Interferon Inhibitory Activity in Patients With Multiple Sclerosis

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Background: Interferon inhibitory activity (IIA) is a logical candidate for explaining neutralizing antibody–negative partial responsiveness to interferon beta in multiple sclerosis (MS), but its role has not been evaluated.

Objective: To investigate the role of IIA and soluble interferon-α/β receptor (sIFNR) in determining response of patients with MS to interferon beta therapy.

Design: Parallel-group, open-label study.

Setting: Baird Multiple Sclerosis Center, Buffalo, NY.

Patients: Blood was obtained before and 24 hours after injection of interferon beta-1a from 38 anti–interferon beta neutralizing antibody–negative patients with relapsing-remitting MS and 16 untreated healthy controls. On the basis of clinical parameters of response to interferon beta therapy, the patients were divided into stable or good-responder (n=20) and active or partial-responder (n=18) groups.

Main Outcome Measures: Quantitative analyses of magnetic resonance imaging were obtained; the IIA and sIFNR levels were measured using bioassay and enzyme-linked immunosorbent assay, respectively.

Results: The IIA and sIFNR levels were elevated in MS patients compared with controls (P<.001). The IIA levels were higher in active or partial responders compared with stable or good responders (P<.001); the sIFNR levels were not different between groups. The Extended Disability Status Score and T2 lesion volumes were higher in the active or partial-responder group compared with the stable or good-responder group. Interferon beta-1a did not have short-term effects on the IIA and sIFNR levels. In univariate general linear model and stepwise regression analyses, IIA levels were associated with T2 lesion volume.

Conclusion: The levels of IIA are associated with increased MS disease activity and with responsiveness to interferon beta therapy in anti–interferon beta neutralizing antibody–negative MS patients.

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Approximately 30% to 40% of patients with multiple sclerosis (MS) respond well to treatment with interferon beta, whereas the remaining patients exhibit varying extents of partial responsiveness. Neutralizing antibodies (NABs), which provide a biologically intuitive mechanism for partial responsiveness, occur in 5% to 25% of MS patients treated with interferon beta-1a. However, most MS patients partially responsive to interferon beta are NAB negative. The molecular mechanism(s) underlying NAB-negative interferon beta partial responsiveness in MS are not well understood.

Our group first hypothesized and later confirmed that the heterogeneity of interferon responses in patients with malignancies was caused by circulating interferon inhibitory activity (IIA) and free-soluble interferon-α/β receptors (sIFNR), whose expression was increased primarily in nonresponsive patients. High levels of IIA were also found in late-stage adenocarcinomas, including those of the colon, prostate, uterus, and breast. When the neoplasm was removed by surgery or reduced with radiation, a significant reduction in IIA occurred. Serum samples from clinical conditions such as vasculitis of systemic lupus erythematosus, Wegener granulomatosis, and AIDS also contain IIA. The highest IIA level was found in patients with AIDS who later developed lymphoma and Kaposi sarcoma. Healthy individuals do not have significant IIA in their circulation. The IIA is a logical candidate for explaining NAB-negative partial responsiveness to interferon beta in MS, but its role has not been evaluated. This research assesses IIA and sIFNR as possible molecular mechanisms contributing to interferon beta treatment response heterogeneity in MS.
**STUDY POPULATION**

All patients included in the study had a diagnosis of MS according to the criteria of McDonald et al, with a relapsing-remitting disease course, and were receiving interferon beta therapy for a minimum of 2 years. All patients had a baseline clinical neurologic evaluation (Extended Disability Status Score [EDSS]) and magnetic resonance imaging (MRI) assessments and tested negative for anti–interferon beta NABs.

