Journal of Immunoassay and Immunochemistry, 35:288–299, 2014
Published with license by Taylor & Francis
ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321819.2013.848815

Taylor & Francis
Taylor & Francis Group

IN VITRO ASSESSMENT OF THE BIOLOGIC ACTIVITY OF INTERFERON BETA FORMULATIONS USED FOR THE TREATMENT OF RELAPSING MULTIPLE SCLEROSIS

Carolina Scagnolari,[1] Carla Selvaggi,[1] Emilia Di Biase,[1] Maurizio Fraulo,[1] Fernando Dangond,[2] and Guido Antonelli[1]

[1]*Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy*
[2]*EMD Serono, Inc., Rockland, Massachusetts, USA*

□ *A new formulation (NF) of subcutaneous (sc) interferon (IFN) β-1a was developed in an attempt to improve injection tolerability and immunogenicity. We compared antiviral and IFNβ-stimulated gene (ISG) activities of IFNβ-1a sc NF with IFNβ-1a sc original formulation and IFNβ-1b sc. When equivalent unit amounts were compared, the IFNβ formulations demonstrated similar antiviral activity and induced similar levels of ISG mRNA. However, on a weight basis (ng/mL), significantly more IFNβ-1b sc was needed to equal the antiviral activity of either IFNβ-1a sc formulation, and both IFNβ-1a sc formulations induced significantly higher levels of ISG mRNA than IFNβ-1b sc.*

Keywords antiviral activity, biologic activity, gene expression activity, *in vitro* assays, interferon beta-1a, interferon beta-1b

INTRODUCTION

Interferon beta (IFNβ) is an antiviral and immunomodulatory agent with demonstrated efficacy and safety in the treatment of relapsing multiple sclerosis (MS).[1–5] In randomized, double-blind, placebo-controlled clinical trials, IFNβ therapy has been shown to reduce relapse rate and brain lesion development in patients with relapsing forms of MS.[2,4] Indeed, a subcutaneous (sc) formulation of IFNβ-1a (Rebif®; Merck Serono S.A., Geneva, Switzerland) administered three times weekly (tiw) and an intramuscular (im) formulation of IFNβ-1a (Avonex®; Biogen Idec/Elan, Cambridge, MA, USA) administered once weekly have also been shown to delay the progression of physical disability in patients with relapsing forms of MS.[6–8]

Address correspondence to Guido Antonelli, Laboratory of Virology, Department of Molecular Medicine, Sapienza University of Rome, Viale di Porta Tiburtina 28, 00185, Rome, Italy. E-mail: guido.antonelli@uniroma1.it

Three formulations of IFNβ are currently approved in the USA for treatment of patients with relapsing forms of MS: IFNβ-1a 22 μg or 44 μg sc tiw (Rebif; original formulation, which is referred to in this article as IFNβ-1a sc), IFNβ-1a 30 μg administered im once weekly (Avonex), and IFNβ-1b 250 μg sc administered every other day (Betaseron®/Betaferon®; Bayer HealthCare Pharmaceuticals, Wayne, NJ, USA; Extavia®, Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA). The response to IFNβ therapy can vary with the type of preparation, and may be impacted by drug formulation, dosing regimen, and route of administration.[1,9,10] For example, head-to-head comparisons of different IFNβ formulations demonstrated that higher and more frequent doses of IFNβ had greater efficacy compared with lower, less frequent dosing regimens (of a different IFNβ formulation) for the treatment of relapsing MS.[2,11–13]

Treatment with IFNβ in patients with relapsing MS has been associated with the development of neutralizing antibodies (NAbs).[14–17] Although the full clinical impact of these anti-IFN antibodies is not completely known, the presence of high titers of NAbs to IFNβ can reduce the therapeutic effects of treatment.[18–20] A new sc formulation of IFNβ-1a (Rebif New Formulation [NF]; Merck, Bari, Italy) that is produced without fetal bovine serum and without human serum albumin as an excipient, with the goals of improving injection tolerability and reducing immunogenicity, was developed.[21,22] *In vivo* administration of IFNβ-1a sc NF in a mouse model has suggested a slower and weaker development of NAbs compared with IFNβ-1a sc.[23]

Biologic activity assessed from *in vitro* assays can supplement *in vivo* data, thereby helping to fully characterize a particular therapeutic agent. Evidence from previous studies indicates that the antiviral and biologic activity of IFNβ may vary among different formulations.[1,10] Specific measures of IFNβ biologic activity include the inhibition of viral replication and the enhanced expression of mRNAs that are induced by IFNβ. The objective of the current *in vitro* study was to compare the antiviral and IFNβ-stimulated gene (ISG) expression activity of IFNβ-1a sc NF with that of the original IFNβ-1a sc formulation; IFNβ-1b sc was used as an additional comparator.

EXPERIMENTAL

IFNβ Formulations

Antiviral activity and induction of ISG expression were evaluated for three formulations of IFNβ: IFNβ-1a sc, 44 μg (Rebif, original formulation, lot number Y09B7770V, Industria Farmaceutica Serono, Rome, Italy); IFNβ-1a sc NF, 44 μg (Rebif, serum-free formulation, lot number Y09B0227,

Industria Farmaceutica Serono); and IFNβ-1b sc, 250 μg (Betaferon, lot number 1560, Bayer Schering, Berlin, Germany).

