAb titre was negatively correlated with MxA level at baseline, post IFNβ administration and also MxA induction [Figure 5-5].. In subjects with NAb titre 5 – 19 NU (n = 12) a correlation existed with baseline MxA level (spearman r = -0.69, p<0.0001) MxA post injection (spearman r = - 0.74, p < 0.0001) and MxA induction (spearman r = - 0.70, p< 0.0001) Similarly in subjects with clinically defined NAb +ve titres (i.e. NAb titre > 20 NU, n = 37) a correlation existed between titre and baseline MxA level (spearman r = -0.72, p<0.0001), MxA post injection (spearman r = - 0.79, p < 0.0001) and MxA induction (spearman r = - 0.67, p = 0.0004) [Figure 5-6]. This indicated that NAb titre is an accurate predictor of MxA level either pre or post IFNβ administration and confirms a titre (dose) dependent suppresson of MxA induction in the presence of NAbs. Using a ROC analysis of Log_{10} MxA post and Nabs the sensitivity and specificity of being NAb negative setting the threshold at Log_{10} MxA > 2 (ie MxA >100) is sensitivity 98 % & specificity 86% (area under curve 0.951 and p<0.0001). Adjusting the threshold to Log_{10} MxA > 2.5 (ie MxA = 316) as a cut point, the sensitivity remains 99 % and specificity falls to 84%. Therefore a single post dose sample would be sufficient to predict IFNβ bioactivity in clinical practice; provided the subject injected themself with bioactive IFNβ
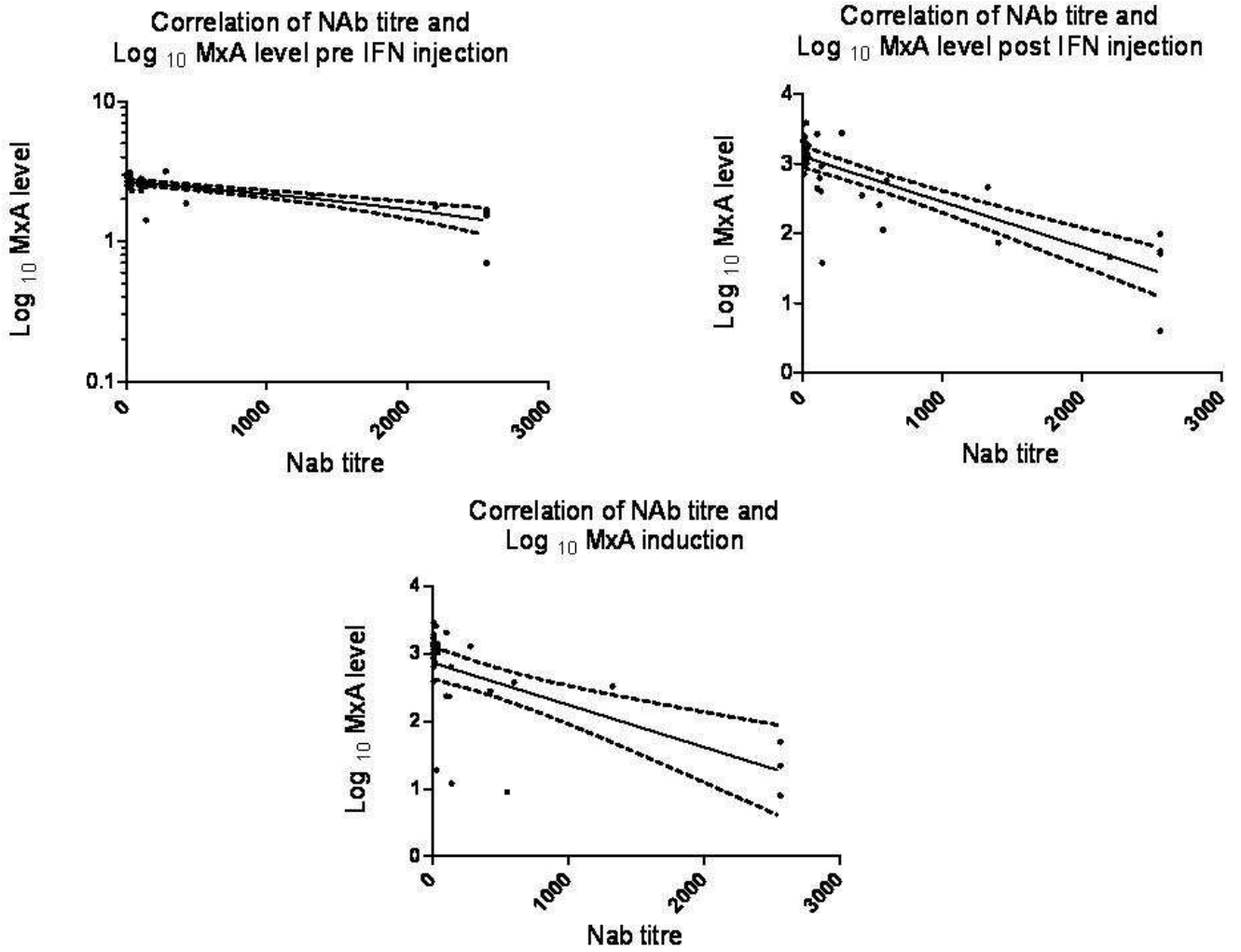
Figure 5-5 Correlation of NAb titre (sample > 5 NU) with MxA level.
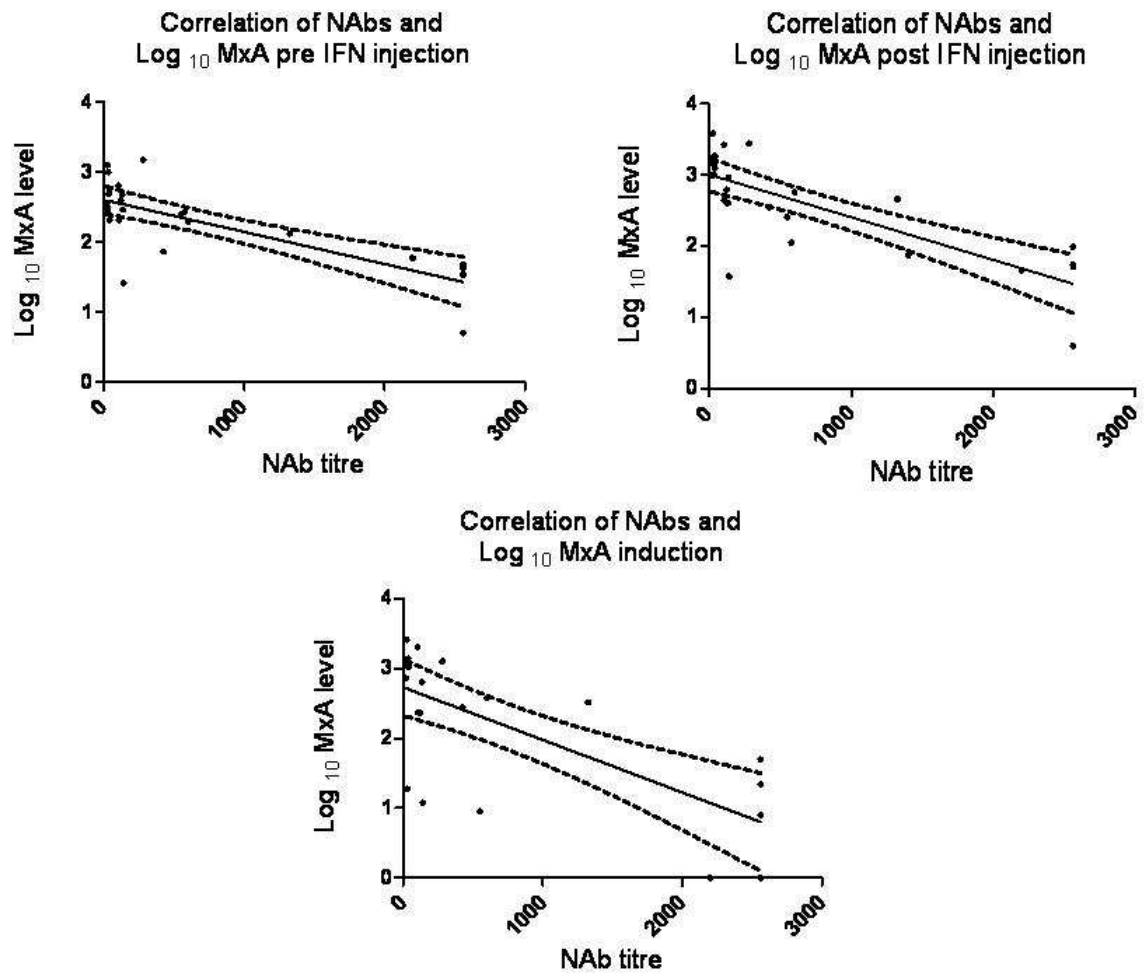**Pre IFNβ injection, B. Post IFNβ injection and C. MxA induction**

**Figure 5-6 Correlation of NAb titre (samples > 20 NU) with MxA level.
A. Pre IFNβ injection, B. Post IFNβ injection and C. MxA induction**

5.7. Discussion

The effect of NAbs at a group level is generally well understood and has been shown to be associated with increased relapse rates and increased disease activity on MRI. What this means however for the individual with NAbs had remained somewhat unclear. Other groups [Sominanda *et al.* 2008] have shown a titre dependent loss of MxA induction after IFNβ administration in subjects with NAbs. In this work the luciferase NAb assay was used to determine NAb status and MxA expression at baseline and after IFNβ administration was quantified. We have found that there is a clear correlation between NAb titre and MxA level in both the pre injection and 12 hours post injection samples. The post dose MxA level had the best correlation with NAb titre and therefore could be a useful adjunct in clinical practice to predict IFNβ bioactivity. Those with low positive NAbs < 100 NU did not show significant loss of bioactivity (as measured by MxA) as compared with those with titres 100 - 600 NU whom experienced a reduction of activity and those > 600 NU who showed little / no response to IFNβ administration (mean post dose MxA expression 101, 95% CI 0 – 224). These results were consistent in those treated with either IFNβ-1a SC (both 22 and 44μg doses) or IFNβ 1b SC. There was no significant difference in NAb titre obtained in samples before or after IFNβ injection and thus a single 12 hour post dose sample would suffice to assess both NAbs and MxA expression. Those with NAbs > 600 NU show significant loss of biological effect and those with NAbs 100 – 600 NU should have MxA expression measured to assess bioactivity. At present it is difficult to determine an exact NAb titre at which one can predict with $> 95\%$ both sensitivity and specificity that there is no biological response due to the small numbers of subjects who were in this category. However using the mean MxA levels post IFNβ administration it is

apparent that a good response is inferred by a level > 1000. If the value is lower than this it probably represents abrogated in vivo bioactivity and a sample should be repeated 6 – 12 weeks later to reassess response. In those with significantly reduced MxA response and persistently positive NAbs consideration should be given to changing to an alternative treatment.

