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Quantitative differences in the immunomodulatory effects of Rebif and Avonex in IFN-β 1a treated multiple sclerosis patients

George P. Christophi[2,5], **Jennifer A. Christophi**[4], **Ross C. Gruber**[3], **Cornelia Mihai**[1], **Luis J. Mejico**[1], **Paul T. Massa**[1,2], and **Burk Jubelt**[1,2,*]

[1]Department of Neurology, SUNY Upstate Medical University, Syracuse NY, USA

[2]Department of Microbiology & Immunology, SUNY Upstate Medical University, Syracuse NY, USA

[3]Department of Neuroscience, SUNY Upstate Medical University, Syracuse NY, USA

[4]Department of Pathology, University of Maryland School of Medicine, Baltimore MD

[5]Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA

Abstract

Interferon-β (IFN-β) is a current effective treatment for multiple sclerosis (MS) and exerts its therapeutic effects by down-modulating the systemic immune response and cytokine signaling. In clinical practice there are several formulations of interferon including a low dose of IFN-β 1a formulation of 30μg IM once weekly (Avonex) and a high dose formulation of 44 μg SC three times weekly (Rebif). Recent studies suggest that Rebif is more efficacious compared to Avonex in preventing relapses and decreasing MRI activity in relapsing remitting MS (RRMS) patients. This study examines whether there are quantitative gene expression changes in interferon-treated RRMS patients that can explain the difference in efficacy and side effects between Rebif and Avonex. Herein, RRMS patients were treated for three months with IFN-β 1a and the levels of plasma cytokines and gene expression in peripheral blood mononuclear cells were examined. Thirty-two normal subjects were compared to thirty-two RRMS patients, of which ten were treated with Rebif and ten with Avonex. Rebif and Avonex both significantly and equally suppressed plasma TNF-α and IL-6 levels. Rebif suppressed IL-13 significantly more than Avonex. Rebif also significantly suppressed the levels of the chemokines CCL17 and RANTES, the protease ADAM8, and COX-2 at a higher degree compared to Avonex. The STAT1-inducible genes IP-10 and caspase 1 were significantly increased with Rebif compared to Avonex. In conclusion, the higher dosed, more frequently administered IFN-β 1a Rebif when compared to IFN β-1a Avonex has more potent immunomodulatory effects. These quantitative results might relate to efficacy and side-effect profile of the two IFN-β 1a formulations and provide prospective practical clinical tools to monitor treatment and adjust dosage.

*Corresponding Author Mailing Address: Department of Neurology Research Laboratories, UH 5820 Upstate Medical University, State University of New York, 750 East Adams Street, Syracuse, NY 13210, Phone Number: (315) 464-6401, Fax: (315) 464-6402, jubeltb@upstate.edu.

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Keywords

Multiple sclerosis; CNS Demyelination; Autoimmunity interferon; dosage; efficacy; cytokines; inflammatory gene expression; Signaling; Signal Transducers and Activators of Transcription STAT; nuclear factor kappa-B (NF-κB); peripheral immune cells; EAE/TMEV

INTRODUCTION

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) that remains a major cause of disability (1). However, disease-modifying agents that decrease exacerbation rates and slow progression of disability are now available for use in treatment (2–6). One of these agents is interferon-β 1a (IFN-β 1a), which reduces the frequency of clinical exacerbations, reduces magnetic resonance imaging (MRI) disease activity, and slows disease progression (7–9).

IFN-β is a pleiotropic cytokine with diverse mechanisms of action including antiviral, immune-stimulating and immunosuppressive actions (10, 11). IFN β primarily signals by phosphorylating the tyrosine residues of the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) proteins like STAT1 and STAT3, which leads to transcription factor translocation into the nucleus promoting gene expression (12). Multiple mechanisms in which IFN-β modulates the immune response have been reported, including down regulation of class II major histocompatiability complex (MHC) molecules and limiting the migration of immune cells across the blood brain barrier (BBB) (13–16). Furthermore, IFN-β acts to decrease the elevated blood levels of T cell-derived cytokines IFN-γ, IL-4/IL13 and TNF-α seen in MS (17–20), which otherwise act to increase the proinflammatory transcription factors STAT 1, STAT 6 and NF-κB in MS patients (12, 14, 21–24).

