

ANTI-INTERFERON ANTIBODIES IN MULTIPLE SCLEROSIS. MOLECULAR BASIS AND THEIR IMPACT ON CLINICAL EFFICACY

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1. ABSTRACT

Low levels of naturally-occurring, high-affinity antibodies directed against cytokines can be found in the circulation of individuals who have never been exposed to exogenously-supplied cytokines. These antibodies are thought to play a regulatory role in the intensity and duration of immune response. Interferon (IFN) beta has been shown to attenuate both relapsing-remitting multiple sclerosis (MS) and secondary progressive MS in several well-powered, randomized, controlled clinical trials. IFN therapy can induce the production of anti-IFN neutralizing antibodies (NAb), usually in the second 6 months of treatment, in 3–45% of treated patients. This variation in the proportion of NAb-positive patients is probably due to the immunogenicity of different formulations of IFN beta, as well as the assay used, which are not currently standardized. The occurrence of NAb appears to be directly correlated with the dose of therapeutic IFN administered, up to a certain dose threshold. If the dose is increased beyond this threshold, the levels of NAb decrease. The biological significance of anti-IFN NAb is not yet known, nor has it been proven conclusively that they affect the clinical response to IFN beta therapy. The presence of NAb is therefore not an indication that treatment should be changed. Indeed, any treatment decision should be based only on the clinical response to therapy.

2. INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous

system believed to result from an autoimmune process that induces demyelination, destruction of oligodendrocytes, and axonal injury (1). The progression of MS is unpredictable, and although about 15% patients have steadily worsening disability over months or years with no periods of remission, a large number of individuals with MS are not as severely affected, and can lead relatively normal lives. The immunology of MS is associated with increased leukocyte migration into the central nervous system (CNS) and the production of several cytokines that can vary with the course of the disease, e.g. levels of proinflammatory cytokines, such as interleukin (IL)-2, IFN gamma and tumor necrosis factor (TNF) alpha, increase during relapses and are down-regulated during the non-active phases of the disease (2, 3). In patients with MS, IFN beta has been shown to decrease leukocyte migration to the CNS (with reduced expression or production of adhesion molecules or matrix metalloproteases) (4, 5), and to shift the immunoregulatory balance from T helper 1 (Th1) lymphocytes (with production of the pro-inflammatory cytokines IFN gamma and TNF alpha) to the immunomodulatory T helper 2 (Th2) lymphocytes (with production of immunosuppressive cytokines IL-4 and IL-10) (6, 7). These immunomodulatory effects have led to the development of IFN beta as a treatment for MS.

The development of recombinant DNA technology has enabled the large-scale production of human homologues of IFN, growth factors and hormones, which has benefited a large number of patients with a range

of diseases. Recently, however, there has been considerable discussion on the potential problem of the induction of antibodies against agents such as recombinant human cytokines, including IFN beta, during prolonged therapy (8, 9). This review will address this issue, and in particular, whether these antibodies have any clinical significance.

3. INTERFERON BETA USE IN MULTIPLE SCLEROSIS

The results of several well-powered, randomized, controlled clinical trials have demonstrated the efficacy of IFN beta in the treatment of relapsing-remitting MS (RRMS) and secondary progressive MS (SPMS) and have ensured its place as the current treatment of choice for both forms of MS (10, 11, 12, 13, 14, 15). Two types of recombinant human IFN beta are currently used in the treatment of RRMS – IFN beta-1a and IFN beta-1b. Recombinant IFN beta-1a is produced in transfected mammalian cells (Chinese hamster ovary), and is a glycosylated polypeptide of 166 amino acids with the same amino-acid sequence, carbohydrate side-chain and structure as the natural human cytokine (16). Recombinant IFN beta-1b is a non-glycosylated peptide of 165 amino acids produced in transfected *Escherichia coli* bacterial cells using a modified human gene sequence that contains a genetically-engineered cysteine-to-serine substitution at position 17 and a methionine-1 deletion. Despite the differences between the two IFN beta subtypes, clinical trials have shown that both are effective in the treatment of patients with MS.

Based on different pharmaceutical formulations, three different recombinant IFN beta products are registered for the therapy of MS: Betaferon® (IFN beta-1b) from Schering AG (Berlin, Germany) (Betaseron® in the United States), Rebif® (IFN beta-1a) from Ares-Serono (Geneva, Switzerland) and Avonex™ (IFN beta-1a) from Biogen (Cambridge, MA). The recommended dosages of IFN beta are 8 million international units (MIU) every other day for Betaferon®, 6 or 12 MIU three times a week for Rebif®, and 6 MIU once a week for Avonex™. Routes of administration of IFN beta also differ between products – Betaferon® and Rebif® are given by subcutaneous injection, while Avonex™ is given by intramuscular injection. Interestingly, the amounts of IFN beta protein used for these preparations differ considerably. Eight MIU of Betaferon® contain 250 mcg protein, 6 MIU Rebif® contain 22 mcg, and 6 MIU Avonex™ contain 30 mcg. The relationship between biologic activity (as expressed by IU) and the absolute weight of protein is termed specific activity and is expressed as IU per weight. Therefore, the specific activity for Betaferon® is 32 MIU/mg, compared with 273 MIU/mg for Rebif®, and 200 MIU/mg for Avonex™. Because the manufacturers use different IFN standards, the specific activities of the different IFN beta preparations may not be directly comparable.

