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# Anti-interferon-β neutralising activity is not entirely mediated by antibodies

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**Abstract**

Many multiple sclerosis (MS) patients treated with interferon-β (IFNβ) develop anti-IFNβ antibodies (BAbs), which can interfere with both *in vitro* and *in vivo* bioactivity of the injected cytokine. Objective of this study was to correlate these measures. Among the 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*.


*Keywords:* Anti-IFNβ antibodies; BAbs; NAbs; Neutralising activity

## 1. Introduction

Interferon-beta (IFNβ) is the first-line treatment for relapsing remitting multiple sclerosis (MS) and several large randomised trials have shown that the drug reduces the frequency and severity of clinical relapses, slows the progression of disability and suppresses signs of disease activity on MRI (Paty et al., 1993; The IFNB Multiple Sclerosis Study Group, 1995; Jacobs et al., 1996; Rudick et al., 1997; Simon et al., 1998; PRISM Study Group, 1998; European Study Group on IFNβ-1b, 1998; Miller et al., 1999; Li and Paty, 1999). However, a proportion of patients fails to respond to IFNβ, mainly because of the development of anti-IFNβ neutralising activities, which abolish both its biological and clinical action (Deisenhammer et al., 1999; Vallittu et al., 2002; Bertolotto et al., 2001, 2003, 2004; Gilli et al., 2004a; Pachner et al., 2003, 2005; Polman et al., 2003; Perini et al., 2004; Malucchi et al., 2004; Kappos et al., 2005; Sorensen et al., 2005).

All previously reported studies on IFNβ neutralisation in MS assumed, but did not verify, that the observed neutralisation was mediated entirely by antibodies [particularly binding antibodies (BAbs) of a neutralising nature (NAbs)]. This assumption, however, disagree with a previous study by Pungor and co-workers (Pungor et al., 1998), where it has been shown that human sera contain non-immunoglobulin mediated components that can inhibit the activity of IFNβ *in vitro*. Recently, we have confirmed this finding in an inter-laboratory study, where low level inhibitions, that were not immunoglobulins-mediated, were observed in sera of patients treated with IFNβ (Gilli et al., 2006a). To date, however, there is no evaluation of the real entity of this phenomenon, as well as no data are available on the loss of *in vivo* IFNβ biological activity related to that non-antibody-mediated neutralisation.

In the present study we demonstrate that a subset of MS patients treated with IFNβ present a non-antibody-mediated neutralisation, which inhibit both *in vivo* and *in vitro* IFNβ biological activity. We also evaluate the real prevalence of this phenomenon in MS patients showing that it involves a significant percentage of subjects.

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## 2. Methods

### 2.1. Regular monitoring of patients treated with IFNβ

By December 1998, 386 patients with MS treated with one of the four available IFNβ preparations/dosing regimens were regularly monitored for their clinical and biological response to treatment, at our centre. For clinical monitoring, subjects were required to visit the clinic for a baseline evaluation and every 3 months for blood collection and nursing visits. In addition, every 6 months, patients came to the MS centre for neurological examination with Expanded Disability Status Scale (EDSS) score and recording of relapses and adverse effects. In addition to clinical evaluation and standard laboratory tests, biological responsiveness to IFNβ treatment was regularly assessed in each patient via *in vitro* neutralising activity (NA) and MxA mRNA measurements. Serum levels of NA were measured at baseline (pre-treatment specimens) and every 3 months throughout the treatment window (post-treatment specimens).

Based on previous data on MxA mRNA measurements (Bertolotto et al., 2003, 2004; Pachner et al., 2003; Gilli et al., 2005, 2006b), it was decided that the best time for obtaining the samples was 12 h (12.4±1.6, range 9–14) after IFNβ injection. Therefore, if patients came to the clinic 12 h after IFNβ injection, an additional matched blood sample was taken for MxA mRNA quantification by real-time PCR.

### 2.2. Study design and patients

In planning this study, we had available regular clinical evaluations as well as matched measurements of *in vitro* NA and MxA mRNA of all those patients ($n$=386). Two hundred fifty-six patients were then selected because they met the following inclusion criteria: (1) no immunosuppressive drug therapy; (2) at least two-years of chronic treatment with IFNβ; (3) no switch of IFNβ type; (4) no glucocorticosteroid therapy <30 days prior to the blood draw; and (5) informed consent.

