Expression and regulation of IFNα/β receptor in IFNβ-treated patients with multiple sclerosis

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ABSTRACT

Background: The cytokine interferon beta (IFNβ) is successfully used in the treatment of multiple sclerosis (MS), although there is a high degree of variability in the response. A common mechanism involved in the modulation of responsiveness to cytokines is represented by regulation of their receptor expression through autocrine ligand–mediated loops. The present study is aimed at investigating the regulation of IFNα/β receptor (IFNAR) during IFNβ therapy in patients with MS and at correlating it with the biologic responsiveness to the cytokine.

Methods: Quantitative PCR measurements of IFNAR-1 and the three IFNAR-2 isoforms were performed in 141 patients after short-term and long-term treatment. Patients were also regularly screened for anti-IFNβ neutralizing antibodies (NAbs). IFN-inducible myxovirus resistance protein A messenger RNA was used as an indicator of bioactivity.

Results: Pretreatment levels of IFNAR-2 in patients were lower overall than in controls ($p = 0.038$), and high levels correlated with greater bioactivity. Upon prolonged treatment, NAb-negative patients displayed a state of decreased transmembrane IFNAR-2 expression ($p \leq 0.025$), whereas levels of soluble IFNAR-2 were slightly increased ($p < 0.0001$). The presence of NAbs reversed these effects ($p \leq 0.0056$). In NAb-positive patients, pretreatment expression levels of both transmembrane IFNAR-2 isoforms were significantly lower than in NAb-negative patients ($p \leq 0.0089$).

Conclusions: Findings show that interferon-α/β receptor (IFNAR)-2 isoforms are important regulators of the responsiveness to endogenous and systemically administered interferon beta (IFNβ). They show a dual action, agonistic and antagonistic, that influences both the magnitude and the nature of the biologic response to IFNβ. Levels of IFNAR-2 are regulated with the aim of keeping the body in a state of equilibrium, even when nonphysiologic stimuli are present. *Neurology*® 2008;71:1940–1947

GLOSSARY

AUC = area under the curve; **BAB** = binding antibody; **CRESM** = Centro Riferimento Regionale Sclerosi Multipla; **GAPDH** = glyceraldehyde phosphate dehydrogenase; **IFNβ** = interferon beta; **IFNAR** = interferon-α/β receptor; **iNAb+** = isolated neutralizing antibody positive; **mRNA** = messenger RNA; **MS** = multiple sclerosis; **MxA** = myxovirus resistance protein A; **NAb** = neutralizing antibody; **NAb+** = neutralizing antibody positive; **NAb–** = neutralizing antibody negative; **NAb-DFS** = neutralizing antibody development–free survival; **NS** = not significant; **PBMC** = peripheral blood mononuclear cell; **pNAb+** = persistent neutralizing antibody positive; **RE** = relative expression; **ROC** = receiver operating characteristic; **sc** = subcutaneous; **TRU** = 10-fold reduction unit.

Because of the existence of different cell patterns, there is a high degree of variability in the response to interferon beta (IFNβ) therapy in multiple sclerosis (MS).[1]

IFNβ activity is mediated by the binding to a cellular receptor (interferon-α/β receptor [IFNAR]). IFNAR comprises two subunits, IFNAR-1 and IFNAR-2, which associate with IFNs to form a ternary complex.[2] The major ligand binding subunit, IFNAR-2, exists in three forms derived

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from alternatively spliced transcripts encoded by the IFNAR-2 gene: soluble (IFNAR-2a), transmembrane short form (IFNAR-2b), and transmembrane long form (IFNAR-2c).[2]

A common mechanism involved in the modulation of cytokine responsiveness is the regulation of their receptor expression through autocrine ligand–mediated loops. With respect to the IFNα/β system, it has been shown that IFNAR is internalized and degraded after ligand binding in several cell lines.[3-5]

From a therapeutic perspective, it has been observed that after prolonged IFNα therapy, patients displayed a state of decreased receptor expression and that lack of IFNAR down-regulation correlated with failure of therapy.[6] Recently, this has also been shown in MS patients treated with IFNβ.[7]

Besides receptor down-regulation, prolonged therapies with IFNs often lead to the development of anti-IFN antibodies (binding antibodies [BABs]). A subset of the BABs is of a neutralizing nature (neutralizing antibodies [NABs]) and has been associated with reduced biologic[8,9] and clinical efficacy of IFNβ therapy.[10-13] Because NAbs block IFNAR stimulation,[13] it is argued that in NAb-positive patients there is a lack of regulation of IFNAR expression.

This research assesses IFNAR-1 and the three IFNAR-2 splice variants as possible molecular mechanisms contributing to IFNβ treatment response in MS.

METHODS

Participants. This study was approved by the Regional Ethical Committee of Piedmont and conducted according to the Declaration of Helsinki. Informed consent was obtained from each patient.

One hundred forty-one patients (82 women and 59 men) with definite relapsing–remitting MS according to the McDonald criteria[14] were retrospectively included in this study. Patients were eligible for the study if they had initiated therapy with one of the available IFNβ products/dosing regimens and had been treated for at least 2 years, with regular screening for NAbs. If patients came to the clinic 12 hours after the last IFNβ injection, an additional matched blood sample was taken for myxovirus resistance protein A (MxA) messenger RNA (mRNA) quantification.

Thirty-two individuals (17 females and 15 males) were included as controls. They were healthy individuals without medical illnesses and without any symptoms of viral infection at least 2 weeks before and after blood donation.