4. NATURALLY OCCURRING ANTI-CYTOKINE AUTO-ANTIBODIES

Several studies have shown that low levels of high-affinity antibodies directed against cytokines can be found in the circulation of both healthy and diseased

individuals who have never been exposed to exogenously-supplied cytokines (17, 18, 19, 20). Indeed, naturally-occurring autoantibodies to cytokines, including IFN alpha, beta, gamma, IL-1alpha, IL-2, IL-6, IL-10 and TNF-alpha, have been reported by several research groups, and their serum levels increase in patients suffering from a variety of immuno-inflammatory diseases. For example, high concentrations of naturally-occurring autoantibody specific for IFN alpha have been found in the sera of patients suffering from the autoimmune disease, systemic lupus erythematosus (21, 22). Furthermore, serum levels of anti-cytokine autoantibodies can vary significantly over the course of an infectious disease, with levels peaking during the most active stage of the disease and reducing considerably after disease resolution (23).

It has been suggested that these ‘naturally occurring’ autoantibodies play a regulatory role in the intensity and duration of an immune response. In fact, there is evidence of a close correlation between production of a cytokine and its autoantibody following immune stimulation. When the cytokine exceeds a critical threshold level, the level of autoantibody may be increased to modulate this response. Although the mechanisms which mediate this regulation have yet not been fully elucidated, particularly *in vivo*, it is known that a proportion of these autoantibodies can reduce the biologic activity of cytokines by preventing the interaction of the cytokine with its cell-surface receptor. They are therefore referred to as NAb, and are thought to modulate the immune response by acting as antagonists of cytokine action at the site of inflammation. Furthermore, autoantibodies that bind to the cytokine but do not interfere with its biologic activity are known as non-neutralizing antibodies. Non-neutralizing autoantibodies are believed to function as carriers, preventing the rapid elimination of cytokines from the circulation and increasing their bioactivity. Alternative mechanisms of cytokine regulation by autoantibodies may involve other factors that control cytokine activity, such as soluble cytokine receptors, cell surface decoy receptors, and receptor antagonists.

The detection of autoantibodies by conventional immunosorbent assays can be difficult, as they may be present in the circulation complexed with anti-idiotypic antibody or their respective cytokine. Moreover, potential neutralizing autoantibodies complexed with endogenously produced cytokines may also escape detection in bioassays.

5. THERAPEUTICALLY-INDUCED ANTI-CYTOKINE AUTOANTIBODIES

A second class of anti-cytokine autoantibody is induced by therapy with cytokines. Therapeutically-induced anti-cytokine autoantibody might be produced as a consequence of chemical modification of the therapeutic cytokine, improper folding, or contaminants bound to the cytokine, all of which would produce new antigenic sites. Alternatively, these autoantibodies might be generated by the immune system as part of a compensatory mechanism to counter-regulate high concentrations of exogenously-supplied cytokines.

Certain cytokines are more likely to induce anti-cytokine antibodies. For example, type I IFNs (IFN alpha and beta) are potent inducers of IL-10 production by peripheral blood mononuclear cells (24, 25). Among its various biologic activities, IL-10 has been shown to enhance proliferation and immunoglobulin production by B cells, most likely by preventing apoptosis of lymph node germinal centers (26, 27). IFN-mediated up-regulation of IL-10 in the germinal center is likely to result in a more effective B-cell response to viral antigens, but concomitantly favors the generation of autoreactive B cells.

Biopharmaceuticals can induce antibodies by two different mechanisms. The classic reaction is induced by foreign proteins, such as biopharmaceuticals of bacterial or plant origin, e.g. streptokinase and asparaginase, and is comparable with the immune response to a vaccine. In the majority of cases, NAb is produced during the classic reaction, often even after a single injection. The NAb persists for a long time and inhibits the efficacy of the biopharmaceutical. The second mechanism of autoantibody production is one that breaks the immune tolerance that normally exists to self-antigens. This mechanism leads to the production of autoantibodies against human erythropoietin. Generally, these autoantibodies appear after prolonged therapy in a minority of patients only, and can disappear during therapy or after treatment cessation. In the majority of cases, there are no consequences from the presence of anti-cytokine autoantibodies, clinical or otherwise. The mechanisms by which tolerance is induced or broken are not yet completely understood. One important method of breaking tolerance to self-antigens is to present them to the immune system in a repetitive manner, e.g. aggregates of protein can efficiently inactivate the naïve or anergic B cells that are involved in tolerance (28, 29). Furthermore, the lack of glycosylation of glycoproteins produced in prokaryotes, such as GM-CSF and IFN beta, may induce autoantibodies because such molecules are less soluble, or because epitopes which are normally hidden by the glycosylation are exposed (30, 31). In addition, the route of administration of the therapeutic cytokine and the characteristics of the individual patient play a role in breaking tolerance to self-antigens (32, 33).

6. ASSAYS FOR ANTI-IFN ANTIBODY TITRATION

To date, two types of assay systems have been used to measure anti-IFN antibodies. Conventional antibody assays, such as ELISA and Western blot, detect antibody that binds to epitopes on the IFN molecule. These assays are a useful pre-screen to the neutralization bioassays as even those antibodies with low affinity will be detected. The presence of NAb in sera is determined in special bioassays that measure the ability of antibodies to reduce IFN beta bioactivity. The biologic assays most frequently employed to determine the levels of NAb to type I IFN are based on antiviral activities such as yield reduction or cytopathic effect or, less frequently, the inhibitory effect of the antibodies on the proliferation of T lymphocytes (34). The antiviral assay (AVA), which measures the residual activity of IFN beta against viral cytopathic effect after the addition of potentially

neutralizing sera, is the most commonly-used biologic assay, and was recommended by the World Health Organization (WHO) (35). The titers of NAb are reported as the reciprocal of the highest dilution of the serum neutralizing 10 laboratory units (LU)/mL IFN beta activity to an apparent 1LU/mL activity. The definition for NAb positivity requires that two consecutive serum samples from a patient exhibit a titer of at least 20 neutralizing units (NU), although not all clinical studies follow this definition as frequent serial sampling of serum is required. The cut-off titer of 20 NU was originally based on the maximum sensitivity (lowest titer detectable) of the AVA. The criterion for two consecutive positive titers was selected empirically as giving the lowest possible NAb-positive frequency among placebo recipients.

