A novel assay to detect low-titred antibodies to interferon beta in multiple sclerosis patients

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Neutralizing antibodies (NAbs) may compromise interferon (IFN) clinical efficacy in patients with multiple sclerosis (MS) receiving IFN-beta treatment. When bioassays are used for anti-IFN-beta antibody detection, they are unable to discriminate between NAbs or other interfering substances with anti-IFN activity. Here we report the development of an anti-IFN-beta Western blot method that facilitates the detection of IFN low-titred antibodies and characterizes such low neutralizing activity as specifically due to the presence of particular IFN antibodies. The assay was characterized using serum samples from patients with MS treated with IFN-beta. It was developed by adding anti-IFN-positive antibody sera to Dynabeads M-280 tosylactivated followed by Western blot analysis. All sera samples from MS patients with IFN-beta1a NAbs (≤ 50 t100) proved to be antibody-positive using this new method and, more importantly, four of 27 binding antibody-negative sera samples were scored as IFN antibody-positive. The method was found to be rapid, specific and sensitive and consistent with respect to well-established antiviral neutralization or commercial enzyme-linked immunosorbent assays.

KEY WORDS: Multiple Sclerosis, Interferon, Interferon Antibody

INTRODUCTION

Interferons (IFNs) are a biologically active family of proteins with antiviral, antitumour and immunomodulatory activities that have been used successfully in the treatment of a variety of infectious and neoplastic diseases (Zein, 1998; Kirkwood, 2002). However, there are many, often unexpected, side-effects associated with IFN therapy. Among them is the development of antibodies able to neutralize (NAbs) and/or bind (BAbs) to IFN molecules. This phenomenon was first observed by Vallbracht and co-workers (1981) in a patient with nasopharyngeal carcinoma treated with IFN-beta. Since then, there have been many reports describing the development of anti-IFN antibodies in healthy individuals, in patients suffering from autoimmune disease and, with greater frequency, in patients undergoing IFN treatment (Meager, 1997; van der Meide & Schellekens, 1997; Antonelli & Dianzani, 1999).

The issue of antibody development in IFN therapy has been highlighted recently in patients suffering from multiple sclerosis (MS) since they are particularly prone to the development of such antibodies when undergoing IFN-beta treatment and also since it has been suggested that the development of NAbs in these patients is significantly associated with reduced clinical effi-
cacy of the administered IFN (for review see Vartanian et al., 2004). From a pharmacodynamic point of view, the presence of NAb s has been shown to correlate with a decrease in levels of IFN-induced markers, such as neopterin, beta2-microglobulin and mRNA-dependent myxovirus-resistance protein A (Rudick et al., 1998; Deisenhammer et al., 1999; Deisenhammer et al., 2000; Cook et al., 2001; Vallittu et al., 2002; Bertolotto et al., 2003; Gilli et al., 2003; Bertolotto et al., 2004). It has also been established that NAb s may develop during treatment with both types of IFN currently available for MS therapy, namely recombinant (r) IFN-beta1a and rIFN-beta1b, and that anti-IFN-beta antibodies developed during therapy with rIFN-beta1b in MS are also able to neutralize rIFN-beta1a, and vice versa (Antonelli et al., 1999).

