JOURNAL OF INTERFERON & CYTOKINE RESEARCH 28:713–724 (2008)

DOI: 10.1089/jir.2008.0144

A Biosensor-Based Characterization of the Affinity Maturation of the Immune Response Against Interferon-β and Correlations with Neutralizing Antibodies in Treated Multiple Sclerosis Patients

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The study of antibodies against Interferon-β (IFN-β) in treated multiple sclerosis (MS) patients has focused primarily on detection and quantification, with no single method of detection providing a comprehensive characterization. We assessed serial samples of 18 MS patients, treated with IFN-β for between 66 and 198 months, to characterize the affinity maturation of these antibodies using a biosensor-based approach (Biacore™). Biacore utilizes the principles of surface plasmon resonance (SPR) with data from the dissociation phase of the antigen–antibody reaction being inversely proportional to relative antibody affinity. In patients with neutralizing antibodies (NAbs+), mean antibody dissociation rates decreased from $0.00118 \pm 0.00030\ s^{-1}$ at month 6 to $0.00021 \pm 0.00008\ s^{-1}$ at month 36, followed by a slight increase to $0.00027 \pm 0.00003\ s^{-1}$ at month 60. In NAb− patients, mean antibody dissociation rates decreased only very slightly from $0.00130 \pm 0.00025\ s^{-1}$ to $0.00105 \pm 0.00020\ s^{-1}$ at month 18, followed by a gradual increase to $0.00243 \pm 0.00099\ s^{-1}$ at month 60. Our study shows little improvement in antibody affinity in NAb− patients, in contrast to a marked increase in antibody affinity over time in NAb+ patients with a significant correlation between NAb titers and relative antibody dissociation rates (Spearman's correlation, $R^2 = -0.374$, $p < 0.001$). The evaluation of relative antibody affinities will contribute to a thorough understanding of the anti-IFN-β antibody response.

Introduction

Antibodies against interferon-β (IFN-β) represent an important aspect in multiple sclerosis (MS) patients undergoing treatment with IFN-β. It has now been conclusively proven that neutralizing antibodies (NAbs) can reduce the clinical efficacy of IFN-β in a proportion of patients (Boz and others 2007). In addition, the biological activity of IFN-β can also be abrogated by the presence of these antibodies (Vallittu and others 2002; Bertolotto and others 2003; Pachner and others 2003), the implications of which can be far reaching, as we recently discovered; an IFN-β-treated patient with NAbs persistent for over 4 years despite cessation of treatment, developed a very rare form of melanoma (Gibbs and others 2008). This we hypothesize could be a consequence of the NAbs neutralizing not only exogenous, but the endogenously produced IFN-β, which among other functions is known to have growth inhibitory and proapoptotic effects (Chawla-Sarkar and others 2001; Parmar and Platanias 2003). Chronologically, in IFN-β-treated patients, peak BAb levels are attained earlier than peak NAb levels, and it is hypothesized that the former, driven by the process of affinity maturation, evolve over time into Nabs (Mayr and others 2003). It has been demonstrated that patients who develop BAbs but no NAbs (BAb+/NAb−), tend to have lower antibody affinities when compared to BAb+/NAb+ patients (Gneiss and others 2006). How this process emerges longitudinally, however, has not been ascertained.

In addition to antibody specificity and titer, the biological effectiveness of an antibody is dependent on its affinity (Steward and Lew 1985). Affinity defines the binding strength between antigen and antibody, and affinity maturation is the overall improvement of affinity overtime. As exemplified by IgG antibodies, this process is well-established and increases the effector functions of IgG antibodies during the adaptive immune response (Roost and others 1995). Initially, during an immune response, the predominant Ig are of the

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IgM isotype with low antibody affinity. As the response proceeds, there is a progressive switch to the IgG isotype with a concomitant increase in affinity. This maturation process involves B-cell proliferation, germinal centre differentiation, somatic hypermutation and the competition for antigen by different clones of B cells.

Biacore™ is a state-of-the-art-technology that utilizes surface plasmon resonance (SPR) to analyze biomolecular interactions between proteins, carbohydrates, nucleic acids, and peptides, as they proceed in real time (Swanson and others 2002). An inherent advantage of this technology is that it is label-free, without the use of enzymatic, fluorescent or radioactive labeling of the interactants or secondary detector molecules. SPR is an optical resonance phenomenon occurring at the interface between a thin gold film and a liquid medium. When light with a wavelength of 760 nm is focused on the film surface of the sensor chip, SPR occurs at an angle. Analyte bound to ligand will result in an additional mass and a change in refractive index with a resultant change in SPR angle that is reported in response units (RUs) (Malmqvist 1993). A plot of RU over time produces a sensorgram, which provides real-time monitoring of the binding kinetics, with 1 RU corresponding to ~1 pg/mL of bound analyte. Curve-fitting software, allows for the calculation of the association constant (k_{on}), the dissociation constant (k_{off}) and the equilibrium constant. Relative antibody affinity (avidity) can be estimated from the k_{off} values obtained from the dissociation phase: the higher the affinity, the slower the dissociation rate (Takacs and others 1999).

In this study, IFN-β of high purity was immobilized onto sensor chips and used in a Biacore™ 3000 instrument to evaluate the binding characteristics and relative dissociation rates, and thus apparent affinities, of anti-IFN-β antibodies. To address the question of how the affinity maturation of the anti-IFN-β antibodies evolves in NAb+ and NAb− patients, we examined serial samples of 18 IFN-β-treated patients, 12 of whom were NAb+ and 6 NAb−. In addition, we report the IgG subclass-specificities of these antibodies, some of which have previously been published (Gibbs and Oger 2007).

Materials and Methods

Patient sera

Serum samples were collected serially from 18 MS patients who had been continuously treated with subcutaneous IFN-β1b ($n = 9$) or subcutaneous IFN-β1a ($n = 9$). Patients were selected from the University of British Columbia (UBC) MS Clinics Serum Bank based on the following criteria: (1) patients were receiving one single type of IFN-β therapy, (2) completion of at least 60 months of IFN-β therapy, (3) at least three serum samples collected with a period of at least 6 months separating these sera, and (4) availability of results for BAbs and NAbs. Treatment duration ranged from 66 to 198 months. These patients were stratified into BAb+ NAb− ($n = 6$) and BAb+ NAb+ ($n = 12$) based on detection of BAbs by enzyme-linked immunosorbent assay (ELISA) (Gibbs and Oger 2007) and NAbs by both the CPE and luciferase reporter gene Assays. A patient was considered BAb+ if the optical density value of the serum sample was greater than the mean + 3 SD of five healthy control sera. Patients were designated as NAb+ if they tested positive [>20 10-fold reduction units (TRUs)) on at least two consecutive samples. Otherwise, patients were considered NAb− but with IFN-β-binding activity (BAb+). Control sera were obtained from patients whose BAb and NAb status had been identified by the BAb/NAb testing program at UBC, and from healthy donors.

Equipment and data analysis software

The binding characteristics of serum antibodies to IFN-β were assessed using Biacore 3000™ instrument (Biacore AB, Uppsala, Sweden) that utilizes SPR technology. Data were analyzed using BiaEvaluation 3.1 software (Biacore AB, Uppsala, Sweden).