There are several problems with the AVA, and its sensitivity, specificity and reproducibility for the detection of IFN activity has been questioned. Indeed, the AVA has a relatively narrow dynamic range between the limit of detection and saturation of the IFN response, and may lack the selectivity to type I IFN when testing human sera due to the presence of inherent antiviral factors (36, 37). In addition, there is significant variation between assays and laboratories. Another problem with the AVA is that the cytopathic effect used as an endpoint is not considered an effective quantitative measure of IFN and anti-IFN antibody, and human sera can cause direct cytotoxicity to the cells used in the assays, increasing the risk of false-positive results and decreasing specificity.

The problems with the AVA have therefore stimulated interest in the development of a new assay to detect anti-IFN antibodies, and a bioassay based on myxovirus resistance-1 (MxA) gene induction in cultured human cells has been validated for determining NAb to IFN (38). The assay is based on the ability of type I IFN to induce intracellular MxA protein production, and is a two-step process. The first stage involves the incubation and co-culture of the test sera, IFN, and a cell line sensitive to induction of MxA protein by IFN. The second step of the assay utilizes a two-site immunochemiluminescent assay specific for MxA protein, which is somewhat similar to a non-competitive antibody-bound ELISA (39). If a test serum contains NAb to IFN, then MxA levels will be reduced. The MxA-induction assay is an attractive alternative to the AVA as it does not require viral infection of the cell line. Good correlation between the results of the AVA and MxA assays have been demonstrated using sera from MS patients receiving Betaferon® therapy, and the frequency of false positives was low in both assays. Moreover, the MxA assay was identified as the more sensitive of the two.

The major problem with most bioassays is the intra-assay and inter-laboratory variability (Figure 1). The endpoints for assays can be different – some laboratories use cytopathic effect, whereas others estimate viral growth or the induction of specific viral or cellular proteins. In addition, many laboratories have their own preferred cell line, viral antigen and methods of diluting the IFN. Furthermore, each laboratory defines its own ‘laboratory

NABs to IFN beta (% positive): inter- and intra-laboratory variability

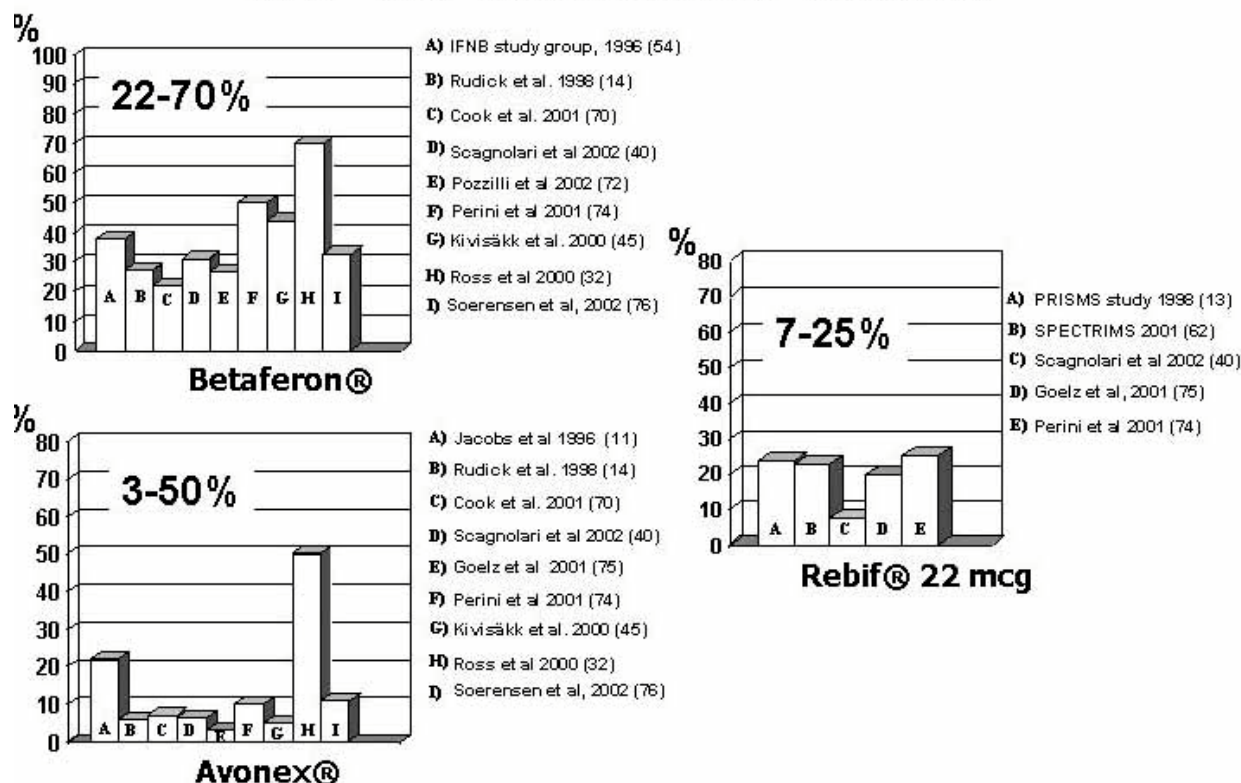


Figure 1. NABs occurring during treatment with the three IFN beta registered for MS treatment. The figure emphasizes inter- and intra-laboratory variability. The bold capital letters on each bar identify the laboratories, reported on the right hand side of each graph with the bibliographical reference between parentheses. % : percent NAb positive patients.

units' (LU), which are based upon a certain dilution of serum that neutralizes a laboratory-defined concentration of IFN. The amount of IFN added to the assay can also differ between assays and laboratories. This variation can affect the sensitivity of the assay, as demonstrated by Ross et al (32) who showed that adding IFN beta at concentrations from 3 to 100 LU/mL varied the detection of anti-IFN beta NAb from less than 10% to 90% of patients. Moreover, all the assays that measure anti-IFN NAb are based upon the inhibition of a biological function of the IFN beta molecule but, as the specific mechanism(s) by which IFN beta influences the course of MS has not yet been identified, the clinical relevance of the bioactivity tested in the assays is unknown.