So far, several assay methods have been used for the detection of IFN antibodies (for a review see Antonelli & Dianzani, 1999; Deisenhammer et al., 2004). These can be grouped into two categories: first, activity-neutralizing assays (also called neutralizing antibody assays or bioassays), which measure the ability of patient’s serum to neutralize the biological activity of exogenous IFN, and, second, those that measure only the ability of the serum to bind to the IFN molecule. The bioassays can detect antibodies directed at the functional epitopes involved in generating the biological activity of IFNs. Thus they detect the so-called NAb s. However, the second type of assay can detect antibodies directed towards any part of the IFN molecule, thus showing the so-called BAbs that include both neutralizing and non-neutralizing antibodies. The sensitivity of individual assay methods can vary considerably. There is a great variability even among those measuring the neutralization of antiviral activity, depending on the amount of IFN added, the cells used in the assay, and the virus used to produce the cytopathic effect. Of particular interest in relation to the anti-IFN-beta antibody issue is the association between the presence of IFN-beta-neutralizing activity and the presence of Ig directed against the IFN-beta molecule. Most papers on the development of NAb s only marginally address issues such as this and the existence of the so-called IFN inactivators or inhibitory factors, which differ from antibodies and can occasionally be found in both untreated or IFN-treat-
ed patients (Ambrus et al., 1997; Mizukoshi et al., 1999; Chadha et al., 2004). This raises important questions on the characterization of NAb s, especially in low-titred sera. Indeed, the IFN-beta neutralizing activity of a serum sample is not usually characterized as specifically associated with the presence of antibodies. Some researchers performed an enzyme immunoassay to confirm the presence of a specific antibody (Antonelli et al., 1999; Deisenhammer et al., 1999; Brickelmaier et al., 1999; Vallittu et al., 2002) in order to attribute the observed inhibition of the IFN activity to the presence of NAb s in serum samples. Occasional attempts have been made to characterize the neutralizing activity, for example, by absorption to protein A-Sepharose (De Maeyer-Guignard et al., 1984; Pungor et al., 1998; Antonelli et al., 1999). However, such procedures can only be properly and routinely performed on sera with a high neutralizing titre, owing to the currently limited availability of serum. At present, this incomplete characterization of neutralizing activity may bias estimates of the incidence of low-titred anti-IFN antibodies in MS patients treated with IFN-beta, as some neutralization may result from the presence of IFN-inhibitors rather than antibodies. This bias may be particularly important in studies on the incidence of antibodies produced by different IFN preparations or the effect of low-titred NAb s on the pharmacodynamic profile of IFN-beta.

The main goal of this study was to address this issue. A method of enriching the concentration of antibodies to IFN was developed, followed by Western blot analysis that allowed detection of low-titred IFN antibodies and the characterization of such low neutralizing activity specifically caused by the presence of specific antibodies against IFN. The results also indicate that immunoglobulin (Ig)M may develop against IFN in patients with MS undergoing treatment with IFN-beta and that sera samples that, using current methods, give negative results for NAb s and BAbs may still contain IFN-beta antibodies.

**MATERIALS AND METHODS**

**Coating Dynabeads with IFN-beta**

Dynabeads M-280 tosylactivated (Dynal Biotech ASA, Oslo, Norway) were coated with recombinant
IFN-beta1b (Betaferon, Schering AG, Berlin, Germany): concentration 8 x 10^6 IUs; 250 µg. 

Briefly, the beads were washed twice in Buffer A (0.1 M Na-phosphate buffer pH 7.5). Then the rIFN-beta1b was dissolved in buffer A and added to the tube containing the washed beads at a concentration of 10 µg IFN/10^7 beads. 

The beads were incubated with a slow tilt rotation for 24 hours at 37°C.

After incubation, the tube was placed in the magnet for 4 minutes and the supernatant removed. In all, 67% of the added IFN was bound, as observed by spectrophotometric protein measurement (Beckman, DU^®-64 spectrophotometer; Global Medical Instrumentation, Minnesota, USA). In order to eliminate unbound IFN, the coated beads were then washed five times: twice in Buffer D [phosphate buffered saline (PBS), pH 7.4, with 0.1% W/v bovine serum albumin (BSA)] for 5 minutes at 4°C; once in Buffer E (0.2 M Tris, pH 8.5, with 0.1% W/v BSA) for 24 hours at 20°C; once in Buffer D for 5 minutes at 4°C; once in Buffer C (0.1 M acetate buffer, pH 4.0).

**Binding of IFN-beta antibodies to IFN-beta coated Dynabeads**

The coated beads were washed three times in 3 ml of PBS. A total of 10^7.5 beads were incubated with 50 µL of MS patients’ serum for 30 minutes at 20–25°C with bidirectional mixing. Sera were pipetted off and the beads were washed three times in 3 ml of PBS.

**Elution of IFN-beta antibodies from IFN-beta coated Dynabeads**

After adding 30 µL of 0.1 M citrate, pH 2.8, to the rIFN-beta1b coated beads, they were well mixed by tilting and rotation for 2 minutes. The tube was placed in the magnet and the supernatant transferred to a clean tube. This step was repeated twice. The supernatants containing IFN-beta antibodies were pooled and the pH value was adjusted to 7 using 5N NAOH.

