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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.