A further consensus paper from the members of the NABINMS consortium will be published in the near future and their recommendations are that patients doing well clinically, NAb and/or MxA bioactivity assessments should be performed. In those with sustained high titer NAbs and/or lack of MxA bioactivity switching to a non-IFNβ therapy should be considered. In patients with intermediate disease activity, continuation of IFNβ therapy could be considered in NAb negative patients, whereas high NAb titers and/or lack of MxA bioactivity should suggest a switch to a non-IFNβ. Patients who are clinically failing therapy should have a change in treatment irrespective of NAb or MxA bioactivity outcomes. The data generated from our study highlights this grey zone and the need for a personalised approach to managing NAbs in people with moderate NAb titres.

Chapter 6

Evolution of the anti-IFNβ antibody response

6 Evolution of the anti-IFNβ antibody response

6.1.   Background

As biological products are known to provoke the production of anti-drug antibodies we sought to understand the maturation process of antibodies to IFNβ and thus provide insights into prevention or treatment of NAbs. Antibodies (immunoglobulins) form the major component of the humoral immune response, serving to protect the extracellular component. They serve two main purposes (i) to bind specifically to molecules from the antigen that elicited the response, (ii) to recruit cells and molecules to destroy the antigen (pathogen) once antibody is bound to it. The immunoglobulin molecule is produced by B cells and consists of four polypeptide chains -two heavy and two light chains, forming a Y shaped structure. Together the light and heavy chains form two antigen binding sites at the tips of the Y. Each molecule has a constant region (C region) which determines its class and a variable region (V region) which determines its antigen specificity. After a B cell is exposed to antigen it must express a receptor which will bind this antigen, as the cells proliferate they undergo somatic hyper-mutation of the variable region genes to improve specificity (clonal selection). The stronger the binding the more likely it is that the B cell clone will survive and proliferate – also called "affinity maturation". Affinity maturation describes the process by which selection of the antibodies with the highest affinity for an antigen occurs.

There are five human immunoglobulin isotypes – IgM, IgG, IgD, IgE and IgA, which are specialised to activate different immune effector mechanisms. The environment

determines the specific antibodies that are produced. IgM is always the first antibody produced in a humoral immune response and is of low affinity. Thus class switching to an antibody of higher affinity; IgG, IgA and IgE, occurs shortly after the initial response. IgG is the principal isotype in the blood and extracellular fluid, whereas IgA is abundant in secretions – mucous epithelium. IgE is bound avidly by receptors on mast cells but is in low volume in the blood. IgG and IgA can also be divided into subclasses; IgG (IgG1, IgG2, IgG3 and IgG4) and IgA (IgA1, IgA2) 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 [Ferrante *et al.* 1990; Siber *et al.* 1980]. Immune responses with substantial IL-4 production (Th2 cytokines) 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]. Type I interferons induce IL-10 secretion by PBMCs which enhances proliferation and immunoglobulin production by B cells [Rousset *et al.* 1992].

6.2.   *Oligoclonal Antibody response*

Oligoclonal bands are discrete bands of immunoglobulins with decreased electrophoretic mobility which may be detected in the serum or CSF in response to any process which generates an immune reaction such as infection (e.g. measles, herpes simples), autoimmunity (e.g. lupus cerebritis), neoplasia (e.g. paraneoplastic syndromes). When the source is systemic these antibodies cross the blood brain barrier and may also be detected in the CSF. Bands may be detected by isoelectric focusing or agarose gel electrophoresis, which separate proteins of different charge and size [**Error! Reference source not found.**]. By using these methods we can see evidence of a (poly) specific humoral immune response to the antigen in question.

Isoelectric focusing

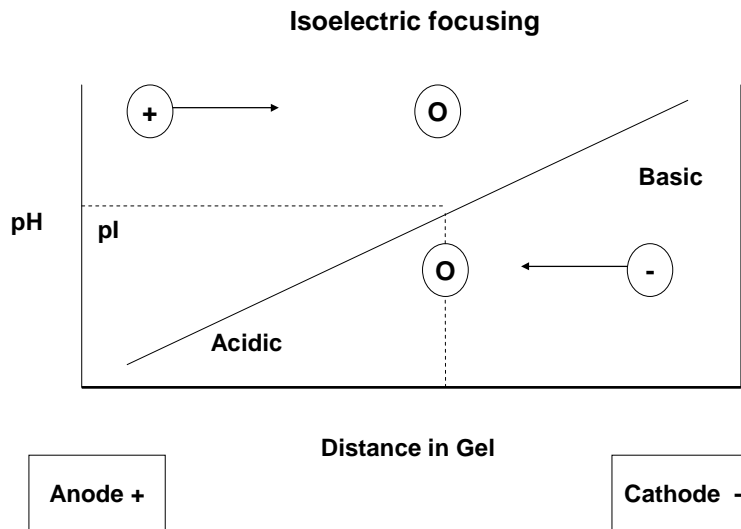**Figure 6-1 Schematic of isoelectric focusing**
The motion of a protein as it approaches it's isoelectric point (pI). The pH gradient and motion of the protein are controlled by the electric field. At a pH lower than pI the protein is positively charged (+) and moves towards the Cathode. Above the pI the protein is negativelt charged (-) and moves towards the Anode. There is no electrical force at the pI.

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6.2.1.   Antibodies to IFNβ

The antibodies generated against IFNβ, as with the majority of protein based biological products, are generally of the IgG isotype. There are conflicting reports in the literature as to which IgG subclass predominates. In one study, in patients treated with IFNβ-1b who developed NAbs, IgG2 and IgG4 were found to occur more frequently than in patients with only BAbs (30% vs. 3%, $p = 0.05$, and 55% vs. 18%, $p = 0.003$, respectively). The NAb titre correlated strongly with the IgG4 titre ($r = 0.53$, $p = 0.02$) [Deisenhammer *et al.* 2001]. Another group reported that the IgG subclass depended on IFNβ product administered and was predominantly IgG1 and IgG3 within the first 6 months of treatment followed by a progressive decline, while IgG4 levels increased and peaked later after 24 months [Gibbs and Oger 2007]. Regardless of the treatment received, NAb +ve patients had higher levels of IgG4 subclass-specific antibodies than NAb −ve patients. These studies suggest that as a result of a high protein therapy (especially those prone to aggregation) the antibody response to IFNβ matures over time with increased IgG4 fractions and may develop a mono or oligoclonal response to specific IFNβ epitopes.

6.2.2.   Immunoblotting

This technique was initially used to investigate antigen specific oligoclonal immunoglobulin G patterns in subacute sclerosing panencephalitis and herpes simplex encephalitis [Moyle *et al.* 1984]. It makes use of the affinity maturation process which selects a few highly specific clones which recognise the antigen in question. Thus by using

isoelectric focusing to separate the protein bands these antibodies can be transferred by passive blotting onto a membrane impregnated with the antigen in question and can then be detected immunologically. If the response to IFNβ is specific, one would expect that one or a few high affinity clones would emerge and be detectable as mono / oligoclonal bands on immunoblots.

Hypothesis

The aim of this chapter was to:

1. Explore the hypothesis of affinity maturation of the anti-IFNβ antibody response and determine if the anti-IFNβ antibody response is oligoclonal using iso-electric focusing and immunoblotting techniques.

2. Quantify the IgG subtypes in NAb +ve and BAb +ve samples and evaluate the role of IgG4 in neutralising activity by subtracting IgG4 fraction from sera and re-evaluating neutralising capacity.

6.3.   *High Affinity immunoblot*

[This method was developed by Dr Miles Chapman as part of his doctoral thesis [Chapman *et al* 2006]. All blots were performed with Dr Miles Chapman-Neuroimmunology Dept, Institute of Neurology, London].

6.3.1.   *Materials*

Ultrabind 450 membrane (Gelman, UK), IFNβ – albumin-free protein (courtesy of Ed Croze, Schering) IFNβ-1b stock (Betaferon® Bayer-Schering), 0.9% Saline (9g sodium chloride (Sigma) in 1 L de-ionised water), trizma base (Sigma) , non-fat dry milk powder (skimmed milk), 0.05M sulphuric acid, 1 M sodium hydroxide, methanol, Goat anti-human IgG (Diasorin), Rabbit anti-goat IgG- HRP conjugate (Dako), 3-amino-9-ethylcarbazole (Sigma), ethanol, acetate buffer, hydrogen peroxide, mouse monoclonal anti-human IFN-β #B-02 Yamasa SAM-260871-1, polypropylene universal 50ml tubes (Sarstedt), slow rocker (Stuart), electrophoresis tank (GE Healthcare), blotting paper (Hollingsworth and Vose), scalpel blade.