Assessment of Antiviral Activity

Antiviral activity was assessed using the A549/vesicular stomatitis virus (VSV) cytopathic effect (CPE) assay, which measured cell viability following infection of A549 human lung carcinoma cells with the lytic VSV (Indiana strain[24]). A549 cells (Zooprophylactic Institute, Brescia, Italy) were maintained in Dulbecco's modified Eagle medium (D-MEM), supplemented with 10% fetal calf serum (FCS; Sial, Rome, Italy), 2 mM L-glutamine (Sial), 50 μg/mL of gentamycin (Sial), and 25 mM of Hepes buffer solution (Sial). Cells were seeded at 3×10^4 cells per well in 96-well plates (Falcon™, Becton Dickinson Labware, Lincoln Park, NJ, USA), and after 24 hr, triplicate cell cultures were incubated with serial dilutions of each of the IFNβ formulations for 18–20 hr. In each test, 12 wells were filled with 0.1 mL of medium to serve as both virus and cell control. A549 cell monolayers were then washed with D-MEM, and both IFN-treated and control viruses were inoculated with VSV at a multiplicity of infection of 0.1 $TCID_{50}$ (50% tissue culture infectious dose)/cell. After adsorption at 37°C for 1 hr, the excess virus inoculum was removed, the cell monolayers were washed with phosphate-buffered saline, and wells were filled with complete medium to a total volume of 0.1 mL/well.

Infection with VSV was allowed to progress for 24 hr according to the time required for the specific cytopathic effects to become clearly visible by optical microscopy. Culture supernatants were then collected, and titration of VSV was carried out in L929 mouse fibroblast cells (Zooprophylactic Institute). L929 cells were maintained in minimal essential medium with 10% FCS (Sial), 2 mM L-glutamine (Sial), and 50 μg/mL of gentamycin (Sial). Titration of VSV was performed by determination of the $TCID_{50}$/mL, according to the method of Reed and Muench.[25] A series of three-fold dilutions of the VSV inocula were added to L929 cell monolayers, and were incubated for 24 hr in order to detect VSV-induced CPE. The cell monolayers were then stained with crystal violet in 20% ethanol. The dye taken up by the cells was eluted with 33% acetic acid, and its absorbance measured at 540 nm with an enzyme-linked immunosorbent assay microplate reader (Varioskan® Flash Spectral Scanning Multimode Reader, Thermo Fischer, Pittsburgh, PA, USA).

The antiviral activity of IFNβ was calculated from a dose-response curve where the viral yields obtained in IFN-treated cells were expressed in terms of percentage of viral inhibition with respect to yield from virus-infected control cells. Results of these analyses were expressed in terms of 50% inhibitory concentrations (IC_{50}) calculated in International Units (IU)/mL and in ng/mL when the specific activities of the different IFNβ formulations were

considered. The specific activities used were 32 MIU/mg for IFNβ-1b sc and 270 MIU/mg for IFNβ-1a sc. These specific activity values were provided by the manufacturers, and were obtained by comparing the antiviral activity of the IFNβ formulations to the World Health Organization (WHO) reference standard of recombinant human IFNβ.[26] Furthermore, the antiviral activities of each IFNβ formulation were also compared by measuring the VSV yield reductions in A549 cells treated with 10 and 100 times the IC_{50} of each IFNβ formulation obtained after performing the experiments described above; these reductions in VSV yield in IFNβ-treated cells were measured relative to the VSV yields in control A549 cells that were not treated with IFNβ.

Assessment of ISG Expression

Induction of ISG expression was assessed using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) for myxovirus resistance protein A (MxA), ADAR1 (adenosine deaminase, RNA-specific), and ISG56 mRNAs. A549 cells were incubated with various concentrations of IFNβ formulations for 24 hr. At the end of the incubation, total cellular RNA was extracted from the cells using Trizol® (Gibco BRL, New York, NY, USA) according to the manufacturer's instructions. RNA was dissolved in 50 μL of RNase-free water, and the quantitation of ISG mRNA was performed by a real time 5' exonuclease RT-PCR Taqman assay using an ABI 7000 sequence detector (Applied Biosystems, Foster City, CA, USA) after generation of cDNA as previously described.[27]

The following primer pair and probe – MxA (MxA F: 5'-CTGCCTGG CAGAAAACTTACC-3'; MxA R: 5'TCTGTTATTCTCTGGTGAGTCTCCTT-3'; MxA P: 6-carboxifluorescein (FAM)-5'CATCACACATATCTGTAAATCTC TGCCCCTGTTAGA3'-6carboxy-tetramethylrhodamin (TAMRA)[28]); ADA R1 (ADAR F: 5'-CCGGCAGGATGACACAGAC-3'; ADAR R: 5'-GCTTGGC AATATTCCAAGGC-3'; ADAR P: 5' FAM-CACTTCCCAGGGAGCACGG GCA-3' TAMRA); ISG56 P56 F: 5'-TGAAGAAGCTCTAGCCAACATG TC-3'; P56 R: 5'-GAGCTTTATCCACAGAGCCTTTTC-3'; P56 P: 5' FAM TATGTCTTTCGATATGCAGCCAAGTTTTACCG-3' TAMRA[29]) – were added to the universal PCR master mix (Applied Biosystems, Foster City, CA, USA) at 300 nM and 100 nM, respectively, in a final volume of 50 μL. Coamplification of the beta-glucuronidase gene (*GUS*; Assay-On-Demand, Hs99999908_m1; Applied Biosystems) was used to normalize the amount of total RNA present using the threshold cycle relative quantification according to the supplier's guidelines (2^-ΔΔCt method). The threshold cycle (Ct) value is the PCR cycle in which the amplification plot crosses the threshold line. The Ct values of each amplification reaction performed in A549 cells before and after *in vitro* IFNβ treatment were used to calculate the difference

