Serial immunoprecipitation assays for interferon – (IFN)-β antibodies in multiple sclerosis patients

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ABSTRACT. We devised a sensitive, radioimmunoprecipitation assay (RIPA) for anti-interferon (IFN)-β – binding antibody (BAB) detection. Our RIPA showed good agreement with a reference RIPA (mean difference, – 3.2 ± 10.6 AU), and detected BAB to both IFN-β-1a and IFN-β-1b. Neutralizing antibodies to IFN-β (NAB) were also determined with a standard method. BAB and NAB were measured in 393 serum samples from 77 multiple sclerosis (MS) patients treated with IFN-β-1a or -1b, who were studied over two years, and subsequently classified as responders and non-responders. BAB were found at higher concentrations, and more frequently detected, in IFN-β-1b- than in IFN-β-1a-treated patients, and, at highest titres, preferentially in patients who were positive for NAB. However, in our series of MS patients, both titres and frequency of detection of BAB or NAB did not differ between IFN-β responders and non-responders.

Keywords: multiple sclerosis, IFN-β, binding antibodies, anti-IFN-β antibodies

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune, demyelinating disease of the central nervous system. Chronic inflammation sustains the immunopathological process, although recent data suggest that pro-inflammatory signals are not always detrimental [1].

Interferon beta (IFN-β)-based therapy of (MS) reduces the number of exacerbations in the relapsing-remitting (RR) forms, and delays progression in the secondary progressive forms. IFN-β anti-inflammatory activity would account for the reduction in inflammatory lesions seen on magnetic resonance imaging. Antibodies to IFN-β can develop during the therapy, and are classified as binding antibodies (BAB) and neutralizing antibodies (NAB). BAB can bind any epitope of the IFN-β molecule, whereas NAB exclusively recognize functional epitopes. Neutralization of functional epitopes prevents the drug from binding to cellular receptors. BAB and NAB increase clearance of immune complexes, and thus decrease serum levels of the drug [2]. Collectively, these mechanisms may decrease IFN-β therapeutic activity.

Several assays have been developed for BAB and NAB determination [3] but, at present, standardization is still ongoing. Moreover, studies on BAB and NAB occurrence in MS patients treated with IFN-β-1a/b are relatively few, in comparison with those on the therapeutic efficacy and mechanism of action of IFN-β-1a/b.

Here, we report the development and validation of a radioimmunoprecipitation assay (RIPA) [4] for BAB detection. The method was applied to a large cohort of IFN-β-1a/b-treated, relapsing-remitting (RR) MS patients, who were longitudinally studied over two years. NAB were also measured with a standard method. The mere presence, or high titers of BAB or NAB were tentatively associated with patients who did not respond to IFN-β therapy, whereas the absence of these antibodies was associated with patients who responded to the therapy. Responder or non-responder MS patients were retrospectively subcategorized after two years of treatment.

PATIENTS AND METHODS

The study enrolled 77 clinically confirmed [5] RR-MS patients from three MS Centers (Barcelona, Spain; Bari and Milan, Italy). Table 1 shows their demographic characteristics and therapeutic regimens. All MS patients enrolled in the study had had at least two relapses in the

two years prior the treatment. They were clinically followed, for up to two years. Sampling was performed before treatments and trimonthly, up to 12, 18 or 24 months (total serum samples, 393). Samples were collected at least 48 hours after the last injection of the drug. Patients who suffered from exacerbations of the disease were treated with short courses of corticosteroids. These patients missed the blood sampling, if it had been scheduled within the two months following the end of the corticosteroid treatment. No other immunomodulatory or immunosuppressive therapies were administered. The local ethics committees approved the protocol. At the end of the follow-up period, MS patients were sub-classified as responders or non-responders. To avoid over-interpretation, we adopted stringent criteria to categorize patients into responders and non-responders. Patients who had no clinical relapses and stable EDSS scores in the two years of treatment were considered to be responders, while patients who had at least one relapse, or an EDSS deterioration ≥ 0.5 point in the two years of treatment, were considered to be non-responders.