**Western blotting**

Aliquots of 5 µg of rIFN-beta1b were added to 5x sodium dodecyl sulphate (SDS) gel loading buffer (Sigma Chemical Corporation, St Louis, MO) and denatured at 100°C for 5 minutes. These aliquots were electrophoresed onto a denaturing 10% polyacrylamide mini-gel (linear range of separation KD 16–68). Prestained SDS-PAGE standard (Bio-Rad Laboratories Srl, Milan, Italy) proteins were used as markers. The run was at 90 volts for 2 hours. The proteins from the SDS polyacrylamide mini-gel to nitrocellulose filter were transferred at 400 mA for 90 minutes using a Bio-Rad (Bio-Rad Laboratories) apparatus. At the end of the run, the membrane was cut into strips. These were agitated for 15 minutes at room temperature in blocking buffer (Bioline Division, Turin, Italy). The blocking buffer was removed and 20 µL of the supernatants containing IFN-beta antibodies in 1 mL dilution buffer (Bioline) added; they were then incubated and agitated for 40 minutes at room temperature.

An internal positive control serum from an MS patient served as the positive control. Serum from an IFN-beta-naive MS patient was used as the negative control. The samples were removed, washed four times, and 1 ml of polyclonal rabbit alkaline-phosphatase conjugated anti-human IgG or IgM, diluted in dilution buffer (Bioline) at 1:100, was added, then incubated and gently agitated for 30 minutes at room temperature. The strips were then washed several times (four times with washed buffer and once with distilled water), and then incubated and agitated with 1 mL of cromogene substrate (Bioline) for 15 minutes at room temperature. The substrate was removed and 1 mL of 2.5% acetic acid [stop solution (Bioline)] added for a few seconds. This stop solution was removed and a further 1 mL of stop solution was added for a further 3 minutes. The strips were then removed and air dried.

**Detection of IFN-beta NAbs**

A serum sample was collected from each patient with MS at least 24 hours after the last injection of IFN-beta and then assayed against rIFN-beta1a regardless of the treatment received. Antibody titres were determined as previously described (Antonelli et al., 1998) by a neutralization test against 10 IU of rIFN-beta1a. The sera samples were routinely inactivated at 56°C for 30 minutes before titration. Two serial dilutions (starting from 1:10) of 60 µL of sample or control sera were incubated at 37°C with 60 µL of 20 IU/ml IFN-beta. After 1 hour, 100 µL of the
individual mixtures were added to duplicate monolayers of human lung carcinoma (A549) cells in 96-well microtitre plates. After 18–24 hours of culture and extensive washing, the cells were challenged with the encephalomyocarditis virus and incubated at 37°C for 24 hours. The controls included a titration of IFN preparations used in the assays and a reference standard antibody to IFN (National Institutes of Health, Bethesda, MD; code GO38-501-572). The antiviral activity and its neutralization were assessed on the basis of the virus-induced cytopathic effect (CPE) and, to quantify this, the cells were stained with crystal violet in 20% ethanol. The dye taken up by the cells was eluted with 33% acetic acid and its absorbance measured in a microdensitometer at 540 nm (OD$_{540}$). The extent of the virus-induced CPE, its inhibition by IFN-beta, and the reversal of this inhibition by NAbs were shown by the amount of dye eluted from each well. Titres were calculated using Kawade’s method and expressed as $t_{1/10}$, that is, the dilution of serum reducing 10 laboratory units (LU)/mL of IFN to 1 LU/mL (Kawade, 1986; Grossberg & Kawade, 1997).

Serum samples were routinely assayed for endogenous or residual IFN activity.