For the purposes of this study, we considered a single post-treatment specimen per patients that had been obtained after 18 months of chronic treatment with the cytokine; the mean duration of therapy was 18.15±1.90 months (median 17.91; range 16–20 months). Besides *in vitro* NA and *in vivo* IFNβ bioactivity analyses, all samples (pre-treatment and post-treatment specimens) were also blindly tested for the presence of BAbs by a capture ELISA (cELISA).

Of the 256 patients, 60 were treated with intramuscular IFNβ-1a (IFNβ-1a$_{im}$) (Avonex: Biogen, Cambridge, USA) 30 μg once a week, 51 were treated with subcutaneous IFNβ-1b (Betaferon: Schering, Berlin, Germany) 250 μg three times a week and 145 were treated with subcutaneous IFNβ-1a (IFNβ-1a$_{sc}$) (Rebif, Serono, Genève, Switzerland) with either 22 μg ($n$=95 patients) or 44 μg ($n$=50 patients) three times a week.

After those analysis, 8 patients, who were representative of each combined BAb and *in vitro* NA category, were selected for repeated sampling analysis. These patients underwent baseline serologic (both BAbs and *in vitro* neutralising activity) and molecular bioactivity measurements prior to therapy and at intervals (every three months) after initiation of IFNβ therapy.

### 2.3. BAbs measurement by cELISA

Serum specimens were examined for BAbs using the cELISA described elsewhere (Brickelmaier et al., 1999; Pachner, 2003). In brief, 96-well microtitre plates were coated overnight with monoclonal anti-human IFNβ IgG antibody BO2 (Yamasa Shoyu Co. Ltd, Tokyo, Japan) at a concentration of 0.1 μg/well in 0.1 M carbonate–bicarbonate. After washing and blockade of wells with non-fat dry milk 50 μL/well of either IFNβ-1a (150 ng/mL) or IFNβ-1b (1.5 μg/mL) was added. The presence of bound antibody was detected using a rabbit anti-human IgG antibody conjugated with horseradish peroxidase (DakoCytomation, Glostrup, Denmark), followed by the addition of substrate. Results were obtained in optical density (OD) units by spectrophotometric analysis and were converted to units by comparison with a standard curve from a known positive specimen.

Serum samples were also tested for the presence of anti-IFNβ IgM, IgE and IgA. For measurement of IgM, IgE, or IgA response, a rabbit anti-human IgM, IgE or IgA (DakoCytomation, Glostrup, Denmark) was used as primary antibody.

### 2.4. BAbs analysis by Western Blot Assay (WBA)

WBA for BAbs was performed as previously described (Deisenhammer et al., 1999). IFNβ was subjected to 10% sodium-dodecyl-sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis and enhanced chemio-luminescence (ECL) western blotting was performed by using as antiserum either respective test serum or a murine control (Yamasa Shoyu Co. Ltd, Tokyo, Japan).

### 2.5. Antiviral cytopathic effect (CPE) assay for neutralising activity

The antiviral CPE assay was used to detect the neutralising activity because it is the assay that has been recommended by the World Health Organization (WHO) (1985).

Briefly, serum samples were diluted, mixed with one of the three IFNβ products at a final concentration of 10 IU/mL, and incubated for 1 h. Thereafter, A549 cells were plated and incubated with serum-IFNβ mixture and then encephalomyocarditis murine (EMC) virus was added. After culture, cells were stained and the absorbance was read. According to WHO recommendations (1985), data from the neutralisation assay are reported as the reciprocal of the highest dilution of serum inducing 50% neutralisation (i.e. neutralising 10 IU/mL of IFNβ activity to an apparent 1 IU/mL of activity). The neutralisation titre of a serum sample was calculated according to Kawade's formula (Kawade, 1986; Kawade et al., 2003) and expressed in 10-fold reduction units per millilitre (TRU/mL) (Grossberg et al., 2001). A level of ≥20 TRU/mL is generally considered the threshold of positivity.

### 2.6. Immunoglobulin depletion

In order to ensure a wider immunoglobulin depletion, a combined purification with both protein L and protein G sepharose was performed.