IFN-β 1a treatment of patients with RRMS *in vivo* and also *in vitro* leukocyte cultures can reduce the activation of the transcription factors NF-κB and STAT6 and decrease downstream inflammatory gene expression regulated by those transcription factors (25). However, IFN-β 1a (Rebif) treatment signals directly to activate the transcription factor STAT1 and increase the expression genes containing STAT1 responsive sites in their promoter. Additionally, IFN-β induces the activation of intracellular regulatory proteins. The inhibitory effects of IFN-β 1a treatment on cytokine signaling are at least partly mediated by the induced expression of the tyrosine phosphatase SHP-1 that acts as a broad negative regulator of STAT-1, STAT6, and NF-κB signaling and results in decreased inflammatory gene expression (25).

IFN-β 1a is approved to treat MS patients with two different dosages and delivery methods. The Interferon-β 1a Avonex is delivered at 30 μg intramuscularly (im) once a week (7), while Interferon-β 1a Rebif is delivered at 44 μg subcutaneously (sc) three times a week (3). Clinically comparative studies have shown that the high-dosed, more frequently administered Rebif is significantly more effective than the low-dosed, less frequently administered Avonex (9, 26). Specifically, after 24 weeks of treatment 75 % of Rebif patients did not have a relapse versus 63% of patients taking Avonex, with an odds ratio of 1.9 (1.3–2.6) and 48% of Rebif treated patients had no new MRI activity versus 33% of patients treated with Avonex (p=0.0001). This superior efficacy of Rebif over Avonex could still be demonstrated at an additional medium time on study of up to 64 weeks (27). In addition, disease activity in the low dosed IFN-β 1a Avonex group could be partially suppressed by switching subjects to the high dosed IFN-β 1a Rebif (28). On the other hand, Rebif is associated with increased side effects compared to Avonex such as injection site

irritation and mild flu-like symptoms (26, 29). Importantly, some studies demonstrate that different formulations and dosages of IFN-β treatment elicit different gene and protein expression profiles that might relate to treatment efficacy (30–32).

The present study investigates whether the pronounced clinical efficacy of high-dosed IFN-β 1a Rebif compared to the low-dosed IFN-β 1a Avonex is reflected in plasma cytokine and inflammatory gene expression levels in PBMCs that have been previously implicated in the pathogenesis of MS and are regulated by interferon treatment. Furthermore, we evaluated whether we could detect deferential and quantitative gene expression changes that could provide a reliable marker for IFN-β 1a response or dosage adjustment. Here we demonstrate that three-month treatment with Rebif had more potent immunomodulatory activity compared to Avonex. Rebif was more effective compared to Avonex at reducing expression of several NF-κB-responsive inflammatory genes including the chemokines CCL17 and RANTES, the protease ADAM8, and COX2, which are implicated in MS pathogenesis. Furthermore, Rebif was significantly more effective compared to Avonex at inducing the STAT1-inducible chemokine IP-10 and caspase 1. The differential modulation of these genes could at least partially contribute to the pronounced clinical efficacy of the high-dosed IFN-β 1a in RRMS and also provide novel molecular tools to monitor therapeutic effects and adjust dosage.

METHODS

Patient selection

Patients were clinically diagnosed as having definite MS (33) of the relapsing-remitting (RR) type (34). All patients selected had not received any disease modifying treatment, IFN-β, glatiramir acetate, steroids, or other immunosuppressive agents, at least three months prior to donating blood. RRMS patients gave blood before and after a three-month treatment with recombinant IFN-β 1a (Rebif or Avonex). Table I provides additional information on the patients and normal subjects used in this study. The average age of the patients was 40 years old, 70% female patients, an average age at onset of 35 years, and an average EDSS score just before treatment of 2.5. There was no significant difference among the various groups and patient characteristics were similar to previous relevant studies (9). The Institutional Review Board of SUNY Upstate University approved all studies and both patients and normal controls granted informed consent before providing blood.