BAB were determined with a newly developed RIPA. The human IFN-β-1a coding sequence was excised from the plasmid pORF-hIFN-β (Invivogen), and cloned into the pSPUTK vector (Stratagene). Purified plasmid DNA was obtained by Quantum Prep spin column preparation (Bio-Rad), and *in vitro* transcribed and translated with the TnT SP6-coupled rabbit reticulocyte lysate system (Promega), in the presence of ^{35}S-methionine (Amersham). Unincorporated ^{35}S-methionine was removed with gel chromatography on a NAP5 column (Pharmacia). The correct IFN-β-1a molecular size was verified with SDS-PAGE and autoradiography. RIPA was then carried out. Briefly, 2 µL of serum were added to 25 µL of PBS, pH 7.2, 1% Tween 20 (PBST), containing 20,000 cpm of ^{35}S-IFN-β-1a, and incubated overnight on ice. Immune complexes were incubated with 1 mg of protein A-Sepharose (Pharmacia), resuspended in 50 µL of PBST, for 1 h at 4°C. Protein A-Sepharose beads were washed five times with PBST, and centrifuged between washes. Protein A-sepharose bound immune complexes, in 100 µL of TBST, were then transferred into a 96-well Optiplate (Packard), and 150 µL of Microscint 40 (Packard) was added. Plates were shaken for 30 min, and counted for 5 min in a TopCount (Packard) scintillation counter. Results were expressed as arbitrary units (AU) relative to a calibration curve obtained by serial dilutions of a reference serum with high BAB levels. High titers BAB were considered when > 20.0 AU, medium titers between 5.0-20.0 AU and low titers < 5.0 AU.

For the study of analytical accuracy, 50 samples of Betaferon®-treated MS patients were also tested with a ^{125}I-IFN-β-1b-based RIPA (A.Vincent and L. Lawrence, University of Oxford, UK, unpublished results).

NAB determinations were performed with a cytopathic assay as previously described [6]. Patients who showed at least two positive, consecutive determinations during the follow-up period were considered to be BAB positive or NAB positive. Two-tailed Fisher exact test for categorical data, and a plot for the assessment of agreement between two methods [7] were used for the statistical analysis.

RESULTS

The detection limit of our RIPA was 1.6 AU, i.e., the BAB concentration corresponding to a signal greater than the 99th percentile of 100 sera from healthy controls. Within- and between-run imprecision was below 10%.

Results from the 50 samples of Betaferon®-treated patients, as tested with our RIPA, showed good agreement with the other RIPA, based on ^{125}I-IFN-β (mean difference, – 3.2 AU; 95% CI for the upper limit of agreement, 12.8 to 23.2, and the lower, – 19.2 to – 29.6) *(Figure 1)*.

During the follow-up, positivity for BAB was detected in 1/20 (5%) of the Avonex™-treated patients, 27/41 (66%)

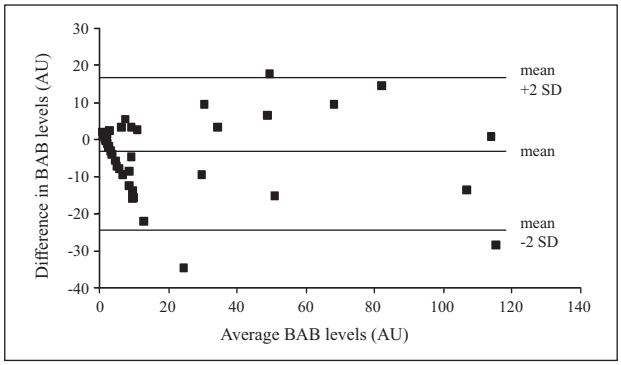

Figure 1
Comparative analysis of our *versus* the reference RIPA for anti-IFN-β-1b antibody (BAB) determination. The respective differences (^{35}S-RIPA – ^{125}I-RIPA) *versus* the means [(^{35}S-RIPA + ^{125}I-RIPA)/2] of the two methods are plotted. The plot gives an idea of how much a new method is likely to differ from the old, when none of them provides unequivocally correct measurements. Solid lines indicate the mean and limits of agreement.