Serum was diluted 1:2.5 with alpha-MEM, 4 mM glutamine, 50 U/mL penicillin G, and 0.05 mg/mL streptomycin sulfate and then mixed with 50 μL of PBS-washed protein G sepharose (Amersham, Freiburg, Germany) for batch adsorption overnight at 4 °C. The resin was centrifuged and supernatant was removed and sterile filtered. Thereafter, a new depletion by protein L was performed, using the NAb™ Spin Kits (Pierce, Rockford, IL, USA) and following the manufacturer's instruction. Finally, supernatant was assayed for neutralisation activity by the CPE assay.

### 2.7. MxA gene expression analysis

Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) using the RNAwiz reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Total RNA (50 ng final concentration) was first incubated with 2 μg random hexamer primers (Perkin Elmer, Shelton, CT) and then reverse transcribed using the Omniscript™ RT-kit (Qiagen, Hilden, Germany). Thereafter, cDNA was used as a template for the real-time PCR analysis based on the 5′-nuclease assay with the ABI Prism 7000 sequence detection system (Applied Biosystems, Monza, Italy).

Glyceraldehyde-phosphate-dehydrogenase (GAPDH) was used for sample normalisation, and Applied Biosystems' TaqMan® Assay-on-Demand pre-designed gene expression products were applied (Gilli et al., 2004b). All reactions were performed in duplicate. Relative quantisation of all targets was calculated by the comparative cycle threshold method outlined in user bulletin No.2 provided by Applied Biosystems.

### 2.8. Dot-blot for detection of soluble IFNα/β receptor

The possible presence in sera of a soluble form of the IFNα/β receptor (IFNAR2) was evaluated by dot-blot assays using nitrocellulose membrane against sera samples from treated patients. Sample proteins were spotted onto nitrocellulose membranes and hybridized with a specific neutralising anti-IFNAR2 antibody probe (USBiological, Swampscott, MA, USA). Thereafter, membranes were incubated with secondary antibody-HRP conjugate solution (in blocking solution) for 1 h. After washings, membranes were developed using 3-aminoethyl carbazol (Sigma, St Louis, MO, USA), until spots were visible.

A549 cell lysates were used as positive control, as well as a patient's serum, that has been evaluated as positive for soluble IFNAR and has been run as control in each assay.

### 2.9. Statistical analysis

For statistical analysis (median, mean, SD, Mann–Whitney *U* test) GraphPad Prism® program version 4.0 (GraphPad Software Inc., San Diego, CA) was used. All reported *p* values are based on two-tailed statistical tests, with a significance level of 0.05.

## 3. Results

### 3.1. Detection of BAbs by WBA and cELISA

In previous reports, WBA for BAbs gave similar results to the ELISA and was shown to have a low false-negative rate when screening for BAb positivity (Deisenhammer et al., 1999). Thus we used WBA for confirmation of cELISA results. For sera which gave positive or negative results by the cELISA, there was an excellent correlation between the results of this test and the WBA. Two samples that were negative for BAbs in the cELISA converted to positive during WBA. Those samples, that were regarded as positive in the whole remaining analysis, were concurrently evaluated as negative by the CPE assay and with low MxA induction. Besides, none of the samples positive by cELISA was negative by WBA.

### 3.2. Analysis of pre-treatment samples

At baseline, all patients ($n=256$) scored negative for both BAbs and *in vitro* neutralising activity and had levels of gene expression for MxA similar to that of normal controls (data not shown). Baseline MxA gene expression was low but always detectable. The mean level±standard deviation (SD) of MxA-specific transcript was 32.25±32.44 relative expression compared to GAPDH (RE) (median=28; range 0.25–122.56). Thus, an upper threshold of normal for untreated patients with MS was calculated as mean+3 SD=130 RE. This value agrees with that of our previous study where the threshold was 133 RE (Gilli et al., 2006b).

### 3.3. Analysis of post-treatment samples

#### 3.3.1. Anti-IFNβ antibodies

Anti-IFNβ antibodies (BAbs) were detectable in 42.9% (110/256) of patients (Table 1). Of these, 17 patients were treated with IFNβ-1a$_{im}$ (28.3% of the IFNβ-1a$_{im}$ treated patients), 37 patients were treated with IFNβ-1b (72.5% of the IFNβ-1b treated patients) and 56 patients were treated with IFNβ-1a$_{sc}$ (38.6% of the IFNβ-1a$_{sc}$ treated patients), either at 22 μg ($n=38$) or at 44 μg ($n=18$).