PBMC isolation

Patients and normal subjects donated 60 ml of blood collected in heparinized tubes. Blood was diluted 1:1 with HBSS and overlaid onto lymphocyte separation medium (Cellgro, Herndon, VA). After centrifugation, the plasma was collected and used to quantify cytokine levels, while the 10 ml of the interface containing the PBMCs were collected and washed twice with HBSS (12). Freshly isolated cells were suspended in STAT- 60 (Tel-Test, Friendswood, TX) for RNA extraction. Plasma was used for cytokine analysis using ELISA.

Real-Time RT-PCR

Total RNA was isolated using RNA STAT-60. RNA was quantified spectrophotometrically and 0.5 μg of total RNA was converted into cDNA. Briefly, total RNA and random primers (Invitrogen, Carlsbad, CA) were incubated at 72 degrees for 10 minutes and Superscript II RT enzyme (Invitrogen, Carlsbad, CA) was used for reverse transcription (35). cDNA was used for quantitative real time PCR using SYBR Green kit (Abgene, Epson, UK). Serial dilutions of cDNA containing a known copy number of each gene were used in each quantitative PCR run to generate a standard curve relating copy number with threshold amplification cycle (36). A blank/negative control (cDNA reaction without RNA) was run

with each RT-PCR assay and the samples always had lower threshold amplification cycle than the negative control. Gene expression levels were calculated during the logarithmic amplification phase by determining the initial mRNA copy number using the standard curve (37). Amplification of each gene specific fragment was confirmed by examination of melting peaks, by agarose gel electrophoresis, and DNA sequencing. The primers used in the study were previously documented (12, 25).

Cytokine ELISA

The levels of the cytokines IFN-γ, IL-4, IL-13, TNF-α and IL-6 were measured using R&D Systems DuoSet ELISA kits (R&D Systems) following the manufacturer's protocol.

Statistical Analysis

Data are presented in means with standard error values. The p-values were generated using the unpaired Student's t-test value of less than 0.05, which was chosen to indicate statistical significance between two sample means.

RESULTS

Normal subjects and RRMS patients who were treated with either Rebif or Avonex donated blood before and three months after treatment. First, we quantified the levels of the plasma cytokines by ELISA in normal subjects, untreated RRMS, Rebif-treated RRMS, and Avonex-treated RRMS patients (Table II). The levels of IFN-γ, a cytokine that induces STAT1 activation, were significantly higher in the plasma of untreated MS patients compared to normal subjects, and IFNβ-1a treatment failed to significantly alter IFN-γ levels. The levels of IL-4 and IL-13, cytokines that signal through STAT6, were significantly higher in the plasma of untreated MS patients as compared to normal subjects and treatment with IFN-β 1a had no effect on IL-4 levels. However, treatment with Rebif caused a significant two-fold decrease in IL-13 levels, while Avonex did not, and importantly there was a significant difference between Rebif-treated and Avonex-treated MS patients. Furthermore, the levels of the NF-κB-inducible cytokines TNF-α and IL-6 were significantly higher in untreated MS patients as compared to normal controls. Both Rebif and Avonex significantly reduced IL-6 and TNF-α levels, but there was not a significant difference between the two treatments.

Next, it was important to quantify the levels of several cytokine-inducible genes that where shown to be modulated in MS by interferon treatment (25, 38, 39). Several genes were quantified by real time RT-PCR in freshly isolated PBMC of normal subjects, RRMS patients and in three-month treated Rebif or Avonex RRMS patients (table II). First, we quantified the expression levels of the STAT1-inducibe genes the chemokine IP-10 (interferon gamma-induced protein)/CXCL10 and caspase 1, which were elevated in PBMC of MS patients as compared to normal subjects, in accordance with previous reports (40–42). Treatment with either IFN-β 1a Rebif or Avonex significantly increased the expression of IP-10 and caspase 1, which is not surprising since IFN-β signals primarily to activate STAT1. Importantly, Rebif significantly induced higher levels of IP-10 mRNA compared to Avonex treatment.