Table 1
Demographic characteristics of MS patients who underwent IFN-β-1a/b therapy and were longitudinally studied

Drug	Molecule	Dose (regimen)	N° of patients (male/female)	R/NR	Age range (mean ± SD)[a]	MS duration range (mean ± SD)[a]	Baseline EDSS (mean ± SD)[b]	Relapse (mean ± SD)[c]	N° of samples
Avonex™	IFNβ-1a	30 µg i.m. (per week)	20 (6/14)	15/5	18 – 57 (30.3 ± 8.5)	1 – 29 (6.2 ± 7.4)	0.0 – 3.5 (1.5 ± 1.5)	2 – 5 (2.0 ± 1.1)	102
Betaferon®	IFNβ-1b	250 µg s.c. (every other day)	41 (12/29)	29/12	16 – 60 (33.1 ± 9.0)	1 – 31 (6.3 ± 6.5)	0.5 – 5.0 (1.5 ± 2.5)	2 – 8 (2.5 ± 2.7)	218
Rebif®	IFNβ-1a	22 µg s.c. (× 3/week)	16 (5/11)	11/5	20 – 62 (35.8 ± 11.4)	1 – 28 (6.8 ± 7.0)	0.5 – 4.0 (1.5 ± 2.0)	2 – 7 (2.5 ± 1.3)	73

R/NR, responders/non-responders. [a] Data are expressed in years. [b] Data represent the range. [c] Data represent the number of relapses in the two years prior to the beginning of the treatment.

of those Betaferon®-treated, and 2/16 (12%) of those Rebif®-treated. Among the Betaferon®-treated patients, 4/41 (10%) had high titers, 9/41 (22%) medium titers and 14/41 (34%) low titers, respectively. Among Rebif®-treated patients, nobody had high titers, 1/16 (6%) medium titers, and 1/16 (6%) low titers, respectively. The sole MS patient showing BAB during Avonex™ treatment had low titer antibodies. Figure 2a shows how BAB fluctuated over time in the whole population of IFN-β-1a/b patients. At month 3, 51% of the Betaferon®-treated patients were BAB positive ($p < 0.0001$ *versus* samples collected before the treatment); BAB positivity peaked at 73% at month 6, and decreased to 33% at month 24 ($p = 0.002$, month 6 *versus* month 24). The presence of BAB did not correlate with the status of non-responders *(Figure 2B)*. In the Betaferon® -treated patients, BAB associated with the non-responder status with a sensitivity of 81%, and specificity of 35%.

NAB were found in 5/41 (12%) of Betaferon®-treated patients, and in 2/16 (12%) of those Rebif®-treated, but in none (0/5) of the Avonex™-treated patients. Two patients were NAB positive and BAB negative. The majority of NAB positive patients (6/7), as well as those with the highest BAB levels (3/4, with BAB > 20.0 AU), were responders.

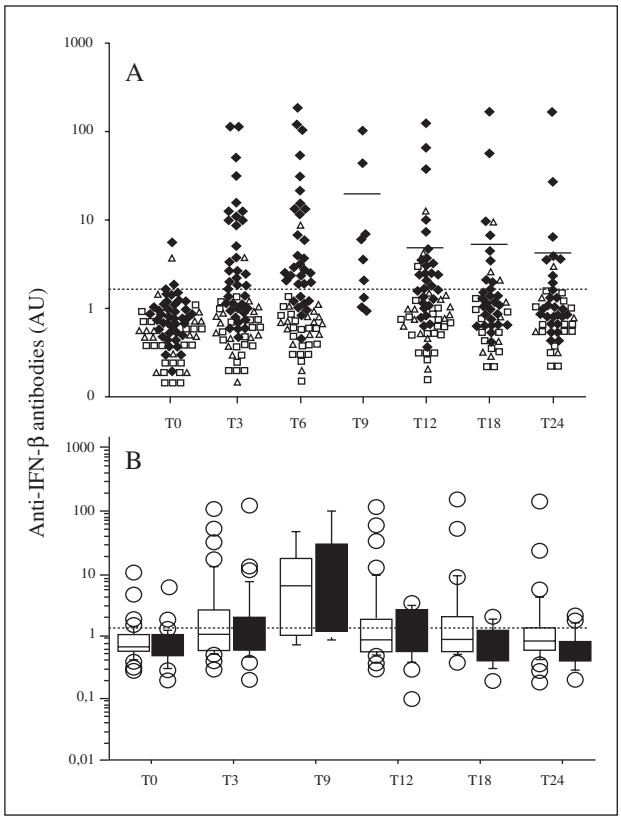

Figure 2

Panel **A**. Anti-IFN-β-1a/b antibody (BAB) levels in the total MS population. In panel A, MS patients treated with Avonex™ (□), Rebif® (△), and Betaferon® (◇) are depicted. Solid lines represent the mean AU values in the total MS population, while the dotted line represents the cut-off point as measured in healthy subjects. Panel **B**. Box plots showing the median levels (± SD) and the 95th percentiles of BAB in the MS population categorized as responders (white boxes) *versus* non-responders (grey boxes). In both panels, measurements performed at different time points (T) during the two years of follow-up are indicated on the x-axis (0, 3, 6, 9, 12, 24 months after the beginning of the treatment).