Table 1
Distribution of patients based on the presence of anti-IFNβ antibodies (BAbs) and *in vitro* neutralising activity (NA)

| | BAbs+ | BAbs− | Total |
|---|---|---|---|
| NA+ | 38 (14.8%) | 11 (4.3%) | 49 (19.2%) |
| NA− | 72 (28.1%) | 135 (52.7%) | 207 (80.8%) |
| Total | 110 (42.9%) | 146 (57%) | 256 |

The threshold for NA positivity was 20 TRU/mL, whereas the threshold for BAbs positivity was 1 U.

Table 2
Distribution of patients according to the presence of anti-IFNβ antibodies (BAbs) and *in vitro* neutralising activity (NA)

| | Intramuscular IFNβ-1a | Subcutaneous IFNβ-1b | Subcutaneous IFNβ-1a 22 μg | Subcutaneous IFNβ-1a 44 μg | Total |
|---|---|---|---|---|---|
| A. | | | | | |
| BAb+/NA+ | 3 | 13 | 16 | 6 | 38 (14.8%) |
| BAb−/NA− | 41 | 14 | 51 | 27 | 135 (52.7%) |
| BAb+/NA− | 16 | 24 | 22 | 12 | 72 (28.1%) |
| BAb−/NA+ | 0 | 0 | 6 | 5 | 11 (4.3%) |
| TOTAL | 60 | 51 | 95 | 50 | 256 |
| B. | | | | | |
| BAb+/NA+ | 5 | 18 | 24 | 10 | 57 (22.3%) |
| BAb−/NA− | 41 | 10 | 48 | 26 | 125 (48.8%) |
| BAb+/NA− | 14 | 19 | 14 | 8 | 55 (21.5%) |
| BAb−/NA+ | 0 | 4 | 9 | 6 | 19 (7.4%) |
| TOTAL | 60 | 51 | 95 | 50 | 256 |

We used a threshold for NA positivity defined as a titre of either (A) 20 TRU/mL or (B) 5 TRU/mL. BAb threshold for positivity was 1 U.A.

### 3.3.2. *In vitro* neutralising activity

*In vitro* neutralising activity was detected in 19.1% (49/256) of patients (Table 1). Of these, three patients were treated with IFNβ-1a$_{im}$ (5% of the IFNβ-1a$_{im}$ treated patients), 13 patients were treated with IFNβ-1b (25.5% of the IFNβ-1b treated patients) and 33 patients were treated with IFNβ-1a$_{sc}$ (22.8% of the IFNβ-1a$_{sc}$ treated patients) either at 22 μg ($n$=22) or at 44 μg ($n$=11).

## 3.4. Samples stratification

Two thresholds for NA positivity were analysed: (1) the traditional threshold of 20 TRU/mL or above, and (2) a more sensitive threshold of 5 TRU/mL. The 20 TRU/mL threshold is generally considered the threshold for positivity and, therefore, it was used for decision making on the CPE assay. The 5 TRU/mL threshold was only used for post-hoc analysis.

Based on these two thresholds, samples were classified into the following groups: (1) BAb/NA-negative (BAb−/NA−), for samples that had neither an inhibition of IFNβ activity in the CPE assay nor measurable total BAbs to IFNβ in the cELISA; (2) BAb-positive/NA-negative (BAb+/NA−), for samples that had positive BAbs but did not meet a criteria for positive neutralising activity in the CPE assay; (3) BAb-positive/NA-positive (BAb+/NA+), for samples that had measurable BAbs and a significant inhibition of IFNβ activity in the CPE assay.

### 3.4.1. *In vitro* neutralising activity: threshold ≥20 TRU/mL

Table 2A shows stratification of patients considering a threshold for positivity of ≥20 TRU/mL. Surprisingly, besides the three groups described above, we found a fourth group of 11 patients (4.3%) showing a significant inhibition of IFNβ activity in the CPE assay at dilutions ≤1:230, but no measurable BAbs (BAb−/NA+). Those samples were all obtained from patients treated with IFNβ-1a$_{sc}$, either at 22 μg ($n$=6), or at 44 μg ($n$=5).