The patients were divided into 2 groups based on their responses to interferon beta treatment. Group 1 consisted of 20 MS patients who were stable or good responders to interferon beta therapy. These patients were relapse free and had stable EDSSs for the preceding 2 years of interferon beta-1a treatment. The patients had no gadolinium-enhancing lesions and no new or enlarging T2 lesions on the MRI performed at study entry compared with the clinical MRI obtained in the previous year. Group 2 consisted of 18 MS patients defined as active or partial responders to interferon beta-1a therapy. These patients had one or more documented relapses in the preceding 12 months with or without concomitant changes in MRI activity.

Of the 38 patients, 35 patients were taking 30 µg, once weekly, of intramuscular interferon beta-1a (AVONEX; Biogen Idec, Cambridge, Mass) and 3 patients from group 2 were taking 44 µg, 3 times weekly, of subcutaneous interferon beta-1a (Rebif; Serono, Rockland, Mass). Serum samples from 16 healthy individuals (8 men and 8 women; mean ± SD age, 35.3 ± 14.6 years; range, 18-65 years) were used as controls.

**LABORATORY PROTOCOLS**

Patient blood and serum samples were obtained before and 24 hours after interferon beta injection. Serum, plasma, and whole blood lysate was obtained and stored at −70°C. All laboratory analyses were conducted on blinded samples.

**INTERFERON ACTIVITY ASSAY**

Interferon antiviral activity was assayed on human foreskin fibroblast (BG-9) cells by the dye uptake method of Finfer. Using vesicular stomatitis virus as a challenge. The BG-9 cells were maintained in minimal essential medium that contained nonessential amino acids and 10% fetal bovine serum. All titers are expressed as international units with reference human interferon alfa provided by the National Institutes of Health.

**IIA ASSAY**

The IIA was measured by mixing serial dilutions of patient serum with 100 IU of recombinant interferon beta-1a, incubating for 2 hours at 37°C, and assaying for the remaining interferon antiviral activity using the dye uptake method previously described. The following controls were used in all inhibitor assays: (1) patient serum to determine any carryover interferon antiviral activity present in the serum, (2) pooled normal human serum plus 100 IU of recombinant interferon beta-1a to document that no IIA was present in the normal human serum, and (3) 100 IU of recombinant interferon beta-1a. All controls were treated identically to patient during inhibitor assays. One unit of IIA is defined to block the antiviral activity of 25 IU of recombinant interferon beta-1a. The inhibitor titer is the reciprocal of the serum dilution needed for 1 U of IIA.

**sIFNR ASSAY**

The details of the sandwich enzyme-linked immunosorbent assay that was used to measure circulating sIFNR levels are described elsewhere. Purified sIFNR receptor protein (for the standard curve) and anti-sIFNR antibodies were provided by Manachem Rubinstein, PhD (Weizmann Institute, Rehovot, Israel). The remaining immunological reagents were from Jackson ImmunoResearch Laboratories Inc (West Grove, Pa) and R&D Systems (Minneapolis, Minn).

**ANTIBODIES TO INTERFERON BETA**

Anti–interferon beta NAB status was evaluated at an independent laboratory blinded to patient status. Serum samples were first tested for binding antibodies (BABs) using a capture enzyme-linked immunosorbent assay. The BAB-positive samples (titers ≥ 0.8 laboratory units [LU]) were analyzed for NABs using a viral cytopathic effect assay. All patients were negative for NABs (ie, they either were BAB negative [titers < 0.8 LU] or had a NAB titer < 20).

**β2-MICROGLOBULIN PROTEIN**

The β2-microglobulin protein levels were measured in pretreatment and posttreatment serum samples using a commercial immunoassay (MP Biomedical, San Diego, Calif).

**MRI ANALYSIS**

Clinical MRI studies were obtained for 37 patients on a 1.5-T scanner (Signa 4X-LX; General Electric, Milwaukee, Wis) using standardized procedures. For each image, T2-weighted imaging, 3-dimensional spoiled gradient echo T1-weighted imaging, conventional spin-echo T1-weighted imaging, and fluid-attenuated inversion recovery images were obtained. The gadolinium-enhanced conventional spin-echo T1-weighted imaging sequence was obtained within 5 minutes after an intravenous bolus injection of gadolinium–diethylenetriamine pentaacetic acid (0.1 mmol/kg).