DISCUSSION

The incidence of BAB and NAB in MS patients treated with commercial beta-IFNs varies significantly, ranging from zero to 95%, and depends on many factors [3]. One of the factors relies on the different methods used to detect them.

Our RIPA allowed us to perform a comparative study on the three IFN-β formulations currently used in MS therapy. The potential to detect BAB to both IFN-β-1a and -1b preparations confirms the data showing that BAB and NAB, in IFN-β-1a- or IFN-β-1b-treated patients, are cross-reactive [8, 9]. Our RIPA is precise, sensitive, as analytically accurate as a reference RIPA, and requires small sample volumes. Its analytical characteristics overcome the shortcomings of the ELISA format, in which IFN-β is directly coated onto the microwells. These shortcomings may lead to an underestimation of the incidence of BAB [9]. Indeed, coating of IFN-β on to plastic results in a non-native mode of antigen presentation. Therefore, some IFN-β epitopes, which are present on the native protein, may not be represented in ELISAs based on directly coated antigen [9]. The advantage of being a solution-phase RIPA, which is able to detect antibodies to the native IFN-β, would compensate for the disadvantage of depending upon radioactivity.

Our results confirm that BAB, NAB, or both occur, with the highest frequency, in IFN-β-1b-treated patients, and less frequently in the IFN-β-1a-treated ones [10-12]. Among the latter, we found that the BAB incidence was higher in the patients who received higher weekly IFN-β-1a doses (Rebif® > Avonex™).

At present, NAB determination is considered to be the only serological test that helps in the decision to discontinue IFN-β-1a/b therapy [10, 11]. NAB can inhibit the bioavailability of IFN-β-1b [12]. However, the precise mechanisms by which IFN-β acts in MS are not clear, and even less clear are those underlying the BAB- and NAB-drug interactions. It is reasonable to presume that BAB have, after meeting the antigen, the functional role of removing it from the circulation through the phagocytic system, mostly in the liver and spleen [2]. The lack of efficacy of IFN-β seems to be more consistently related to the occurrence of NAB, rather than of BAB. Recent data confirm that NAB abolish the bioavailability of IFN-β, although only in patients with high titers or persistent positivity [13]. The Authors also show that NAB-negative patients could have low indexes of IFN-β bioavailability, and include the presence of BAB among the possible explanations. Clinical data were not included in the study. Moreover, BAB and NAB can even fluctuate unpredictably over time [14], representing a transient phenomenon. This is another event that complicates the interpretation of the issue from the immunological and, above all, clinical point of view.

Our data show that BAB were unrelated to the clinical efficacy of IFN-β, the highest titers paradoxically occurring in responders, and disappeared at 24 months, in a significant proportion of patients. BAB titers did not correlate with the non-responder status either. Responder or non-responder patients were retrospectively subcategorized after two years of treatment. The definition of the "non-responder" was particularly stringent, since patients with even one relapse within the two years were

considered to be non-responders. Moreover, we considered to be BAB, or NAB positive those patients with at least two consecutive, positive samples. These criteria are not commonly used in clinical practice. However, this categorization allowed us to really assess the relevance, from a clinical point of view, of having or not anti-IFN-β antibodies. At the same time, the above-mentioned criteria yielded data on responders and non-responders that are not directly comparable with those from other settings [10, 11, 15, 16]. It is also important to highlight that the readout of IFN-β therapeutic efficacy in MS is activity of disease, which is assessed with difficulty and high variability.

NAB were associated with the high titer BAB, except in two cases. In these cases, we cannot exclude that the results of the assay were due to non-antibody, IFN-β-inactivating/inhibiting factors that occasionally occur in both treated and untreated MS patients [3]. An alternative hypothesis is that our RIPA is not sensitive enough to detect all NAB-positive samples, as NAB recognize only a few of the IFN-β epitopes. In any case, these findings suggest that the procedure for testing for NAB only BAB-positive samples may give false-negative results.