(ΔCt values) between the ISGs and the housekeeping gene *GUS* (ΔCt = $Ct_{ISG} - Ct_{GUS}$). The fold-change in IFNβ-induced expression of ISGs (MxA, ADAR1, or ISG56) compared with untreated A549 cells was estimated using the formula, $2^{-(\Delta\Delta Ct)}$, where ΔΔCt = [Ct *ISG* (+IFN) − Ct *GUS* (+IFN)] − [Ct *ISG* (–IFN) − Ct *GUS* (–IFN)]. All experiments were performed in triplicate.

Statistical Analysis

Two aspects of biologic activity were investigated: antiviral activity and induction of ISG expression. The three IFNβ formulations were compared both on the basis of units (IU/mL) and weight (ng/mL). Three replicates in each of two experiments were run to measure antiviral activity in terms of IC_{50}, providing six observations of each formulation on a unit basis and six observations on a weight basis. The mean and standard deviation were calculated for each formulation and displayed in a bar chart. After the IC_{50} of each IFNβ formulation had been measured, six replicates for each IFNβ formulation were run from independent observations to measure VSV yield reductions in A549 cells treated with 10 and 100 times the IC_{50} of each IFNβ formulation. The antiviral activities of IFNβ formulations were compared using Student's *t*-test. Analysis was performed using SPSS version 13.0 for Windows (SPSS, Inc., Chicago, IL, USA).

For each of four different concentrations of each IFNβ formulation, MxA gene expression activity was measured in three replicates, and ADAR1 and ISG56 expression were measured in six replicates. Since the concentrations differ by factors of 10, a logarithmic scale was used to present the results so that they could be displayed meaningfully on the same graph. Student's *t*-test was used to compare relative gene expression of MxA, ADAR1, and ISG56 among the three IFNβ formulations at the same amount of dilution.

RESULTS

Antiviral Activity

The three IFNβ formulations demonstrated similar antiviral activity against A549 cells infected with VSV when equivalent unit amounts of the IFNβ formulations were compared. There were no differences in mean IC_{50} (IU/mL) values ± standard deviation among the IFNβ-1a sc (2.13 ± 0.46 IU/mL), IFNβ-1a NF sc (2.20 ± 0.46 IU/mL), and IFNβ-1b sc (2.31 ± 0.51 IU/mL) formulations (Figure 1A). Importantly, antiviral activity was similar for both IFNβ-1a sc and IFNβ-1a sc NF. When the formulations were

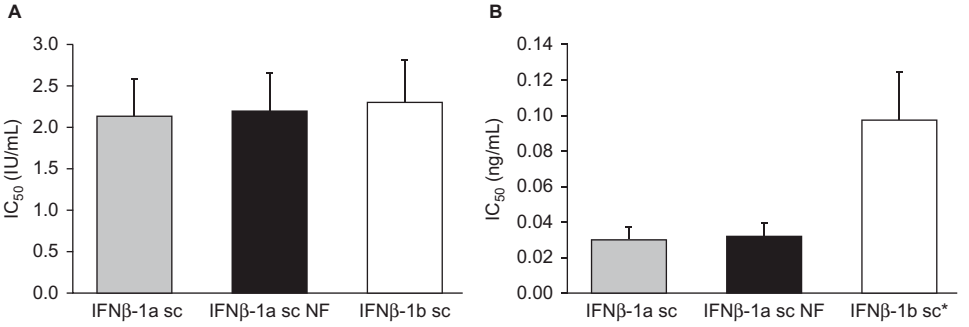

FIGURE 1 Evaluation of antiviral activity of IFNβ formulations using the A549 human lung carcinoma cells/VSV CPE reduction assay. (A) All three IFNβ formulations demonstrated the same activity against A549 cells infected with VSV when equivalent unit amounts were compared. (B) When the formulations were compared on a weight basis (ng/mL), significantly more IFNβ-1b sc was needed to equal the antiviral activity of IFNβ-1a sc or IFNβ-1a sc NF. *$P < 0.05$ for both comparisons using Student's *t*-test. CPE, cytopathic effect; IC_{50}, 50% inhibitory concentrations; IFNβ, interferon beta; IU, International Units; NF, new formulation; sc, subcutaneous; VSV, vesicular stomatitis virus.