Furthermore, we examined the expression of NF-κB responsive genes: the chemokine RANTES (Regulated upon Activation, Normal T-cell Expressed)/CCL5, and Secreted, the metalloprotease MMP9, cyclooxygenase-2 (COX-2), and vascular cell adhesion protein 1 (VCAM1) (Table II). All these genes have been previously implicated in the pathogenesis of MS and have been shown to be elevated in leukocytes and in the CNS of MS patients (12, 43–46). As expected, RANTES, MMP9, COX-2, and VCAM1 were significantly elevated in

leukocytes of untreated MS patients as compared to normal subjects. The levels of RANTES and COX2 were suppressed following treatment with either Rebif or Avonex compared to untreated patients. Importantly, Rebif treatment reduced RANTES and COX2 to a greater degree compared to Avonex treatment. MMP9 and VCAM1 mRNA levels were significantly reduced by Rebif treatment but not by Avonex treatment compared to untreated patients.

We also quantified the expression of the STAT6/NF-κB responsive genes, the chemokine TARC (Thymus and Activation Regulated Chemokine)/CCL17 and the MBP-cleaving protease ADAM8, and the STAT6 responsive gene arginase I (12, 47–50). The expression levels of these genes were significantly higher in untreated MS patients as compared to normal subjects (Table II). Rebif-treated MS patients had significantly lower levels of CCL17, ADAM8 and Arginase I as compared to untreated MS patients. Avonex-treated MS patients had significantly reduced ADAM8 and Arginase 1 levels as compared to normal subjects. Importantly, the reductions seen in CCL17 and ADAM8 were significantly greater in Rebif-treated MS patients as compared to Avonex-treated MS patients.

The phosphatase SHP-1 is deficient in untreated MS patients (12) and Rebif and Avonex treatment resulted in a significant upregulation of SHP-1 (Table II). Although no significant differences were observed between the two different treatments, there was a trend showing Rebif treatment inducing higher levels of SHP-1 compared to Avonex treatment. In addition, we did not observe any differences in the expression levels of the chemokine receptors CXCR3 or CCR4, which are not directly regulated by STAT1, STAT6 or NF-κB. Finally, the levels of the housekeeping control genes beta-actin and GAPDH were similar in all groups serving as an important internal control.

DISCUSSION

The high-dosed, high-frequency IFN-β 1a Rebif is more effective than low-dosed, low-frequency IFN-β 1a Avonex in reducing relapses and MRI activity in patients with RRMS (9, 26). This study aimed to establish an immunological/molecular basis for this difference in efficacy and document collectively possible biomarkers that can potentially explain the difference in efficacy and can be monitor treatment and adjust dosage.

Multiple sclerosis is an immune-mediated disease and leukocytes in the blood and in CNS lesions have increased activation of the transcription factors STAT1, STAT6, and NF-κB that might be the result of increased plasma levels of the corresponding cytokines IFN-γ, IL-4/IL13, and TNF-α (12, 14, 20–24, 51–57). In turn, these elevated transcription factors drive the expression of several inflammatory genes that might contribute to the enhanced demyelinating activity of CNS-infiltrating leukocytes. Here, we demonstrate that three-month treatment of RRMS patients with Rebif compared to Avonex results in differential and quantitative changes in cytokines and their downstream inflammatory gene expression.

First, we examined and quantified the levels of the plasma cytokines that were shown to be upregulated in MS and correlated with the acute phase of MS (17–20). IFN-β 1a treatment significantly decreased TNF-α and IL-6 levels but there were no significant differences between Rebif and Avonex treatment. The levels of the cytokines IFN-γ and IL-4 were not significantly affected following treatment with either agent. However, Rebif treatment caused a significant reduction in plasma IL-13 compared to Avonex treatment. Importantly, IL-13 is highly elevated in lymphocytes of MS patients during relapse and return near baseline during remission (54). Interestingly, mast cells are a main source for IL-13 and are believed to play a major role in lesion formation in MS and EAE (58, 59).