Blood samples. At 2 years, we had available regular clinical evaluation as well as regular measurements of NAbs of all 141 patients. On the contrary, matched blood samples for gene expression analyses were only taken from part of those patients. Hence, in planning this study, we had availability of mRNA samples taken from each patient at different time points (table e-1 and figure e-1 on the *Neurology*® Web site at www.neurology.org).

Blood samples for gene expression analyses had been taken from all 141 patients before IFNβ therapy. In 120 of the 141 patients, mRNA samples had been also taken at +3, +12, and +24 hours after the first IFNβ injection. Those samples were used for testing the IFNβ first-dose effect on the transcriptional regulation of IFNAR.

Because 93 of the 141 patients always had come to the clinic 12 hours (12.4 ± 1.6 [range 9–14]) after their last IFNβ injection, they also had been sampled at intervals (+6, +12, +24 months) after initiation of IFNβ therapy. These samples were used for testing the effect of long-term treatment of IFNβ on the transcriptional regulation of IFNAR.

Gene expression analysis. Peripheral blood mononuclear cells (PBMCs) were collected from whole blood by density gradient centrifugation and were subjected to RNA extraction. Thereafter, 0.5 μg of total RNA was reverse transcribed with the High Capacity Reverse Transcription Kit (Applera Italia, Monza, Italy), and PCR was performed using the ABI Prism 7000 Sequence Detection System.

Levels of IFNAR-1 and the three IFNAR-2 isoforms were normalized using the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) as reference. The relative expression (RE) levels of targets were calculated by the comparative cycle threshold method outlined in user bulletin number 2 provided by Applied Biosystems.

For the three IFNAR-2 isoforms, primers and probes were generated on either side of alternative splice events using the Primer Express Software. IFNAR-2 amplicons obtained by PCR were checked using DNA sequencing (ABI Prism 3100 Genetic Analyzer).

For IFNAR-1, MxA, and GAPDH, Applied Biosystems' TaqMan Assay-on-demand gene expression products were used.

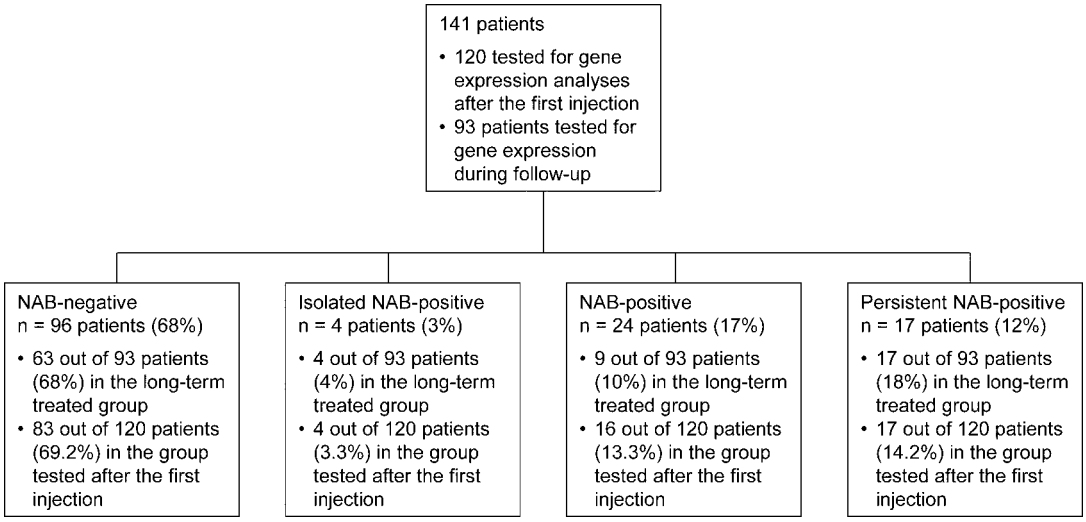
Cytopathic effect assay. Patients were evaluated for the presence of NAbs from study entry and every 3 months during IFNβ treatment. The antiviral cytopathic effect assay was used to detect NAbs because this assay has been recommended by World Health Organization,[15] and it was performed as previously described.[8,10]

The neutralization titer of a serum sample was calculated according to Kawade[16,17] and expressed in 10-fold reduction units per milliliter (TRU/mL).[18] A level of ≥20 TRU/mL was considered the threshold of positivity.

Four categories of patients were identified: NAb-negative (NAb−) patients had no positive samples during follow-up; isolated NAb-positive (iNAb+) patients had a single NAb+ sample or sporadic positivity during follow-up; NAb-positive (NAb+) patients had at least two, but less than seven, consecutive samples positive for NAbs; and persistent NAb-positive (pNAb+) patients had seven consecutive samples positive for NAbs.

Statistical analyses. Data were analyzed using nonparametric statistical tests. Comparisons were performed by using either the Mann–Whitney *U* test or the Wilcoxon signed-ranked test. Multiple group comparisons were performed with either the Kruskal–Wallis test or the Friedman test. Proportions were com-

Figure 1 Flowchart highlighting the overall NAb status



The flowchart outlines the four neutralizing antibody (NAb) categories within the different study groups, i.e., NAb negative, isolated NAb positive, NAb positive, and persistent NAb positive.

pared by using the Fisher exact test, and correlations were calculated with the Spearman rank test.