Immobilization of IFN-β antigen

Under conditions of continuous flow of running buffer HEPES buffered saline (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% P-20 surfactant) (HBS-EP, 0.05% P-20), pure recombinant IFN-β1a (Avonex, Biogen, Cambridge, MA) was immobilized onto a CM-5 sensor chip (Biacore, Uppsala, Sweden). Using an amine coupling kit (Biacore AB), the active flow cell was first activated with a 7-min injection of equal volumes of 0.05 M *N*-hydroxysuccinimide(NHS) and 0.2 M *N*-ethyl-*N*′-(3 diethylaminopropyl) carboiimide (EDC), according to the manufacturers instructions. The EDC/NHS mixture converts the carboxymethyl groups of the dextran matrix on the sensor chip to succinimide esters. Subsequent injection of IFN-β1a (7.5 µg/mL in HBS-EP, 0.005% P-20, pH 7.4) over the surface results in the formation of amide bonds between NH2 groups on the IFN-β and succinimide groups of the activated surface. Finally, unreacted succinimide groups were blocked by a 7-min injection of 1 M ethanolamine hydroxide (pH 8.5) (Fig. 1A). The amount of IFN-β1a immobilized on the active flow cell ranged typically from 135 to 155 response units. On the reference flow cell, following activation by NHS/EDC mixture, the surface was immediately blocked with ethanolamine hydroxide (Fig. 1B). All immobilization procedures were carried out at 25°C.

Biacore analysis of serial serum samples

A 96-well microtiter plate format was used to perform the Biacore assays, at a temperature of 37°C, under conditions of continuous-running buffer flow (except otherwise stated). Serum samples were diluted 1 in 10 in HBS-EP, 0.005% P-20 containing 1 mg/mL carboxymethyl dextran (CM-D). All samples originating from each patient were included in the same assay. Each assay also contained a positive and a negative control, and a blank control consisting of the sample diluent. Each sample was sequentially injected over the active IFN-β1a-coated surface and the reference control surface (no IFN-β1a) at a flow rate of 60 µL/min for 2 min during which time binding was assessed in the association phase. Subsequently, sample injection was stopped and antibody dissociation was allowed for 5 min during which time only running buffer was injected over the sensor chip surface

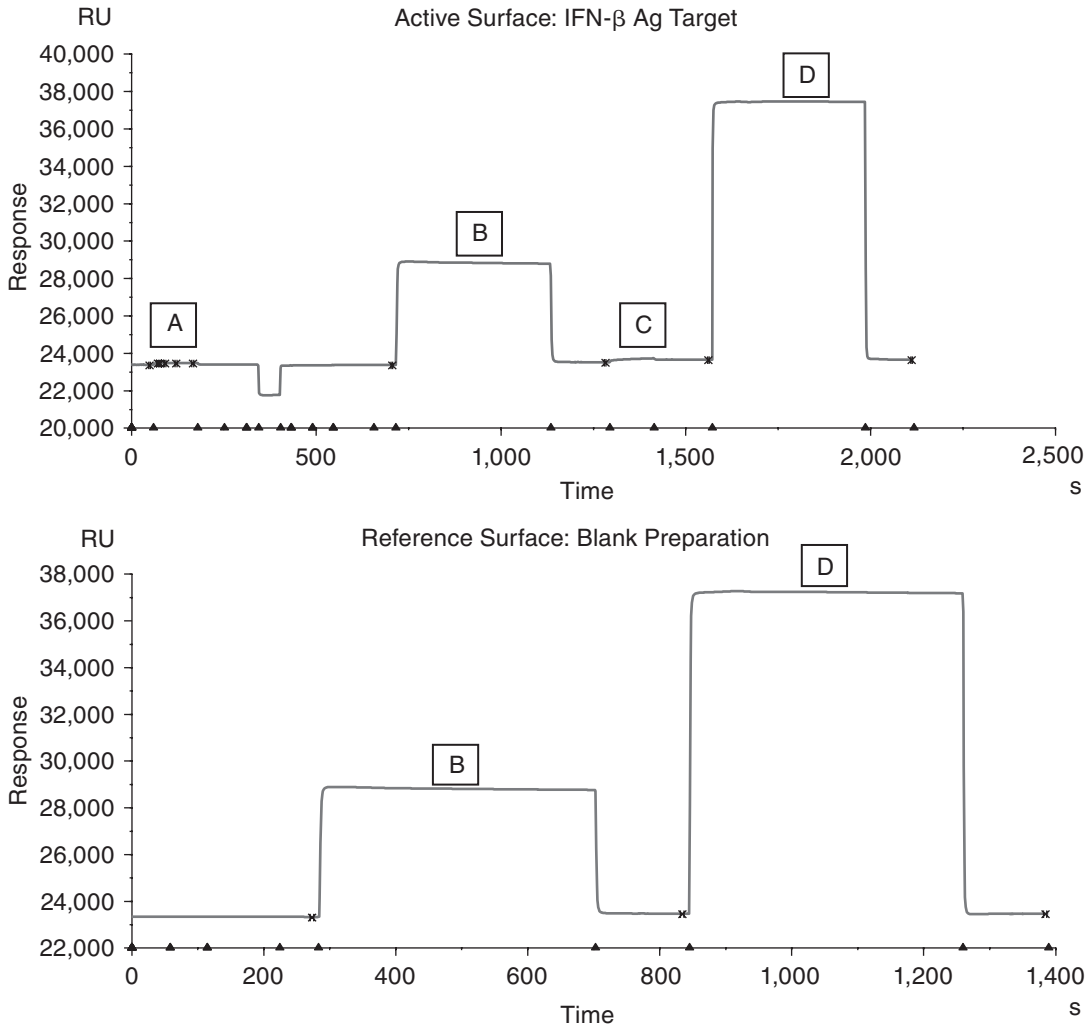

FIG. 1. Sensorgrams showing the procedures for the preparation of the active and reference surfaces. (**A**) Preconcentration of IFN-β1a antigen, a process by which IFN-β1a is electrostatically attracted to the negatively charged dextran matrix and the target level of immobilization is tested. (**B**) Activation of surface with mixture of 0.2 M EDC and 0.05 M NHS (v/v) to generate succinimide groups. (**C**) Injection of 7.5 µg/mL of pure IFN-β1a in HBS-EP, 0.005% P-20 (pH 7.4). During this process, the amine groups of IFN-β1a couple with reactive succinimide groups of the activated surface to form covalent amide bounds. (**D**) One molar Ethanolamine hydrochloride is used to block the uncoupled succinimide groups.

(Fig. 2A). The binding response was measured in response units (RU) with 1 RU typically corresponding to a surface mass change of 1pg/mm^2 protein, and the dissociation phase was measured in seconds^{-1} (s^{-1}). After the completion of a cycle, association and dissociation, the sensor chip surface was then regenerated with a pulse of 50 mM NaOH (15 µL at 60 µL/min).