6.3.2.   *Methods*

6.3.2.1.   *Coating the membrane*

Ultrabind 450 membrane (Gelman, UK) 8cm x 4cm ($32cm^2$) was coated with IFNβ solution ($5ug/cm^2$ = 160ug of IFNβ in 11 mls 0.9% saline) and placed in a dry

polypropelene universal tube. This was sealed and placed on a slow rocker overnight at 4ºC. The following morning the saline was decanted and the membrane washed twice in tap water. This was blocked in 50mL of 0.9% saline/2% skimmed milk/1% TRIS for at least 1 hour. The blocking solution was decanted and the membrane washed in saline and allowed to air dry. These could be frozen in a sealed plastic bag at -20ºC for up to a week.

6.3.2.2. Isoelectric focusing

The prepared gel was allowed to equilibrate to room temperature. The electrophoresis tank (GE Healthcare) was coupled to a circulating water cooler at 12ºC. This was started ~ 30 minutes before focusing was run to reach temperature. Wicks of blotting paper had been prepared; one was soaked in 0.05 M sulphuric acid and the other in 1 M sodium hydroxide. The cooling plate was coated with ~ 1ml of 50 % methanol/50 % water solution prior to placing the gel onto it to ensure an airtight seal between the gelbond and the plate. Excess methanol was blotted away with dry tissue. The edges of the gel were trimmed using a scalpel blade to remove any "meniscus effect". An application foil was laid on the gel approximately 3cm from the bottom edge of the gel. This was pressed firmly onto the gel to ensure an airtight seal so that when samples were applied they would not be drawn away from the wells. Samples were diluted 1:100 in de-ionised water and applied from right to left. Blank wells were filled with deionised water. The wicks were then placed on the gel; the sulphuric acid-soaked wick at the anode and the sodium hydroxide-soaked wick at the cathode. Paper towels were placed at the edges of the gel to absorb any moisture generated.

The electrodes were secured in place using screw clamps and those on the glass plate were adjusted so the wire ran along the centre of the wick. The leads from the electrodes

were connected and the lid of the apparatus put in place. The gel was run at 800 volts, maximum 150 watts and 20 mA for 900 volt hours. During the run a pause was made to remove the application foil, replace the soaked blotting paper and remove any moisture.

6.3.2.3. Blotting the gel

After IEF of samples was complete the blotting paper and wicks were discarded. Excess water was removed with nitrocellulose. A membrane either pre-impregnated with IFNβ or only with block (as control) was then placed on the gel, ensuring no air bubbles were trapped. Once in place 6 sheets of blotting paper were placed on top, followed by a glass plate and 1kg weight, for 10 minutes.

6.3.2.4. Development of the membrane

A 1:1000 dilution of goat anti-human IgG was made by adding 25µL of the antibody to 25 mls of 0.2% milk in 0.9% saline. The membrane was immersed in this and incubated, covered, overnight at 4ºC on a slow rocker. The following day the membrane was washed six times with 0.01% Tween/0.2 % milk/0.9 % saline. The membrane was then incubated with 25 ml rabbit anti-goat IgG- HRP conjugate (diluted 1:1000 in 0.2 % milk/0.9 % saline) for 2 hours and visualised using ethylaminocarbazole (50µg of 3-amino-9-ethylcarbazole (Sigma)) dissolved in 20ml ethanol, 100ml working strength acetate buffer and 100µL 30% v/v hydrogen peroxide added before use). The membrane was developed for at least 10 minutes after which it was removed, washed in tap water to prevent further colour development and dried between two sheets of blotting paper under a hairdryer.

6.3.3. Sample identification

10 samples were identified from 8 subjects, which had been screened for BAbs and NAbs. The first set of 5 sera consisted samples which were high BAb +ve / NAb +ve. The second set of 5 sera were included one which was BAb -ve/NAb -ve , one BAb +ve/NAb -ve and 3 BAb +ve / NAb +ve of different titres from the same individual. Six subjects were treated with Rebif® and two with Betaferon®. The results of BAb and NAb testing and immunoblot of patient sera are shown [Table 6-1 & Figure 6-1].

Sample no	Product	Patient no	BAb titre	NAb titre
724	R	1	234	>2560
876	B	2	132	>2560
888	R	3	165	2435
893	B	4	197	>2560
964	R	5	126	3550
3751	R	6	<3	negative
5202	R	7	8	37
2733	R	8	21	56
3737	R	8	64	785
3486	R	8	265	>2560

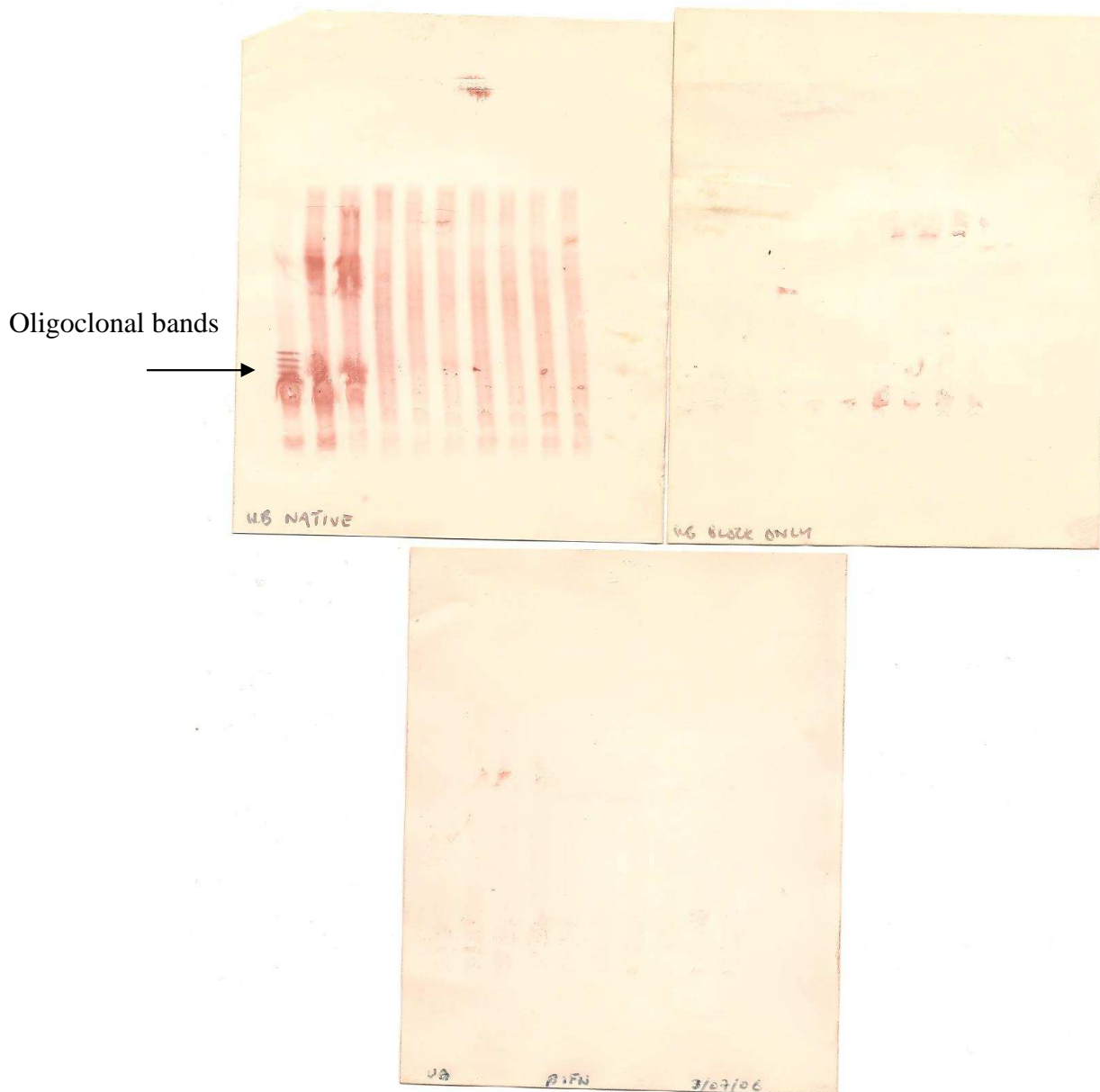**Table 6-1 Patient samples used in immunoblots**

Oligoclonal bands →

Figure 6-2 Immunoblot of patient sera (I)
A. Patient sera. Note bands in far left sample.
B. Blot using antigen free membrane
C. Blot with IFNβ impregnated membrane – no bands visable

6.4. Results

On three occasions blots were performed with minor modifications to the method as follows;

The initial immunoblot [Figure 6-3] was performed using commercial IFNβ (Betaferon®) and samples 1-5 (all high positive NAbs). This showed a polyclonal response with no clear band(s) of anti-IFNβ antibodies. The blot was repeated in the same way with samples 6-10 using albumin free IFNβ but the result was similar and no clear clonal response was found in the sera and no binding to the IFNβ protein was detected [Figure 6-4]. This was repeated (blot not shown) but again did not detect an oligoclonal pattern in NAb sera or any binding to the IFNβ protein.