Blinded MRI analysts at the Buffalo Neuroimaging Analysis Center in Buffalo, NY, conducted computations of T2 and T1 lesion volume (LV) and brain atrophy. The T2LV was calculated using a reproducible, semiautomated local thresholding technique for lesions. The lesions were outlined on fluid-attenuated inversion recovery images on each axial section (T2-weighted images were used to increase confidence in lesion detection). A conservative approach for the calculations of lesions on T1-weighted images was used, as previously described.

For brain extraction and tissue segmentation, Hybrid SIENAX, a modified, fully automated version of the SIENAX cross-sectional brain atrophy analysis tool (Oxford Centre for Functional Magnetic Resonance Imaging of the Brain, Oxford, England), was used. The brain parenchymal fraction (BPF) was calculated from the gray matter (GM), white matter (WM), and cerebrospinal fluid (CSF) as follows:

\[ BPF = \frac{\text{GM} + \text{WM}}{\text{GM} + \text{WM} + \text{CSF}} \]

The measured scan-rescan variability for BPF was 0.1%.

**DATA ANALYSIS**

The SPSS statistics program (SPSS Inc, Chicago, Ill) was used. The t test was used for interferon activity, IIA, and sIFNR; the Mann-Whitney U test was used for EDSS.
Repeated-measures analysis was used to compare the pre– and post–interferon beta treatment levels of interferon activity, IIA, and sIFNR between the stable or good-responder and active or partial-responder groups. The univariate general linear model (GLM) was used for statistical analysis of MRI variables: all models included sex as a factor and disease duration and treatment duration as covariates; additional variables, such as interferon beta response status, IIA, sIFNR, and interferon activity, were included in certain analyses. The regression procedure included sex, age, disease duration and treatment duration, posttreatment interferon activity, pretreatment IIA, and sIFNR levels as variables. A forward stepwise procedure with \( P = .05 \) for entry and \( P = .10 \) for exit was used.

**RESULTS**

**DEMOGRAPHIC AND CLINICAL CHARACTERISTICS**

The Table summarizes the characteristics of the entire sample (n=38) and the stable or good-responder and the active or partial-responder groups. The 2 groups are representative, and their mean male-female ratios \( (P = .43) \) and treatment \( (P = .45) \) and disease durations \( (P = .82) \) were similar; however, patients in the active or partial-responder group had higher EDSSs \( (P = .01; \text{Mann-Whitney } U \text{ test}) \), were younger \( (P = .002, t \text{ test}) \), and had earlier age at onset \( (P = .01; t \text{ test}) \). These characteristics are consistent with the findings of Waubant et al.2

**IIA, sIFNR, AND INTERFERON ACTIVITY**

**Figure 1 A and B** compares the IIA and sIFNR levels, respectively, in MS patients and controls. Fifteen controls were analyzed for IIA and sIFNR. The IIA levels in all control samples were below the limit of detection of the assay, and these were assigned a value of 0.08 U/mL, the lower limit of detection, for statistical analysis. The IIA and sIFNR expression in controls (sIFNR in controls: mean ± SD, 18.7±9.4 ng/mL) was significantly lower than in stable or good responders \((P<.001; t \text{ test})\).

