Chapter 3.2

Long-term blocking of endogenous IFN-β by persisting NAbs after IFN-β treatment discontinuation

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Abstract

Objectives: To confirm that neutralizing antibodies (NAb) to Interferon-beta (IFN-β) can persist after therapy withdrawal and to evaluate whether persisting NAb are associated with a worse clinical disease course in Multiple Sclerosis (MS).

Design: Retrospective study of the association of persisting NAb and clinical disease course in MS.

Setting: Tertiary Referral Center in the Netherlands

Patients: 71 relapsing-remitting MS patients treated with IFN-β in the past

Main outcome measures: Persisting NAb after therapy withdrawal were tested using the cytopathic effect assay. Patients with and without persisting NAb were compared on several outcomes: the change in annualized relapse rate from prior to IFN-β initiation to after cessation of treatment, time to sustained disability on the Kurtzke’s Expanded Disability Status Scale (EDSS) and the use of disease modifying treatments after IFN-β cessation.

Results: 17/71 patients (24%) tested NAb positive after a median interval of 25 months (IQR 10-51) after IFN-β cessation. 11 of these 17 patients (15%) were high titre NAb positive (>150 TRU/ml). Persisting NAb were associated with an increase in the annualized relapse rate (p = 0.040) and a reduction in time to reach a sustained EDSS score of 6.0, i.e. the need for unilateral assistance to walk 100m (p = 0.018). Moreover, NAb positive patients were significantly more often treated with second line therapy, especially mitoxantrone (p = 0.006).

Conclusion: Anti-IFN-β NAb can persist after IFN-β withdrawal and are associated with overt clinical disease activity. This is apparent by an increase in relapse rate and faster disability progression and supported by the observed need for more aggressive therapy after IFN-β cessation. Prospective studies are warranted to confirm these results.
Endogenous type I IFN pathways and disease activity in MS

Introduction

Multiple Sclerosis (MS) is a chronic immune mediated disorder of the CNS and one of the leading causes of permanent disability in young adults\(^1\). Interferon beta (IFN-\(\beta\)) has been shown to be a safe and effective treatment for relapsing-remitting MS and is widely used as a first-line treatment\(^2,3,4\). The chronic use of recombinant protein based therapeutics, such as IFN-\(\beta\), insulin, growth hormone and factor VIII, can lead to the generation of an immune response directed against the drug\(^5\) which is mainly based on breaking B cell tolerance\(^6\). Anti-drug antibodies may cross-react with their endogenous counterpart\(^7\), influence therapeutic efficacy of the drug\(^8\), or may be associated with allergic reactions\(^9\). The clinical significance of anti-IFN-\(\beta\) neutralizing antibodies (NAb) continues to be a controversial issue within the MS community\(^10\). Increasing evidence suggests that, during treatment, bioactivity of IFN-\(\beta\) is influenced by NAb and efficacy of the treatment is decreased with persisting NAb\(^6,8,11,12,13\). Some studies have shown that NAb can persist even after cessation of IFN-\(\beta\) treatment. Dynamics and clinical impact of persisting NAb, however, are largely unknown\(^14,15\). In this study, our first aim was to confirm the occurrence of persisting NAb after withdrawal of IFN-\(\beta\) therapy and to evaluate potential predisposing factors. Secondly, as persistent NAb after cessation of IFN-\(\beta\) therapy will probably inhibit endogenous IFN-\(\beta\), we correlated persisting NAb status to measures of clinical disease activity.