These results suggest that either the antibody response to IFNβ is polyclonal or that the method with which we tried to show an oligoclonal response is not sufficiently optimised or sensitive. The commercial product of IFNβ contains albumin which may encourage aggregation and interfere with the binding of IFNβ molecules to the membrane. IFNβ itself is highly prone to aggregation in high concentration (>250µg/ml) thus limiting the concentration we could impregnate the membrane with. In view of this the experiment was repeated using albumin-free protein; however, a polyclonal response was still detected. The method was further altered to focus the patients' sera in duplicate and blot directly to the membrane. One copy was blotted onto a membrane which was coated with IFNβ which would bind to any adherent anti-IFNβ antibodies, however no binding was detected.
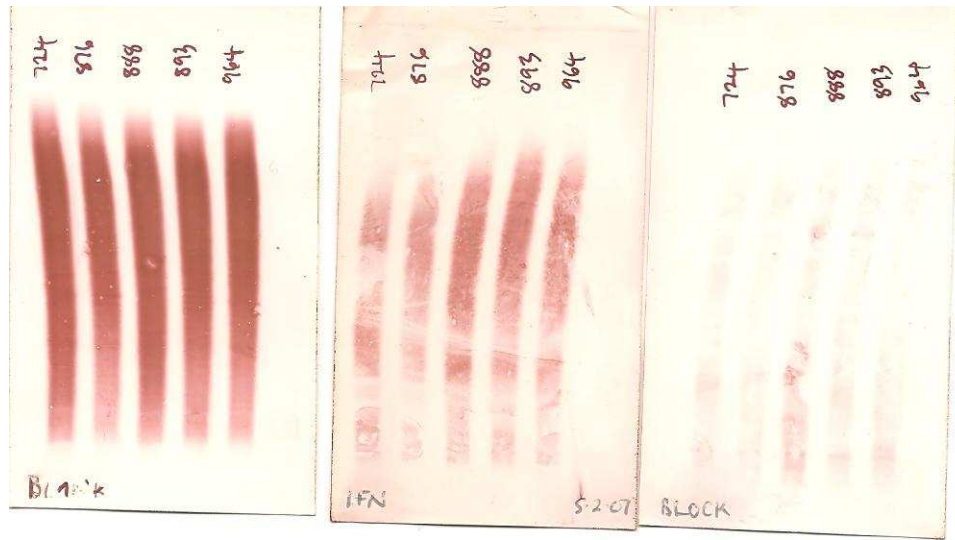
Figure 6-3 Immunoblot patient sera (II)
A. Focused sera of IFNβ treated patients with known high positive NAbs
B. Immunoblot with membrane impregnated with commercial IFNβ (Betaferon®)
C. Blot using an antigen free membrane (control)

CONTROL

IFN
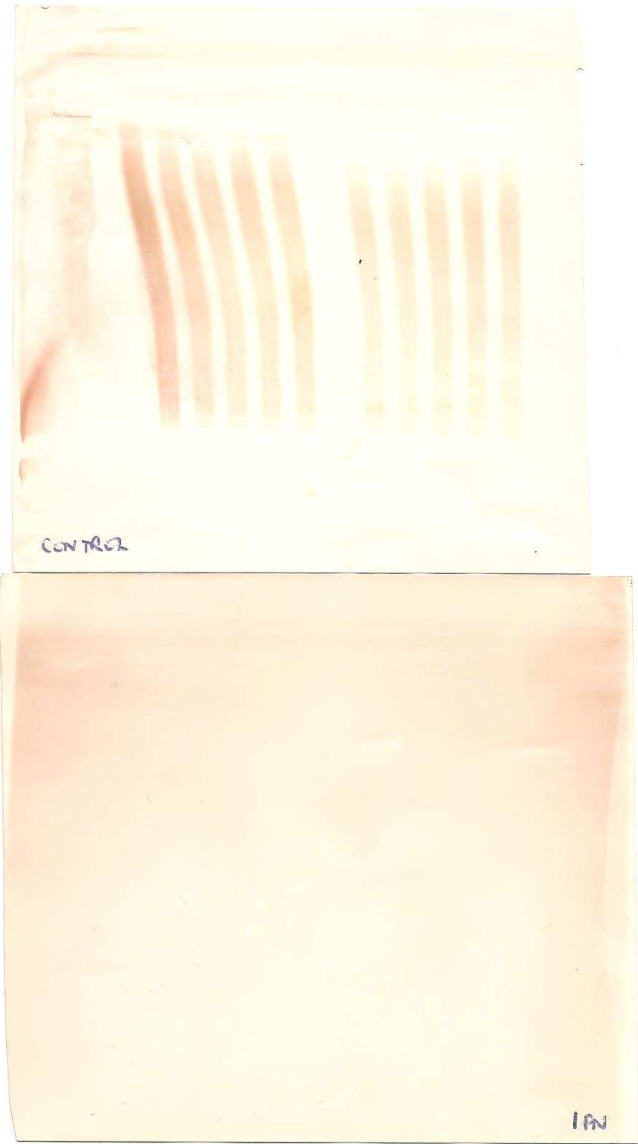
Figure 6-4 Immunoblot of patient sera (III)
A. immunoblot of patient sera
B. no binding to IFNβ protein

6.5.   Immunoglobulin subclass quantification

The purpose of these experiments was to measure the neutralising capacity of a sample pre and post IgG4 extraction to evaluate if IgG4 subclass was largely responsible to neutralising capacity:

- IgG4 extraction column preparation

- IgG4 extraction

- Measurement of IgG subtypes in pre and post IgG4 extraction, sera

- NAb quantification in pre and post IgG4 extraction, sera

6.5.1.   Materials

MicroLink™ Protein Coupling Kit (PIERCE #20475) contains:

AminoLink Plus Coupling Gel spin columns, coupling buffer: BupH™ phosphate buffered saline pack (0.1M sodium phosphate, 0.15M NaCl; pH 7.2), µLtrapure water, quenching buffer, 1M Tris HCl 0.05% NaN$_3$, pH 7.4, Sodium cyanoborohydride (5M), 0.01M NaOH, wash solution 1M NaCl, 0.05% NaN$_3$, elution buffer pH 2.8 (contains primary amine), microcentrifuge collection tubes. Bindarid™ Human IgG subclass single dilution kits: Radioimmune diffusion plates (specific for IgG 1, 2, 3 or 4), calibrators IgG 1, 2, 3 and 4, 7% bovine serum albumin (BSA) solution, control serum, gel dividers

Additional materials: Pipettes: 10 µL, 200 µL, 1000 µL, 30-300 µL multichannel adjustable volume pipettes - Eppendorf Research®, pipette tips 10 µL, 20 -200 µL, 1000

µL, centrifuge, slow rocker, polypropylene universal 50ml tubes, polypropylene 1.5mL

microtubes, plastic ruler to measure to 0.1mm. Mouse monoclonal antibody to human IgG4

(ZYMED laboratories)

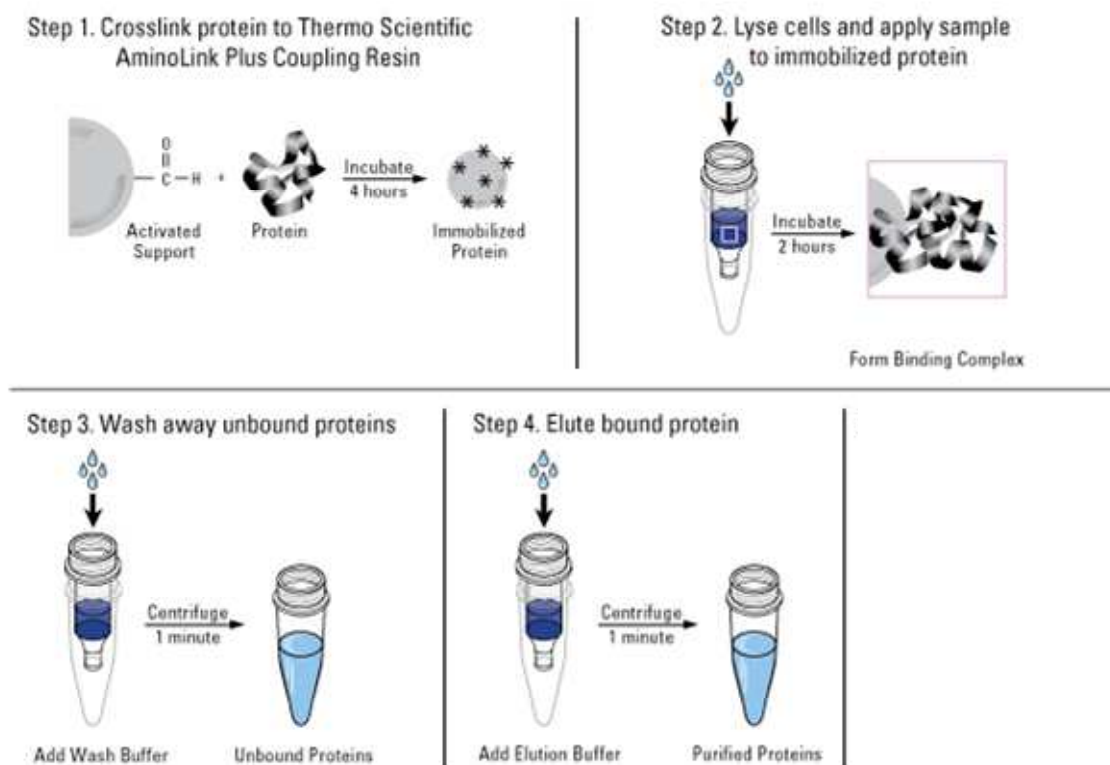## *6.5.2.   Methods - IgG4 immuno-subtraction*

Figure 6-5 Protein coupling to subtract IgG4
Adapted from Pierce microprotein coupling kit information sheet (www.piercenet.com)

6.5.2.1. Column Preparation

All reagents were equilibrated to room temperature. The coupling buffer was prepared by dissolving the dry-blend buffer with 500mls ultrapure water. 50 µL of mouse monoclonal anti- IgG4 antibody was added to 150 µL of coupling buffer. The column cap was loosened and the bottom plug removed. The column was then placed in collection tube and spun at 1000g for 2 minutes. The column cap was removed and plug re-inserted. To re-suspend the gel 300µL coupling buffer was added. Again the plug was removed and column placed in a collection tube. This was centrifuged @ 1000g for 1 minute and flow through was discarded. This was repeated twice.