MxA expression was used as an indicator of biologic responsiveness: patients were monitored for MxA expression after the first IFNβ injection, and line plots were generated showing the change at each time point related to the baseline value. Data were analyzed as area under the curve (AUC) from the line plots, and a Spearman rank correlation of IFNAR expression levels with MxA AUC was calculated.

Finally, we investigated the prognostic value of IFNAR mRNA levels in predicting NAb development by using a threshold of expression generating "high"- and "low"-level groups. This threshold was calculated by receiver operating characteristic (ROC) analysis. Estimates of the optimal cutoff values were made, taking into account sensitivity and specificity, as well as statistical characteristics of the ROC curves. NAb development–free survival (NAb-DFS) was defined as the time from the beginning of IFNβ treatment to NAb development as a result of therapy. An analysis for NAb-DFS was determined using the log-rank test and plotted as a Kaplan–Meier curve.

Analyses were performed using GraphPad PRISM version 4.00 (San Diego, CA), and all reported p values are based on two-tailed statistical tests, with a significance level of 0.05.

RESULTS

NAb status. At baseline, all patients scored negative for NAbs. In the course of 24 months of therapy, NAbs were detectable in 41 of 141 patients (29.1%). These patients were categorized as either pNAb+ (n = 17) or NAb+ (n = 24) (figure 1).

Considering the 93 patients in the long-term treated group, 30 (32.2%) tested positive for NAbs: 17 were pNAb+, 9 were NAb+, and 4 were iNAb+ (figure 1).

Table 1 shows the incidence of NAbs for patients by treatment group. The incidence of pNAb+ was 18% in patients treated with IFNβ-1b, 17% in patients treated with subcutaneous (sc) IFNβ-1a 44 μg, 13% in patients treated with sc IFNβ-1a 22 μg, and 2.5% in patients treated with IM IFNβ-1a. The incidence of pNAb+ was lower in patients treated with IM IFNβ-1a compared with patients treated with all other IFNβ preparations (IM IFNβ-1a vs IFNβ-1b, $p = 0.004$; IM IFNβ-1a vs both sc IFNβ-1a, $p \leq 0.009$; and IFNβ-1b vs both sc IFNβ-1a, $p \geq 0.161$).

IFNAR gene expression analysis. IFNAR-1 expression was comparable between patients and controls ($p = 0.844$). On the contrary, transcripts for both IFNAR-2b and IFNAR-2c were significantly lower in patients than in controls ($p \leq 0.038$). There was no difference in IFNAR-2a expression ($p = 0.0626$).

Expression of IFNAR-1 and of IFNAR-2 variants did not show significant acute phase changes after IFNβ first injection ($p \geq 0.137$).

Dividing long-term treated patients (n = 93) on the basis of their future NAb status, there was a difference in both IFNAR-2b and IFNAR-2c basal expression between pNAb+ and NAb− patients ($p \leq 0.0089$), with approximately 2.6-fold lower expression levels in pNAb+ than in NAb− patients. In NAb− patients, levels at baseline were similar to those in controls ($p \geq 0.934$), whereas levels in pNAb+ patients were lower ($p \leq 0.0021$). In contrast, there were no differences in the basal expression of both IFNAR-1 and IFNAR-2a ($p \geq 0.494$) among NAb− patients, pNAb+ patients, and controls.

In long-term treated, NAb− patients (n = 63), IFNβ administration induced a time-dependent decrease in both IFNAR-2b and IFNAR-2c levels throughout the observation period, with significant suppression by 12 and 24 months ($p \leq 0.025$). In contrast, IFNAR-2a expression levels were signifi-

Table Treatment regimens and NAb status of different groups of patients analyzed in each study step

	IM IFNβ-1a 30 μg given once a week*		sc IFNβ-1b 250 μg given three times a week†		sc IFNβ-1a 22 μg given three times a week‡		sc IFNβ-1a 44 μg given three times a week‡		Total	
	No. of patients	NAb status	No. of patients	NAb status	No. of patients	NAb status	No. of patients	NAb status	No. of patients	NAb status
Basal IFNAR gene expression analysis	40	38 NAb− (95%)	39	21 NAb− (54%)	32	19 NAb− (59%)	30	18 NAb− (60%)	141	96 NAb− (68%)
		1 NAb+ (2.5%)		1 iNAb+ (2%)		2 iNAb+ (6%)		1 iNAb+ (3%)		4 iNAb+ (3%)
		1 pNAb+ (2.5%)		10 NAb+ (26%)		7 NAb+ (22%)		6 NAb+ (20%)		24 NAb+ (17%)
				7 pNAb+ (18%)		4 pNAb+ (13%)		5 pNAb+ (17%)		17 pNAb+ (12%)
First-dose effect analysis	36	34 NAb− (94%)	31	15 NAb− (48%)	28	18 NAb− (64%)	25	16 NAb− (60%)	120	83 NAb− (69%)
		1 NAb+ (3%)		1 iNAb+ (3%)		2 iNAb+ (7%)		1 iNAb+ (8%)		4 iNAb+ (3.3%)
		1 pNAb+ (3%)		8 NAb+ (26%)		4 NAb+ (14.5%)		3 NAb+ (12%)		16 NAb+ (13%)
				7 pNAb+ (23%)		4 pNAb+ (14.5%)		5 pNAb+ (20%)		17 pNAb+ (14%)
Long-term effect analysis	29	27 NAb− (93%)	25	15 NAb− (60%)	20	10 NAb− (50%)	19	11 NAb− (58%)	93	63 NAb− (68%)
		1 NAb+ (3.5%)		1 iNAb+ (4%)		2 iNAb+ (10%)		1 iNAb+ (5%)		4 iNAb+ (4%)
		1 pNAb+ (3.5%)		2 NAb+ (8%)		4 NAb+ (20%)		2 NAb+ (11%)		9 NAb+ (10%)
				7 pNAb+ (28%)		4 pNAb+ (20%)		5 pNAb+ (26%)		17 pNAb+ (18%)