7. NAb OCCURRENCE IN MS PATIENTS TREATED WITH IFN BETA: BIOLOGICAL AND CLINICAL SIGNIFICANCE

The frequency of anti-IFN beta antibody in MS patients before IFN beta therapy is low and is similar to that of a healthy population (0.1%) (32). The development of anti-IFN beta antibodies during treatment has been reported in variable numbers of MS patients in all published clinical trials which tested for the presence of

anti-IFN antibodies. All antibodies that bind to the IFN beta molecule are defined as 'binding antibodies' (BAb). NAb are a subset of BAb thought to bind to the portion of the IFN beta molecule which interacts with its receptor in the target cells.

The issue of NAb occurrence during IFN beta therapy has been addressed by two different approaches, although no study has yet been designed specifically to address this subject. Several studies, often performed on small patient samples, addressed the issues of the frequency of NAb, the different immunogenicity of various IFN beta preparations, the influence of dose frequency and route of administration, as well as their effect on biological markers, thought to be 'surrogate' markers of IFN clinical efficacy in MS. These studies are usually short-term, mostly lasting between 1 year, and a maximum of 18 or 24 months. The second approach involved well-powered studies that primarily evaluated the efficacy of IFN beta therapy on clinical and magnetic resonance imaging (MRI) parameters. These clinical trials also investigated the occurrence of NAb and their impact on these clinical and MRI parameters. However, these analyses were typically performed post hoc, and as a result, the statistical methodology was not always well designed. Nevertheless,

Table 1. Occurrence of NAb and their impact on clinical and MRI outcomes in the randomized multicenter trials on IFN beta in MS

	Follow-up years	IFN beta subtype	IFN beta dose	NAb positivity %	Reduced CLINICAL efficacy	Reduced MRI efficacy
IFNB MS group (54)	3	1b	50 mcg tiw sc	42	No	No
		1b	250 mcg tiw sc	38	Yes	Yes
MSRCG (14)	2	1a	30 mcg ow im	6	No	Yes
PRISMS (13)	2	1a	22 mcg tiw sc	23,8	No	-
		1a	44 mcg tiw sc	12,5	No	-
PRISMS-4 (64)	4	1a	22 mcg tiw sc	23,7	-	-
		1a	44 mcg tiw sc	14,3	Yes	Yes
INCOMIN (50)	2	1a	30 mcg ow im	6	No	No
		1b	250 mcg tiw sc	22	No	No
EVIDENCE (51)	1	1a	30 mcg ow im	2	No	-
		1a	44 mcg tiw sc	25	No	Yes
EUSPMS (55)	3	1b	250 mcg tiw sc	27,8	No	No
SPECTRIMS (62)	3	1a	22 mcg tiw sc	20,6	No	-
		1a	44 mcg tiw sc	14,7	No	-
IMPACT (77)	2	1a	60 mcg ow im	3,3	-	-

these studies have contributed important information about the influence of NAb on IFN clinical efficacy.

8. TIMING OF NAb APPEARANCE

Generally, anti-IFN beta antibodies appear during the second 6 months of treatment with IFN beta-1a or -1b (32, 40, 41), although they have been reported at 3 months after the initiation of therapy (42, 43). There is no clear significant difference in the time of appearance between the various formulations of IFN beta. Nevertheless, in NAb-positive patients, a decreased biologic response to IFN beta (measured as the production of MxA protein) (44) was reported in the months preceding seroconversion. This could mean that, at least in some patients, either low or undetectable levels of NAb might already impair the biologic response to IFN beta. Alternatively, other unknown factors may render these patients poor responders to IFN beta therapy, independent of the presence of NAb. A subgroup of patients might therefore be both poor responders to IFN beta therapy and produce low or undetectable levels of anti-IFN beta NAb.

9. BAb AND NAb OCCURRENCE AND CORRELATIONS

BAb can be detected in almost all treated patients (50–80%), usually just before the appearance of NAb (40, 44, 45, 46). Currently, the clinical relevance of BAb in patients with MS is not known. It is possible that BAb induce organ damage by the formation of immunocomplexes with IFN beta. In addition, they may increase the bioactivity of IFN beta by preventing its rapid elimination.

Some NAb-positive samples have a very low BAb titer. There is a wide variation in the levels of BAb and NAb between individuals, and this suggests that the neutralization of IFN beta depends on both the quantity and the quality of antibody. BAb and NAb IgG subclasses differ

(47): BAb are predominantly IgG₁, and NAb are IgG₂ and IgG₄. Patients with high-titer IgG₁ BAb are more likely to develop NAb, although other patients with a low titer of BAb, may also subsequently develop IgG₂, or more frequently, IgG₄ NAb. There is probably clonal selection of NAb with a higher affinity for IFN beta epitopes, and it is thought that BAb-negative patients may eventually develop NAb (46).