Detection of IFN-beta BAbs
Antibodies binding to IFN-beta were detected by a non-competitive enzyme-linked immunosorbent assay (ELISA) using an enzyme immunoassay kit specific to IFN-beta (Bühlmann Laboratories AG, Switzerland), following the manufacturer’s recommendations. Briefly, serum samples obtained from patients with MS were diluted and incubated in a 96-well microtitre plate previously coated with a mixture of natural human IFN-beta, rIFN-beta1a and rIFN-beta1b. This assay permits the detection of BAbs directed against any type of IFN-beta. The presence of bound antibody was detected using a horse-radish peroxidase-conjugated antiserum to human IgG, allowing the addition of a buffered tetramethylbenzidine chromogen/substrate solution (TMB). The results were obtained in optical density units using spectrophotometric analysis. A cut-off value of 50 BTU (Bühlmann titre units) was used, as recommended by the manufacturer. Indeed, the BTU value of normal donor sera we recently tested never exceeded 48 BTU which represents the mean value ± 3SD of 200 sera from healthy donors calculated by the manufacturer.

RESULTS

Description of the assay for detection of IFN-beta antibodies
A new method for anti-IFN-beta-binding antibody detection is described. Figure 1 is a schematic representation showing how the assay is used to detect qualitatively the IFN-beta antibodies present in the serum of patients with MS.

In detail, Dynabeads M-280 tosylactivated (hereinafter called ‘beads’) were incubated with IFN-beta for 24 hours at 37°C (1st step). During this step, IFN binds covalently to the beads. Then the patient serum, previously inactivated at 56°C for 30 minutes, was added to beads and, if present, any IFN-beta antibody binds to the IFN present on the beads. This allows the formation of ‘immunocomplex’ IFN-antibodies (2nd step). After several washings, an elution process was used (see Materials and Methods) to obtain the anti-IFN antibody free of other serum proteins (3rd step). This antibody was transferred to nitrocellulose and a Western blot analysis performed using anti-human IgG (see Material and Methods). Since the positive band represents the binding of the anti-human IgG to the IFN beta antibody on the nitrocellulose strip, the results indicate that the above assay permits qualitative detection of IFN antibodies and at the same time a tentative association between the neutralizing activity and the presence of human Ig, in this case, IgG.

Such a procedure was first used with a high NAb-positive serum (1000 $t_{1/10}$ to rIFN-beta1a) and an NAb-negative serum (<10 $t_{1/10}$ to rIFN-beta1a) from patients with MS treated with rIFN-beta1a. The results are shown in Figure 2. When the positive sample was examined, a band corresponding to IFN-beta (at an approximate molecular weight of 20,000 daltons) was present; there were no bands in the negative sample or washing buffer.

Specificity of the assay
To verify whether the assay was specific and if the elution process yielded human Ig specifically
directed to IFN-beta, competitive experiments were performed adding an excess of ‘free’ IFN-beta to the collected serum antibodies before their addition onto the nitrocellulose strip to which the IFN-beta had previously been transferred (data not shown). Both low-titred (52 t<sub>1/10</sub> against rIFN-beta1a) and high-titred (3446 t<sub>1/10</sub> against rIFN-beta1a) sera were assayed by Western blot analysis performed as above in the presence or absence of an excess (double quantity of that run on the gel) of ‘free’ IFN-beta. When soluble IFN-beta was added, the human sera showed no reactivity with the IFN present on the nitrocellulose strip. These findings demonstrate that free IFN-beta can block the human serum binding to the nitrocellulose, thus confirming that the assay allows the specific detection of IFN-beta antibodies.

**Sensitivity of the assay**
To test the sensitivity of the method and, at the same time, to verify its reliability, several sera were assayed. The sensitivity of the assay was determined by testing sera of patients with low-titred antibodies to interferon beta in multiple sclerosis patients.
samples from MS patients with titres lower than 50 \( t_{1/10} \) against rIFN-beta1a were assayed using the method described. The results are shown in Figure 3, where it can be seen that all NAb-positive samples showed, to different extents, a positive reaction. A similar positive reaction can also be obtained using a well-established antiviral neutralization assay when the samples tested have a positive titre of 10 \( t_{1/10} \). This demonstrates that our method is reliable and sensitive.

To further verify the sensitivity of the assay, serial dilutions of a NAb-positive serum with a titre of 1104 \( t_{1/10} \) against rIFN-beta 1a were tested as detailed above. The result was still positive at 1:250 dilution (data not shown), thus demonstrating that the Western blot method allows the detection of NAbs titred at 4 \( t_{1/10} \) and confirming that the assay is highly sensitive.