### 3.4.2. *In vitro* neutralising activity: threshold ≥5 TRU/mL

Table 2B presents patients' stratification with the threshold defined as a titre ≥5 TRU/mL. As expected, lowering the threshold criteria from a detection limit of ≥20 TRU/mL to ≥5 TRU/mL increased the incidence of neutralising activity in each treatment group. In fact, a higher percentage of BAb+/NA+ patients ($n$=57; 22.3%), as well as BAb−/NA+ patients ($n$=19; 7.4%) was observed. These latter BAb−/NA+ samples were obtained from 15 patients treated with IFNβ-1a$_{sc}$ either at 22 μg ($n$=9) or at 44 μg ($n$=6). The remaining 4 samples were obtained from patients treated with IFNβ-1b.

### 3.4.3. Correlation of BAbs with *in vitro* and *in vivo* bioactivity

Samples from IFNβ-treated patients showed a mean MxA gene expression of 238.6±222.15 (median=201; range 4–2025), which was about 7-fold higher than the level observed with treatment-naïve samples and two-fold higher than the upper threshold of normal. MxA mRNA (values ≥130 RE) was significantly increased in 198/256 (77.3%) treated patients, whereas in the remaining 58/256 patients (22.7%) MxA mRNA was unaffected by IFNβ administration (<130 RE).

Fig. 1 shows MxA gene expression based on BAb/NA categorisation. In BAb−/NA+ patients, mean MxA gene expression was 114.8±263.8 (median=103.0; range 12–810). Although mean MxA expression in these patients was 2.3 higher than in BAb+/NA+ patients (76.15±80.34; median=53.0; range 5–450), the statistical comparison failed to show a significant difference

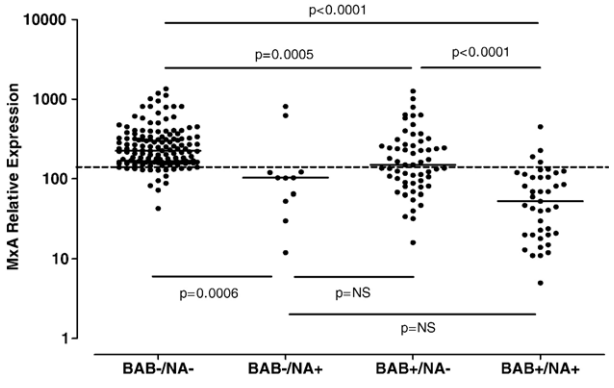


Fig. 1. Comparison of MxA gene expression levels in different MS patient groups subdivided based on both anti-IFNβ antibodies (BAbs) and *in vitro* neutralising activity (NA). An upper normal threshold was calculated as mean expression of untreated samples ±3 SD=130 relative expression (RE): the dotted line represents the threshold level, whereas the median values are indicated by the horizontal bars. $p$ values are reported.