10. FREQUENCY OF NAb IN PATIENTS TREATED WITH IFN BETA

Anti-IFN beta NAb are detected in 3–45% of treated patients, a frequency that is lower than that of BAb. The levels of anti-IFN beta NAb positivity differ among published studies, depending on the formulation of IFN beta used (Table 1). This variation probably results from the differing immunogenicity of the IFN beta preparations, as well as different dosage regimens. Nevertheless, the assays used to detect the antibodies are not standardized and will most likely contribute to the variation in antibody frequency. Comparative studies performed in a single laboratory are therefore essential to our understanding of the role of anti-IFN antibodies during IFN therapy (32, 42, 45). These studies have demonstrated that the probability of developing NAb is higher for Betaferon® (37–50%) than Rebif® 22 mcg (19%) or Avonex™ (4–5%). Although the difference between IFN beta-1b and beta-1a may be due both to the different chemical structures (48) and dosages, Ross et al (32) found that the occurrence of anti-IFN antibody almost doubled with Rebif® 22 mcg compared with Avonex™. In this case, the variation might result from the different route of administration or manufacturing processes. Indeed, improved processes to manufacture Avonex™ decreased the frequency of NAb by five-fold (14), while it has been postulated that the subcutaneous route is more immunogenic (14, 32), but this has not been confirmed by others (42). In the Once Weekly IFN for MS (OWIMS) study (49) 5% of patients administered Rebif® 22 mcg once-weekly developed NAb, a figure which

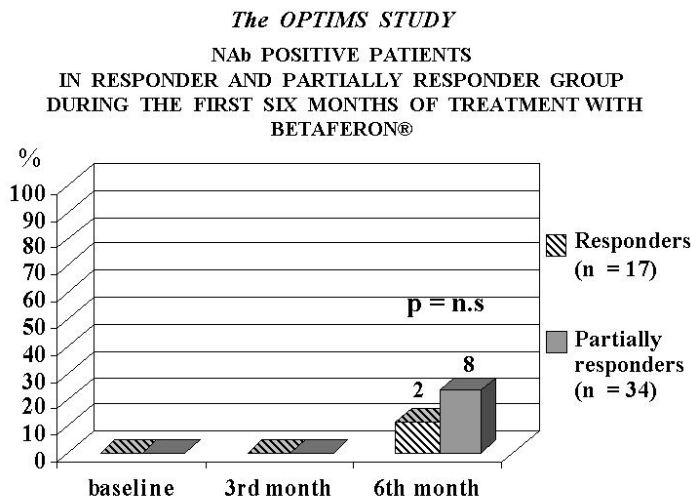


Figure 2. The OPTIMS STUDY, a multicenter study actually ongoing in Italy. Preliminary results on NAb occurrence: NAb positive patients in responder and partially responder group during the first six months of treatment with Betaferon®.

compares well with data reported for Avonex[™]-treated patients (14). It is, however, important to note that the high dose/frequent administration protocols of some of the IFN products can increase the rate of some side effects (NAb occurrence included), but have more pronounced clinical and MRI effects (50, 51). Increased bioavailability of both Betaferon® and Rebif® may explain the increase in efficacy and adverse events (52).

11. RELATIONSHIP BETWEEN IFN DOSE AND THE DEVELOPMENT OF NAb

The occurrence of NAb appears to be directly correlated to the dose of therapeutic IFN administered, up to a certain dose threshold. When the dose is increased above this threshold, a high-zone tolerance may be induced, and the frequency of NAb decreases (53). In fact, it is known that the quantity of antigen may influence the intensity of the immune response, and that repeated and higher doses of the antigen may inhibit the production of antibody. Indeed, in the Prevention of Relapses and Disability by Interferon beta-1a Subcutaneously in Multiple Sclerosis (PRISMS) study (13), which compared Rebif® 22 mcg and 44 mcg with placebo, the proportion of patients with NAb was significantly lower and almost reduced by half in patients receiving the higher dose of Rebif® (Table 1). This study confirms the results from the IFNB Multiple Sclerosis Study Group (10, 54), which demonstrated that patients treated with the lowest dose of Betaferon® (50 mcg) had a slightly higher frequency of NAb positivity compared with those receiving Betaferon® 250 mcg (Table 1).

Recently, we have addressed the issue of the relationship between IFN beta dose and the occurrence of NAb in the Optimization of Interferon for MS (OPTIMS) trial, which was completed in Italy during the second half of 2003. OPTIMS is a multicenter study involving 24 Italian MS centers, which aimed to test the efficacy of two different doses of Betaferon® (250 or 375 mcg

subcutaneously, every other day) in RRMS patients who had responded partially to the standard Betaferon® dose (250 mcg) after 6 months of treatment, i.e. patients with persistent MRI activity or relapses. Patients were randomized to continue treatment with Betaferon® 250 mcg or 375 mcg, every other day. NAb levels were assessed every 3 months using the IFN beta-induced MxA production neutralization assay against 10 IU/mL of IFN beta, with titers ≥ 20 considered positive. Preliminary data from the study during the first year of follow-up on NAb occurrence and titer has shown that after 3 months of treatment, no patients were NAb positive. At 6 months, there were 10 NAb-positive patients, with a greater frequency in patients who were partial responders (approximately 20% versus 10%) (Figure 2). This difference is probably not significant due to the small sample size. During the second 6 months of the trial, when only partial-responder patients were treated with the two different doses of Betaferon®, there was no difference in the frequency of NAb positivity in the two groups. When the changes in NAb titer over time (Figure 3) were analyzed, however, a clear trend was observed for an early decrease of NAb titer in the group treated with the highest dose of Betaferon®.