Determination of binding responses and relative antibody affinities

Antibody binding to immobilized IFN-β was measured as the increase in SPR signal, in response units (RU), from 15 s prior to sample injection (baseline) to 15 s prior to end of sample injection. The true antibody-binding response was obtained by an online reference method that subtracts the SPR signal obtained on the reference surface from the active surface, resulting in normalized sensorgrams (Fig. 2B). For the analysis of relative antibody affinities, the BiaEvaluation 3.1 Software (Biacore AB, Uppsala, Sweden) was used. First the normalized sensorgrams were X-transformed to start at the same sample injection start point, followed by Y-transformations at 0.00 RU and subtraction of the sample diluent. The sensorgrams were then fitted to Langmuir 1:1 binding model (single site binding) from 10 to 310 s postcessation of sample injection (5 min dissociation). The resultant dissociation rates (s^{-1}) are reflective of the relative antibody affinity, with high affinity antibodies having a relatively slower dissociation rate and low-affinity antibodies with fast dissociation rates. Due to the polyclonal nature of serum however, the relative dissociation rates and affinities are an apparent estimate, representing an average of the multiple populations and interactions of the different antibody clones (Sem and others 1999). In order to make comparisons

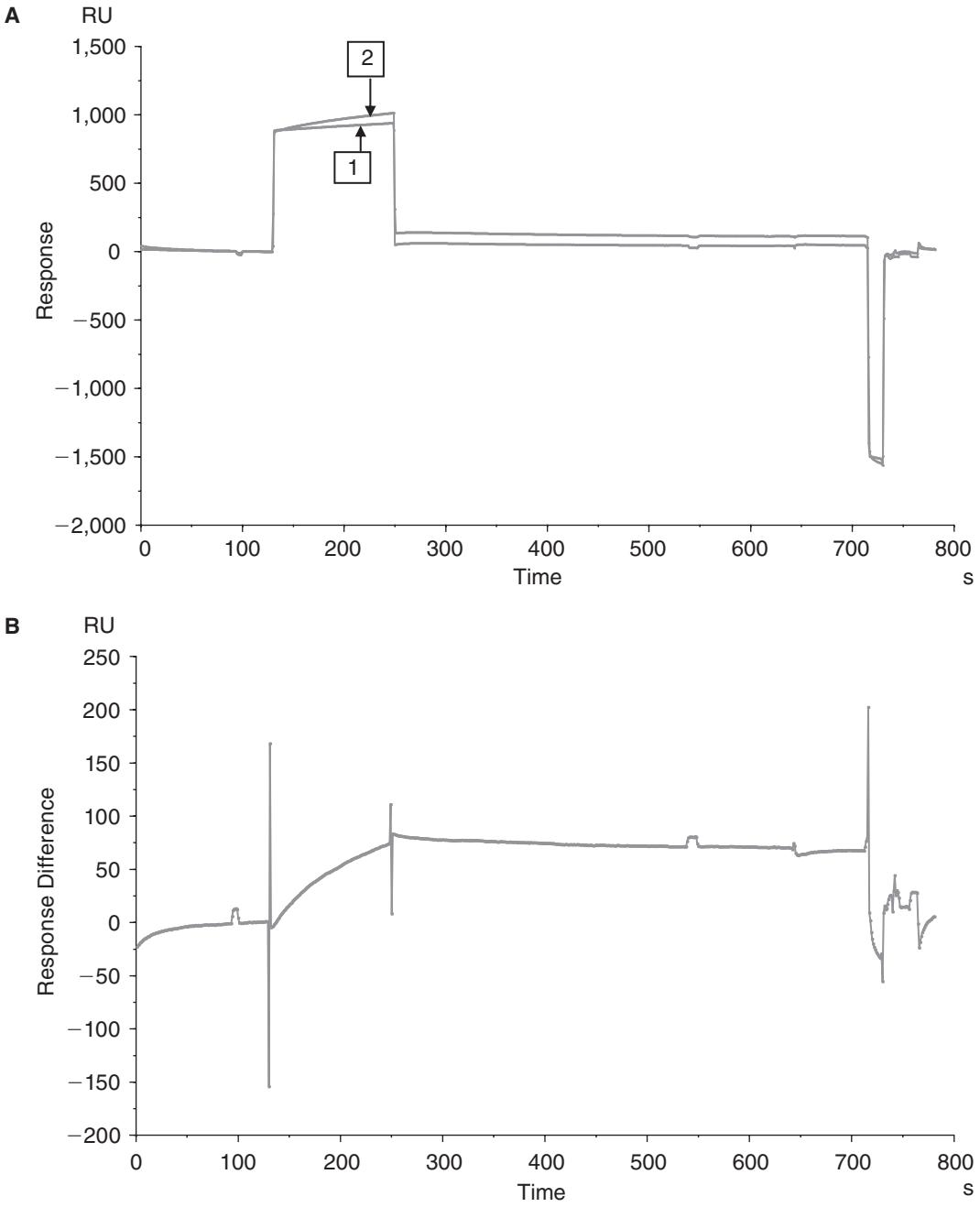

FIG. 2. (**A**) Sensorgram plots of a representative anti-IFN-β antibody positive serum sample binding to the reference (1) and active (2) surfaces. (**B**) Sensorgram obtained by subtraction of 1 from 2.

between patients, the dissociation rate of pretreatment serum was further subtracted from subsequent serum samples for each patient.

Determination of IgG subclass-specificities of anti-IFN-β antibodies

The IgG subclasses of these antibodies were previously analyzed by a capture ELISA as part of a larger cohort of patients (Gibbs and Oger 2007). Briefly, 96-well microtiter plates (Costar 3576, Cambridge, MA.) were coated with a capture antibody, mouse anti-human IFN-β monoclonal antibody (Chemicon MAb416, Temecula, CA.), and incubated overnight at 4°C. Plates were then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and nonspecific sites blocked with 1% bovine serum albumin (BSA) at 37°C for 1 h. After washing with PBS/Tween, diluted commercially available IFN-β1b (Berlex, Canada) or IFN-β1a (Serono, Canada) was added and plates incubated further overnight at 4°C. Following three washes, diluted patient sera were added and incubated at 37°C for 1 h. Serial samples of each patient were assayed together on the same

plate. After washing, horseradish peroxidase–conjugated mouse monoclonal anti-human IgG1, Fc region–specific (clone HP6069), antihuman IgG2, heavy chain Fd region–specific (clone HP6014), antihuman IgG3, heavy chain hinge region–specific (clone HP6047), and antihuman IgG4, heavy chain Fc region–specific (clone HP6025), all obtained from Zymed Laboratories (San Francisco, CA), were added in the appropriate wells for 1 h 37 C. Bound IgG antibodies were then detected by addition of *o*-phenylenediamine substrate and optical densities measured at 490 nm wavelength in an MRX ELISA Microplate Reader (Dynex Technologies, Chantilly, VA).

Statistical analysis

Statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL) The descriptive statistics consisted of the mean and standard error of the mean for each group of patient and at different time points. We determined the significance of the differences in Biacore-binding responses and relative antibody dissociation rates between NAb negative and NAb positive patients using the Mann–Whitney test. The relationship between Biacore binding and NAb titers, and between relative antibody dissociation rates and NAb titers were determined by Spearman's correlation test.