compared on a weight basis (ng/mL), substantially more IFNβ-1b sc was needed to equal the antiviral activity of either IFNβ-1a sc or IFNβ-1a sc NF. Specifically, mean IC_{50} (ng/mL) values ± standard deviation were: IFNβ-1a sc (0.030 ± 0.0077 ng/mL); IFNβ-1a NF sc (0.032 ± 0.0082 ng/mL); and IFNβ-1b sc (0.098 ± 0.028 ng/mL) (Figure 1B). There was no significant difference between IFNβ-1a sc and IFNβ-1a sc NF in antiviral activity when the formulations were compared on a weight basis. We also measured VSV yield reductions in A549 cells treated with 10 and 100 times the IC_{50} of each

TABLE 1 VSV yield reductions in A549 cells treated with 10 and 100 times the IC_{50} of IFNβ preparations

	VSV yield reduction, mean ± SD[a]			
	10 × IC_{50}[b] IU/mL	10 × IC_{50}[c] ng/mL	100 × IC_{50}[b] IU/mL	100 × IC_{50}[c] ng/mL
IFNβ-1b sc	1.47 ± 0.10	1.66 ± 0.31[d]	2.48 ± 0.36	3.56 ± 0.33
IFNβ-1a sc	1.50 ± 0.09	2.25 ± 0.24	2.49 ± 0.29	3.69 ± 0.36
IFNβ-1a sc NF	1.50 ± 0.13	2.29 ± 0.24	2.44 ± 0.32	3.78 ± 0.17

IC_{50}, 50% inhibitory concentration; IFNβ, interferon beta; IU, International Units; NF, new formulation; sc, subcutaneously; SD, standard deviation; $TCID_{50}$, 50% tissue culture infectious dose; VSV, vesicular stomatitis virus.

[a]VSV yield reduction was calculated as [VSV titer in untreated cells (log $TCID_{50}$/mL) − VSV titer in cells treated with IFNβ (log $TCID_{50}$/mL)].

[b]IC_{50} values measured in A549 cells infected with VSV are expressed as IU/mL (2.31 ± 0.51 IU/mL for IFNβ-1b sc, 2.13 ± 0.46 IU/mL for IFNβ-1a sc, and 2.20 ± 0.46 IU/mL for IFNβ-1a sc NF).

[c]IC_{50} values measured in A549 cells infected with VSV are expressed as ng/mL (0.098 ± 0.028 ng/mL for IFNβ-1b sc, 0.030 ± 0.0077 ng/mL for IFNβ-1a sc, and 0.032 ± 0.0082 ng/mL for IFNβ-1a sc NF).

[d]$P < 0.05$ for both IFNβ-1b sc vs. IFNβ-1a sc and IFNβ-1b sc vs. IFNβ-1a sc NF, using Student's *t*-test.

IFNβ formulation. At both 10 and 100 times the IC_{50}, VSV yield reductions did not significantly differ between IFNβ-1a sc and IFNβ-1a sc NF (Table 1). VSV yield reductions also did not significantly differ between IFNβ-1b sc and either IFNβ-1a sc formulation when concentrations corresponding to 10 or 100 times the IC_{50} of each IFNβ formulation were compared on the basis of IFN unit amount (IU/mL). However, when concentrations corresponding to 10 times the IC_{50} of each IFNβ formulation were compared on a weight basis (ng/mL), the VSV yield reduction was higher with both IFNβ-1a sc and IFNβ-1a sc NF than with IFNβ-1b sc. When concentrations corresponding to 100 times the IC_{50} of each IFNβ formulation were compared on a weight basis (ng/mL), VSV yield reductions did not significantly differ between IFNβ-1b sc and either IFNβ-1a sc formulation.

ISG Expression

Differences in ISG expression, as determined by the induction of MxA, ADAR1, and ISG56 mRNAs, were consistent with those observed for antiviral activity of the IFNβ formulations. When equivalent unit amounts of the three IFNβ formulations were compared, they all induced similar transcript levels of MxA, ADAR1, and ISG56. At both 10 IU/mL and 100 IU/mL, there were no significant differences between the mean fold-changes in ISG mRNA expression for IFNβ-1a sc, IFNβ-1a sc NF, and IFNβ-1b sc (Figures 2A, 2C, and 2E). ISG transcript levels were similar for both the original IFNβ-1a sc formulation and the IFNβ-1a sc NF. When the formulations were compared by weight, at 0.5 ng/mL or 0.05 ng/mL, both IFNβ-1a sc and IFNβ-1a sc NF induced higher transcript levels of MxA, ADAR1, and ISG56 compared with IFNβ-1b sc (Figures 2B, 2D, and 2F), with no significant difference in ISG expression between IFNβ-1a sc and IFNβ-1a sc NF.

DISCUSSION

The objective of this current article was to compare the biologic activity of IFNβ-1a sc NF with that of IFNβ-1a sc; IFNβ-1b sc was used as an additional comparator. The antiviral and biologic activities of these formulations were compared by measuring: (1) the reduction of CPE by IFNβ on A549 human lung carcinoma cells following infection with VSV and (2) the levels of IFNβ-induced MxA, ADAR1, and ISG56 mRNAs, which encode antiviral proteins and are commonly assayed in cell cultures exposed to Type I IFN as an *in vitro* measure of IFN biologic activity.[30–33] When equivalent unit amounts of the formulations were compared, IFNβ-1a sc, IFNβ-1a sc NF, and IFNβ-1b sc demonstrated similar levels of antiviral activity in terms of IC_{50}. However, when the formulations were compared on a weight basis (ng/mL), significantly more IFNβ-1b sc was required to equal the antiviral activity of either