The column was plugged and 200 µL of sample was added directly onto the gel. The cap was replaced and gently vortexed. 2 µL of sodium cyanoborohydride solution was added to the column in fume hood, the cap replaced and mixed again. The column was incubated at room temperature for 4 hours, vortexing gently every hour (gentle end over end mixing). The column cap was loosened and plug removed. The column was placed in a fresh collection tube and centrifuged. The column was uncapped and plug re-inserted. 300µL of coupling buffer was added, the cap replaced and mixed. The column was placed in a collection tube and centrifuged twice.

To block remaining active binding sites 300µL of quenching buffer was added onto the gel and mixed. The column cap was loosened and plug removed. The column was placed in a collection tube and centrifuged. This step was repeated again. The column plug was re-inserted and 200µL of quenching buffer directly added onto gel. The cap was replaced and column vortexed gently again. 4 µL of sodium cyanoborohydride solution was added to the

column in the fume hood. This was mixed again and incubated at room temperature for 30 minutes mixing every 15 mins. The cap and plug were removed and the column placed in a collection tube and centrifuged.

6.5.2.2. Wash and store

300µL of wash solution was added to the column and plug reinserted. The cap was replaced and solutions mixed. The cap and plug were removed and the column placed in a collection tube which was centrifuged and the flow through discarded. This was performed twice more.

The column was plugged and 300 µL of coupling buffer added, the cap was replaced and column was mixed. The cap and plug were removed and the column was centrifuged, the flow through was discarded. Again this was repeated twice. Finally the column was plugged and 300µL coupling buffer added along the sides of column to wash down gel. The cap was replaced and the column stored at 4˚C for up to 2 weeks.

Thereafter the serum sample was placed in the column and incubated on a rocker for 2 hours at room temperature. The column was centrifuged to yield the IgG4 depleted sample.

6.5.2.3. Quantification of IgG subclasses

The principle of radial immunodiffusion is based on the ability of antigen to diffuse through an agarose gel containing an appropriate mono-specific antibody. The antigen-

antibody complexes are formed and a precipitin ring forms. The ring size increases until the equilibrium between complex formation and breakdown is reached. At this stage a linear relationship between the ring diametre and antigen concentration exists. A concentration curve was produced with known antigen concentrations and thus the antigen content of unknown samples was calculated.
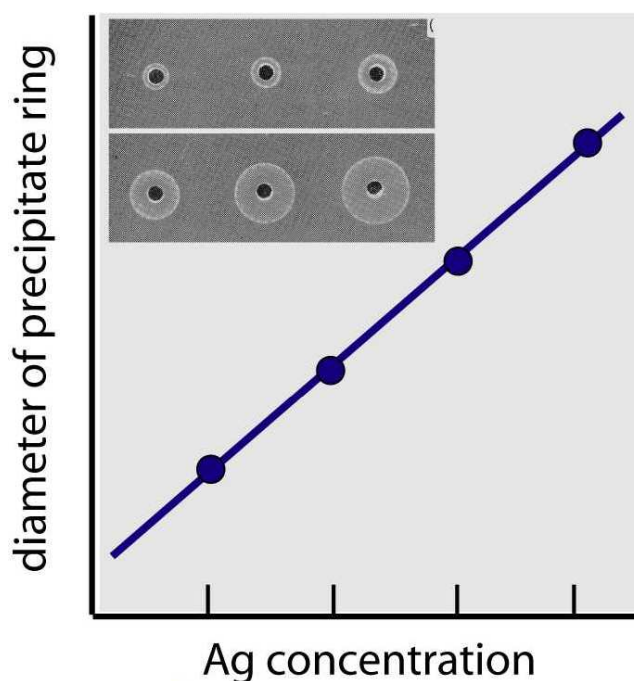

Figure 6-6 Principle of Radial immunodiffusion.
Equilibrium of antigen-antibody complexes forming precipitant rings

6.5.2.4. Methods

The four RID plates (IgG1, 2, 3, 4) were removed from the foil pouches and equilibrated for ~ 15 minutes to room temperature to ensure no condensation was present. These were kept upside-down until any moisture had evaporated. The plates were

partitioned into 4 sections with gel dividers. Calibrators and controls were mixed gently before use. Dilutions of 60% (moderate) and 10% (low) of the calibrators were made to create a standard curve. Due to the expected higher concentrations of IgG1 and 2, a 1:10 dilution of control IgG1 and IgG2 were prepared using 25µL control serum with 225µL of diluent to maintain accuracy. Dilution of IgG3 and IgG4 controls was not required.

Point of curve	Calibrator µL	Diluent µL
High	100	0
Moderate	60	40
Low	25	225

Table 6-2 Standard curve radial immunodiffusion assay

6.5.2.5. *Patient serum samples*

8 patient samples were chosen, 1 pooled normal serum and one treatment naive patient were tested. The neat patient sample and the serum post IgG4 subtraction were prepared for use in the RID plates. A 1:10 dilution of the patient's samples were made for application to the IgG1 and 2 plates, neat sera was added to IgG3 and 4 plates.

6.5.2.6. *IgG 1 and 2 plates*

5 µL of calibrator dilutions – neat, moderate and low, were added to the first 3 wells (1-3). The next well (4) was filled with 5 µL of control sera @ 1:10 dilution. The remaining wells were filled with patient samples @ 1:10 dilutions.

6.5.2.7. IgG 3 and 4 plates

5 µL of calibrator dilutions – neat, moderate and low, were added to the first 3 wells (1-3). The next well (4) was filled with 5 µL of neat control sera. The remaining wells were filled with neat patient samples.

The lids were replaced tightly on the 4 plates and were incubated at room temperature for 72 hours to incubate and the diffusion rings to appear.

6.5.3. *Calculation of results*

Lids were removed and using a ruler and magnifying glass the diameter of the precipitin rings were measured. Figure 6-7 shows an example of the appearance of the precipitin rings. The ring diameters were measured to the nearest 0.1mm using a magnifying glass. The squares of the diameters (mm^2) of the rings were calculated. The known concentration f the IgG subclass was plotted on the x-axis vs. the D^2 of calibrators (3 dilutions) on the y-axis vs). A trendline was applied and the IgG concentrations of each sample were calculated from the equation [Figure 6-8].
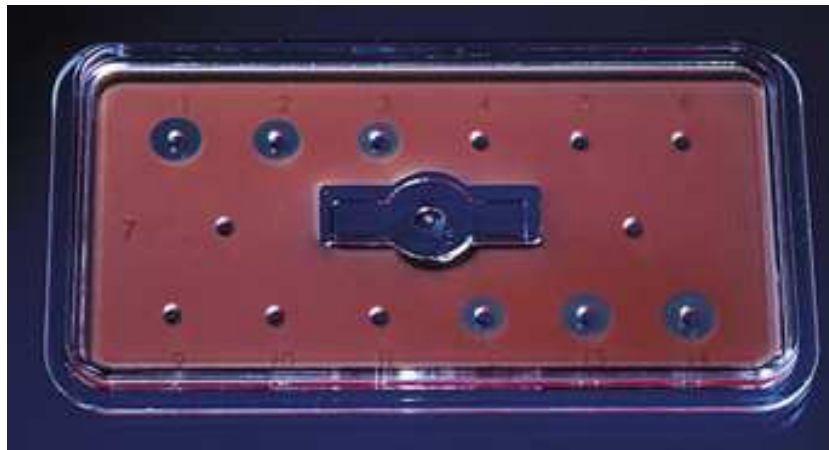
Figure 6-7 Radial immunodiffusion plate with IgG precipitant rings
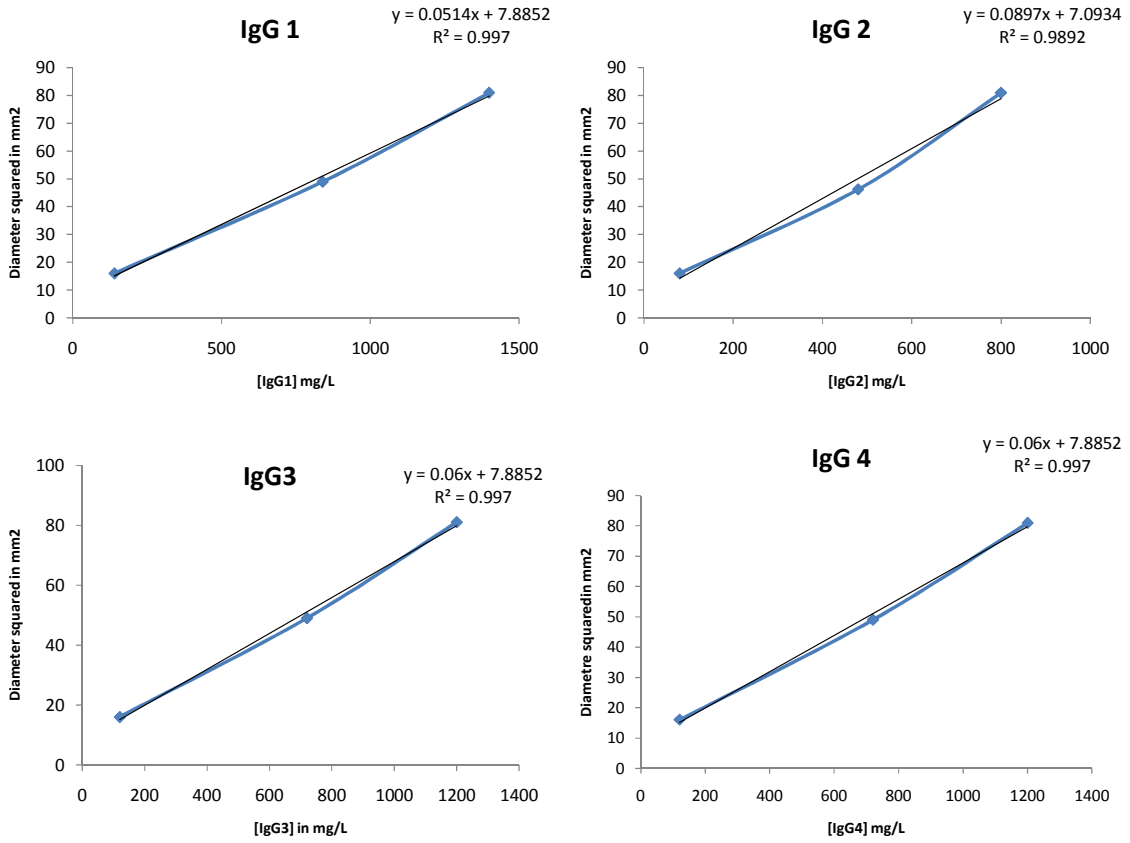
Figure 6-8 Standard curves of each IgG subclass with trendline equation.