Results

Evaluation of the assay

The specificity of the Biacore assay to detect antibodies to IFN-β was assessed using serum samples from 43 healthy donors and 11 IFN-β-treated MS patients. Figure 3 shows that the 43 samples from healthy donors had no detectable binding to immobilized IFN-β1a, whereas 5 of 11 IFN-β-treated patients' samples had a detectable binding response above baseline (serum diluent blank). Thus these data indicate the specificity of the Biacore assay in detecting serum antibodies from treated MS patients that bind to immobilized IFN-β1a. To address the sensitivity of the assay, two representative sera (anti-IFN-β positive and anti-IFN-β negative) were serially diluted (2-fold) and injected over immobilized IFN-β1a. Even at the lowest concentration (1:160 dilution) of the positive serum sample, the binding response was still greater than the negative serum (Fig. 4).

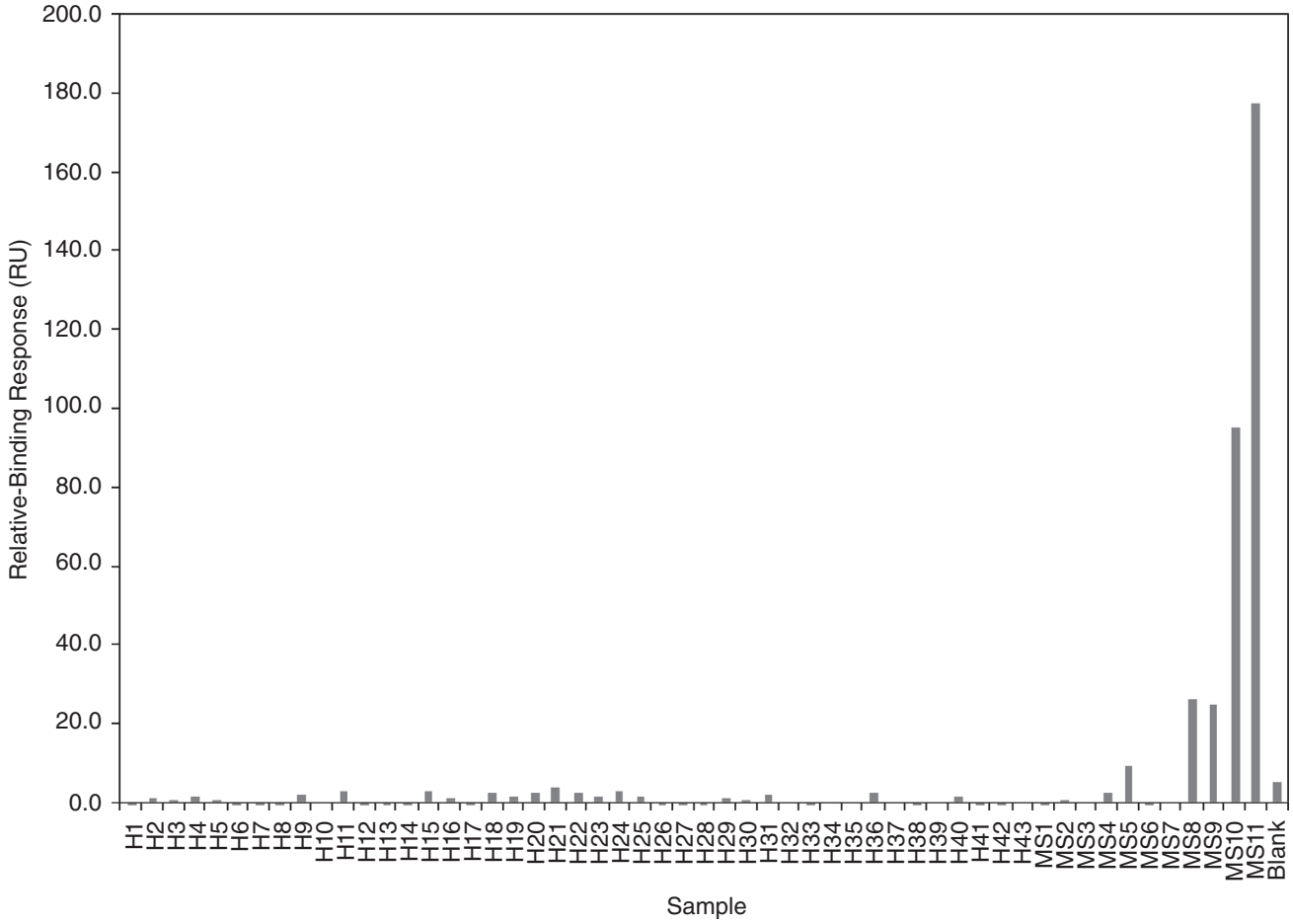

FIG. 3. Biacore reactivity of 54 serum samples against immobilized IFN-β1a. Sera, diluted 1:10, were obtained from healthy donors (H1–H43) ($n = 43$) and IFN-β-treated MS patients (MS1–MS11) ($n = 11$).

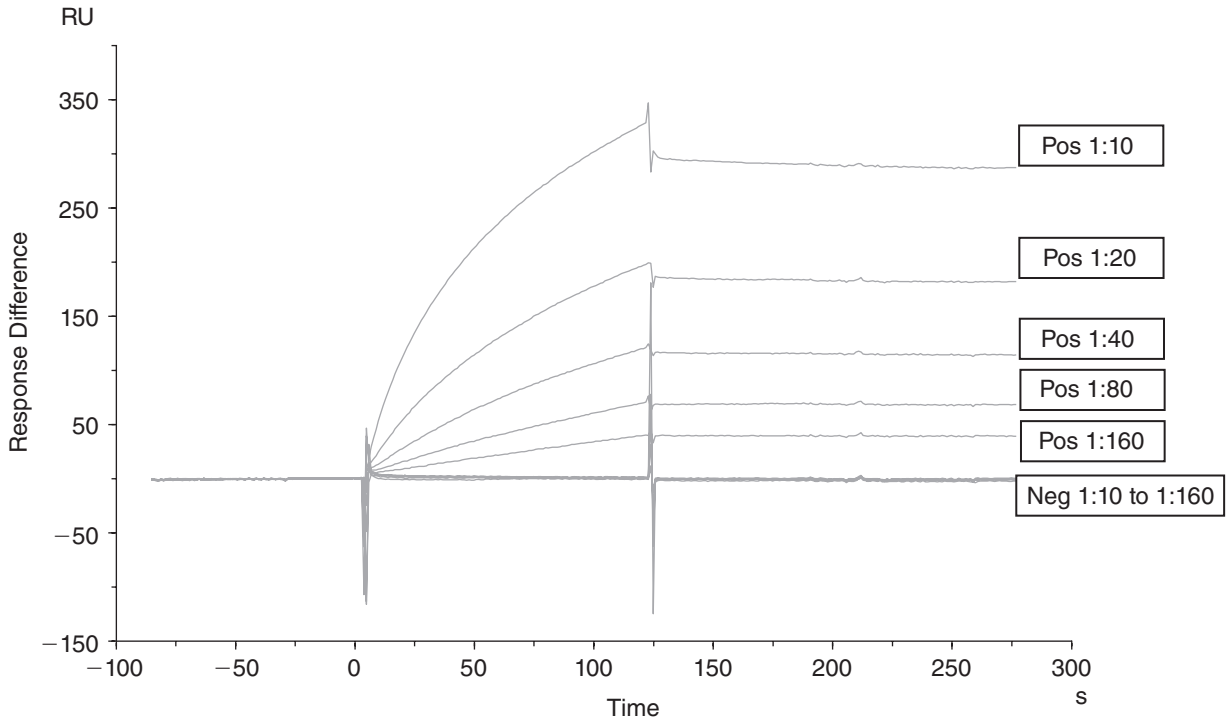

FIG. 4. Sensorgram overlay plots obtained when 2-fold serial dilutions of an anti-IFN-β antibody positive and anti-IFN-β antibody negative sera bind to immobilized IFN-β1a on the sensorchip surface.