12. DO NAb PERSIST OVER TIME?

The majority of studies that have been discussed were conducted for only 12–18 months, a relatively short time to evaluate whether NAb persists. Two studies (32, 45), however, have reported results from a number of patients who were followed up for 2 years. Both these trials confirmed that there are two different trends for different IFN beta preparations. While NAb positivity to Betaferon® increases during the first year of follow up, stabilizing during the first half of the second year, it tends to decrease by about 30–50% at the end of second year (Figure 4). NAb positivity to once-weekly 22 mcg IFN beta subcut, however, shows a progressive increase over time, during both the first and second years. By the end of the second

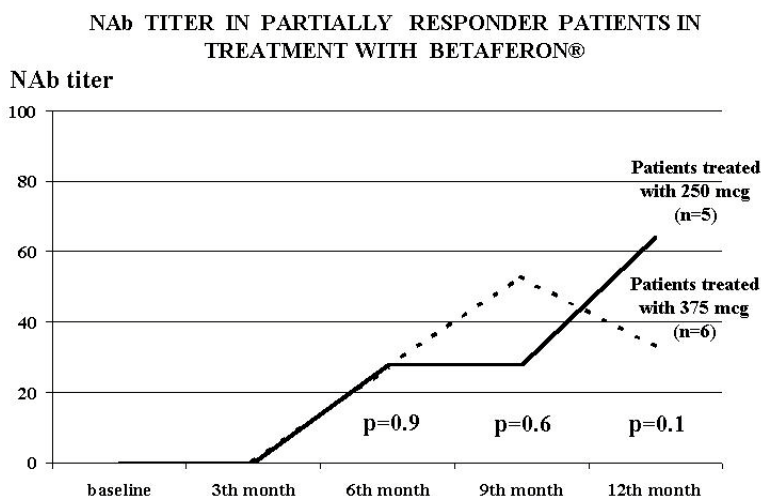
The OPTIMS STUDY

Figure 3. The OPTIMS study. Preliminary results on NAb titer. Titer of patients treated with the higher dose of Betaferon® (375 mcg, every other day) showed a trend for an early decrease compared to that of patients treated with the standard dose (250 mcg, every other day).

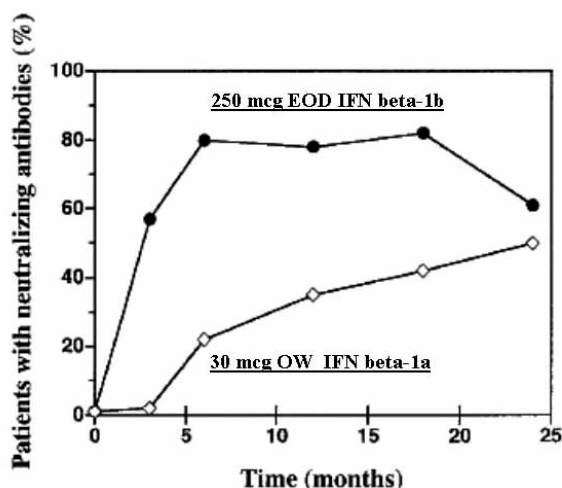


Figure 4. Percent of NAb-positive patients during prolonged treatment with 250 mcg every-other-day (EOD) Betaferon® or 30 mcg once-weekly (OW) Avonex™. (From: Ross et al, ref. No. 32, with permission of John Wiley & Sons, Inc.).

year, the difference in the occurrence of NAb between the groups of patients treated with the two IFN beta products is no longer very pronounced (Figure 4).

In long-term MS clinical trials, NAb have often been tested frequently over periods of 3–6 years. In the IFNB Multiple Sclerosis trial (10) of Betaferon® in patients with RRMS, NAb levels were tested every 3 months for up to 5 years (12). More than 60% of the patients classified as NAb positive in this study were subsequently classified as being NAb negative in at least one test (J. Petkau et al., personal communication). In the European Study Group on

IFN beta-1b in Secondary Progressive MS trial (EUSPMS) (15), which randomized 718 patients to treatment with Betaferon® or placebo for 3 years, approximately 50% of the NAb-positive patients reverted to NAb negative status at least once, and of these patients, almost 80% had no further positive titer (55). A similar trend of a reduced proportion of NAb-positive patients with prolonged treatment was observed in patients treated with Rebif® in the 6-year open-label continuation of the PRISMS study (56), although the result was not so pronounced as that observed with Betaferon®. NAb usually persists over time if present at a high titer [$\geq 1:45$, Bertolotto et al, 2003 (43); $\geq 1:100$, J. Petkau, personal communication; $\geq 1:500$, Paszner et al, 1999 (46); $\geq 1:7000$, Rice et al, 1999 (57)], although this effect is still controversial, particularly since the threshold titer for persistence is unclear. The disappearance of NAb with time is particularly evident with Betaferon®. This may be due to the more rapid induction of B cell tolerance as a greater quantity of IFN beta protein in Betaferon® is administered (875 mcg per week) relative to the other IFN beta products (30–132 mcg per week). A more prolonged follow-up study with Betaferon® on NAb positivity has been conducted by Rice et al. (57) who tested serum samples from patients in the London, Ontario, and Vancouver cohorts of the IFNB MS Study Group trial. Fifty-nine patients were followed up for 9 years; 40% were NAb positive during the pivotal trial, but only 3 patients (5% of the total) remained NAb positive at the end of the study. No long-term studies on the variability of NAb positivity with Avonex™ have been performed.