6.5.4. Results

Paired samples (pre and post IgG4 subtraction) of 4 patients were run on each plate. The concentration of IgG1, 2, 3 and 4 was calculated in each case to evaluate if IgG4 had been specifically extracted [Table 6-3]. Paired samples (pre and post IgG4 subtraction) of 4 patients were run on each plate. The mean proportion of amount of IgG4 extracted was 90% range [53 - 100]. Although only IgG4 should have been extracted in the column reductions were seen in other IgG subtypes also; IgG1 22% [13 – 33], IgG2 15% [0 - 50], IgG3 47% [0 – 98]. The pre and post IgG4 extraction samples were then tested for neutralising activity using the procedure detailed in chapter 3. Although reduction of neutralising antibody titre was noted in most samples this showed no correlation to the reduction of total IgG4 [Figure 6-9].

Sample	IgG1	IgG2	IgG3	IgG4
Control 1 **% reduction**	23	0.2	21	**94**
Control 2 **% reduction**	26	4.7	25	**85**
S 1 **% reduction**	33	18	82	**100**
S 2 **% reduction**	16	0	98	**100**
S 3 **% reduction**	13	24	0	**53**
S 4 **% reduction**	17	50	32	**100**
S 5 **% reduction**	27	5	24	**94**
S 6 **% reduction**	35	21	83	**100**
S 7 **% reduction**	13	5	98	**100**
S 8 **% reduction**	16	26	4	**75**

Table 6-3 Percentage reduction of Immunoglobulin subclass.

	Neutralising activity PRE	Neutralising activity POST	Reduction in neutralising activity (%)	Reduction in IgG4 (%)
1	3928	1180	70	100
2	2092	558	73	100
3	13051	6494	50	53
4	>10240	>10240	NA	100
5	438	321	27	94
6	1572	976	38	100
7	4239	3290	22	100
8	970	548	44	75

Table 6-4 Neutralising antibody titre for each sample pre and post IgG4 extraction.
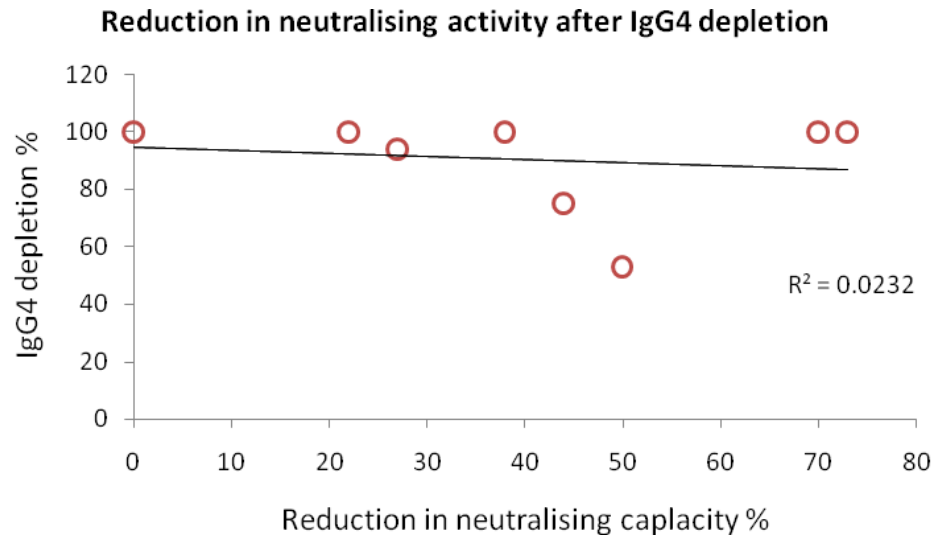

Figure 6-9 Correlation of neutralising activity and IgG4 subtraction

6.6.   Conclusions

In this work we were unable to detect an oligoclonal response to IFNβ. This might indicate that (i) the antibody response to IFNβ is indeed polyclonal (ii) that the method with which we tried to show an oligoclonal response is not sufficiently optimised or sensitive. The commercial product of IFNβ contains albumin which may encourage aggregation and interfere with the binding of IFNβ molecules to the membrane. IFNβ itself is highly prone to aggregation in high concentration (>250μg/ml) thus limiting the concentration we could impregnate the membrane with. In view of this the experiment was repeated using albumin-free protein; however, a polyclonal response was still detected. We therefore chose to alter the method to focus the patients' sera and blot directly to the membrane. We then incubated one copy with IFNβ which would bind to any anti-IFNβ antibodies adherent to the membrane, however no binding was detected. It may be that the response is oligoclonal but due to the limitations of the techniques and the nature of the IFNβ molecule we were unable to show this.

It is possible however that the phenomenon known as "scrambling" of the IgG4 antibody fraction is the reason that we are unable to detect the oligoclonal immune response using immunoblotting techniques. An antibody is composed of two identical dimers with identical Fab binding arms. However IgG4 subtype antibodies are able to exchange dimer partners to form antibodies which have different Fab arms and thus crosslink different antigens. This switch of Fab arms is called scrambling [Burton and Wilson 2007].

Regarding the effect of IgG4 subclass extraction we were unable to specifically remove IgG 4 without also reducing the amount of other subclasses. In view of this any apparent reduction of neutralising capacity of the patient's sera could not be attributed to IgG 4. No correlation was found between the reduced neutralsing capacity and the reduction in IgG 4. It would be of interest to extract IFNβ specific antibodies thereafter subtract the IgG4 fraction. Further work to evaluate affinity maturation and the role of individual IgG subtypes in neutralising IFNβ activity is required but was not undertaken within the remit of this thesis.

In conclusion, it appears that NAbs are found in the IgG4 and non-IgG4 components of the IgG response. Although we showed that the NAb response to IFNβ was polyclonal this is likely to be due to the biology of IgG4. Technical factors in relation to the chemistry of recombinant human IFNβ may explain our inability to detect oligoclonal bands in the other IgG subtypes.

Chapter 7

Incorporating NAbs and NAb testing into

clinical practice

7 Incorporating NAbs and NAb testing into clinical practice

7.1. Diffusion of innovation

Diffusion of innovation is a theory of "how, why, and at what rate new ideas and technology spread through cultures". Everett Rogers introduced this theory in 1962 in his book, *"Diffusion of Innovations"*, writing that "Diffusion is the process by which an innovation is communicated through certain channels over time among the members of a social system". Rogers theorised that innovations would spread through a community in a sigmoidal curve. The early adopters select the innovation first (which may be a technology or in the scientific world the cause of a disease), followed by the majority, until a technology or innovation (or opinion) has reached its saturation point in a community. He also described the process of innovation adoption as a process that occurs over time through five stages: Knowledge, Persuasion, Decision, Implementation and Confirmation. Accordingly, the innovation-decision process is the process through which an individual or other decision-making unit passes (i) from first knowledge of an innovation, (ii) to forming an attitude toward the innovation, (iii) to a decision to adopt or reject, 4, (iv) to implementation of the new idea and (v) to confirmation of this decision [Rogers 2003].

This theory can be applied easily to medical practice and also to the response of the MS community to NAbs.

Interferon beta (IFNβ) has been widely used for more than ten years in patients with

relapsing multiple sclerosis (MS). Funding of disease modifying treatments such as interferons and glatiramer acetate have been controversial in many countries, especially in the UK [Pickin *et al.* 2009]. The unpredictable course of MS and its potential to cause severe disability explains the high demand for disease-modifying agents – but independent bodies such as Cochrane and the National Institute of Clinical Excellence (NICE) www.nice.org.uk/TA32) have highlighted the lack of clear-cut evidence of their clinical efficacy. Over 560 papers are currently indexed on PubMed when search terms "interferon beta & neutralizing antibodies" are entered. Conflicting results of clinical trials are reflected in equivocal guidelines regarding NAbs issued by European, American and British Neurological bodies [EFNS, AAN ABN guidelines]. There has been much controversy with respect to the significance of these antibodies in patients with multiple sclerosis treated with IFNβ and how to manage them [Hartung and Munschauer, III 2004; Farrell and Giovannoni 2007]. Ambivalent data underpins the controversy relating to NAbs and this is largely due to highly variable methodology between trials, many of which are of insufficient duration to show an effect on primary endpoints. The influence of the pharmaceutical industry should not be underestimated as they have certainly played a large role in encouraging doubt over the relevance of NAbs.