Serial Biacore analysis of antibody-binding responses

Serial serum samples from 18 IFN-β-treated MS patients were evaluated for binding to a low density immobilized IFN-β1a. All samples from each patient were assayed on the same 96-well microtiter plate, and a depiction of sensorgrams of serial samples of one patient is shown in Figure 5; the magnitude of the binding responses as well as the relative dissociation rates clearly changed as a function of the duration of IFN-β treatment. The mean-binding response peaked at month 18 among the NAb− patients at 28.5 ± 14.9 RU followed by a fast decline. In contrast, in NAb+ patients, a mean peak response of 54.8 ± 21.2 RU occurred later at month 36, followed by a second peak of 56.9 ± 19.5 RU at month 60. The mean-binding response was also higher during the first 12 months in the NAb− patients (month 6 = 24.3 ± 8.5 RU, month 12 = 25.4 ± 11.2 RU) compared to the NAb+ patients (month 6 = 10.0 ± 2.9 RU, month 12 = 15.6 ± 4.8 RU); this difference in binding response was statistically significant at month 6 (Mann–Whitney U, 13.0, $p = 0.044$). However, with continued treatment, the antibodies generated in the NAb+ patients developed a higher binding response after month 12 compared to the NAb− patients (Fig. 6), this being significant at month 48 (Mann–Whitney U, 0.0, $p = 0.017$) and month 60. (Mann–Whitney U, 0.0, $p = 0.021$).

Serial determination of the IgG subclass-specificities of anti-IFN-β antibodies

As shown in Figure 7A, among the NAb− patients, overall there was no predominant expression of any particular IgG subclass, with all subclasses being expressed. As the immune response progressed, the relative levels of all subclasses decreased, such that by month 36 only low levels of IgG3 and IgG4 could be detected in the NAb− group. In contrast, NAb+ patients demonstrated a distinct pattern of IgG subclass distribution (Fig. 7B); there was a relative predominance of IgG1 during the first 6 months of IFN-β therapy, but as the immune response evolved, IgG4 levels increased markedly and became the predominant subclass, with peak levels at month 18. Relative levels of IgG2 and IgG3 were generally low and did not feature prominently in the immune response, although there was a notable peak of IgG3 at month 6, but which precipitously declined thereafter.

Serial Biacore analysis of relative antibody dissociation rates

On average NAb+ patients had slower rates of antibody dissociation than NAb− patients. Over time, the mean antibody dissociation rate continued to decrease in the NAb+ patients, whereas there was only a very slight decrease and a subsequent increase in the NAb− patients (Fig. 8). In NAb+ patients the mean antibody dissociation rates decreased from 0.00118 ± 0.00030 s^{-1} at month 6 to 0.00021 ± 0.00008 s^{-1} at month 36, and followed by a slight increase to 0.00027 ± 0.00003 s^{-1} at month 60. In NAb− patients, there was a negligible decrease in mean dissociation rate from 0.00130 ± 0.00025 s^{-1} to 0.00105 ± 0.00020 s^{-1} at month 18, followed by an increase to 0.00244 ± 0.00099 s^{-1} at month 60. Thus NAb+ patients generally had lower antibody dissociation rates than NAb− patients, and this was significant

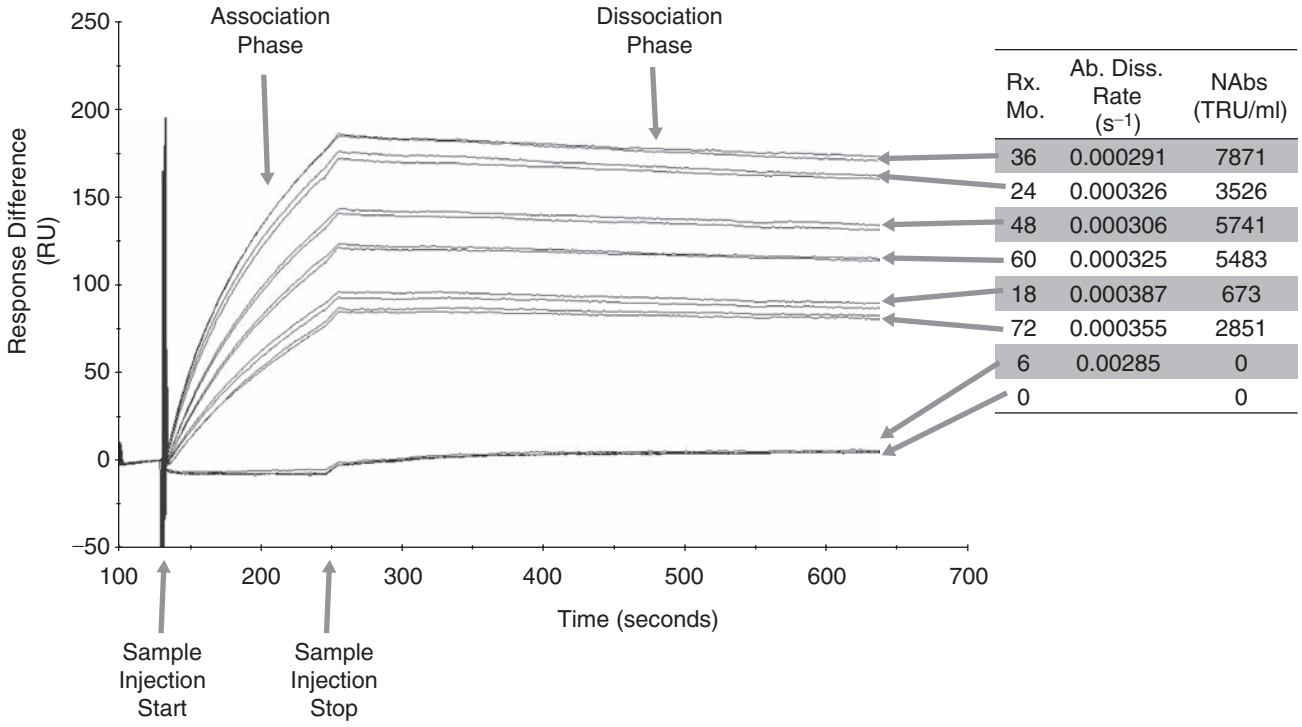

FIG. 5. A representative plot of sensorgrams from serial samples of one patient, showing the interaction between anti-IFN-β antibodies and immobilized IFN-β1a. Serum samples were taken at different time points during IFN-β treatment. Samples were analyzed in duplicate over the Active Surface (containing 143 RU IFN-β1a) and the Reference Surface (no IFN-β1a). The sensorgrams were evaluated with a BIAevaluation Software 3.1 (Biacore AB) by first X-transforming all curves to start at the same injection startpoint, followed by Y-transformations at 0.0 RU and subtraction of the sample diluent. Curves were then fitted by the 1:1 Langmuir model to obtain relative dissociation rates (s^{-1}) between 255 and 555 seconds.

at month 24 (Mann–Whitney U, 10.0, $p = 0.030$), month 36 (Mann–Whitney U, 0.0, $p = 0.025$), and month 60 (Mann–Whitney U, 0.0, $p = 0.014$).