13. NAb EFFECTS ON IFN BETA BIOLOGICAL MARKERS

The occurrence of NAb has been associated with reduced levels of IFN-induced biological markers. Indeed,

compared with NAb negative patients, NAb-positive Avonex[™]-treated patients (14) had reduced serum levels of neopterin, a macrophage protein which is induced by all types of IFN (58), and MxA mRNA in their peripheral blood mononuclear cells (43). In addition, NAb-positive patients treated with Betaferon[®] or Rebif[®] exhibited decreased serum levels of neopterin (41, 44) and reduced leukocyte MxA mRNA production (43). The reduction of the biologic effects of IFN beta seemed more consistent when NAb were present at a high titer ($\geq 1:45$) (43).

Unfortunately the relationships between these biological markers and the clinical efficacy of IFN beta are still unclear. To date, only a single study (59) has demonstrated that the serum level of MxA, which increases a few months after starting IFN beta treatment, is higher in patients without relapses and decreases during the active phase of the disease, although it does not return to the baseline level. Other studies have reported a correlation between the occurrence of NAb and a change in levels of immunological markers, which is most likely related to the mechanism of action of IFN beta in MS. NAb positivity reverses the biological marker and cytokine profile induced by IFN beta therapy. Indeed, decreased serum levels of the soluble form of the intercellular cell adhesion molecule-1 (ICAM-1) and increased activity of matrix metalloproteases have been associated with NAb positivity when compared with the previous NAb-negative period (60). Furthermore, NAb positivity was related to decreased levels of IL-4 and IL-10, and with increased concentrations of IFN gamma and TNF alpha in myelin basic protein-reactive T lymphocytes (61). All these studies are, however, of short duration, have small patient numbers, while sera samples were tested only once or a few times after starting treatment. Moreover, no definite conclusion can be made on the long-term effect of NAb on the biological activities of IFN beta as several of the biological parameters studied that change during the first months of IFN beta treatment, tend to spontaneously return to baseline levels.

14. IMPACT OF NAb ON THE EFFICACY OF IFN BETA

The randomized, placebo-controlled trials that have been conducted to evaluate the efficacy of IFN beta on clinical and MRI parameters have enrolled large numbers of patients and followed them up for 2–3 years in the placebo controlled phase and for 5–6 years during the open-label extension phases. Several of these trials have tried to address the issue of NAb and their clinical impact on the efficacy of IFN beta. Regrettably, the frequency of blood sampling was often inadequate, the NAb detection technique varied between studies and the statistical methods used were unsatisfactory. Indeed, most analyses approached the problem of the clinical impact of NAb with a cross-sectional comparison, i.e. the analyses addressed the relapse rate, disease progression, or MRI activity of NAb-positive and NAb-negative patients after 2 or 3 years. If patients with higher relapse rates and a more aggressive form of MS, however, are more likely to become NAb positive, and seroconversion to NAb positive would have little effect on clinical and MRI outcomes, there will

therefore be a greater proportion of patients with a high rate of relapse in the NAb-positive than NAb-negative group, biasing the results. A more accurate approach would be a longitudinal statistical method which uses each individual patient as its own control, comparing the changes in the rate of outcomes, e.g. relapse, disease progression, MRI activity, during NAb-positive or -negative periods, and correcting for the baseline clinical and MRI parameters of each patient. Although the longitudinal statistical analysis requires complex methods, it corrects for several factors that would be associated with long-term follow-up.

The advantages of the longitudinal statistical method are exemplified by the IFNB Multiple Sclerosis Study Group (10), which initially analyzed NAb status with sera sampled every 3 months from RRMS patients treated with Betaferon[®], using a cross-sectional statistical approach (10, 54). This approach reported an attenuation of treatment effects with respect to relapse rates and T2 lesion load in patients who had developed NAb, but mean scores on the Expanded Disability Status Scale showed a trend toward worsening only among patients without NAb (54). Data were subsequently reanalyzed using a longitudinal statistical approach (J. Petkau et al., personal communication). In contrast to the cross-sectional results, these longitudinal analyses did not show NAb-associated attenuation for the 250 mcg dose of Betaferon[®] on relapse rates, EDSS, or MRI measures. Attenuating effects of NAb were found only for relapse rates in the low-dose (50 mcg) group. Analyses considering both low- and high-titer periods simultaneously indicated that attenuating effects appeared to be more prominent in periods with higher NAb titers, when high titers were considered as $\geq 1:100$. In studies of IFN beta-1a (11,14,62,63) there were trends toward reduced treatment effect on relapses and on MRI activity in NAb-positive compared with NAb-negative patients. In these trials, serum samples were drawn only every 6 months, and analyses were restricted to the cross-sectional approach. Of particular interest when investigating treatment effect and NAb on IFN-1a and -1b are two comparative trials which demonstrated that the treatment regimen had an impact on efficacy –the Independent Comparison of Interferon (INCOMIN) study and the Evidence for Interferon Dose Effect: European-North American Comparative Efficacy (EVIDENCE) trial.

The INCOMIN study compared the effects of Betaferon[®] 250 mcg every other day with Avonex[™] 30 mcg once a week over 2 years (50). Patients treated with Betaferon[®] had significantly better clinical and MRI responses than those treated with Avonex[™], although the frequency of NAb-positive patients was clearly higher in the former group. The risk of relapse was significantly reduced by over 30% in the Betaferon[®] compared with the Avonex[™] group (Table 2). NAb positivity, on the other hand, did not affect the risk of relapse as the risk ratio was not significantly reduced in NAb-positive patients. Using a logistic regression model, the risk of relapse was analyzed according to the treatment plus NAb status. The calculated adjusted odds ratio further increased the statistical significance as the risk of relapse for patients with the same NAb status was reduced by nearly 60% in Betaferon[®]-