The predose levels of IIA in active or partial responders \( (\text{mean} ± SD, 3.53±0.20 \text{ U/mL}) \) were significantly higher \( (P<.001; t \text{ test}) \) than in the stable or good responder group \( (\text{mean} ± SD, 1.94±0.19 \text{ U/mL}) \). Predose sIFNR levels in the active or partial-responder group \( (\text{mean} ± SD, 51.9±7.2 \text{ ng/mL}) \) vs the stable or good-responder group \( (\text{mean} ± SD, 67.4±0.20 \text{ ng/mL}) \) were
not statistically significantly different \((P = .12; \ t\ test)\). Figure 2A and B summarizes IIA and sIFNR levels, respectively, in the stable or good-responder and active or partial-responder groups before and 24 hours after interferon beta-1a administration. There were no significant acute effects of interferon beta treatment on either IIA \((P = .23)\) or sIFNR \((P = .45)\) for time effects in repeated-measures analysis. Separate statistical comparisons of the 44-µg subcutaneous interferon beta-1a regimen are not feasible because these patients were all in the active or partial-responder group and the sample size was small. The acute effect of interferon beta treatment on mean IIA and sIFNR levels in the patient subset treated with interferon beta-1a was qualitatively similar to the remainder of the active or partial-responder group.

Four of 38 patients (3 from the active or partial-responder group and 1 from the stable or good-responder group) had interferon beta–binding antibodies greater than 8 LU; none of these samples were NAB positive. There were no associations between BAB titers and the IIA (Spearman correlation coefficient, \(\rho = 0.14; P = .39\)) or sIFNR levels (Spearman correlation coefficient, \(\rho = -0.07; P = .70\)).

At 24 hours after treatment, modest but statistically significant \((P < .001\) for within-sample time effects in repeated-measures analysis) increases in interferon activity were observed in both the stable or good-responder (Figure 3A) and active or partial-responder (Figure 3B) groups. However, both groups had similar interferon activity \((P = .91\) in repeated-measures analysis). The interferon activity in the interferon beta-1a–treated subgroup was qualitatively similar to the other active or partial-responder patients. The increases in interferon activity were observed in all but 2 patients. Interestingly, both patients without the interferon activity increase after dosing were active or partial responders. Although a detailed pharmacokinetic profile is unavailable, this finding is consistent with the possibility that factors related to interferon beta disposition (eg, low rates of absorption from the site of injection and/or high rates of elimination or inactivation) could be a contributing factor in a subset of active or partial responders.

\(\beta_2\)-MICROGLOBULIN UP-REGULATION

Figure 4 summarizes the serum \(\beta_2\)-microglobulin levels before and 24 hours after interferon beta-1a administration. There was a significant increase in \(\beta_2\)-microglobulin levels in the stable or good-responder group \((P = .03; \text{paired } t\ test)\) but not in the active or partial-responder group \((P = .64; \text{paired } t\ test)\). These findings are consistent with an attenuated response to the induction of the \(\beta_2\)-microglobulin biomarker in the active or partial-responder group.

MRI ASSOCIATIONS

Quantitative MRI parameters were available for 37 patients (Table). The mean T2 LV was higher in the active or partial-responder group compared with the stable or good-responder group \((P = .04; \text{Mann-Whitney } U\ test)\).
The associations of MRI parameters with pretreatment IIA, sIFNR, and posttreatment interferon activity levels were assessed by GLM and regression analyses. The posttreatment IIA and sIFNR and pretreatment interferon activity levels were strongly correlated with the respective pretreatment IIA and sIFNR and posttreatment IFN activity levels and were not included as covariates.

In the GLM analysis, the T2 LV was associated (F=5.89; \( P = .001 \) for final overall model) with the pretreatment IIA levels (\( P = .03 \)) and disease and treatment durations (\( P = .02 \) and \( P = .04 \), respectively). Regression analyses also indicated a significant association (\( R = 0.56; F = 7.22; P = .003 \) for the final model) between T2 LV and the pretreatment IIA (\( P = .04 \); standardized \( \beta = 33 \)) levels and disease duration (\( P = .02 \); standardized \( \beta = 40 \)). The magnitudes of the standardized \( \beta \)-coefficients indicate that IIA makes an important contribution to T2 LV heterogeneity. The 3-dimensional graph in Figure 5 shows the dependence of T2 LV on IIA levels and disease duration; the regression plane with pretreatment IIA levels and disease duration is superimposed. The graph visually demonstrates that T2 LV increases with increased IIA. The IIA, sIFNR, and IFN activity levels were not significantly associated with the T1 LV and BPF measures. These findings are consistent with an adverse association between IIA levels and T2 LV in MS.