What are the "known facts" relating to NAbs? The detrimental effect on clinical outcomes is only seen in patients followed for more than two years. The frequency of NAbs is product dependent with IFNβ-1b SC being more immunogenic than IFNβ-1a SC and IFNβ-1a IM [Jacobs *et al.* 1996; Francis *et al.* 2005; IFNβ MS Study group 1996]. The loss of clinical effect is titre dependent [Sominanda *et al.* 2008; Pachner *et al.* 2009b] (and confirmed in this thesis) and those with low titres may revert to NAb negative status over

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time. This was shown in the IFNβ-1b follow-up study in which 80% of NAb positive patients converted to antibody-negative status after 8 years despite continued therapy [Rice *et al.* 2001]. Another known fact is that NAbs to one IFNβ product cross-react with the others, so switching therapy to another IFNβ is not likely to be an effective strategy in the NAb positive patient [Khan and Dhib-Jalbut 1998; Bertolotto *et al.* 2000] .

In the UK no NAb testing was readily available and those who wished to test would send samples to research laboratories or elsewhere in Europe. Thus using the Rogers model of diffusion of innovation adoption of routine testing for NAbs has been arrested in the 3rd or 4th phase in the UK and indeed many other countries. Early adopters have championed the significance of NAbs but there remain many who oppose incorporating them into clinical decision making and routine testing has only appeared in a handful of countries.

7.2. Existing guidelines.

7.2.1. American Academy of Neurology (AAN)

The AAN reported in 2007 their recommendations focusing on the effect of NAbs on clinical and radiologic outcomes [Goodin *et al.* 2007]. The committee concurred that there is probably a reduction in efficacy of treatment because of NAbs and there is likely to be greater antibody production in response to IFNβ-1b than to IFNβ-1a, and that IM IFNβ-1a is clearly less immunogenic than other interferon therapies. Despite the consistent finding of NAb levels greater than 1:200 being associated with a reduction of efficacy, the committee was unable to make definite recommendations for changing therapy.

7.2.2. European Federation of Neurological Societies (EFNS)

The EFNS recommended that patients treated with IFNβ are tested for the presence of NAbs at 12 and 24 months of therapy (Level A recommendation). In those whom remain NAb negative at 18 – 24 months further testing is not routinely required (Level B recommendation). There is class I evidence that the presence of NAbs significantly reduces the effect of IFNβ on relapse rate and active lesions and burden of disease seen with MRI. In patients who are NAb+ve measurements should be repeated at intervals of 3–6 months and therapeutic options should be re-evaluated (Level A recommendation). Therapy with IFNβ should be discontinued in patients with high titres of NAbs at repeated measurements with 3- to 6-month intervals (Level A recommendation) [Sorensen *et al.* 2005a]. New guidelines are currently in preparation.

7.2.3. Association of British Neurologists (ABN)

Recently the ABN have issued updated guidelines pertaining to use of IFNβ in subjects with RRMS and CIS superseding those published in 2001. This included a statement relating to NAbs stating that positive tests for NAbs to beta-interferon strengthen the case for discontinuation of treatment when clinical or MRI features of treatment failure are present. NAb testing should be performed in a competent lab using a reliable assay www.theabn.org .

Despite the body of evidence confirming the impact of NAbs on treatment efficacy opinions regarding NAbs differ greatly. In Denmark since IFNβ became available NAbs have been routinely tested in all subjects and much of what we know has been published by this group [Ross *et al.* 2000; Sorensen *et al.* 2003c; Vartanian *et al.* 2004; Ross *et al.* 2006; Hesse *et al.* 2009; Sellebjerg *et al.* 2009]. In the UK and USA however opinion is less certain with many doubting the significance of NAbs. The modest average efficacy of IFNβ in MS (~ 30% reduction of relapses) also contributes to the uncertainty as MS is a disease of relapses and remissions in the majority of patients, the natural history being that relapses decrease with time, thus apparent treatment response may occur in the presence of NAbs and may be a model of benign MS rather than treatment efficacy.

7.3. Delivery

The purpose of this work was to address the need to develop a simple, time and cost efficient assay, which could be reliably used in clinical practice. For this purpose we have developed the Luciferase assay which has been validated against gold standard assays in Europe [chapter 3]. The assay has been used to illustrate the increased relapse rate and treatment failure in subjects who are NAb positive [chapter 4] and titres obtained with this assay predict bioactivity of IFNβ as predicted by MxA expression [chapter 5]. As part of the European consortium to standardise Nab testing 40 blinded samples were exchanged between laboratories in the UK, Denmark and Canada. The three laboratories showed excellent agreement with the "gold standard" assay (CPE in Dr Antonio Bertolotto's lab in Italy) and the Luciferase assay showed excelled interlaboratory validity [Figure 7-1].
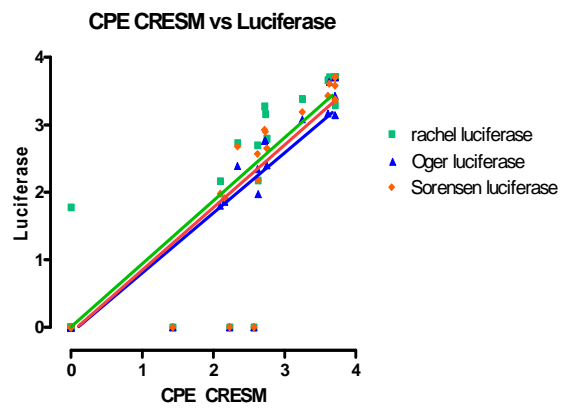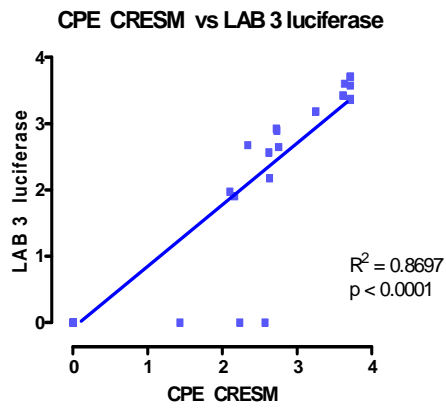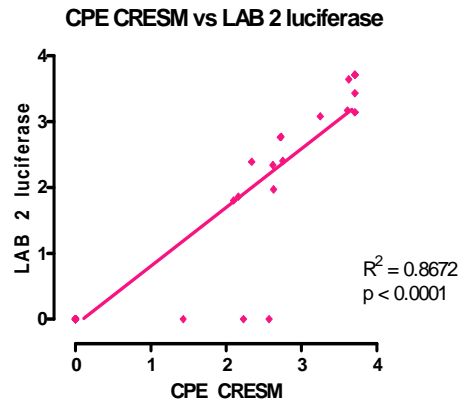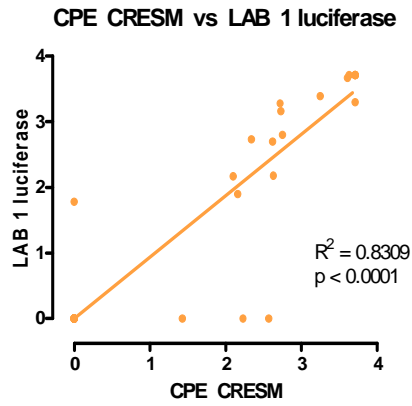
Figure 7-1 Agreement of Luciferase assay with CPE

7.3.1. *Clinical practice*

The luciferase NAb assay [chapter 3] was initially developed in 2006 and was approved for routine NHS use in June 2007, and became available to all prescribing centres. To assess the opinion of Neurologists to NAbs we performed an anonymous online survey using SurveyOnNet www.surveyonnet.com initially surveying opinion in the UK. Thereafter we extended the survey to North American and European countries using surveymonkey www.surveymonkey.com [chapter 7.3.2]. Neurologists involved in NAb testing in 17 countries were invited to take part and 9 agreed to circulate the questionnaire. However after three months only the UK, USA, Canada and Austria had generated sufficient data.

7.3.2. Questionnnaire - Neutralising anti-interferon beta antibodies (Nabs) in patients with Multiple Sclerosis.

1. Do you treat patients with MS?

2. Do you prescribe disease modifying therapies (please tick relevant treatments):
    A. Interferon beta
    B. Glatiramer Acetate
    C. Mitoxantrome
    D. Natalizumab
    E. None
    F. Others (please list)……………………

3. Have you heard about neutralising antibodies (NAbs) to Interferon beta?
    A. Yes
    B. No

4. Are you aware of International Guidelines pertaining to NAbs?
    A. Yes - EFNS
    B. Yes - AAN
    C. Yes - Both
    D. No

5. Do you counsel patients about NAbs when educating them about treatment options?
    A. Yes
    B. No

6. Do you take NAbs into consideration when starting an Interferon beta preparation?
    A. Yes
    B. No
    C. Some times

7. Do you think that high levels of NAbs abrogate the efficacy of the drug:
   A. Yes
   B. Probably
   C. No
   D. Don't know

8. Which statement best describes your practice with respect to NAbs?
   A. I test for NAbs routinely in all patients receiving IFNβ
   B. I only test for NAbs in those who have relapses
   C. I never test for NAbs
   D. I test for NAbs when the patient specifically requests it.

9. If a patient is NAb positive do you:
   A. Do nothing
   B. Retest in 3 months, with view to changing therapy
   C. Discuss change of therapy with patient.
   D. Acknowledge result but only change therapy if patient is actively relapsing
   E. Request MRI and change therapy if Gadolinium enhancing lesions present.