Serial measurement of NAbs and correlations with relative antibody dissociation rates and Biacore-binding responses

In the NAb+ patients, mean NAb titer increased from 79 ± 26 TRU at month 6 reaching peak levels of 3800 ± 1371 TRU at month 36, followed by a gradual decline to 2007 ± 1180 RU at month 60. Figure 9 demonstrates that as IFN-β treatment duration continued, there was an increase in mean NAb titers and a concomittant decrease in mean antibody dissociation rates, such that peak NAb titer coincided with the lowest rate of antibody dissociation rate at month 36. Additionally, as mean NAb titer decreased after month 36, so too did the mean antibody dissociation rate increase. Thus there was a significant inverse correlation between NAb titers and relative antibody dissociation rates (Spearman's correlation, $R^2 = -0.374$, $p < 0.001$).

Similarly, Figure 10 demonstrates the relationship between NAb titers and Biacore-binding responses in NAb+ patients. There was a strong positive relationship between the two (Spearman's correlation, $R^2 = 0.537$, $p < 0.001$), both parameters increasing in parallel and with their peak levels coinciding at month 36.

Discussion

In this study, the assessment of the antibody-binding characteristics to IFN-β by SPR technology was effective in providing both quantitative binding responses and qualitative antibody dissociation rates. The results were sensitive and specific enough to distinguish between anti-IFN-β antibodies in treated MS patients and the absence of anti-IFN-β antibodies in healthy donors or in anti-IFN-β antibody negative treated MS patients. However it is well documented that autoantibodies against IFN-β do exist in healthy donors, albeit extremely rare (0.1%), provided antibody assays are sufficiently sensitive (Bendtzen 2003). Using the 96-well microtiter plate format, instead of tubes, enabled the rapid analysis of serial samples of multiple patients.

Initially, it was necessary to determine the appropriate concentrations of IFN-β1a for immobilization, analyte (antibody) flow rate and regeneration buffer. To efficiently immobilize the IFN-β1a antigen to the sensorchip surface, it first had to be electrostatically attracted toward the hydrophilic negatively charged dextran matrix surface. This process of preconcentration was achieved by imparting a net positive

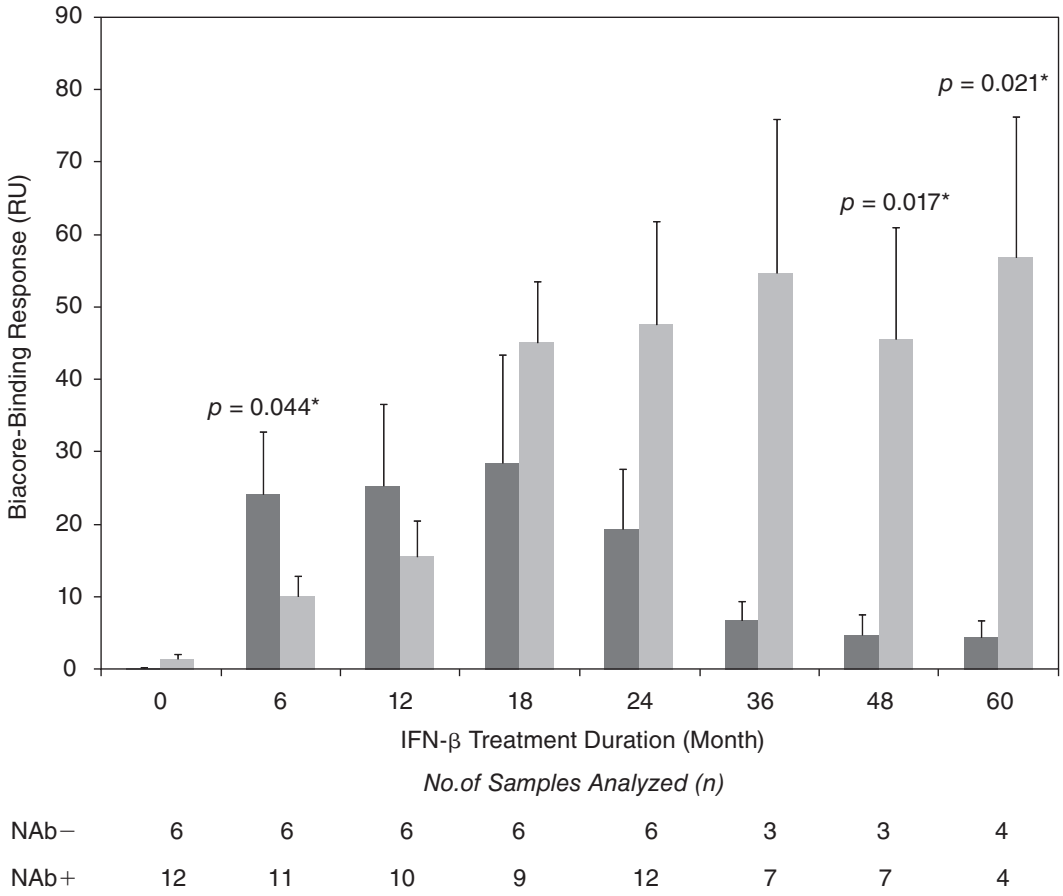

FIG. 6. Antibody-binding responses to IFN-β, as measured by Biacore, discriminate between NAb− ($n = 6$) and NAb+ ($n = 12$) samples. Mean (+SEM) Biacore-binding responses are plotted over time. Differences in antibody binding were statistically significant (Mann–Whitney U test) as indicated (*). [NAb− (black bars), NAb+ (grey bars)].

charge on the IFN-β1a molecule; the isoelectric point (pI) of IFN-β is 9.8, and therefore dissolving it in HBS-EP, P-20 (pH7.4) results in a net positive charge. We used a low surface antigen density of between 135 and 155 RUs of IFN-β1a for immobilization after preliminary experiments showed that a high surface antigen density of 3000 RUs resulted in different serum samples having similar antibody dissociation rates. However, under the ideal conditions of limiting antigen and high analyte (antibody) flow rates, only high-affinity antibody molecules would bind long enough for association and dissociation data to be collected. Low-affinity antibodies are out-competed and would not bind under such conditions. The IFN-β1a was also of high purity, and unlike the commercial available forms administered to patients, it did not contain BSA. This was an absolute necessity as any additives with amine groups would also bind to the sensorchip surface and cause nonspecific binding of antibodies. Nonspecific binding was further reduced by an online referencing system that subtracts the binding curve (sensorgram) obtained by flowing serum sample through the reference surface (no IFN-β1a) from the active surface that contains immobilized IFN-β1a. Sensorgrams were further double-referenced by subtracting out the blank serum diluent curve, and for each patient the pretreatment curve was subtracted from all other subsequent curves. The latter was necessary in order to make comparisons between patients.