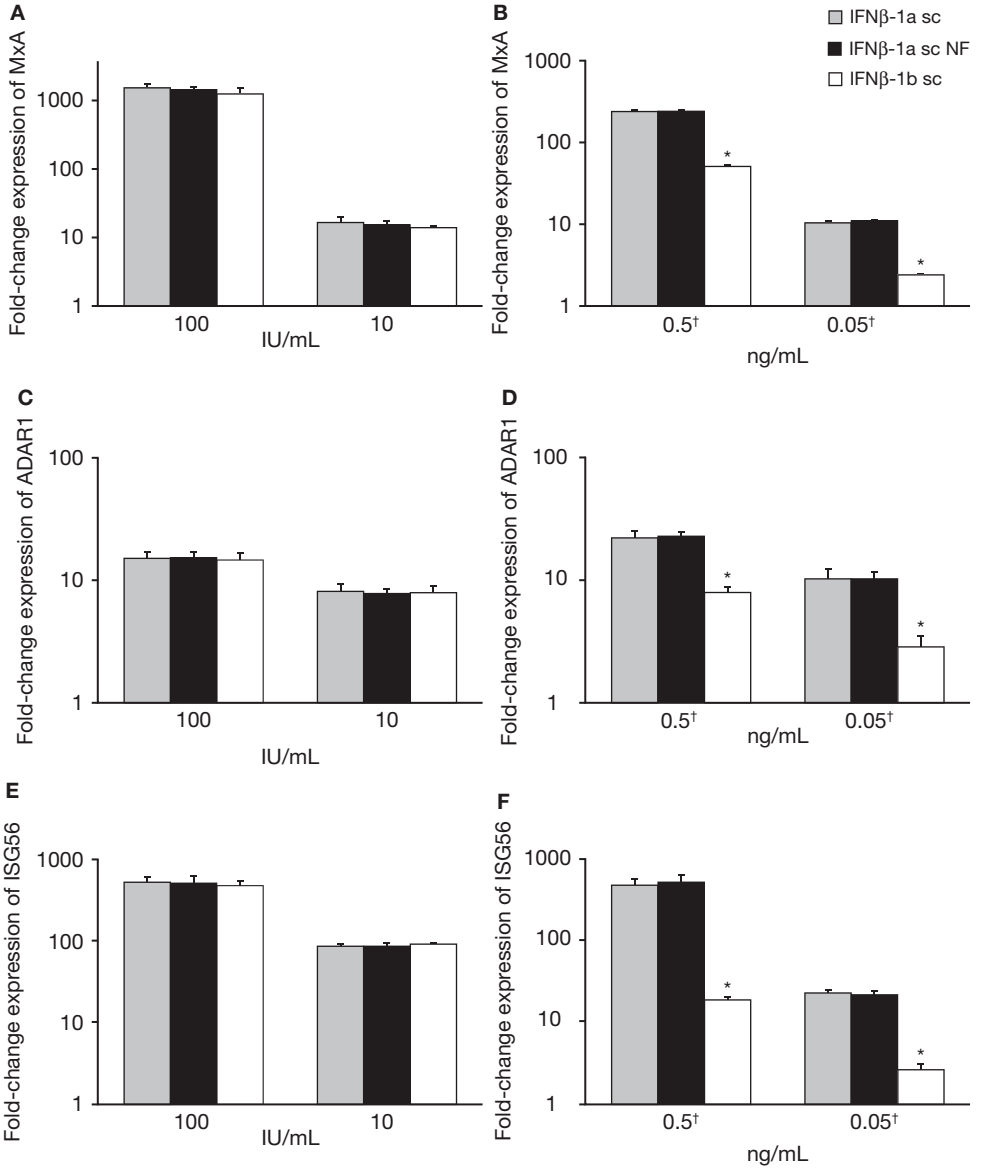

FIGURE 2 Evaluation of MxA, ADAR1, and ISG56 mRNA induction by IFNβ formulations in A549 human lung carcinoma cells. All three IFNβ formulations induced similar mRNA levels of (A) MxA, (C) ADAR1, and (E) ISG56, when equivalent unit amounts were compared (100 or 10 IU/mL). When the formulations were compared on a weight basis, at 0.5 ng/mL and 0.05 ng/mL, IFNβ-1a sc and IFNβ-1a sc NF induced significantly higher mRNA levels of (B) MxA, (D) ADAR1, and (F) ISG56 compared with IFNβ-1b sc. *$P < 0.05$ for both comparisons using Student's *t*-test. †Calculated according to the specific activities of IFNβ-1b sc (32 MIU/mg) and IFNβ-1a sc (270 MIU/mg). ADAR1, adenosine deaminase, RNA-specific; IFNβ, interferon beta; ISG56, interferon stimulated gene 56; IU, International Units; MxA, myxovirus resistance protein A; NF, new formulation; sc, subcutaneous.

formulation of IFNβ-1a sc in terms of IC_{50}. Similarly, when concentrations corresponding to 10 times the IC_{50} of each IFNβ formulation were compared on a weight basis, a smaller reduction in VSV yield was observed with IFNβ-1b sc than with either IFNβ-1a sc formulation. However, when concentrations corresponding to 100 times the IC_{50} of each IFNβ formulation were compared on a weight basis, VSV yield reductions were similar with each of the three IFNβ formulations, probably because at this concentration the plateau phase of the dose–response curve has been reached, and so differences in antiviral activity between IFNβ formulations are no longer observed.