In conclusion, we show that the frequency and titers for BAB differed between different IFN-β preparations, did not differ between responders and non-responders, and spontaneously decreased over time. Neither BAB, nor NAB were useful for clinical purposes, when stringent criteria were applied to categorize responders and non-responders. The indirect detection of IFN-β bioavailability [13] — independently from antibody response to the drug, interfering IFN-β receptor-related mechanisms [17], or both — will probably help neurologists care for MS patients on IFN-β therapy.

REFERENCES

1. Martino G, Adorini L, Rieckmann, Hillert J, Kallmann B, Comi G, Filippi M. 2002. Inflammation in multiple sclerosis: the good, the bad, and the complex. *Lancet Neurology* 1: 499.
2. Pachner AR. 1997. Anticytokine antibodies in beta interferon-treated MS patients and the need for testing: plight of the practicing neurologist. *Neurology* 49: 647.
3. Antonelli G, Dianzani F. 1999. Development of antibodies to interferon beta in patients: technical and biological aspects. (Review) *Eur. Cytokine Netw.* 10: 413.
4. Bonifacio E, Lampasona V, Bingley PJ. 1998. IA-2 (islet cell antigen 512) is the primary target of humoral autoimmunity against type 1 diabetes – associated tyrosine phosphatase autoantigens. *J. Immunol.* 161: 2648.
5. Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, Johnson KP, Sibley WA, Silberberg DH, Tourtellotte WW. 1983. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann. Neurol.* 13: 227.
6. Scagnolari C, Bellomi F, Turriziani O, Bagnato F, Tomassini V, Lavolpe V, Ruggieri M, Bruschi F, Meucci G, Dicuonzo G, Antonelli G. 2002. Neutralizing and binding antibodies to IFN-beta: relative frequency in relapsing-remitting multiple sclerosis patients treated with different IFN-beta preparations. *J. Interferon Cytokine Res.* 22: 207.
7. Bland JM, Altman DG. 1986. Statistical methods for assessing agreement between two methods of clinical measurements. *Lancet* I: 307.
8. Khan OA, Dhib-Jalbut SS. 1998 Neutralizing antibodies to interferon beta-1a and interferon beta-1b in MS patients are cross-reactive. *Neurology* 51: 1698.
9. Brickelmaier M, Hochman PS, Baciu R, Chao B, Cuervo JH, Whitty A. 1999. ELISA methods for the analysis of antibody responses induced in multiple sclerosis patients treated with recombinant interferon-beta. *J. Immunol. Methods* 227: 121.
10. The IFNB Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group. 1999. Neutralizing antibodies during treatment of multiple sclerosis with interferon beta-1b: experience during the first three years. *Neurology* 47: 889.
11. PRISMS Study Group and the University of British Columbia MS/MRI Analysis Group. 2001. PRISMS-4: Long-term efficacy of interferon-beta-1a in relapsing MS. *Neurology* 56: 1628.
12. Deisenhammer F, Reindl M, Harvey J, Gasse T, Dilitz E, Berger T. 1999. Bioavailability of interferon beta-1b in MS patients with and without neutralizing antibodies. *Neurology* 52: 1239.
13. Bertolotto A, Gilli F, Sala A, Capobianco M, Malucchi S, Milano E, Melis F, Marnetto F, Lindberg RL, Bottero R, Di Sapio A, Giordana MT. 2003. Persistent neutralizing antibodies abolish the interferon β bioavailability in MS patients. *Neurology* 60: 634.
14. Rice GP, Paszner B, Oger J, Lesaux J, Paty D, Ebers G. 1999. The evolution of neutralizing antibodies in multiple sclerosis patients treated with interferon-; -1b. *Neurology* 52: 1277.
15. Kivisäkk P, Alm GV, Fredrikson S, Link H. 2000. Neutralizing and binding anti-interferon-β; (IFN-β) antibodies. A comparison between IFN-β-1a and IFN-β-1b treatment in multiple sclerosis. *Eur. J. Neurol.* 7: 27.
16. Bertolotto A, Malucchi S, Sala A, Orefice G, Carrieri PB, Capobianco M, Milano E, Melis F, Giordana MT. 2002. Differential effects of three interferon betas on neutralizing antibodies in patients with multiple sclerosis: a follow up study in an independent laboratory. *J. Neurol. Neurosurg. Psychiatry* 73: 148.
17. Dupont SA, Goelz S, Goyal J, Green M. 2002. Mechanisms for regulation of cellular responsiveness to human IFN-beta 1a. *J. Interferon Cytokine Res.* 22: 491.