Our study demonstrates the dynamics of the binding responses of antibodies to IFN-β, as measured by Biacore, in NAb− and NAb+ patients. A quantitative comparison of the two groups of patients shows that binding antibody responses were higher in NAb− patients, significantly so at month 6 ($p = 0.044$), than in NAb+ patients during the first year of IFN-β therapy. Thereafter, the antibody-binding responses became higher in NAb+ patients compared to NAb− patients, with significant differences being observed at months 48 and 60. Furthermore, whereas the binding response in NAb− patients peaked at month 18, peak antibody-binding responses were attained at month 36 in the NAb+ patients. This pattern of antibody binding, as measured by Biacore, in NAb− and NAb+ patients, is reminiscent of the temporal profile of BAbs, as measured by ELISA, and NAbs, with BAbs peaking early and NAbs attaining peak levels later (Gibbs and others 2002).

We have also examined the IgG subclass distribution of anti-IFN-β antibodies, and our results show a very dynamic pattern in NAb+ patients, with a predominant expression of IgG1 antibodies during the first year of IFN-β therapy,

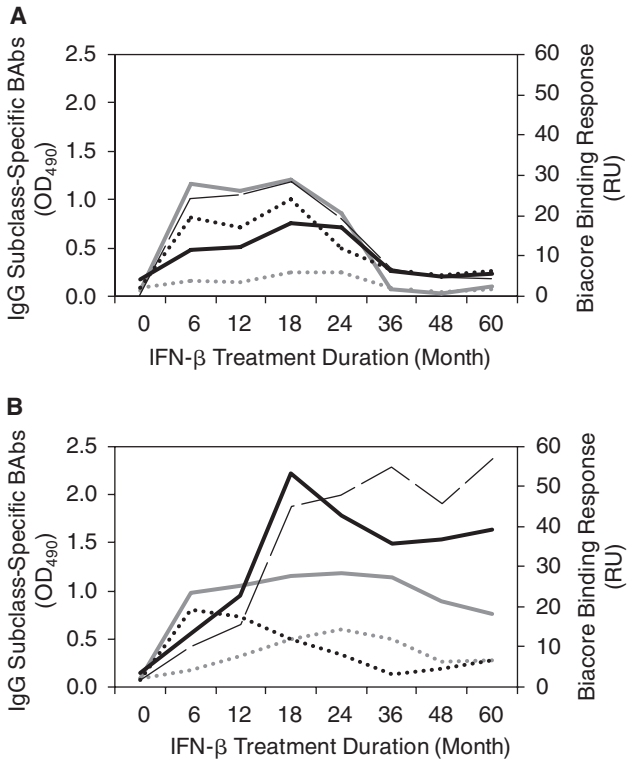

FIG. 7. The patterns of changes in IgG subclass-specificities in relation to Biacore-binding response of anti-IFN-β antibodies in (**A**) NAb− patients ($n = 6$) and (**B**) NAb+ patients ($n = 12$). Data represent mean values. IgG subclasses were measured by ELISA. [Biacore binding (black broken line), IgG1 (grey unbroken line), IgG2 (grey dotted line), IgG3 (black dotted line), and IgG4 (black unbroken line)].

followed by a relative predominance of IgG4 antibodies after month 12. In contrast, IgG subclass distribution in NAb− patients was less dynamic, as no subclass featured prominently. This suggests that there is a shift from IgG1 to IgG4 in NAb+ patients, and not so in NAb− patients.

Not surprisingly, the mean relative antibody affinities among NAb− patients were lower (faster dissociation rates) than among NAb+ patients (slower dissociation rates), confirming previous findings (Gneiss and others 2006). However, using serial samples, our study revealed for the first time the temporal differences in affinity distributions of anti-IFN-β antibodies in NAb− and NAb+ patients. In the same vein, our longitudinal study documented the marked shift from low- to high-affinity antibodies (affinity maturation) in NAb+ patients compared to minimal changes in affinity in NAb− patients. Our study was limited by the fact that as the actual concentration of anti-IFN-β antibodies is unknown, absolute affinity could not be calculated. Thus we measured relative affinity based on relative dissociation rates. Dissociation rates were obtained by fitting curves in a 1:1 Langmuir-binding model which is a simplistic approach for the modeling of the antigen–antibody reaction, as we know that antibodies are bidentate in nature and that in any given serum, there are subpopulations of different antibodies. Thus our analyses of dissociation rates were an average of all the different antibody clones in polyclonal serum. Our Biacore studies were performed with a common antigen, IFN-β1a, even though 50% of the patients were treated with IFN-β1b. This may have resulted in higher antibody-binding responses than if a homologous antigen challenge was used, considering that NAb titers measured using IFN-β1a as challenge antigen are generally higher than titers obtained with IFN-β1b as antigen, regardless of whether the sera are from IFN-β1a or

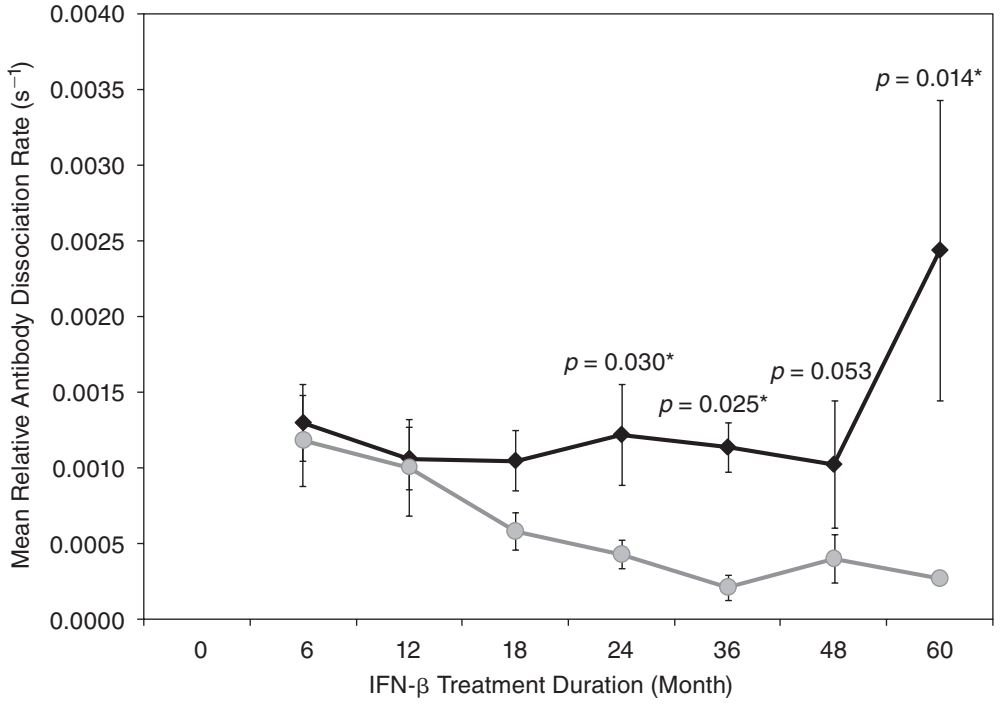

FIG. 8. Rates of relative antibody dissociation distinguish between NAb− and NAb+ sera. These differences are statistically significant (Mann–Whitney U test), as indicated (*). Data represent the mean (±SEM) at different time points during treatment with IFN-β. [NAb− (black line), NAb+ (grey line)].