Importantly, differences in the IFNβ-1a sc and IFNβ-1b sc formulations on a weight basis were demonstrated by measuring the expression of well-established ISGs. In particular, IFNβ-1a sc and IFNβ-1a sc NF induced similar levels of ISG expression, and both induced significantly higher mRNA levels of ISGs compared with IFNβ-1b sc. These findings suggest that, on a weight basis, biologic activity is equivalent for both formulations of IFNβ-1a sc and greater than that of IFNβ-1b sc. Thus, a greater weight of IFNβ-1b sc may be needed to achieve the same biologic response as IFNβ-1a sc, despite the fact that IFNβ-1b sc has a molecular weight (approximately 18,500 daltons[34]) that is approximately 82% of that of IFNβ-1a sc (approximately 22,500 daltons[35]). Antonetti and colleagues examined the antiviral activity of IFNβ-1a for sc use and IFNβ-1b for sc use using the same CPE assay system[1] and showed that IFNβ-1a has an antiviral activity approximately 14 times greater than that of IFNβ-1b (0.236 ng/mL versus 3.333 ng/mL, respectively).[1] Similar differences in biologic activity between IFNβ-1a and IFNβ-1b formulations have been reported previously.[9,10]

The findings of this pilot study emphasize the potential importance of determining the relative biologic activity of different IFNβ formulations, and suggest the amount of protein required to achieve a biologic response might be higher for IFNβ-1b than IFNβ-1a formulations. A notable limitation of this study was that IFNβ-1a im (Avonex) was not included for comparison with the other available IFNβ formulations. To date, there are no published studies that have compared within the same assay system the biologic activity of all three IFNβ formulations currently approved for treatment of relapsing MS. A second potential limitation of the present study is that only a single virus and cell model system (VSV/A549) was used to study the biologic activity of the IFNβ formulations. Therefore, additional studies are required that compare the biologic activity of all approved IFNβ formulations; such studies should employ not only the VSV/A549 model system, but also other assay systems with different IFN-sensitive viruses, as well as types of IFN-sensitive cells.

CONCLUSIONS

In conclusion, the biologic activity of IFNβ has been found to vary among different IFNβ formulations. The results of this article indicate that, with respect to the specific assays used, IFNβ-1a sc and IFNβ-1a sc NF exhibit equivalent biologic activity. Furthermore, on a weight basis, the biologic activity of both IFNβ-1a sc formulations is greater than that of IFNβ-1b sc (as measured by these methods), suggesting that a greater amount of IFNβ-1b sc may be needed to achieve the same *in vitro* response as IFNβ-1a sc. The relevance of these *in vitro* findings to the clinical effects of the IFNβ formulations in relapsing MS is unknown; however, examination of *in vitro* biologic responses demonstrates distinct properties of these compounds, the relevance of which needs further investigation.

ACKNOWLEDGMENTS

The authors wish to thank David Gibson Ph.D., CMPP, Callie Grimes Ph.D., and Reza Sayeed M.A. of Caudex Medical, who provided writing and editing assistance with the manuscript, funded by EMD Serono and Pfizer Inc. All authors had significant input to the content of and revisions to the manuscript and received no compensation for their contributions. The Rebif® new formulation used in this *in vitro* study is not approved for clinical use in the U.S.

FUNDING

This article was supported by both EMD Serono, Inc. (Rockland, MA, USA), a subsidiary of Merck KGaA (Darmstadt, Germany), and Pfizer Inc. (New York, NY, USA).

REFERENCES

1. Antonetti, F.; Finocchiaro, O.; Mascia, M.; Terlizzese, M.G.; Jaber, A. A Comparison of the Biologic Activity of Two Recombinant IFN-beta Preparations Used in the Treatment of Relapsing-Remitting Multiple Sclerosis. *J. Interferon Cytokine Res.* **2002**, *22*(12), 1181–1184.
2. Goodin, D.S.; Frohman, E.M.; Garmany, G.P.; Halper, J.; Likosky, W.H.; Lublin, F.D.; Silberberg, D.H.; Stuart, W.H.; van den Noort, S. Disease Modifying Therapies in Multiple Sclerosis: Report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology and the MS Council for Clinical Practice Guidelines. *Neurology* **2002**, *58*(2), 169–178.
3. IFNB Multiple Sclerosis Study Group; University of British Columbia MS/MRI Analysis Group. Interferon Beta-1b in the Treatment of Multiple Sclerosis: Final Outcome of the Randomized Controlled Trial. *Neurology* **1995**, *45*, 1277–1285.
4. Gonzalez-Andrade, F.; Alcaraz-Alvarez, J.L. Disease-Modifying Therapies in Relapsing-Remitting Multiple Sclerosis. *Neuropsychiatr. Dis. Treat.* **2010**, *6*, 365–373.
5. Markowitz, C.E. Interferon-Beta: Mechanism of Action and Dosing Issues. *Neurology* **2007**, *68*(24 Suppl 4), S8–11.