To further investigate the method from a more applicable point of view, the assay was performed on 27 sera samples from MS patients that had given negative results when tested by antiviral neutralization or commercial ELISAs. The results showed that 15% (4/27) of the sera samples were positive for IFN antibodies when examined by this new method.

Furthermore, we had the opportunity to test serially collected sera samples from two MS patients undergoing rIFN-beta 1a therapy whose NAb assay results were positive, with the aim of characterizing the class of Ig developed during IFN-beta therapy. The results (not shown) indicated that after 3 months of treatment one patient developed IgM. This was followed by the development of IgG anti-IFN-beta after 6 months of treatment. In the other patient, only IgG anti-IFN-beta was present after 3 months of treatment.

**DISCUSSION**

The development of IFN antibodies has become particularly relevant in the treatment of MS patients, in whom a high percentage of those undergoing IFN-beta therapy develop NAbs and BAbs, depending on the type of IFN-beta used for treatment (Vartanian et al., 2004). Many assays for the detection of anti-IFN antibodies have been developed and performed, including the gold standard bioassay suggested by World Health Organization (OMS/WHO) and The International Society for Interferon and Cytokine Research (ISICR) (for review see Antonelli & Dianzani, 1999; Deisenhammer et al., 2004). When bioassays are used to test human sera, they may not be able to discriminate between NAbs and any other substances with anti-IFN activities that may be present in sera (Kawade, 1986). Many of these substances, which include non-specific inhibitors (Ambrus et al., 1997), soluble receptors (Mizukoshi et al., 1999; Ambrus et al., 2003), cytokines (Khabar et al., 1997; Mihm et al., 2004), prostaglandin E\(_2\) (Ambrus et al., 1992; Chadha et al., 2004) and cAMP phosphodiesterases (Chadha et al., 2004), still await further characterization, and no assay is available that will readily discriminate between them and antibodies specific to IFN-beta. This is of particular importance when patient serum contains only low-titred neutralizing activity. Indeed, owing to the usual limited availability of serum from a patient, it is very difficult to associate any neutralizing activity with the presence of specific IFN antibodies.

The development and characterization of a new method for the analysis of the antibody response...
to IFN induced in MS patients treated with IFN-beta is described, which addresses both the above issues and, at least in part, solves them. The method allows the concentration of IFN antibodies to be increased and characterizes them using a Western blot analysis without the presence of interfering substances present in the serum. This assay method allows a tentative association between the presence of neutralizing activity and the presence in serum of Ig directed against the IFN-beta molecule. Indeed, all the NAb-positive sera samples that had been examined using a well-established antiviral neutralization assay were also positive when examined using the new method, which gave specific and highly sensitive results. Moreover, the new assay method detected IFN antibodies even when present at very low concentrations and also, more importantly, in sera samples that were negative for the presence of antibodies when measured using the current antiviral neutralization method or commercial ELISAs.

However, it can be argued that such low-titred IFN antibodies may not have any clinical or biological relevance – and with this we would agree. Nevertheless, to understand a biological phenomenon whose biological meaning has yet to be defined in detail, it is important to consider any point that may give a new insight. The method also allowed us to describe the pattern of the Ig response in two MS patients who had developed a humoral response to IFN-beta. After 3 months of therapy, one had developed IgM against IFN-beta; however, after 6 months only IgG was present in the patient's serum. Such a finding could not be obtained by testing serum samples for the presence of IFN-beta NAbs using standard bioassay techniques. It may also be argued that the method is complicated from a technical viewpoint and can only be performed in specialised laboratories. We consider this to be true but we are not proposing the use of this assay for screening purposes; the current assays to detect NAbs and BAbs are considered much more useful because they are easy to perform and can be done in unspecialised laboratories. However, in certain circumstances our method should be of value in assessing the specific behaviour of the humoral response against IFN, and its use could help to detect the low-titred positivity of NAb activity to IFN and differentiate between the presence of biological antagonist to IFN and the presence of specific anti-IFN Ig. This is probably more applicable to cancer patients in whom such inhibitors have been repeatedly reported (Ambrus et al., 1992; Ambrus et al., 2003).

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