*Avonex (Biogen Idec, Cambridge, MA).
†Betaferon (Shering, Berlin, Germany).
‡Rebif (Serono, Geneva, Switzerland).
NAb = neutralizing antibody; IFNβ = interferon beta; sc = subcutaneous; IFNAR = interferon-α/β receptor; NAb− = NAb negative; NAb+ = positive; iNAb+ = isolated NAb positive; pNAb+ = persistent NAb positive.

cantly up-regulated over the first year of therapy ($p < 0.0001$). While IFNβ administration also caused a reduction in IFNAR-1 expression, the effect was not as marked as for IFNAR-2 and did not reach statistical significance ($p > 0.0978$). A different trend of expression was observed in long-term treated patients with NAbs (pNAb+; n = 17), in whom we observed an increase in IFNAR-2b and IFNAR-2c expression over time ($p \leq 0.0056$). Differently, the expression of both IFNAR-1 and of IFNAR-2a remained unchanged over the follow-up period (figure 2).

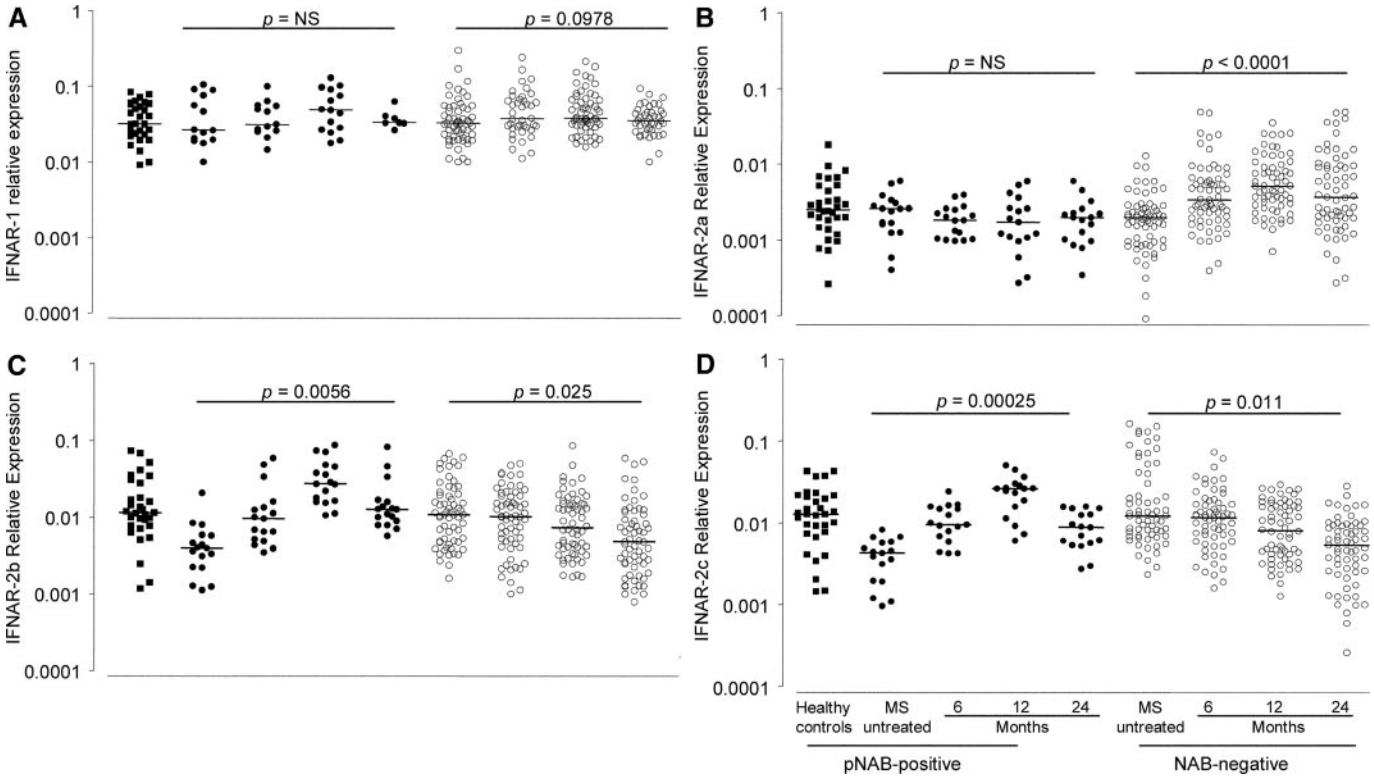
There was no difference between the various types and dosages of IFNβ in regulating both IFNAR-1 and IFNAR-2 expression.

