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TITLE:

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

ABSTRACT:

□ 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 IFN β -stimulated gene (ISG) activities of IFN β -1a sc NF with IFN β -1a sc original formulation and IFN β -1b sc. When equivalent unit amounts were compared, the IFN β formulations demonstrated similar antiviral activity and induced similar levels of ISG mRNA. However, on a weight basis (ng/mL), significantly more IFN β -1b sc was needed to equal the antiviral activity of either IFN β -1a sc formulation, and both IFN β -1a sc formulations induced significantly higher levels of ISG mRNA than IFN β -1b sc.

INTRODUCTION:

Interferon beta (IFN β) 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, IFN β 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 IFN β -1a (Rebif® Merck Serono S.A., Geneva, Switzerland) administered three times weekly (tiw) and an intramuscular (im) formulation of IFN β -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]

Three formulations of IFN β are currently approved in the USA for treatment of patients with relapsing forms of MS: IFN β -1a 22 μ g or 44 μ g sc tiw (Rebif; original formulation, which is referred to in this article as IFN β -1a sc), IFN β -1a 30 μ g administered im once weekly (Avonex), and IFN β -1b 250 μ g sc administered every other day (Betaseron®/Betaferon®; Bayer HealthCare Pharmaceuticals, Wayne, NJ, USA; Extavia®, Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA). The response to IFN β 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 IFN β formulations demonstrated that higher and more frequent doses of IFN β had greater efficacy compared with lower, less frequent dosing regimens (of a different IFN β formulation) for the treatment of relapsing MS.[2,11–13]

Treatment with IFN β 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 IFN β can reduce the therapeutic effects of treatment.[18–20] A new sc formulation of IFN β -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 IFN β -1a sc NF in a mouse model has suggested a slower and weaker development of NAbs compared with IFN β -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 IFN β may vary among different formulations.[1,10] Specific measures of IFN β biologic activity include the inhibition of viral replication and the enhanced expression of mRNAs that are induced by IFN β . The objective of the current in vitro study was to compare the antiviral and IFN β -stimulated gene (ISG) expression activity of IFN β -1a sc NF with that of the original IFN β -1a sc formulation; IFN β -1b sc was used as an additional comparator.

IFN β Formulations ::: EXPERIMENTAL:

Antiviral activity and induction of ISG expression were evaluated for three formulations of IFN β : IFN β -1a sc, 44 μ g (Rebif, original formulation, lot number Y09B7770V, Industria Farmaceutica Serono, Rome, Italy); IFN β -1a sc NF, 44 μ g (Rebif, serum-free formulation, lot number Y09B0227, Industria Farmaceutica Serono); and IFN β -1b sc, 250 μ g (Betaferon, lot number 1560, Bayer Schering, Berlin, Germany).

Assessment of Antiviral Activity ::: EXPERIMENTAL:

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 (Zooprophyllactic 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 IFN β 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 TCID₅₀ (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 (Zooprophyllactic 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 TCID₅₀/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 IFN β 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 (IC₅₀) calculated in International Units (IU)/mL and in ng/mL when the specific activities of the different IFN β formulations were considered. The specific activities used were 32 MIU/mg for IFN β -1b sc and 270 MIU/mg for IFN β -1a sc. These specific activity values were provided by the manufacturers, and were obtained by comparing the antiviral activity of the IFN β formulations to the World Health Organization (WHO) reference standard of recombinant human IFN β . [26] Furthermore, the antiviral activities of each IFN β formulation were also compared by measuring the VSV yield reductions in A549 cells treated with 10 and 100 times the IC₅₀ of each IFN β formulation obtained after performing the experiments described above; these reductions in VSV yield in IFN β -treated cells were measured relative to the VSV yields in control A549 cells that were not treated with IFN β .

Assessment of ISG Expression ::: EXPERIMENTAL:

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 IFN β 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'-CTGCCTGGCAGAAACTTACC-3'; MxA R: 5'TCTGTTATTCTCTGGTGAGTCTCCTT-3'; MxAP: 6-carboxyfluorescein (FAM)-5'CATCACACATATCTGTAAATCTCTGCCCCCTGTTAGA3'-6carboxy-tetramethylrhodamin (TAMRA)[28]); ADA R1 (ADAR F: 5-CCGGCAGGATGACACAGAC-3'; ADAR R: 5-GCTTGGAATATTCCAAGGC-3'; ADAR P: 5' FAM-CACTTCCCAGGGAGCACGGGCA-3' TAMRA); ISG56 P56 F: 5-TGAAGAAGCTCTAGCCAACATGTC-3'; P56 R: 5-GAGCTTTATCCACAGAGCCTTTTC-3'; P56 P: 5' FAMTATGTCTTTTCGATATGCAGCCAAGTTTACCG-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^{-(ΔΔCt)}, where ΔΔCt = [Ct ISG (+IFN) – Ct GUS (+IFN)] – [Ct ISG (–IFN) – Ct GUS (–IFN)]. All experiments were performed in triplicate.