Patients and methods

Potential participants were retrospectively identified by a chart review of all well-monitored patients who had started IFN-\(\beta\) treatment from 1994 until 2006 at the MS center Amsterdam. Consecutive patients were invited to participate when treated with IFN-\(\beta\) for at least 12 months and subsequently ceased treatment for at least 3 months. In general, patients were seen at baseline, 1, 3, 6 and 12 months and annually thereafter. Patients had additional visits when indicated. We collected data concerning age at disease onset, gender, disease duration at start IFN-\(\beta\) treatment, treatment-duration and IFN-\(\beta\) product used. Furthermore, we evaluated the use of disease modifying treatment (DMT) after IFN-\(\beta\) treatment cessation. The number of relapses was assessed in the two years before IFN-\(\beta\) initiation and in the period after IFN-\(\beta\) cessation and both were converted into annualized relapse rates. Disability status was determined at the start of IFN-\(\beta\) treatment for all subjects by using the Kurtzke’s Expanded Disability Status Scale (EDSS)\(^16\) and repeated at the most recent visit to the outpatient clinic. NAb titre levels were measured by a previously described cytopathic effect assay (CPE) in “Centro Riferimento Regionale Sclerosi Multiplo”, Orbassano, Italy. Titres were calculated according to the Kawade’s formula and expressed in ten-fold reduction units per millilitre (TRU/mL)\(^17,18\). Measurements were performed with the same type of IFN-\(\beta\) as used in individual patients for therapy. For this study, NAb titres of \(\leq\)20 TRU/mL were considered NAb negative, NAb titres >20 TRU/mL were considered NAb
positive and NAb titres >150 TRU/mL were considered high titre NAb positive\textsuperscript{13,10}. Clinical researchers were blinded for NAb titre results and laboratory colleagues for the clinical evaluation of patients. This study was carried out with the approval of the Medical Ethical Committee of the VUmc and written informed consent was obtained from all participants.

**Statistical analysis**

Differences in demographic characteristics and disability status (EDSS) at IFN-β treatment initiation between patients with persisting antibodies after cessation of therapy and NAb negative patients were measured with the Kruskal-Wallis, Mann-Whitney, chi-square and Fisher’s exact test, where appropriate. The association between post-IFN-β treatment regimens and NAb status were analyzed with Fisher’s exact test. Relapse rates before IFN-β therapy and after IFN-β therapy cessation were compared using Wilcoxon signed-rank test for related samples. The proportion of patients with a stable or improved relapse rate after IFN-β cessation was compared to patients with a rise in relapse rate in NAb status groups using logistic regression analysis with treatment regimen after IFN-β as a factor. For the evaluation of disability progression we used time to event analysis, in which the event was defined as reaching EDSS 6, sustained for at least 6 months, corresponding with the need for unilateral assistance to walk at least 100m. We used Cox regression analysis with NAb status as main independent variable and corrected for the EDSS score at initiation of IFN-β therapy.

**Results**

**Patients**

About 525 MS patients had started IFNb therapy at the MS center Amsterdam between 1994 and 2006. We examined the medical charts of those 342 patients who had systematic clinical evaluations at least annually. Ninety-seven patients fulfilled the pre-specified selection criteria. Seventy-one of these patients, 51 women and 20 men, gave written informed consent (for baseline characteristics see Table 1). The reasons for exclusion were either that patients were still on IFNb treatment or that patients had been treated with IFNb for less than 12 months. Twenty-six patients fulfilled criteria but preferred not to participate because of either work-responsibilities (n = 6), or because of MS related conditions (n = 11). Nine patients declined participation in research without pointing out a specific reason. Patients were treated with intramuscular (i.m.) IFN-β-1a (Avonex\textsuperscript{®}) 30 μg once weekly (n = 20), subcutaneous (s.c.) IFN-β-1a (Rebif\textsuperscript{®}) 22 μg (n = 13) or 44 μg (n = 5) three times weekly and s.c. IFN-β-1b (Betaferon\textsuperscript{®}) 250 μg every other day (n = 33).
Persisting NAb after cessation of IFN-β therapy

Seventeen patients (24%) were found to be NAb positive (NAb titres median 320 TRU/mL, range 22-5120) and 11 of these 17 patients were high titre positive (range 152-5120 TRU/mL). NAb positive patients were tested after cessation of IFN-β therapy with a median interval of 25 months (IQR 10-51). No differences were found with respect to age of onset, gender, disease-duration, duration of IFN-β treatment, EDSS at start of IFN-β treatment and MS subtype (Table 1). Patients using IFN-β-1a s.c. (Rebif®) 22 or 44 μg three times weekly were more often persisting NAb titre positive (p = 0.054) and high titre positive (p = 0.003) than patients using either IFN-β-1b (Betaferon®) or IFN-β-1a i.m. (Avonex®) (Table 1).

Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>NAb negative (≤ 20 TRU/mL)</th>
<th>NAb positive (&gt;20 TRU/mL)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td>54 (76)</td>
<td>17 (24)</td>
<td>71 (100)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>40 (74)</td>
<td>11 (65)</td>
<td>51 (72)</td>
</tr>
<tr>
<td>Age at disease onset in yrs: median (IQR)</td>
<td>30.8 (25.5-37.5)</td>
<td>37.0 (27.1-40.6)</td>
<td>31.7 (25.9-39.0)</td>
</tr>
<tr>
<td>EDSS at start IFN-β: median (IQR)</td>
<td>3.0 (2.0-4.0)</td>
<td>3.0 (2.0-4.0)</td>
<td>3.0 (2.0-4.0)</td>
</tr>
<tr>
<td>Disease duration at start IFN-β in yrs: median (IQR)</td>
<td>5.6 (1.3-10.2)</td>
<td>3.1 (1.2-5.6)</td>
<td>4.9 (1.3-9.6)</td>
</tr>
<tr>
<td>IFN-β treatment duration in yrs: median (IQR)</td>
<td>2.4 (1.2-4.4)</td>
<td>2.9 (2.2-5.1)</td>
<td>2.6 (1.3-4.4)</td>
</tr>
<tr>
<td>IFN-β-1a 30μg IM n (%)</td>
<td>18 (33)</td>
<td>2 (12)</td>
<td>20 (28)</td>
</tr>
<tr>
<td>IFN-β-1a 22/44μg SC n/n (%)</td>
<td>8/2 (19)</td>
<td>5/3 (47)</td>
<td>13/5 (25)</td>
</tr>
<tr>
<td>IFN-β-1b 250μg SC n (%)</td>
<td>26 (48)</td>
<td>7 (41)</td>
<td>33 (47)</td>
</tr>
</tbody>
</table>

NAb, neutralizing antibodies; IFN-β, Interferon beta. TRU/mL, ten-fold reduction units per millilitre; IQR, interquartile range; SD, standard deviation; EDSS, Extended disability Status Scale; IM, intramuscular; SC, subcutaneous; DMT, disease modifying treatment. A statistical trend was found for an association between NAb positivity and the IFN-β product used (p = 0.054).

Persisting NAb and DMT after IFN-β treatment

Most patients stopped IFN-β treatment because of disease breakthrough (relapses and/or disability progression) under therapy. Of these 41 patients, 13 (37%) tested positive for NAb after cessation of IFN-β therapy. A smaller group of patients stopped treatment because of side-effects or because of a desire to become pregnant, 4 out of these 26 patients (15%) were positive for persisting NAb. Notably, treatment decisions were made without any knowledge of NAb status during therapy, as NAb measurements were not performed in our clinic during that period. After IFN-β withdrawal, 13 patients (18%) switched to glatiramer acetate (Copaxone®), 13 (18%) to natalizumab (Tysabri®) and 10 (14%) of patients received courses of intravenous mitoxantrone dihydrochloride (Mitoxantrone®). Forty-nine percent (35/71) of patients remained untreated during follow-up (Table 2). Some patients that ceased IFN-β treatment because of perceived treatment failure, remained untreated afterwards (15/41 (37%)). The majority of these patients were considered to be not eligible for other
immunomodulatory treatment because they progressed to the secondary progressive phase of MS. NAb status was associated with the choice of treatment after abortion of IFN-β treatment (table 2; $p = 0.006$). Persisting NAb positive patients were more often switched to second line therapy (mitoxantrone or natalizumab) than NAb negative patients. This difference was mainly driven by the choice for mitoxantrone in these patients.

Table 2. Disease-modifying treatment after cessation of Interferon beta (IFN-β).

<table>
<thead>
<tr>
<th>Treatment after IFN-β</th>
<th>N (%) NAb negative (≤ 20 TRU/mL)</th>
<th>N (%) NAb positive (&gt;20 TRU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natalizumab</td>
<td>8 (62)</td>
<td>5 (38)</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>4 (40)</td>
<td>6 (60)</td>
</tr>
<tr>
<td>Glatiramer acetate</td>
<td>11 (85)</td>
<td>2 (15)</td>
</tr>
<tr>
<td>No treatment</td>
<td>31 (89)</td>
<td>4 (11)</td>
</tr>
</tbody>
</table>

Percentage of neutralizing antibodies (NAb) for NAb positivity (>20 ten-fold reduction units per millilitre (TRU/mL)) in different treatment regimes after cessation of IFN-β. The occurrence of persisting NAb was associated with a treatment switch after IFN-β ($p = 0.006$).