10. Are you aware that NAb testing is available in …………
    A. Yes
    B. No

11. In which city / country do you practice?

12. Please add any further comments with regards to your views on NAbs to IFNβ

7.3.3. Survey Results

UK: One hundred and three British neurologists responded, representing all regions of the UK and Northern Ireland. All were regular prescribers of IFNβ and aware of NAbs (100%) but only 78% were aware that NAb testing was available in the UK funded by the NHS. When commencing IFNβ treatment 61% of neurologists counselled patients about NAbs and 24% felt it influenced product choice. When asked if NAbs abrogate the efficacy of IFNβ 48% of neurologists answered "yes", 48% answered "probably" and only 4% said that they thought NAbs have no effect. To assess the testing patterns of neurologists we asked when they usually tested for NAbs; 17% routinely screened all subjects for NAbs, 38% only tested following a relapse and 11% when specifically requested by the patient, 35% of respondents never tested patients for NAbs. Concerning action following a positive result; 35% re-tested 3 months later to assess persistence, 30% discussed alternative treatments and 42% would only recommend change if patient actively relapsing.

USA: One hundred and thirty two people replied, 95% of whom regularly prescribed disease modifying drugs. Of these 99.1% were aware of NAbs and 56% believe they abrogate the efficacy of IFNβ treatment and 36% replied "probably". In total 87% were aware of guidelines from AAN +/- EFNS and 86% generally counselled patients about NAbs before initiating treatment. Only 13% routinely tested all patients for NAbs, 70% in those who were actively relapsing and 15% never tested. In response to a positive test 46% will retest to evaluate persistence with a view to changing treatment, 24% discuss alternative treatments amd 10% will request and MRI to assess lesion load basing treatment

change on this.

Canada: Twenty three neurologists replied and all (100%) regularly prescribed disease modifying drugs and all were aware of NAbs (100%). 100% believed NAbs do (63.6%) or probably (36.4%) abrogate the efficacy of IFNβ treatment. In total 82% were aware of guidelines from AAN +/- EFNS and 90% generally counselled patients about NAbs before initiating treatment, but generally this did not influence product choice. Routine testing of all patients was described by 30%, 55% tested those who were actively relapsing and 15% never tested. In response to a positive test 48% would retest to evaluate persistence with a view to changing treatment, 19% discuss alternative treatments amd 10% will request and MRI to assess lesion load basing treatment change on this.

Austria: Seventy seven neurologists answered however only 90% prescribed DMDs. Of these 96% were aware of NAbs and 95% thought Nabs do (37%) or probably (48%) affect the efficacy of IFNβ. Routine testing was performed in 25% with a further 67% testing in actively relapsing patients, only 5% said they never test for NAbs. In response to a positive test 39% would repeat the test to evaluste persistence and then consider treatment change. 33% would only consider an alternative treatment if the patient was actively relapsing and 7.4% would base the choice on an MRI.

When comparing responses of all 4 counties awareness regarding Nabs was high and most thought they play a role in reducing the efficacy of the drug. However routine testing was

rarely pursued and most only tested if a patient was actively relapsing. In the UK 35% of those who answered *never* test for NAbs. This is over double the proportion in other countries surveyed. Results are summarised in Table 7-1.

	UK	USA	CANADA	AUSTRIA
Prescribe IFNβ	100%	95%	96%	90%
Aware of NAbs	100%	99%	100%	96%
NAbs reduce IFNβ efficacy				
Yes	48%	56%	63.3%	37%
Probably	48%	36%	36.4%	48%
No	4%	2.6%	0%	5.4%
NAb testing				
All	17%	13%	30%	25%
Relapsing only	38%	70%	55%	67%
Never	35%	15%	15%	5%

Table 7-1 Results of NAbs survey

7.3.4. Uptake of the Luciferase Assay in the UK

It is estimated that more than 10,000 people with MS are treated with IFNβ in the UK. Since the Luciferase assay became available, 3478 samples (representing 2853 individuals) from 117 centres have been tested in our unit (figures from 25th January 2010). Of these 2283 (66%) were negative [titre < 20] and 1185 (34%) positive [327 (9.4%) were low, 331 (9.5%) moderate and 523 (15%) high positive]. This reflects the probable number of patients screened for NAbs in the UK and highlights that only ~ 15% of subjects are in the category that predicts no biological response to the IFNβ injection.

Chapter 8

Conclusions

8 Conclusions

The purpose of this work was to address the need to develop a simple, time and cost efficient assay, which could be reliably used in clinical practice both here in the UK but also to distribute it to other laboratories. We have achieved this in developing the luciferase assay which has been validated against gold standard assays in Europe [chapter 3]. The assay has also been widely distributed thoughout Europe and North America and has shown excellent inter-laboratory validity. The assay has been used to investigate the association of NAbs with clinical status and in chapter 4 the increased relapse rate and treatment failure in subjects who are NAb positive was shown. The reduced clinical efficacy was also shown to be titre dependent and those with highest NAb titres having the greatest risk of relapse. One hundred and fourty four subjects were recruited prospectively whom were treated for a minimum of 6 months with IFNβ [Chapter 5]. Samples were taken before and after IFNβ administration and MxA was measured as an established marker of IFNβ bioactivity. The NAb titres obtained with the Luciferase assaywere found to predict the MxA response with those who were NAb positive showing a reduced MxA induction in a titre dependent fashion.

Over the past two years after validation of the assay this work has been presented at several conferences and the assay methods published [Farrell *et al.* 2008; Farrell & Lam *et al.* 2008]. After submission of a successful business case to UCLH the luciferase assay became the routine assay used in the Neuroimmunology laboratory at the NHNN, London for routine NAb testing on the NHS. We have distributed the assay to several countries including Germany, Austria, Sweden, Denmark, Canada, Italy, Poland, Switzerland, Hungary and in many countries it has become the National Reference Assay.

As we have found in several cohorts of patients tested at NHNN ~ 25 % of all subjects have NAbs. In many these are at a low level and unlikely to affect the bioactivity of the drug significantly. However consideration should be given to identifying those who are responders and non-responders to IFNβ, and alter management accordingly. Problems arise making treatment decisions in patients who are NAb +ve but are doing well clinically. In these cases NAb status should be used as a predictive marker of risk of future relapse and to identify subjects who are more likely to fail IFNβ therapy. The odds of having a relapse in a NAb +ve period compared to NAb -ve is between 1.51 and 1.58 (p< 0.03) and the time to first relapse is prolonged by an average of 244 days in subjects who are NAb -ve at 12 months (p<0.009) [Sorensen *et al.* 2003]. Some clinicians argue that due to the high reversion rates seen in subjects treated with IFNβ-1b in particular, there is no need to stop treatment. It is important to note however that not all will revert and that it is generally only seen in those with low titres. During the period of NAb positivity they will also be at increased risk of relapse and may incur further disability through incomplete recovery. As NAbs are known to be cross-reactive across IFNβ products switching between 1a and 1b is not sufficient [Antonelli *et al.* 1999; Bertolotto *et al.* 2000]. It is due to this that we recommend that those with persistently high titres should be considered for alternative therapy.

An alternative strategy to manage NAbs is to consider directly targeting them by either preventing their formation or treating them after they have developed. Recently studies have explored the effect of corticosteroids in preventing the development of neutralising anti-IFNβ antibodies (NAbs) or minimising their levels. It has been shown that treatment

with concomitant steroids delays the appearance of NAbs and lower titres develop when they do appear [Pozzilli *et al.* 2002; Pozzilli *et al.* 2004]. Concomitant use of immunosuppressive therapies in combination with various biological products is associated with a lower rate or the reversal of NAbs. In an open labelled study of 161 MS patients, receiving IFNβ-1b (Betaferon®, 8MIU s.c. on alternate days), randomised to receive either intravenous methyl-prednisolone 1g monthly for 12 months compared to no corticosteroids the prevalence of NAbs at 15 months in the prednisone treated group was 12.1% compared to 26.8% in untreated group, a relative reduction of 54.9% [Pozzilli *et al.* 2002b]. However results from a number of studies have not shown consistent results [Calabresi *et al.* 2002; Patti *et al.* 2001]. The most feasible option however is to switch to another class of DMD altogether: Glatiramer Acetate, Natalizumab and Mitoxantrone are well established available treatments and it is anticipated that in the next 12 months new oral agents should be available.

We recommend that once a person is established on IFNβ treatment they should be routinely screened for NAbs at 12 and 24 months. If a person is NAb -ve at 24 months it is unlikely that they will become NAb +ve in the future and therefore further routine testing will not be of benefit. In those with titre ~ 100 – 600 NU quantification of MxA expression is useful in determining the individual's response to IFNβ administration. Those who show expression of MxA are likely to be partial responders despite NAbs whereas those will very low levels of MxA suggest the drug is not inducing the required biological response. New guidelines are anticipated for the Eurpoean consortium regarding NAbs and should also be adopted in the UK.

Future research planned following the work described in this thesis will focus on establishing a combined NAb and MxA induction assay as part of routine NAb testing for NHS patients. As sample numbers increase it will enable us to further define the Nab titre at which the biological response is reduced and the titre at which no response is found. Due to the small number of subjects with titres > 600 NU in the cohort described here, the study was underpowered to establish a sensitive and specific "cut point". We must stress that a specific cut-point may not exist and in vivo bioactivity and NAb titre may need to be interepreted together. We also plan to use NAbs as a tool to investigate the treatment effect of IFNβ on viruses which have been linked to MS (eg EBV, HHV6) either in relation to aetiology or disease activity. Longitudinal and cross-sectional studies are underway evaluating the serological response to and reactivation of viruses in subjects which were recruited in the NAb studies described.

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