Predictive discriminating value. To confirm the predictive discriminating value of IFNAR-2 basal expression for NAb development, we performed a ROC analysis, determining optimized cutoffs. ROC analysis for both IFNAR-2a and IFNAR-2b were not significant (AUC ≤ 0.471; $p \geq 0.452$), and thus, for those isoforms no cutoff was established. On the contrary, for the IFNAR-2c isoform, the ROC analysis was significant ($p = 0.0031$), giving an AUC of 0.8028 (figure 3A).

The best optimization of sensitivity and specificity was found with an IFNAR-2c expression level of 0.0049 RE, which yielded both sensitivity and specificity of 69%. Of the 141 patients, 74 (52.5%) had a basal IFNAR-2c expression above the cutoff; of these, 13 patients (17.6%) became either pNAb+ (n = 5) or NAb+ (n = 8) during treatment. Of the remaining 67 patients (47.5%), who showed a basal IFNAR-2c expression below 0.0049 RE, 28 (41.8%) developed NAbs (pNAb+, 12; NAb+, 16). Incidence of patients with NAbs was different in the two groups ($p = 0.0047$; odds ratio = 3.167). Lower basal expression of IFNAR-2c was clearly associated with a higher risk of NAbs development ($p = 0.0061$; figure 3B). Interestingly, patients treated with both sc IFNβ-1a preparations and with low IFNAR-2c levels had a higher probability of developing NAbs than did patients treated with IFNβ-1b (sc IFNβ-1a, $p \leq 0.0085$; IFNβ-1b, $p = 0.065$). IM IFNβ-1a–treated subjects were not considered because of the paucity of pNAb+ patients (n = 2).

MxA gene expression. MxA expression levels in untreated patients were similar to those in controls ($p = 0.523$), whereas the IFNβ first injection led to a significant increase of MxA expression (i.e., 29.5-fold) 12 hours later ($p < 0.0001$). Accordingly, in long-term treated NAb− patients (n = 63), after 6 months of chronic treatment, the median MxA expression level was approximately 14-fold higher than the baseline value ($p < 0.0001$), but less than half compared with that observed in treatment-naive pa-

Figure 2 Comparison of IFNAR-1 (A), soluble IFNAR-2a (B), IFNAR-2b (C), and IFNAR-2c (D) messenger RNA levels in 32 healthy controls and 93 long-term IFNβ-treated patients with MS



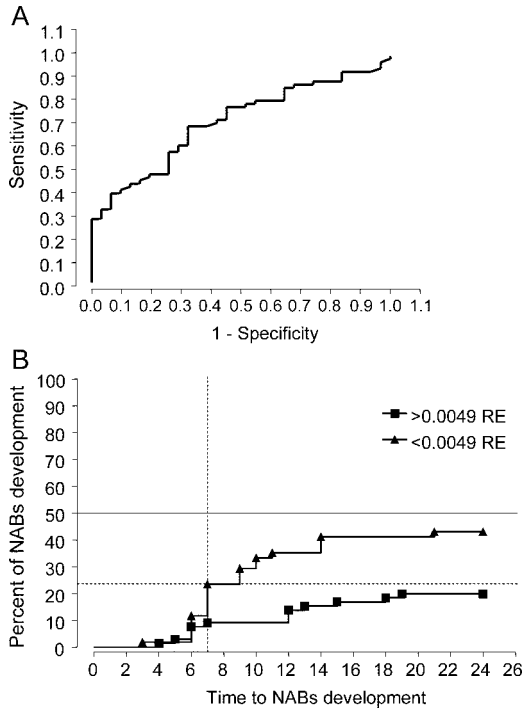
Samples were obtained 12.4 ± 1.6 hours (range 9–14 hours) after the last interferon beta (IFNβ) injection, and patients were tested before and 6, 12, and 24 months after the start of treatment. Patients were subdivided based on their neutralizing antibody (NAb) status: persistent NAb (pNAb) positive (n = 17) and NAb negative (n = 63). In NAb-negative patients, IFNβ administration induced a significant time-dependent decrease in interferon-α/β receptor (IFNAR)-2c and IFNAR-2b transcript levels throughout the observation period. In contrast, expression levels of soluble IFNAR-2a were significantly up-regulated over the first year of therapy. A different trend of expression was observed in pNAb-positive patients, in whom an increase in IFNAR-2b and IFNAR-2c expression over time was detected. On the contrary, the expression level of IFNAR-1 remained unchanged over the whole follow-up in both NAb-negative and pNAb-positive patients. Median values are indicated by the horizontal bars, and *p* values are shown. MS = multiple sclerosis; NS = not significant.

tients after the first injection ($p = 0.0002$). The attenuation of MxA up-regulation observed here in the first months of treatment has already been previously demonstrated.[19-22] Conversely, in the later course of treatment, they showed no change in MxA median level observed at 12 and 24 months of treatment, compared with MxA expression measured at 6 months ($p = 0.2893$). On the contrary, in long-term treated pNAb+ patients, median MxA levels were lower than those of NAb− patients ($p < 0.0001$) and similar to those of untreated patients (figure 4). Particularly, low or moderate levels of NAbs diminished expression markedly, whereas levels greater than 109 TRU/mL resulted in levels not statistically different from those of untreated patients and controls.

