The measurement of antibodies binding to IFNβ in MS patients treated with IFNβ

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The intensity of the antibody response to injected interferons (IFNs) depends on many factors: route of injection, dose injected, frequency of injections, and duration of treatment. The optimal assay or approach for testing for anti-IFN antibodies has not been determined. Neutralizing antibody (NAb) determinations are most commonly used but have intrinsic problems when used as clinical assays. They are time consuming, expensive, and only indirectly measure antibodies. Thus, false-positive results can occur because of other serum factors. In contrast, binding assays measure antibodies directly; they are mainly used as an initial screen to detect NAbs. ELISA methodologies for binding antibodies have been used, but direct adhesion of the antigen (i.e., IFNβ) to the plate has resulted in false-negative and false-positive results, presumably because of changes in antigenicity.[1] This limitation is circumvented by using the capture ELISA in which a first antibody is used to capture the antibody and hold it in an antigenic position, mimicking that of IFNβ in its natural state.

Radioimmunoprecipitation assays (RIPAs) are used routinely to measure pathogenic autoantibodies, including antibodies to the acetylcholine receptor and voltage-gated calcium and potassium channels, with good sensitivity and specificity. The RIPA also has been used to measure antibodies to another biologic substance, botulinum toxin, in patients treated with this toxin.

The experience of groups in the United States (University of Medicine and Dentistry of New Jersey) and Canada (University of British Columbia) with improved ELISAs and in the United Kingdom (Oxford University) with RIPA is summarized below.

Materials and methods. *Direct ELISA.* The assay has been previously described.[2,3] In brief, IFNβ was directly coated onto an ELISA plate, followed by serial incubations with serum, conjugate, and substrate, with washes in between.

Capture ELISA. United States. The assay has been previously described.[1] Experience with this assay in a broad spectrum of patients with MS has recently been published.[4] In brief, 96-well microtiter plates were coated overnight with 50 μL/well of the monoclonal antihuman IFNβ immunoglobulin (Ig) G antibody BO2 (Yamasa-Shoyu Co. Ltd., Tokyo, Japan). After plate washing and blockade with nonfat dry milk, wells were coated with either buffer or IFNβ (1a as Avonex or 1b as Betaseron) at a dilution of 1.5 μg/mL. Subsequent incubation with serum samples, conjugate, and development was the same as the direct ELISA outlined previously. Calculation of units was identical with the direct ELISA outlined previously, except optical densities (ODs) were calculated by subtracting ODs of the wells lacking IFN from the ODs of the IFNβ-treated wells.

Canada. Binding antibodies (BAbs) were detected using a sandwich ELISA based on the capturing of IFNβ with an anti-IFNβ monoclonal antibody.

Radioimmunoprecipitation assay. United Kingdom. This assay was performed as previously described.[5] In brief, 10 μL iodinated IFN (approximately 50,000 cpm) was added to 5 μL serum in 0.02 phosphate buffer with 0.1% triton X100 and incubated for 2 hours at room temperature. Fifty microliters sheep antihuman IgG (The Binding Site, Birmingham, UK) was added and centrifuged, and the precipitate was washed in PTX buffer and counted on a gamma counter (Packard, Berkshire, UK). Three healthy control sera and a high-positive serum were tested in each assay. The mean of the healthy control values was subtracted from every test value, and the results were expressed as a percentage of the value obtained with the high-positive serum.

Results. *United States.* Four hundred fifty-three serum samples were obtained from patients with MS or control subjects and were then aliquoted and stored at −70 °C. The number of patients taking each medication, with the total samples in parentheses, was as follows: Betaseron, 145 (168); Avonex, 189 (214); and Rebif, 17 (17). In 30 patients taking IFNβ, the preparation was not noted. In 23 serum samples, the source of the specimen was a patient about to begin medication. Sixty-nine percent of the patients with MS were women. The mean age of the patients was 37 years, and the mean duration of IFNβ therapy was 1.3 years.

Two hundred eighty-five serum samples from patients with MS taking IFNβ had cELISA values of less than 2.3 U. Most patients with high antibody levels began to develop antibodies within the first 3 months after initiation of therapy. Because no sera with cELISA values of less than 8 U had neutralizing activity, the range of 2.3 U to 8 U was termed "moderate;" there were 52 serum samples in this range. In sera with cELISA values in the range of 8 to 55 U, called "high" levels, the frequency of NAbs was 53% (39/74). The 19 serum samples with cELISA values greater than 55 U were NAb positive, and these were labeled "very high." These data are summarized in the table.

The Pearson correlation coefficient was 0.750; the correlation was significant at 0.01 (two tailed). The mean cELISA values for Avonex, Betaseron, and Rebif were 2.5, 18.4, and 48.4 U; the percentages of NAb positivity for these therapies were 6, 34, and 14. Only 17 samples were available from Rebif-treated patients.

Canada. The same inclusion criteria of two relapses during the preceding 2 years were applied throughout because they have become the condition for medication reim-

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Supported by grants from Berlex Canada/Shering AG (Canada) and from the Foundation of UMDNJ (United States).