Statistical Analysis ::: EXPERIMENTAL:

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 IC₅₀, 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 IC₅₀ 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 IC₅₀ 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.

Antiviral Activity ::: RESULTS:

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 IC₅₀ (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 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 IC₅₀ (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 IC₅₀ of each IFNβ formulation. At both 10 and 100 times the IC₅₀, 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 IC₅₀ of each IFNβ formulation were compared on the basis of IFN unit amount (IU/mL). However, when concentrations corresponding to 10 times the IC₅₀ 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 IC₅₀ 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 ::: RESULTS:

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 IFN β -1b sc (Figures 2B, 2D, and 2F), with no significant difference in ISG expression between IFN β -1a sc and IFN β -1a sc NF.

DISCUSSION:

The objective of this current article was to compare the biologic activity of IFN β -1a sc NF with that of IFN β -1a sc; IFN β -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 IFN β on A549 human lung carcinoma cells following infection with VSV and (2) the levels of IFN β -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, IFN β -1a sc, IFN β -1a sc NF, and IFN β -1b sc demonstrated similar levels of antiviral activity in terms of IC₅₀. However, when the formulations were compared on a weight basis (ng/mL), significantly more IFN β -1b sc was required to equal the antiviral activity of either formulation of IFN β -1a sc in terms of IC₅₀. Similarly, when concentrations corresponding to 10 times the IC₅₀ of each IFN β formulation were compared on a weight basis, a smaller reduction in VSV yield was observed with IFN β -1b sc than with either IFN β -1a sc formulation. However, when concentrations corresponding to 100 times the IC₅₀ of each IFN β formulation were compared on a weight basis, VSV yield reductions were similar with each of the three IFN β formulations, probably because at this concentration the plateau phase of the dose–response curve has been reached, and so differences in antiviral activity between IFN β formulations are no longer observed.

Importantly, differences in the IFN β -1a sc and IFN β -1b sc formulations on a weight basis were demonstrated by measuring the expression of well-established ISGs. In particular, IFN β -1a sc and IFN β -1a sc NF induced similar levels of ISG expression, and both induced significantly higher mRNA levels of ISGs compared with IFN β -1b sc. These findings suggest that, on a weight basis, biologic activity is equivalent for both formulations of IFN β -1a sc and greater than that of IFN β -1b sc. Thus, a greater weight of IFN β -1b sc may be needed to achieve the same biologic response as IFN β -1a sc, despite the fact that IFN β -1b sc has a molecular weight (approximately 18,500 daltons[34]) that is approximately 82% of that of IFN β -1a sc (approximately 22,500 daltons[35]). Antonetti and colleagues examined the antiviral activity of IFN β -1a for sc use and IFN β -1b for sc use using the same CPE assay system[1] and showed that IFN β -1a has an antiviral activity approximately 14 times greater than that of IFN β -1b (0.236 ng/mL versus 3.333 ng/mL, respectively).[1] Similar differences in biologic activity between IFN β -1a and IFN β -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 IFN β formulations, and suggest the amount of protein required to achieve a biologic response might be higher for IFN β -1b than IFN β -1a formulations. A notable limitation of this study was that IFN β -1a im (Avonex) was not included for comparison with the other available IFN β formulations. To date, there are no published studies that have compared within the same assay system the biologic activity of all three IFN β 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 IFN β formulations. Therefore, additional studies are required that compare the biologic activity of all approved IFN β 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 IFN β has been found to vary among different IFN β formulations. The results of this article indicate that, with respect to the specific assays used, IFN β -1a sc and IFN β -1a sc NF exhibit equivalent biologic activity. Furthermore, on a weight basis, the biologic activity of both IFN β -1a sc formulations is greater than that of IFN β -1b sc (as measured by these methods), suggesting that a greater amount of IFN β -1b sc may be needed to achieve the same in vitro response as IFN β -1a sc. The relevance of these in vitro findings to the clinical effects of the IFN β 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.