Correlation of IFNAR expression with pharmacologic responsiveness measured by AUC on the MxA response. To determine whether it might be possible to predict the responsiveness to IFNβ based on the relative levels of IFNAR expression, we examined the relationship between expression of various IFNAR subunits and responsiveness to stimulation by IFNβ first dose. IFNAR-2c levels correlated with the cumulative MxA induction ($r_{Spearman} = 0.799$; $p < 0.0001$), whereas there was no correlation between MxA induction and expression levels of both IFNAR-1 and IFNAR-2b ($r_{Spearman} \leq 0.1639$; $p \geq 0.0912$). IFNAR-2a expression levels negatively correlated with MxA inducibility ($r_{Spearman} = -0.651$; $p = 0.035$; figure e-2). The correlation was even stronger when IFNAR levels were correlated with MxA levels after 1 year of therapy (data not shown).

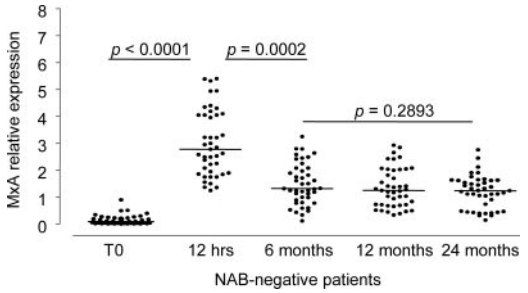
DISCUSSION It is clear from our study that biologic response to IFNβ may vary considerably between patients who express different levels of the IFNAR receptor. Indeed, high levels of IFNAR-2c expression correlated with greater biologic response to IFNβ, whereas such correlation was not true for IFNAR-1 and for both the other IFNAR-2 isoforms. The correlation observed between the levels of expression of IFNAR-2c and the induction responsiveness to IFNβ supports the hypothesis that the biologic response of a tissue type to a specific ligand is dependent on the receptor expression profile in that tissue. In particular, the binding capacity

Figure 3 Predictive value interferon α/β receptor mRNA levels for NAb development



(A) Predictive discrimination of interferon-α/β receptor (IFNAR)-2c messenger RNA levels in 141 untreated patients with multiple sclerosis (MS) vs 63 neutralizing antibody (NAb)-treated patients. Taking into account both sensitivity and specificity, an optimal cutoff was calculated at 0.0049. (B) Thereafter, the NAb development–free survival (NAb-DFS) of patients with MS was evaluated, according to IFNAR-2c basal expression. Analyzing 141 patients with MS, the low (≤ 0.0049 relative expression [RE]) IFNAR-2c expression group (n = 67; 28 events) showed significantly poorer NAb-DFS rates than the high (> 0.0049 RE) IFNAR-2c expression group (n = 74; 13 events) ($p = 0.0047$, log-rank test). Continuous and dotted lines represent the estimated 50th (median) and 25th percentile times.

Figure 4 Analysis of the non-antibody-induced tachyphylaxis in the MxA response



Sixty-three neutralizing antibody (NAb)-negative patients underwent myxovirus resistance protein A (MxA) measurement before and after (+12 hours) first injection and then were sampled at intervals (+6, +12, +24 months) after initiation of interferon beta (IFNβ) therapy. A clear attenuation of MxA up-regulation is observed in the first 6 months of chronic IFNβ treatment, whereas no changes in MxA median levels are shown in the later course of IFNβ treatment, i.e., at 12 and 24 months of treatment. Median values are indicated by the horizontal bars, and p values are shown.

(IFNAR-2c) of the receptor rather than the transcriptional capacity (IFNAR-1) seems to be important for the modulation of biologic responsiveness. Similar results have been obtained in studies that have evaluated the biologic response to other cytokines (e.g., interleukin 10)[23] and growth factors (e.g., epidermal growth factor).[24]

Along with this observation, we have found that IFNAR-2c expression is down-regulated in long-term treated NAb− patients, i.e., patients with a prolonged and fully active stimulation of the IFNAR receptor. Such receptor down-regulation was concurrent with an attenuation of MxA up-regulation, being, therefore, a likely reason for it. Interestingly, we have also observed over time a slight increase in soluble IFNAR-2a expression. Recent studies showed that soluble IFNAR-2a has both agonistic and antagonistic proprieties on IFN activity,[25] suggesting that this isoform could be a potential key regulator for the actions of IFNs. Because of the kinetics of IFNAR-2 induction, it is reasonable to suggest that the induction of soluble IFNAR-2a acts as a classic negative feedback loop whereby IFNβ induces the expression of an inhibitor of itself.

Unlike IFNAR-2, IFNAR-1 expression only showed a slight down-regulation along the treatment period, highlighting that the expression of the IFNAR-2c might be more highly regulated by IFNs than expression of IFNAR-1. This hypothesis agrees with the results of a study where a differential regulation of the expressions of IFNAR-1 and IFNAR-2 subunits was shown in dendritic cells during their maturation.[26] On the other hand, this hypothesis seems to partly contradict earlier work where IFNAR-1 was shown to play an important role for IFN sensitivity, as well as IFNAR-2.[27,28] However, it has been reported that IFNAR-1 expression is regulated by post-transcriptional rather than transcriptional mechanisms, as observed for IFNAR-2.[29] Therefore, mRNA measurements, as used here, do not allow a correct evaluation of IFNAR-1 regulation.

A different modulation was observed in NAb+ patients, in whom the presence of NAbs reversed all these effects, i.e., increased IFNAR-2c without modifying both IFNAR-1 and IFNAR-2a expression. NAbs interfere with the interaction between IFNβ and its receptor, which in turn blocks downstream IFN signaling and the expression of IFN-stimulated gene products. The loss of bioactivity was confirmed by the lack of MxA expression detected in NAb+ patients. In those patients, there was no stimulation of the receptor, which in turn increases its binding capacity. Thus, for the formation of an effective

IFNβ-mediated response, the antagonistic effects of NAbs need to be neutralized by a concomitant increase in IFNAR-2c expression.