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Table *Neutralizing antibodies are found only in patients with high or very high levels of binding antibodies as detected by cELISA (US group)*

Range	cELISA value (units)	No. of sera	Frequency of NAbs (%)
Absent/low	<2.5	308	0/131 (0)
Moderate	2.5–8	52	0/45 (0)
High	8–55	74	39/74 (53)
Very high	>55	19	19/19 (100)

NAbs = neutralizing antibodies.

bursement in the British Columbia health care system where this study was carried out. One hundred twenty-nine patients received IFNβ-1b subcutaneously (Betaseron), and 88 patients received IFNβ-1a subcutaneously three times weekly (Rebif).

Serum samples were obtained as follows from the different groups of patients, who were also monitored clinically for a minimum of 2 years and up to 12 years. Ninety-six patients receiving 250 μg IFNβ-1b subcutaneously every other day were part of two groups: 44 were monitored prospectively and sampled every month for 2 years; the other group of 52 was captured retrospectively and chosen as having volunteered their blood samples between 4 and 10 times during a 2-year period. Thirty-three patients participating in the Betaseron Pivotal Trial receiving 8 MIU (250 μg; n = 16) or 1.6 MIU (50 μg; n = 17) IFNβ-1b subcutaneously thrice weekly provided samples every 6 weeks for 5 years and then approximately every other year for 7 more years (at which time they were all switched to high dose). Twenty patients receiving 44 μg IFNβ-1a subcutaneously thrice weekly and 16 patients receiving 22 μg IFNβ-1a subcutaneously thrice weekly as part of the extension of the PRISMS Study provided blood samples every 6 months. Of the 39 patients with secondary progressive MS who took part in the SPECTRIMS study, 17 received 44 μg and 22 received 22 μg IFNβ-1a subcutaneously thrice weekly.

In this report, samples were considered positive if their binding to IFN-coated plates was above the cut-off point determined as mean of 39 healthy control subjects + 3 SD (value = 18.2 Lab units). BAbs developed in 91 of 129 patients (70.5%) receiving 250 μg IFNβ-1-b subcutaneously every other day for more than 2 years, whereas of 88 patients treated with IFNβ-1a thrice weekly (Rebif), 26 (30%) became BAb positive. There was a difference in persistence of BAbs between Betaseron and Rebif, as shown in figure 1. BAbs in Betaseron-treated patients tended to decrease over time.

To correlate BAbs and clinical outcome, charts from IFNβ-1b–treated patients were assessed independently of BAb status, and patients were segregated into two groups: "clinical successes" (patients totally stable or improving, i.e., no relapse and no worsening on Expanded Disability Status Scale [EDSS]) and "nonclinical success" (all the others). Clinical data and BAb results were obtained from 121 IFNβ-1b–treated patients, corresponding to 88 patients from group A and 33 patients from group B. There were 47 stable patients (successes) and 74 failures (relapse or EDSS worsening). Among the 84 BAb-positive patients, only 27 (32%) were successes, whereas among the 37 from the BAb-negative group, 20 (54%) were successes (χ^2 = 7.51, $p < 0.005$).

United Kingdom. The Oxford group, in collaboration with the Vancouver group, found the RIPA to be highly specific for patients receiving IFNβ treatment with no false-positive results. The RIPA, like other binding assays, became positive earlier and showed fewer fluctuations than the neutralizing assays. Importantly, there was only a modest correlation between the RIPA and NAb titers, suggesting that they measure different antibody populations. A cohort of 50 patients with MS from a randomized study of IFN-1b was tested for BAbs by the RIPA and the

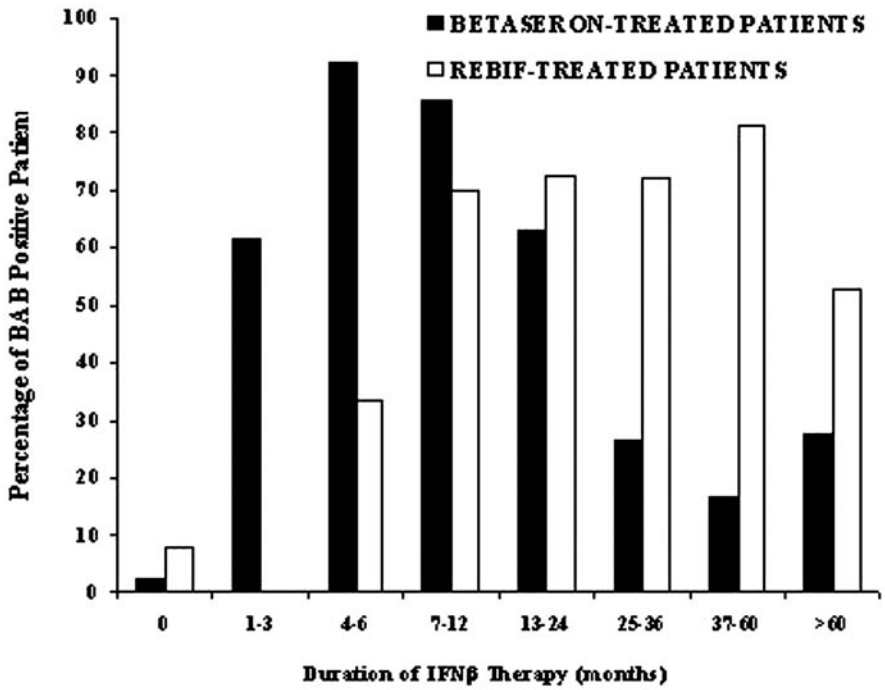

Figure 1. Kinetics of the proportion of binding antibody (BAb)-positive patients in Betaseron and Rebif-treated patients.