Table 2. The INCOMIN Trial : Univariate and logistic regression analysis of relapse risk using therapy and NAb status as independent variables

	n/N (%)	Univariate Analysis		Logistic Analysis
		Crude RR	p	ADJUSTED OR
IFN beta-1a	49 / 73 (67%)	1		1
IFN beta-1b	34 / 76 (45%)	0.67 (0.5-0.9)	0.006	0.41(0.2-0.8)
NAB -	67 / 115 (58%)	1		1
NAB +	16 / 34 (47%)	0.81 (0.55-1.19)	0.25	0.91 (0.4-2)

Comparing patients with the same NAb status further reduce relapse risk for IFN beta-1b-treated patients

EVIDENCE: NAb's have no effect on relapse occurrence over 48 weeks in MS patients treated with 44 mcg Rebif®

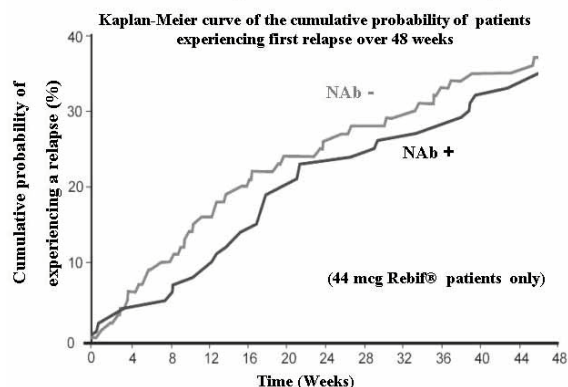


Figure 5. EVIDENCE trial: NAb positivity had no effect on relapse occurrence. Cumulative probability of experiencing a relapse in NAb-positive and NAb-negative Rebif®-treated patients. (From Panitch et al, 2002, ref. No. 51, with permission of Lippincott Williams & Wilkins, Inc.).

Design of PRISMS - 4 year follow-up of patients treated with Rebif®

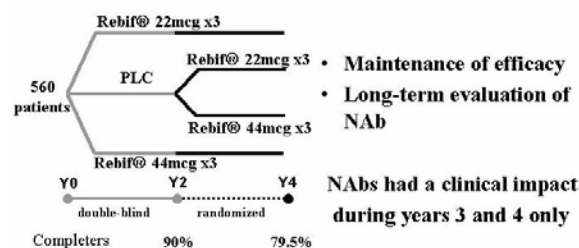


Figure 6. Study design of PRISMS – 4; four year follow-up of patients treated with Rebif®; (ref. N. 64).

reated patients when compared with the Avonex™ group. In addition, when the occurrence of NAb was stratified for patients with or without relapses, disease progression, or MRI activity during the trial, no significant differences in the NAb frequency were observed within each treatment arm.

The EVIDENCE trial (51) compared Rebif® 44 mcg three times a week with Avonex™ 30 mcg once weekly over 1 year. The high dose/high frequency protocol produced better clinical and MRI results than the once-

weekly administration. NAb did not appear to have any impact on clinical response, and the cumulative probability of experiencing a relapse in Rebif®-treated patients, was almost identical whether they were NAb positive or NAb negative (Figure 5). In addition, patients treated with the high dose/high frequency IFN beta-1a protocol had fewer relapses, even when NAb-positive Rebif®-treated patients were compared with NAb-negative patients treated with once-weekly Avonex™.

Although both the INCOMIN and EVIDENCE trials were well-powered studies, they did not demonstrate any effect of NAb positivity on MRI activity. It is probable that studies needed to be conducted longer than the 1 and 2 years of the INCOMIN and EVIDENCE trials to clarify this issue. In fact, the PRISMS-4 study was designed to investigate whether the beneficial effects of IFN beta therapy were maintained over 4 years, and focused on the clinical impact of NAb (Figure 6) (64). NAb-positive patients had a greater number of relapses than NAb-negative individuals, but only in those patients who were persistently NAb positive during years 3 and 4. During the last 2 years of this study, the relapse rate was 0.5 for NAb-negative patients and 0.8 for NAb-positive individuals, a significant difference. It can therefore be concluded that NAb against IFN beta may eventually have a clinical impact on MS if they persist for several years at a high titer.

15. HOW TO COUNTERACT THE DEVELOPMENT OF NAb

As NAb has the potential to eventually negate the benefits of long-term IFN beta therapy, the important question is whether it is possible to reverse the attenuating effects of NAb by switching to a different IFN beta subtype. The precedent for this course of action is based upon clinical efficacy having been restored by switching treatment with one subtype of IFN alpha to another after NAb developed against the initial therapy (65, 66). The major problem of treatment with IFN beta subtypes, however, is that NAb to one IFN beta subtype cross-react with other subtypes, thereby prolonging, or even increasing, their neutralization activity when therapy is switched (67, 68, 69, 70). There appears therefore to be no rationale for switching from one IFN beta subtype to another. Furthermore, as the immune system has a long memory, a 'drug holiday' would not seem appropriate. Strategies to prevent NAb may include the use of higher IFN beta doses or combination therapy with another immunosuppressant, such as azathioprine or

methylprednisolone (71, 72). Indeed, the addition 1 g of methylprednisolone each month to IFN beta therapy was well tolerated and reduced the development of NAb by over 50% (72).

16. PERSPECTIVES

Our conclusion concurs with the American Academy of Neurology Clinical Practice Treatment Guidelines (73), which state that several long-term, controlled randomized studies have demonstrated that although NAb develop during IFN beta treatment, their biologic significance is yet unknown. In addition, it has not been proven conclusively by any sufficiently-powered, randomized, controlled study that NAb can affect the clinical response to IFN beta therapy. Furthermore, measuring the titer of NAb does not appear to have any benefit in the management of patients, nor should the presence of NAb bring about a change in therapy. Any treatment decision, therefore, must be based only on the clinical response of each individual to IFN beta.

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