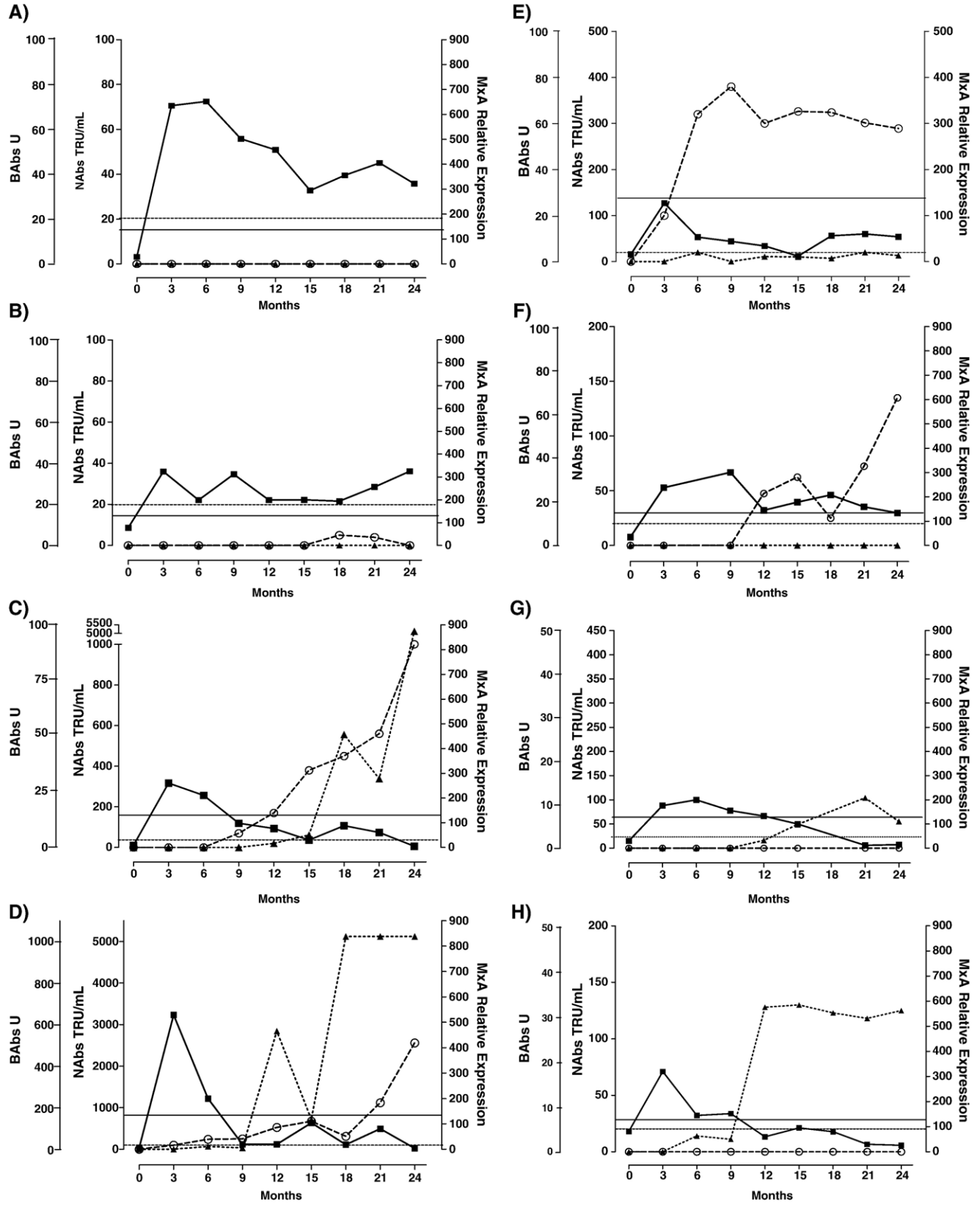


Fig. 2. Repeated sampling analysis of anti-IFNβ antibodies (BAbs) (○), *in vitro* neutralising activity (NA) (▲) and MxA gene expression (■) in patients categorised as BAb−/NA− (A and B), BAb+/NA+ (C and D), BAb+/NA− (E and F) and BAb−/NA+ (G and H). There are three different *y*-axis showing BAb levels in laboratory units (U), NA levels in ten-fold reduction units per millilitres (TRU/mL) and MxA mRNA levels as relative expression compared to GAPDH (RE).

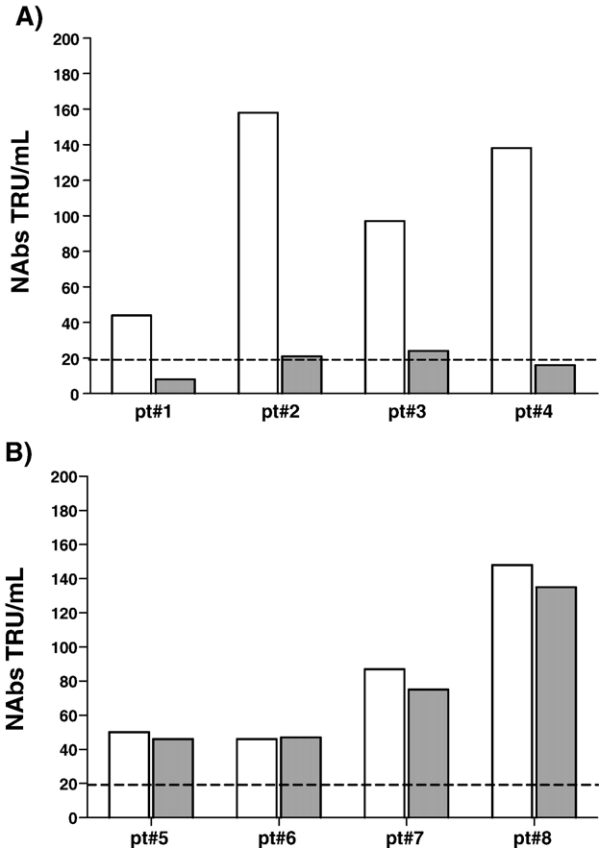


Fig. 3. *In vitro* neutralising activity detected in sera obtained from 8 different MS patients. Samples were tested in duplicate, before (white columns) and after (grey columns) Ig depletion. Pt # 1, 2, 3, and 4, who are BAbs+/NA+, show a significant decrease in neutralising titres after treatment with protein G sepharose (A). In contrast, pt # 5, 6, 7, and 8, who are BAbs−/NA+, did not present significant changes in neutralising titres before and after Ig depletion.