We have investigated for the first time, to our knowledge, the levels and potential contributions of IIA and sIFNR to the heterogeneity of interferon beta treatment responses in NAB-negative MS patients. Our results demonstrate that IIA and sIFNR are increased in MS patients treated with interferon beta relative to controls and that IIA is associated with clinical outcome and T2 LV.

Clinical studies of interferon beta in MS indicate a 30% to 40% general clinical benefit, and 40% to 80% of patients have complete suppression of new gadolinium-enhancing lesions, depending on the dosing regimen. In the type 1 interferon treatment of hepatitis, viral load and hepatic enzyme levels allow monitoring of treatment response, but the treatment response variables for interferon beta in MS have been difficult to ascertain because of the interindividual variability in disease severity.

Our results require larger clinical studies for validation because they were obtained in a relatively small number of MS patients (\( n = 38 \)). Our responder definition combined available clinical and MRI evidence and is similar to those used in recent trials of combination therapies for patients with active disease treated with interferon beta (eg, the AVONEX Combination Trial [ACT] for methotrexate and the Safety and Efficacy of Natalizumab IN Combination With AVONEX [Interferon Beta-1a] in Patients With RELapsing-Remitting MS [SENTINEL] trial for natalizumab). Longer-term observations of interferon beta partial responsiveness may be more reliable but are ethically problematic given the continuous and irreversible nature of MS. Thus, classification of MS patients into responders and nonresponders is always based on incomplete information, and alternative definitions, such as those based on within-patient relapse rate, MRI, and disability changes, potentially might provide a different view of the role of the IIA.

The maximum interferon beta levels after intramuscular administration are 3 to 30 IU/mL, whereas the mean IIA level in active or partial responders (3.53 U/mL) has the potential to inhibit 88 IU/mL of interferon. This may explain the attenuation with interferon beta effects in the presence of high IIA. Additionally, we note that there was overlap in the IIA distributions of stable or good-responder and active or partial-responder groups, with some active patients having low IIA and some stable patients having high IIA. The exact temporal dependence of IIA and MS activity and interferon beta treatment effect is not known. It is possible that the high IIA outliers in the stable or good-responder group may reflect subclinical activity preceding an eventual change in clinical status, and likewise, the active or partial responders with low levels of IIA could have included individuals whose disease was better controlled at the time of sampling. However, a role for other contributing factors cannot be excluded.

Despite the lack of serial MRI in our study, the association of T2 LV with IIA also provides evidence that IIA is clinically consequential. Although the abnormality at the T2 lesion sites can extend from potentially revers-
mible changes to severe irreversible damage. In AIDS, the responses as measured by increased CD4 T-cell levels were inversely correlated with IIA. Furthermore, in systemic lupus erythematosus, the IIA was independent of the anti-interferon alfa antibody levels. Medenica et al also identified an IIA-like factor that was decreased by several cycles of combined plasmapheresis, corticosteroid, cyclophosphamide, and interferon alfa treatment in patients with active MS; the decrease of this factor was linked to clinical improvement. Overall, our results, which are similar to those for dysimmune disease states such as systemic lupus erythematosus and AIDS, suggest a possible association between the IIA and dysimmunity or disease activity of MS. We have conducted several experiments to characterize IIA. The IIA is specific for interferon alfa, beta, and gamma, and the inhibition of interferon alfa is approximately equal to interferon beta greater than interferon gamma. In electrophoresis and chromatography analyses, IIA is an approximately 70-kDa protein. Experiments to obtain preliminary amino acid sequences of the proteins in the IIA-enriched fractions are planned.

In conclusion, our results indicate that IIA levels can explain unaccounted-for clinical and MRI heterogeneity in MS. Thus, monitoring IIA could potentially be a useful first step toward more individualized interferon beta treatment regimens for MS patients.

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