**Persisting NAb and clinical outcome measures**

Overall, the mean relapse rate after IFN-β cessation was significantly lower than the relapse rate of the pre-treatment phase ($p = 0.001$). However, within subgroups only NAb negative patients had a decrease in relapse rate ($p < 0.001$) whereas the relapse rate in NAb positive patients remained unchanged ($p = 0.88$). When comparing the proportion of patients with an increased relapse rate after cessation of IFN-β therapy, correcting for the use of mitoxantrone, a higher proportion was found for NAb positive patients ($p = 0.04$, Table 3). Compared to patients without persisting NAb the risk of an increased relapse rate after IFN-β treatment was almost five times higher in patients positive for NAb (OR = 4.55; 95% CI 1.10 - 19.23). This was confirmed when analysing with cut-offs for high titres (>150 TRU/ml; OR = 5.99; 95% CI 1.21 - 29.41; $p = 0.028$). Of the 11 patients that showed an increased relapse rate, 5/11 (45%) were NAb positive. NAb positive patients who did not show this increase in relapse rate were more frequently treated with mitoxantrone (six out of the remaining 12 NAb positive patients).

In both NAb status groups approximately half of the patients reached an EDSS score of 6.0 at the time of study assessment (Table 3). Cox regression analysis, correcting for EDSS at start of IFN-β treatment, showed that patients positive for persisting NAb after IFN-β withdrawal, progress faster than patients who are NAb negative (HR = 2.94; 95% CI 1.20 – 7.14; $p = 0.018$, Figure 1). For patients who are high-titre NAb positive, a statistical trend was found (HR = 2.36; 95% CI 0.86-6.49; $p = 0.096$).
Table 3. Clinical outcome measures for patients with and without persisting NAb after cessation of IFN-β treatment.

<table>
<thead>
<tr>
<th>Clinical outcome measures related to NAb (TRU/mL) status</th>
<th>NAb negative (≤ 20)</th>
<th>NAb positive (&gt;20)</th>
<th>Total n=71</th>
</tr>
</thead>
<tbody>
<tr>
<td>n= 54</td>
<td>n= 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annualized relapse rate before IFN-β mean (SD)</td>
<td>0.7 (0.6)</td>
<td>1.0 (0.8)</td>
<td>0.8 (0.7)</td>
</tr>
<tr>
<td>Annualized relapse rate after IFN-β mean (SD)</td>
<td>0.3 (0.4)*</td>
<td>1.2 (1.6)</td>
<td>0.5 (0.9)</td>
</tr>
<tr>
<td>Annual relapse rate before/after IFN-β proportion (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>decrease or stable</td>
<td>48 (89)</td>
<td>12 (71)</td>
<td>60 (85)</td>
</tr>
<tr>
<td>increase</td>
<td>6 (11)</td>
<td>5 (29)**</td>
<td>11 (15)</td>
</tr>
<tr>
<td>patients reaching EDSS score of 6, n (%)</td>
<td>27 (50)</td>
<td>9 (53)</td>
<td>36 (51)</td>
</tr>
<tr>
<td>time to EDSS score of 6, mean (SD), y</td>
<td>12.5 (1.3)</td>
<td>6.7 (1.0)</td>
<td>12.3 (6.5)</td>
</tr>
</tbody>
</table>

*In NAb negative patients there was a significant decrease in relapse rate after IFN-β cessation compared to before start of treatment (p < 0.001). **The proportion of patients with an increased relapse rate after cessation of IFN-β therapy is higher in NAb positive patients, when correcting for the use of mitoxantrone (OR = 4.55; 95% CI 1.10 - 19.23). NAb, neutralizing antibodies; TRU/mL, ten-fold reduction units per millilitre; SD, standard deviation; EDSS, Extended disability Status Scale.

Figure 1. Survival probabilities for reaching an Expanded Disability Status Scale (EDSS) score of 6 in a Cox proportional hazards model corrected for EDSS at the start of interferon beta treatment. The black continuous line represents the regression curve of patients positive for persisting neutralizing antibodies (NAb) after cessation of Interferon-beta treatment. The dashed line represents the regression curve of patients negative for persisting NAb. Patients positive for NAb progress faster to an EDSS of 6.0 than patients who are NAb negative (hazard ratio 2.94; 95% confidence interval, 1.20-7.14).
Discussion