between the two groups (Mann–Whitney test: $p=0.060$). At the same time, MxA gene expression was shown to be significantly lower in these patients than in BAb−/NA− (Mann–Whitney test: $p=0.021$).

### *3.5. Longitudinal antibody/bioactivity profiles of patients treated with IFNβ*

Repeated sampling analysis was performed in 8 patients on any commercially available IFNβ therapy, who were representative of the different serologic status, i.e. patients with and without serologic inhibitory activities.

Fig. 2 provides examples of the profiles over time of the 8 chosen patients. Profiles of 2 patients without any inhibitory activity are shown in Fig. 2A and B; levels of both anti-IFNβ antibodies and *in vitro* neutralising activity are absent/low and MxA gene expression is maintained over time. The profiles of patients shown in Fig. 2C and D are representative of patients with high titres of anti-IFNβ antibodies and *in vitro* neutralising activity by time and, as a consequence, a low biological activity of IFNβ. In both examples, the profile shows a normal MxA response prior to the development of antibodies (BAbs) and *in vitro* neutralising activity, whereas MxA gene expression is significantly inhibited when BAb and NA levels increase. In the profiles shown in Fig. 2E and F, anti-IFNβ antibodies levels are high but *in vitro* neutralising activity is negative; in both those patients, biological activity is low although the absence of detectable *in vitro* neutralising activity. Both these profiles confirm previous statement that patients may have complete absence on IFNβ biological activity in absence of *in vitro* neutralising activity, but in the presence of high levels of BAbs (Pachner et al., 2003, 2005). Finally, in the profiles shown in Fig. 2G and H, the biological activity is inhibited over time in association with increase *in vitro* neutralising activity, but not increases in BAb levels.

### *3.6. Non-antibody mediated neutralisation*

#### *3.6.1. IgA, IgE and IgM evaluations*

cELISA was used to determine if in BAb−/NA+ samples the observed neutralisation was mediated by a different class of immunoglobulin, including IgA, IgE and IgM. Any of the Ig classes were detectable in any specimens.

#### *3.6.2. Immunoglobulin depletion by combined protein G–L sepharose treatments*

Samples positive for *in vitro* neutralising activity (NA+ patients included in both BAb+/NA+ and BAb−/NA+ groups) were re-tested with the CPE assay after combined protein G–L sepharose treatments, which presumably removes most of the Ig (all IgG and protein G sepharose 4B treatment abolished the *in vitro* neutralising activity in all sera, that were evaluated as positive for anti-IFNβ antibodies (i.e. BAb+/NA+ patients). On the contrary, protein G sepharose 4B treatment did not reduce the neutralisation in samples showing no anti-IFNβ antibodies (i.e. BAb−/NA+ patients) (Fig. 3).

#### *3.6.3. Detection of soluble IFNAR2 by dot-blot assay*

Serum samples were tested for the presence of a soluble form of the IFNα/β receptor (sIFNAR) by dot-blot assay.

None of both the BAb−/NA− and BAb+/NA− patients, was dot-blot positive for sIFNAR. On the contrary, the dot-blot assay gave a positive results in 10 (91%) of the 11 BAb−/NA+ patients, as well as in 8 (21%) of the 38 BAb+/NA+.

## 4. Discussion

It is a well documented phenomenon that patients may develop neutralising antibodies (NAbs) against IFNβ. However, in all related studies the detection of NAbs has been performed by neutralising assays (e.g. CPE assay), which may not discriminate between anti-IFNβ antibodies and non-immunoglobulin inhibitory factors present in sera. In general, it has been considered sufficient to perform an ELISA to confirm the presence of a specific antibody and consequently the observed inhibition of the IFNβ activity.