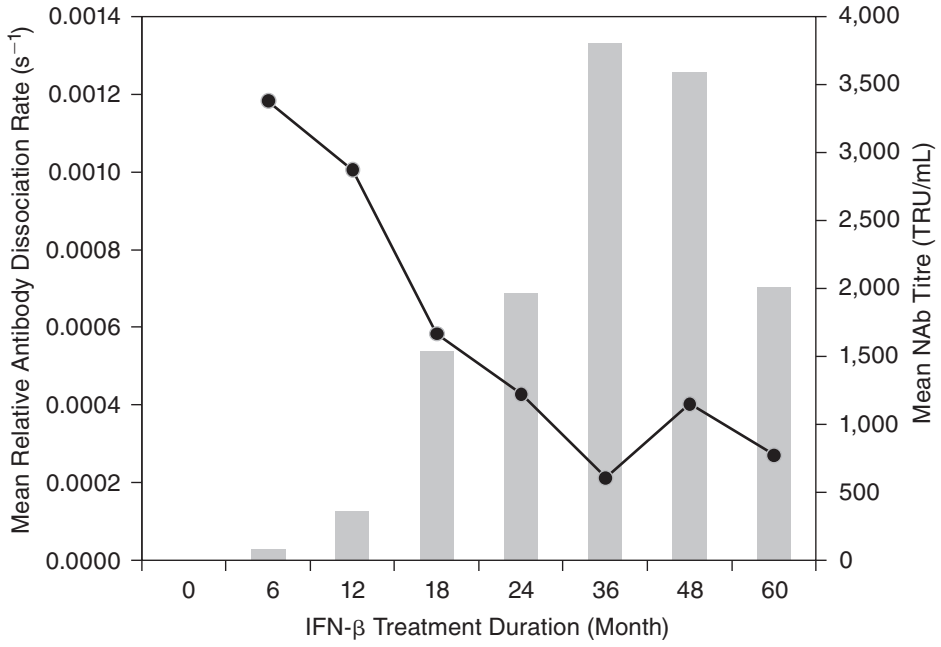

FIG. 9. Changes in NAb titers and relative antibody dissociation rates of the anti-IFN-β immune response in 12 NAb+ patients. There is a strong negative correlation between the two parameters (Spearman's correlation, $R^2 = -0.374$, $p < 0.001$). Data represent the mean of 12 NAb + patients at different time points during treatment with IFN-β. [NAb titers (grey bars), relative antibody dissociation rates (black line)].

IFN-β1b-treated patients (Scagnolari and others 2002; Files and others 2007).

Furthermore, to ascertain whether high NAb titers were indicative of the maturation of the IFN-β immune response, or vice versa, a correlation analysis was performed between NAb titer and antibody dissociation rates. We found a significant relationship between the two (Spearman's correlation, $R^2 = -0.374$, $p < 0.001$). We reveal that there is parallelism between the the NAb titer and affinity of anti-IFN-β antibodies, and that they peak at month 36, a period which coincides with notable reduction in IFN-β clinical efficacy in NAb+ patients, as demonstrated by increased relapse rates and expanded disability scale scores (Boz and others 2007).

A closer examination of the relationship between antibody-binding response, as measured by Biacore, and NAb titers shows that the two are inextricably linked, and that there is a positive correlation between them (Spearman's correlation, $R^2 = 0.537$, $p < 0.001$).

A summation of the profiles of the binding responses and relative antibody dissociation rates of NAb+ and NAb− patients obtained by Biacore analysis warrants us to also conclude that during the first year of IFN-β treatment, NAb− patients possess a higher level of low-affinity antibodies, as opposed to the prevalence of higher levels of high-affinity antibodies among the NAb+ patients after year 1. Thus our biosensor-based approach for the characterization of the

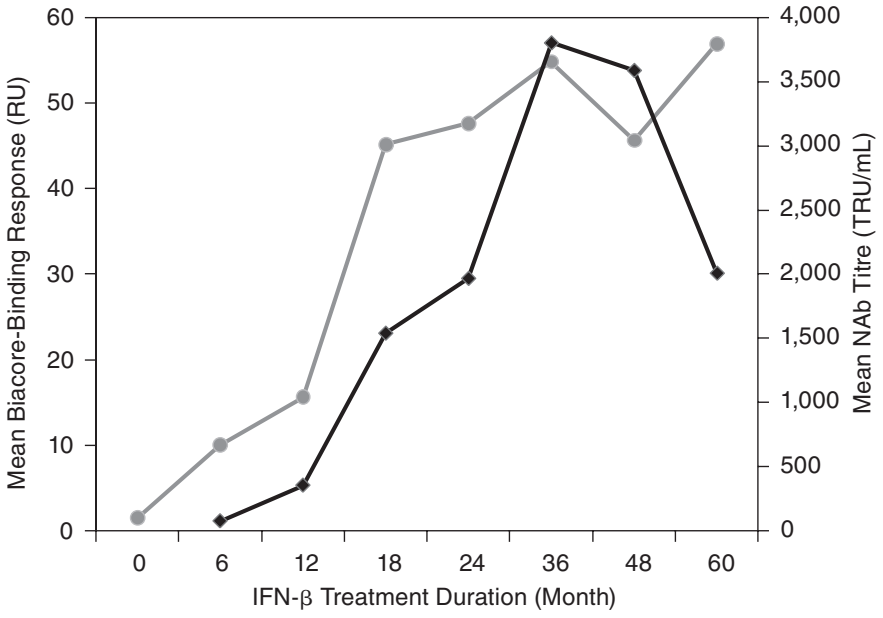

FIG. 10. Profile of binding antibodies as measured by Biacore (Biacore-binding response) and neutralizing antibodies as measured by bioassay, showing the significant positive relationship between the two (Spearman's correlation, $R^2 = 0.537$, $p < 0.001$). Data represent the mean (±SEM) at different time points during treatment with IFN-β, in NAb+ patients ($n = 12$). [Biacore-binding response (grey line), NAb titers (black line)].

immune response against IFN-β reveals a quantitative and qualitative maturation of a subset of BAbs into NAbs. Our study also demonstrates, for the first time, that the affinity maturation of anti-IFN-β antibodies can be objectively monitored in IFN-β-treated MS patients, and that measuring relative antibody affinity contributes to the comprehensive profiling of anti-IFN-β antibodies.

Acknowledgments

We thank Regina Lam and Tariq Aziz for their excellent technical assistance, and members of my Ph.D. Supervisory Committee (Drs. Sidney Grossberg, Susan Goelz, Vincent Duronio, Steven Pelech and Edwin Gong) for their support and direction. We also extend our thanks to the University of British Columbia Biothermodynamics Laboratory for use of the Biacore™ 3000. This work was supported by a Research Studentship (to E.G.) from the Multiple Sclerosis Society of Canada.

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Received 17 December 2007/Accepted 13 May 2008