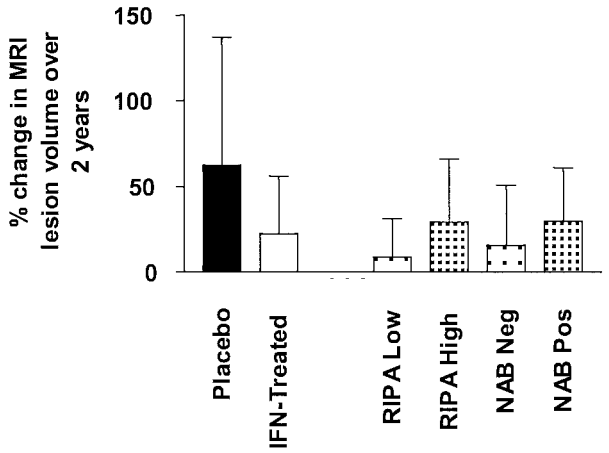

Figure 2. Effect of binding antibody (BAb) status as measured by radioimmunoprecipitation assays (RIPA) on MRI. (Reproduced from Lawrence et al.,[5] by permission from the BMJ Publishing Group.)

neutralizing assay, using the MxA assay, during a 2-year period. Within this group, a treatment effect was only demonstrable on MRI outcomes (figure 2), and thus the effect of the antibody status on these measures was evaluated. Although no effect of antibody status was seen on the number of new and extending lesions during the 2-year period with either assay, a greater attenuation was seen on the change in MRI lesion volume when using the RIPA assay results compared with the MxA NAb results. The conclusion of this group was that the RIPA to detect anti-IFNβ antibodies has advantages over the neutralizing assays not only because it is cheap, reliable, and sensitive but also because it may be more relevant to the effects of the treatment on outcome measures.

Discussion. These studies demonstrate that binding assays can serve as excellent assays to screen for anti-IFNβ antibodies. This is an important finding because binding assays are relatively inexpensive, fast, and easily performed by most clinical laboratories. Whether the assays described previously can serve as stand-alone assays or whether they need to be followed with either NAb assays or bioactivity studies, such as the MxA assays described in another article in this supplement, needs to be determined.

The capture ELISA format allowed the native structure of the IFN to be maintained; this is in contrast to the direct ELISA, in which the IFNβ is directly stuck to the plate and the native structure is deformed. When these two ELISAs are compared, direct-binding ELISA underreports BAbs, and because it is frequently used as a screening test before assaying for NAbs, NAbs themselves may be missed. The US and Canadian groups found that the capture ELISA significantly outperformed the direct ELISA. The capture ELISA and the RIPA should thus be considered as "second-generation" BAb assays, much improved over the "first-generation" BAb assay, in which the IFNβ is directly stuck to the plate.

There were a number of other findings that were consistent in the three countries. The US and Canadian groups found that using the same IFNβ preparation in the assay as was used in treating the patient improved test reliability. The consensus of the groups was that IFNβ-1b was considerably more immunogenic than IFNβ-1a, either given once a week or three times a week. The US and Canadian groups also found that BAbs might have effects on bioactivity independent of their NAb status, the latter defined as the levels of neutralization high enough to be detected by NAb assays. These data are consistent with Dr. Bendtzen's theory, as described in his presentation: NAb activity is a continuum, and all sera that have significant BAb activity have NAb activity if the sensitivity of the assays is set appropriately. Thus, the Canadian group demonstrated for the first time that BAb-positive patients had a greater chance than BAb-negative patients of being treatment nonsuccesses, having more frequent relapses, and increasing EDSS.

Another important finding by the Canadian group was that there was a difference in persistence of BAbs between Betaseron and Rebif. Despite the relatively lower incidence of BAbs in the Rebif group, anti-IFNβ antibodies appeared to persist more in the Rebif group than in the Betaseron group.

In conclusion, the data from the three groups demonstrate that BAb assays can be used clinically to detect anti-IFNβ antibodies. At a minimum, the role of BAb can be that of a screening assay for NAbs. However, they may be able to function as stand-alone assays for antibody, especially when combined with an IFNβ bioactivity assay, such as mRNA or protein assays for MxA.

Acknowledgment

The authors thank Donna Dail and Marie Hurd (United States) and Ebrima Gibbs and Tariq Aziz (Canada).

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