This is the first study reporting that patients with anti-IFN-β neutralizing antibodies that persist after cessation of therapy have a more active disease course. This was indicated by an increase in annualized relapse rate after IFN-β withdrawal and a faster progression to an EDSS score of 6.0. Furthermore, persisting NAb are associated with different treatment regimen after IFN-β withdrawal; less patients remained untreated and more patients switched to second-line treatment, especially mitoxantrone. Anti-IFN-β NAb develop in a significant proportion of MS patients using IFN-β. Median or low titres tend to disappear spontaneously in some patients despite continuous therapy, but high NAb titres persist and are clearly associated with a decrease in treatment efficacy during treatment. In absence of antigen exposure, NAb were expected to resolve within a few months after IFN-β withdrawal. Surprisingly, two small studies described NAb persisting long after cessation of IFN-β therapy. One of the studies reported NAb titres persisting in two patients up to 54 months after IFN-β-1b s.c. was withdrawn. In a Danish retrospective follow-up study in 37 MS patients, NAb were demonstrated after a mean follow-up of 22 months and up to 59 months. High-titre NAb tended to persist over time, as only one of 18 high-titre NAb positive patients reverted to NAb negativity. Furthermore, the chance of reverting differed between the IFN-β products. Similar to their results we found a small percentage of patients with persisting NAb after cessation of IFNb treatment. The majority of NAb positive patients were high-titre NAb positive and persisting high NAb titres were most frequently found with the use of IFN-β-1a subcutaneously. How NAb are able to persist independently from antigen exposure remains unclear. Repeated antigen presentation (during therapy) can induce long-living plasma cells in the bone marrow which continue to secrete antibodies, outlasting the antigen challenge. Alternatively, a cross reaction between recombinant IFN-β and endogenous IFN-β could be the cause of persisting NAb. Endogenous IFN-β produced in response to viral infections could maintain NAb production by activated B cells. In this study, both the decision to stop IFN-β treatment and the treatment-choice afterwards was made without any knowledge of NAb status, as NAb measurements were not performed in our clinic during that period. The results of this study clearly suggest that persisting NAb are associated with a more aggressive treatment strategy; as mitoxantrone, a treatment reserved for patients with aggressive inflammatory disease in our center, was significantly more prescribed to patients who turned out to be positive for persisting NAb. However, it must be noted that the majority of patients that discontinued IFNb treatment because of perceived efficacy failure were not NAb positive. This suggests that treatment failure is often determined by factors other than the persistence of NAb. In our cohort, the majority of these NAb negative patients were progressing under treatment without superimposed exacerbations and were therefore not eligible for alternative immunomodulatory treatment. Another interesting finding of our study is that in NAb positive patients, who were treated...
with mitoxantrone, the immunosuppressive properties of this chemotherapeutic agent were not able to abolish persisting anti-IFN-β NAb. One suggested approach to treat NAb positive patients on IFN-β has been the induction of combination therapies with strong immunosuppressive compounds like mitoxantron\textsuperscript{25}. Our data suggest that this approach, at least for the use of mitoxantrone, is not very likely to be successful. This is in line with a recent study, which shows that treating NAb - after IFN-β therapy withdrawal - with monthly pulsed oral methylprednisolone for a period of six months has no beneficial effect on NAb status or IFN-β bioactivity\textsuperscript{26}. The mechanisms through which persisting NAb exert their impact on MS disease activity are unknown. However, anti-IFNb antibodies probably have an effect on endogenous IFN pathways which may result in a more pro-inflammatory modification of the immune system and subsequently to an increase in MS disease activity. Alternatively, the tendency to develop and sustain anti-IFNb NAb might be a reflection of a more active immune system. In our study patients with persisting NAb were treated somewhat earlier and the pre-treatment relapse rate was somewhat higher. This could support the hypothesis, that these patients have more active disease due to a pre-existent more active immune system. However, although this alternative explanation cannot be fully excluded, it must be emphasized that there were no significant differences between NAb positive and NAb negative patients for any of the clinical features at baseline, including disability status. Obviously, the retrospective nature and the small sample of our study do not allow for definite conclusions to be drawn and causality can not be proven. On the other hand, for the purpose of this analysis, the retrospective nature also provides some strength, because it guarantees that all clinical assessments and treatment decisions were not influenced by knowledge of Nab status. Altogether, our findings suggest that NAb that persist after treatment discontinuation may negatively influence the subsequent course of the disease and may lead to the requirement for more aggressive treatment. Systematic long-term follow-up of patients exposed to NAb titres that persist after termination of IFN-β therapy is lacking and given the possible impact on MS disease course, conclusive studies on persisting anti-IFN-β NAb are warranted.

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References