In addition to cytokine levels, IFN-β modulates the activation of the transcription factors STAT1, STAT6, and NF-κB and their downstream inflammatory gene expression (25). IFN-β signals to primarily activate STAT1, which is widely considered an inflammatory transcription factor inducing several genes that mediate CNS demyelination (25). This seems paradoxical in the light that IFN-β has therapeutic effects in MS; however the activation of STAT1 might be related to the incomplete action of IFN-β and its side-effect profile. Importantly, unlike IFN-γ, IFN-β also activates the transcription factor STAT3 (10, 11). In turn, STAT3 signaling could mediate many of the anti-inflammatory effects of IFN-β treatment in MS though mediating and augmenting the anti-inflammatory IL-10/STAT3 signaling pathway, inducing expression of intracellular molecules like SHP-1 that can inhibit cytokine signaling, and mediate NF-κB and STAT6 inhibition (60–63). Furthermore, augmented NF-κB activation through either increased TNF-α, IL-1b, IL-17 levels or aberrant signaling is considered to be central in MS pathogenesis and an important target of IFN-β therapy (12, 64, 65). Therefore, the fact that Rebif has more potent inhibitory effect on NF-κB-inducible genes might contribute to enhanced clinical benefit through decreased immune cell infiltration and activation, dismissed expression of neurotoxic molecules, and reduced oligodendrocyte injury.

We examined the expression of STAT1 responsive genes, the chemokine IP-10 that chemo-attracts primarily T-cells into CNS, and caspase 1 that is involved in apoptosis and proteolytic processing of IL-1β (40, 66). Expression levels of IP-10 and caspase 1 were elevated in leukocytes of RRMS patients compared to normal subjects. IP-10 levels were significantly further increased following three-month Rebif treatment as compared to Avonex treatment. These data suggest that the higher frequency and high-dosed of Rebif translates into more potent long-term immunomodulatory effects compared to low-dosed Avonex. Interestingly, the increased levels of the chemokine IP-10 following IFN-β treatment closely correlated with the flu-like symptoms occurring in treated MS patients (40) and several studies report that that Rebif treatment is associated with slightly more frequent flu-like symptoms compared to Avonex (26, 29).

Both Rebif and Avonex treatment had a suppressive effect on the mRNA levels of several inflammatory genes regulated by the transcription factors NF-κB and STAT6 in leukocytes from RRMS patients. However Rebif treatment resulted in a significantly greater reduction in the expression of RANTES, COX2, CCL17 and ADAM8 compared to Avonex. In addition to serving as biomarkers to disease activity, these genes play a major role in MS pathogenesis. For example chemokines play an important role in attracting immune cells into the CNS, enzymes like COX-2 process neurotoxic molecules, and proteases like ADAM 8 can directly degrade myelin (46, 49, 67). The genes that showed differential regulation among Rebif and Avonex are induced through the NF-κB activation, suggesting that Rebif treatment more effectively inhibits the NF-κB activation. These data in all suggest that Rebif treatment is associated with an augmented immunosuppressive response compared to Avonex, which is likely to translate into clinical benefits and at least partly explain the increased efficacy seen in treating MS patients (9).

This study demonstrated that indeed there are underling gene expression changes that parallel the difference in clinical efficacy between the two interferon-β 1a formulations, Rebif and Avonex. At the same time, the study has certain limitations including the small sample size of patients, the fact that gene expression was examined only at a single three-month time point after treatment, and the lack of direct correlation of gene expression levels to clinical outcomes of the patients used in this study. These limitations might partially explain why not all the results were homogeneous and why significant differences were not reached with all the genes examined. Nonetheless, this study clearly demonstrates that Rebif and Avonex treatment result in differential gene expression changes that correlate to

previously documented clinical responses. More studies are needed to firmly establish a direct correlation between clinical outcomes and gene expression profiles following interferon-β 1a treatment. Therefore, it is important that this study can provide the groundwork and contribute in developing molecular tools that can objectively and promptly monitor the therapeutic effects of interferon β-1a treatment and possibly help adjust dosages in clinical practice.

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Table I

Biometric data of MS patients and normal subjects used in the study.