In this study we show that the neutralisation observed by the CPE assay, is primarily mediated by the IgG fraction, but about the 4% of patients present a non-antibody components that inhibit *in vitro* the IFNβ bioactivity. In these patients, the neutralisation

assay indicated reduced antiviral activity of IFNβ on A549 cells, although ELISAs failed to reveal the presence of any kind of anti-IFNβ antibodies, suggesting the presence of a different inhibitory factor. Immunoglobulin depletion did not remove the inhibitory activity in these samples at any dilution, which further confirms a non-antibody mediated neutralisation effect. This is well in line with the results of other studies, where low level of non-Ig-mediated inhibition of IFN activity was observed in sera of patients treated with both IFNα and IFNβ (Pungor et al., 1998; Antonelli et al., 1999; Lampasona et al., 2003; Chadha et al., 2006).

The data presented here are the first to show that these non-antibody inhibitory factors are able to affect the IFNβ biological response not only *in vitro*, but also *in vivo*. As previous studies have shown that MxA levels are increased in IFNβ-treated patients (Deisenhammer et al., 1999; Vallittu et al., 2002; Bertolotto et al., 2001, 2003, 2004; Gilli et al., 2004a,b, 2006b; Pachner et al., 2003, 2005), gene expression of this protein has been used as biomarker to evaluate the responsiveness of the host to IFNβ. Hence, we have shown that 82% of patients with non-antibody mediated neutralising activity have MxA gene expression below the detection limit, confirming the importance of such inhibitory factor on the biological response.

Interestingly, the non-antibody mediated neutralisation was observed only in samples obtained from patients treated with a three times a week regimen (i.e. IFNβ-1b and IFNβ-$1a_{sc}$), but in none of the samples obtained from patients treated once a week (IFNβ-$1a_{im}$). However, it should be noted that the amount of *in vitro* neutralisation against IFNβ-1a was higher than that against IFNβ-1b. Indeed, in IFNβ-1b treated patients the neutralisation was detectable only by decreasing the threshold for positivity from 20 TRU/mL to 5 TRU/mL, whereas IFNβ-$1a_{im}$ treated patients were still negative, although the lowered threshold for positivity. This is well in line with our previous data showing that NAbs neutralise a three-fold higher quantity of IFNβ-1a molecules than IFNβ-1b molecules (Bertolotto et al., 2000).

As a whole, the presence of BAb−/NA+ patients only among individuals treated with either IFNβ-$1a_{sc}$ or IFNβ-1b, suggests that such a non-antibody mediated neutralising phenomenon might be related to the frequency of administration of the drug.

Since several cytokine receptors (e.g. TNFR, IL4R, IL6R) were shown to be released as a feedback regulation mechanism from the cell surface upon exposure to either their cognate ligands or agonists (for review see Kiessling and Gordon, 1998), it can be hypothesised that a similar change induced by the high frequency of administration may also involve the IFNβ receptor. This hypothesis seems to be proven correct in that we found immunoreactivity to the IFNα/β receptor (IFNAR), in the great majority (91%) of the BAb−/NA+ serum samples, when analysed by dot-blot.

Besides to the characterisation of the non-antibody mediated neutralisation, it is essential to assess the importance of the phenomenon for the clinical outcome of IFNβ-treated patients. This was not possible in the present investigation, as our study was not adequately powered to evaluate the effect on clinical measures. Such a study would require larger cohorts due to a low frequency of patients presenting the non-antibody mediated neutralisation.

As a whole, our study demonstrates that a non-antibody mediated neutralisation develops in 4% of IFNβ-treated patients. This phenomenon is an important, but so far overlooked, regulator of IFNβ biological responses *in vivo*. Moreover, as the non-antibody mediated neutralisation was found in the 18.4% of patients who were positive for *in vitro* neutralisation by CPE assay, but negative for anti-IFNβ antibodies (BAbs), our study does not support the usefulness of total BAb-ELISA assay as a screening tool for a selection of samples for further investigation (e.g. for *in vitro* neutralising activity and/or for *in vivo* bioactivity). Otherwise, BAb-ELISA assay might be used both as a confirmation test indicating that the decrease of the biological effects is due to specific anti-IFNβ antibodies and as a way to predict the persistency of loss of bioactivity.

Further investigations of the non-antibody mediated neutralisation is needed to better understand the mechanism of the phenomenon. This, hopefully, will lead more accurate monitoring tools for the efficacy of IFNβ treatment and thus a better patient management.

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