Another interesting result was that pretreatment expression levels of IFNAR-2c in MS patients were overall lower than in controls; this result is in accord with a previous study.[14] Nevertheless, as the expression levels in MS patients were rather widely distributed, it might be more accurate to say that there was a fraction of patients whose PBMCs have lower IFNAR-2c expression levels than controls or other MS patients. This statement seems to be confirmed by the fact that initial low IFNAR-2c expression was found in a group of patients showing a significantly higher risk of developing NAbs, also suggesting that IFNβ immunogenicity may be, at least in part, related to IFNAR-2 expression.

A possible explanation for the higher risk of developing NAbs observed in patients with a lower pretreatment IFNAR-2c expression might be the longer circulation time of the injected IFNβ molecules. The lower expression of the IFNAR-2c subunit on the cell surface could significantly decrease the binding of the IFNβ molecules, increasing their circulating time. This longer circulation time could then lead to an increase of immunogenicity, particularly in patients treated with high-dose and high-frequency regimens, in whom there are greater circulating concentrations of IFNβ.

Another fascinating explanation might be the existence of a negative feedback acting through the production of specific autoantibodies. In patients with a low pretreatment IFNAR-2c expression, cells could be physiologically unable to respond to high concentrations of IFNβ. As a consequence, the immune system could mount a "beneficial" autoantibody response to IFNβ. This response counteracts, to a certain extent, the hyperstimulation. This natural counteraction is illustrated in animal models of autoimmunity,[30-32] and evidence is provided that it occurs in humans too.[33]

In both instances, findings indicate that the regulation of IFNAR-2 expression is an important way of modulating the responsiveness to endogenous and systemically administered IFNβ. IFNAR-2 isoforms show a dual action, agonistic and antagonistic, that influences both the magnitude and the nature of the biologic response to IFNβ. Importantly, these data suggest that the levels of IFNAR-2 are regulated with the aim of keeping the body in a state of equilibrium, even when nonphysiologic stimuli are present.

Nevertheless, it is noteworthy that both host-related and product-related factors have impact on immunogenicity, because part of the anti-IFNβ antibodies are surely due to the foreign nature of the drug, i.e., either impurities or aggregates that break B-cell tolerance.[34] This statement agrees with the previous observation that by improved recombinant IFNβ formulations, immunogenicity decreases, but does not disappear completely.[35-37]

AUTHOR CONTRIBUTIONS

Statistical analyses were conducted by F.G.

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REFERENCES

1. Rio J, Nos C, Tintoré M, et al. Defining the response to interferon-beta in relapsing-remitting multiple sclerosis patients. Ann Neurol 2006;59:344–352.
2. Uze G, Luftalla G, Morgensen KE. α and β interferons and their receptors and their friends and relations. J Interferon Cytokine Res 1995;15:3–26.
3. Cleveland MG, Lane RG, Klimpel GRR. Enhanced IFN-α/β and defective IFN-γ production in chronic GVHD: a potential mechanism for immunosuppression. Cell Immunol 1987;110:120–130.
4. Zoon KC, Arnheiter H, Nedden DZ, Fitzgerald DJP, Willingham MC. Human interferon alpha enters cells by receptor-mediated endocytosis. Virology 1989;130:195–203.
5. Lau AS, Hannigan GE, Freedman MH, Williamo BRG. Regulation of interferon receptor expression in human blood lymphocytes in vitro and during interferon therapy. J Clin Invest 1986;77:1632–1638.
6. Billard C, Ferbus D, Diez RA, et al. Correlation between the biological and therapeutic effects of interferon-alpha in low-grade nodular non-Hodgkin's lymphoma: lack of in vivo down-regulation and reduced affinity of IFN-alpha receptors in unresponsive patients. Leuk Res 1991;15:121–128.
7. Oliver B, Mayorga C, Fernandez V. Interferon receptor expression in multiple sclerosis patients. J Neuroimmunol 2007;183:225–231.
8. Bertolotto A, Gilli F, Sala A, et al. Persistent neutralizing antibodies abolish the interferon β bioavailability in MS patients. Neurology 2003;60:634–639.
9. Deisenhammer F, Reindel M, Harvey J, Gasse T, Dilitz E, Berger T. Bioavailability of interferon beta 1b in MS patients with and without neutralizing antibodies. Neurology 1999;52:1239–1243.
10. Malucchi S, Sala A, Gilli F, et al. Neutralizing antibodies reduce the efficacy of betaIFN during treatment of multiple sclerosis. Neurology 2004;62:2031–2037.
11. Kappos L, Clanet M, Sandberg-Wollheim M, et al. European Interferon Beta-1a IM Dose-Comparison Study Investigators. Neutralising antibodies and efficacy of interferon beta-1a: a 4-year controlled study. Neurology 2005;65:40–47.
12. Francis GS, Rice GP, Alsop JC, PRISMS Study Group. Interferon beta-1a in MS: results following development of neutralizing antibodies in PRISMS. Neurology 2005;65:48–55.