Patient Category	Number of Subjects	Age (Years)	Gender	Age at Onset	Disease Duration	EDSS Score
Normal Subjects	32	41±12	22F, 10M	---	---	---
RR MS	32	41±9	29F, 13M	36±9	5.9± 5	2.5±1.4
REBIF Tx	10	38±9	7F, 3M	34±8	4.0±2	2.4±1.7
AVONEX Tx	10	43±8	7F, 3M	35±9	6.8±4	2.5±0.9

RR MS are untreated Relapsing Remitting multiple sclerosis patients. The data are shown in mean value ± SD. For the gender, F stands for females and M stands for males. The age at onset of clinically diagnosed MS and disease duration are shown in years. The clinical symptoms were measured on Kurtzuke's Expanded Disability Status Scale (EDSS) at the time the initial blood was drawn. Patients with RR MS gave blood before and after a three-month treatment with recombinant interferon β-1a (Rebif or Avonex) and EDSS scores shown here were calculated just before treatment with interferon β-1a.

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Table II

Inflammatory profile of normal subjects, untreated RR MS patients, and *in vivo* three-month treated RR MS patients with IFN β-1a Rebif or Avonex.

	Gene	Normal Subjects	Untreated MS Patients	Rebif MS Patients	Avonex MS Patients
Cytokine Levels in Plasma (pg/mL)	**IFN-γ**	10±4	569±207†	467±262	512±311
	IL-4	12±7	201±81†	207±102	143±78
	IL-13	67±26	523±125†	212±82$^{*\$}$	381±113
	TNF-α	33±8	390±156†	243±92*	267±125*
	IL-6	18±5	152±28†	97±25*	83±42*
mRNA levels (relative to normal subjects	**IP-10**	1±0.2	3.2±1.3†	12.4±3.3$^{*\$}$	6.3±2.4*
	Caspase 1	1±0.3	2.5±0.6	6.3±2.0*	4.2±2.6
	RANTES	1±0.1	12.3±2.7†	4.4±1.9$^{*\$}$	8.7±2.3*
	MMP9	1±0.2	4.2±0.9†	2.2±0.7*	3.3±1.0
	VCAM1	1±0.4	6.4±2.1†	2.5±1.2*	4.1±1.7
	COX-2	1±0.1	7.2±1.8†	3.8±1.3$^{*\$}$	5.1±0.6*
	CCL17	1±0.2	4.1±0.6†	1.6±0.5$^{*\$}$	3.0±0.9
	ADAM8	1±0.4	4.9±1.0†	2.3±1.2$^{*\$}$	3.8±0.7*
	Arginase I	1±0.2	3.7±0.6†	2.1±0.9*	2.4±0.8*
	CXCR3	1±0.2	1.7±0.5	1.3±0.2	1.9±0.6
	CCR4	1±0.3	1.5±0.4	1.2±0.7	1.3±0.4
	SHP-1	1±0.1	0.5±0.1†	2.2±0.4*	1.7±0.4*
	Actin	1±0.1	1.0±0.2	1.1±0.3	1.2±0.3
	GAPDH	1±0.1	1.1±0.2	0.9±0.2	1.1±0.3

Several inflammatory indicators were quantified in the plasma or in freshly isolated PBMCs of normal subjects (n=32), untreated RR MS patients (n=32), and Rebif-treated RR MS patients (n=10) or Avonex (n=10). The levels of the cytokines IFN-γ, TNF-α, IL-6, IL-4, and IL-13 were quantified in the plasma by ELISA and are recorded in pg cytokine per mL plasma. The mRNA levels of chemokine IP-10/CXCL10, Caspase 1, the T-cell chemokine RANTES, the metalloprotease MMP9, the vascular adhesion molecule VCAM 1, the cyclooxygenase 2 (COX-2), the chemokine CCL17/TARC, the protease ADAM8, the enzyme arginase I, the chemokine receptors CXCR3 and CCR4, the tyrosine protein phosphatase SHP-1, and the housekeeping control genes beta-actin and GAPDH were quantified by real-time RT-PCR in freshly isolated PBMCs of normal subjects, MS patients, and IFN-β treated MS patients. The mRNA levels were normalized to the levels of normal subjects that are shown as 1. The values indicate statistical means ± standard error values,

†indicates significance of P<0.05 between normal subjects and untreated MS patients and

* indicates significance of P<0.05 between untreated and IFN-β treated MS patients, and

$ indicates significance between Rebif treated and Avonex treated patients.