6. PRISMS Study Group. Randomised Double-Blind Placebo-Controlled Study of Interferon Beta-1a in Relapsing/Remitting Multiple Sclerosis. *Lancet* **1998**, *352*(9139), 1498–1504.
7. PRISMS Study Group; University of British Columbia MS/MRI Analysis Group. PRISMS-4: Long-Term Efficacy of Interferon-Beta-1a in Relapsing MS. *Neurology* **2001**, *56*(12), 1628–1636.
8. Jacobs, L.D.; Cookfair, D.L.; Rudick, R.A.; Herndon, R.M.; Richert, J.R.; Salazar, A.M.; Fischer, J.S.; Goodkin, D.E.; Granger, C.V.; Simon, J.H.; Alam, J.J.; Bartoszak, D.M.; Bourdette, D.N.; Braiman, J.; Brownscheidle, C.M.; Coats, M.E.; Cohan, S.L.; Dougherty, D.S.; Kinkel, R.P.; Mass, M.K.; Munschauer, F.E., 3rd; Priore, R.L.; Pullicino, P.M.; Scherokman, B.J.; Weinstock-Guttman, B.; Whitham, R.H. Intramuscular Interferon Beta-1a for Disease Progression in Relapsing Multiple Sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). *Ann. Neurol.* **1996**, *39*(3), 285–294.
9. Deisenhammer, F.; Mayringer, I.; Harvey, J.; Dilitz, E.; Gasse, T.; Stadlbauer, D.; Reindl, M.; Berger, T. A Comparative Study of the Relative Bioavailability of Different Interferon Beta Preparations. *Neurology* **2000**, *54*(11), 2055–2060.
10. Runkel, L.; Meier, W.; Pepinsky, R.B.; Karpusas, M.; Whitty, A.; Kimball, K.; Brickelmaier, M.; Muldowney, C.; Jones, W.; Goelz, S.E. Structural and Functional Differences between Glycosylated and Non-Glycosylated Forms of Human Interferon-Beta (IFN-beta). *Pharm. Res.* **1998**, *15*(4), 641–649.
11. Coyle, P.K.; Hartung, H.P. Use of Interferon Beta in Multiple Sclerosis: Rationale for Early Treatment and Evidence for Dose- and Frequency-Dependent Effects on Clinical Response. *Mult. Scler.* **2002**, *8*(1), 2–9.
12. Durelli, L.; Verdun, E.; Barbero, P.; Bergui, M.; Versino, E.; Ghezzi, A.; Montanari, E.; Zaffaroni, M. Every-Other-Day Interferon Beta-1b versus Once-Weekly Interferon Beta-1a for Multiple Sclerosis: Results of a 2-Year Prospective Randomised Multicentre Study (INCOMIN). *Lancet* **2002**, *359*(9316), 1453–1460.
13. Panitch, H.; Goodin, D.S.; Francis, G.; Chang, P.; Coyle, P.K.; O'Connor, P.; Monaghan, E.; Li, D.; Weinshenker, B. Randomized, Comparative Study of Interferon Beta-1a Treatment Regimens in MS: The EVIDENCE trial. *Neurology* **2002**, *59*(10), 1496–1506.
14. Durelli, L.; Ricci, A. Anti-Interferon Antibodies in Multiple Sclerosis. Molecular Basis and Their Impact on Clinical Efficacy. *Front. Biosci.* **2004**, *9*, 2192–2204.
15. Goodin, D.S.; Frohman, E.M.; Hurwitz, B.; O'Connor, P.W.; Oger, J.J.; Reder, A.T.; Stevens, J.C. Neutralizing Antibodies to Interferon Beta: Assessment of Their Clinical and Radiographic Impact: An Evidence Report: Report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. *Neurology* **2007**, *68*(13), 977–984.
16. Ross, C.; Clemmesen, K.M.; Svenson, M.; Sorensen, P.S.; Koch-Henriksen, N.; Skovgaard, G.L.; Bendtzen, K. Immunogenicity of Interferon-Beta in Multiple Sclerosis Patients: Influence of Preparation, Dosage, Dose Frequency, and Route of Administration. Danish Multiple Sclerosis Study Group. *Ann. Neurol.* **2000**, *48*(5), 706–712.
17. Sorensen, P.S.; Ross, C.; Clemmesen, K.M.; Bendtzen, K.; Frederiksen, J.L.; Jensen, K.; Kristensen, O.; Petersen, T.; Rasmussen, S.; Ravnborg, M.; Stenager, E.; Koch-Henriksen, N. Clinical Importance of Neutralising Antibodies against Interferon Beta in Patients with Relapsing-Remitting Multiple Sclerosis. *Lancet* **2003**, *362*(9391), 1184–1191.
18. Perini, P.; Calabrese, M.; Biasi, G.; Gallo, P. The Clinical Impact of Interferon Beta Antibodies in Relapsing-Remitting MS. *J. Neurol.* **2004**, *251*(3), 305–309.
19. Petkau, A.J.; White, R.A.; Ebers, G.C.; Reder, A.T.; Sibley, W.A.; Lublin, F.D.; Paty, D.W. Longitudinal Analyses of the Effects of Neutralizing Antibodies on Interferon Beta-1b in Relapsing-Remitting Multiple Sclerosis. *Mult. Scler.* **2004**, *10*(2), 126–138.
20. Sorensen, P.S.; Deisenhammer, F.; Duda, P.; Hohlfeld, R.; Myhr, K.M.; Palace, J.; Polman, C.; Pozzilli, C.; Ross, C. Guidelines on Use of Anti-IFN-Beta Antibody Measurements in Multiple Sclerosis: Report of an EFNS Task Force on IFN-Beta Antibodies in Multiple Sclerosis. *Eur. J. Neurol.* **2005**, *12*(11), 817–827.
21. Giovannoni, G.; Barbarash, O.; Casset-Semanaz, F.; King, J.; Metz, L.; Pardo, G.; Simsarian, J.; Sørensen, P.S.; Stubinski, B.; on behalf of the Rebif® New Formulation Group. Safety and Immunogenicity of a New Formulation of Interferon β-1a (Rebif® New Formulation) in a Phase IIIb Study in Patients with Relapsing Multiple Sclerosis: 96-Week Results. *Mult. Scler.* **2009**, *15*(2), 219–228.