13. Sorensen PS, Deisenhammer F, Duda P, et al. for the EFNS Task Force on Anti-IFNβ Antibodies in Multiple Sclerosis. Guidelines on use of anti-IFNβ antibody measurements in multiple sclerosis: report of an EFNS Task Force on IFNβ antibodies in multiple sclerosis. Eur J Neurol 2005;12:817–827.
14. McDonald WI, Compston A, Edan G, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the international panel on the diagnosis of multiple sclerosis. Ann Neurol 2001;50:121–127.
15. WHO Expert Committee on Biological Standardisation. Thirty-Fifth Report. WHO Technical Report Series 725. Geneva: World Health Organization; 1985.
16. Kawade Y. Quantitation of neutralization of interferon by antibody. Meth Enzymol 1986;119:558–573.
17. Kawade Y, Finter N, Grossberg SE. Neutralization of the biological activity of cytokines and other protein effectors by antibody: theoretical formulation of antibody titration curves in relation to antibody affinity. J Immunol Methods 2001;278:127–144.
18. Grossberg SE, Kawade Y, Kohase M, Klein JP. The neutralization of interferons by antibody, II: neutralizing antibody unitage and its relationship to bioassay sensitivity—the tenfold reduction unit J Interferon Cytokine Res 2001; 21:743–755.
19. Vallittu AM, Halminen M, Peltoniemi J, et al., the Finnish Beta-Interferon Study Group. Neutralizing antibodies reduce MxA protein induction in interferon beta-1a-treated MS patients. Neurology 2002;58:1786–1790.
20. Vallittu AM, Salmi AA, Erälinna JP, the Finnish Beta-Interferon Study Group. MxA protein induction in MS patients treated with intramuscular IFNbeta-1a. Neurol Sci 2006;26:438–443.
21. Gniadek P, Aktas O, Wandinger KP, et al. Systemic IFN-beta treatment induces apoptosis of peripheral immune cells in multiple sclerosis patients. J Neuroimmunol 2003; 137:187–196.
22. Gilli F, Bertolotto A, Sala A, et al. Neutralizing antibodies against IFN-beta in multiple sclerosis: antagonization of IFN-beta mediated suppression of MMPs. Brain 2004; 127(pt 2):259–268.
23. Mathurin P, Xiong S, Kharbanda KK, et al. IL-10 receptor and coreceptor expression in quiescent and activated hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol 2002;282:G981–G990.
24. Sundaresan S, Roberts PE, King KL, Sliwkowski MX, Mather JP. Biological response to ErbB ligands in non-transformed cell lines correlates with a specific pattern of receptor expression. Endocrinology 1997;139:4756–4764.
25. Hardy MP, Owczarek CM, Trajanovska S, Liu X, Kola I, Hertzog PJ. The soluble murine type I interferon receptor Ifnar-2 is present in serum, is independently regulated, and has both agonistic and antagonistic properties. Blood 2001;97:473–482.
26. Gauzzi MG, Canini I, Eid P, Belardelli F, Gessani S. Loss of type I IFN receptors and impaired IFN responsiveness during terminal maturation of monocyte-derived human dendritic cells. J Immunol 2002;169:3038–3045.
27. Dupont SA, Goelz S, Goyal J, Green M. Mechanisms for regulation of cellular responsiveness to human IFN-beta 1a. J Interferon Cytokine Res 2002;22:491–501.
28. Colamonici O, Yan H, Domanski P. Direct binding to and tyrosine phosphorylation of the alpha subunit of the type I interferon receptor by p135tyk2 tyrosine kinase. Mol Cell Biol 1994;14:8133–8142.
29. Fantuzzi L, Eid P, Malorni W, et al. Post-translational up-regulation of the cell surface-associated component of the human type I interferon receptor during differentiation of peripheral blood monocytes: role in the biological response to type I interferon. Eur J Immunol 1997;27:1075.
30. Youssef S, Maor G, Wildbaum G, Grabie N, Gour-Lavie A, Karin N. C-C chemokine-encoding DNA vaccines enhance breakdown of tolerance to their gene products and treat ongoing adjuvant arthritis. J Clin Invest 2000;106: 361–371.
31. Salomon I, Netzer N, Wildbaum G, Schif-Zuck S, Maor G, Karin N. Targeting the function of IFN-gamma-inducible protein 10 suppress ongoing adjuvant arthritis. J Immunol 2002;169:2685–2693.
32. Wildbaum G, Netzer N, Karin N. Tr1 cell-dependent active tolerance blunts the pathogenic effects of determinant spreading. J Clin Invest 2002;110:701–710.
33. Wildbaum G, Nahir MA, Karin N. Beneficial autoimmunity to proinflammatory mediators restrains the consequences of self-destructive immunity. Immunity 2003;19: 679–688.
34. Schellekens H. Bioequivalence and the immunogenicity of biopharmaceuticals. Nat Rev Drug Discov 2002;1:457–462.
35. Jacobs LD, Cookfair DL, Rudick RA, et al. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. Ann Neurol 1996;39:285–294.
36. McKeage K, Wagstaff AJ. Subcutaneous interferon-beta-1a: new formulation. CNS Drugs 2007;21:871–876.
37. Giovannoni G, Barbarash O, Casset-Semanaz F, et al., RNF Study Group. Immunogenicity and tolerability of an investigational formulation of interferon-beta 1a: 24- and 48-week interim analyses of a 2-year, single-arm, historically controlled, phase IIIb study in adults with multiple sclerosis. Clin Ther 2007;29:1128–1145.