22. Jaber, A.; Driebergen, R.; Giovannoni, G.; Schellekens, H.; Simsarian, J.; Antonelli, M. The Rebif New Formulation Story: It's Not Trials and Error. *Drugs R. D.* **2007**, *8*(6), 335–348.
23. Bellomi, F.; Muto, A.; Palmieri, G.; Focaccetti, C.; Dianzani, C.; Mattei, M.; Jaber, A.; Antonelli, G. Immunogenicity Comparison of Interferon-Beta-1a Preparations Using the BALB/c Mouse Model: Assessment of a New Formulation for Use in Multiple Sclerosis. *New Microbiologica* **2007**, *30*, 241–246.
24. Basu, M.; Maitra, R.K.; Xiang, Y.; Meng, X.; Banerjee, A.K.; Bose, S. Inhibition of Vesicular Stomatitis Virus Infection in Epithelial Cells by Alpha Interferon-Induced Soluble Secreted Proteins. *J. Gen. Virol*. **2006**, *87*(9), 2653–2662.
25. Reed, L.J.; Muench, H. A Simple Method of Estimating 50 Per Cent End-Points. *Amer. J. Hygiene* **1938**, *27*, 493–497.
26. Meager, A.; Das, R.G. Biological Standardization of Human Interferon Beta: Establishment of a Replacement World Health Organization International Biological Standard for Human Glycosylated Interferon Beta. *J. Immunol. Methods* **2005**, *306*(1–2), 1–15.
27. Scagnolari, C.; Zingariello, P.; Vecchiet, J.; Selvaggi, C.; Racciatti, D.; Taliani, G.; Riva, E.; Pizzigallo, E.; Antonelli, G. Differential Expression of Interferon-Induced MicroRNAs in Patients with Chronic Hepatitis C Virus Infection Treated with Pegylated Interferon Alpha. *Virol. J*. **2010**, *7*, 311.
28. Scagnolari, C.; Vicenzi, E.; Bellomi, F.; Stillitano, M.G.; Pinna, D.; Poli, G.; Clementi, M.; Dianzani, F.; Antonelli, G. Increased Sensitivity of SARS-Coronavirus to a Combination of Human Type I and Type II Interferons. *Antivir. Ther*. **2004**, *9*(6), 1003–1011.
29. Scagnolari, C.; Midulla, F.; Selvaggi, C.; Monteleone, K.; Bonci, E.; Papoff, P.; Cangiano, G.; Di Marco, P.; Moretti, C.; Pierangeli, A.; Antonelli, G., Evaluation of Viral Load in Infants Hospitalized with Bronchiolitis Caused by Respiratory Syncytial Virus. *Med. Microbiol. Immunol*. **2012**, *201*(3), 311–317.
30. Pachner, A.; Narayan, K.; Price, N.; Hurd, M.; Dail, D. MxA Gene Expression Analysis as an Interferon-Beta Bioactivity Measurement in Patients with Multiple Sclerosis and the Identification of Antibody-Mediated Decreased Bioactivity. *Mol. Diagn*. **2003**, *7*(1), 17–25.
31. Diamond, M. S.; Farzan, M. The Broad-Spectrum Antiviral Functions of IFIT and IFITM Proteins. *Nat. Rev. Immunol*. **2013**, *13*(1), 46–57.
32. Samuel, C.E. Adenosine Deaminases Acting on RNA (ADARs) are Both Antiviral and Proviral. *Virology* **2011**, *411*(2), 180–193.
33. Scagnolari, C.; Antonelli, G., Antiviral Activity of the Interferon Alpha Family: Biological and Pharmacological Aspects of the Treatment of Chronic Hepatitis C. *Expert Opin. Biol. Ther*. **2013**, *13*(5), 693–711.
34. Bayer Inc. Betaseron® (interferon beta-1b) Prescribing Information. Revised January 2013. Available at http://berlex.bayerhealthcare.com/html/products/pi/Betaseron_PI.pdf (accessed July 26, 2013).
35. EMD Serono, Inc. Rebif® (interferon beta-1a) US Prescribing Information. Revised February 2013. Available at http://www.emdserono.com/cmg.emdserono_us/en/images/rebif_tcm115_19765.pdf (accessed July 26, 2013).