Immunogenicity comparison of Interferon Beta-1a preparations using the BALB/c mouse model: assessment of a new formulation for use in multiple sclerosis

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SUMMARY

The *in vivo* immunogenicity of a new interferon (IFN) beta-1a product (Rebif® New Formulation; RNF) was compared with that of two approved recombinant human IFN beta-1a products (Rebif® and Avonex®). Immunogenic potential was assessed based on time to development of neutralizing antibodies (NAbs) and NAb titer. Female BALB/c mice (six in each group) received RNF, Rebif® or Avonex® (1.0 µg/mL subcutaneously three times weekly), and serum samples collected on Days 7, 21, and 35 (Study 1), or 28, 42, 49, and 60 (Study 2) were assayed for NAbs. In Study 1, no mice had NAbs at Day 7, but by Day 21 one mouse in the RNF group had NAbs, compared with three and four mice in the Rebif® and Avonex® groups, respectively. Results were similar in Study 2. All control mice were NAb negative; all actively treated mice had NAbs by day 35 or 42. Throughout Study 1, NAb titers were lowest in the RNF group and highest in the Avonex® group, and at day 35, NAb titers were significantly lower in the RNF group than the Rebif® group ($p = 0.037$). Results indicate that, on a gram-for-gram basis, RNF appears less immunogenic than Rebif® or Avonex®.

KEY WORDS: Antibodies, Immunogenicity, Interferon beta-1a, Mice, Multiple sclerosis, Rebif® new formulation

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INTRODUCTION

The treatment of multiple sclerosis (MS) has been revolutionized in recent years by the introduction of disease-modifying drugs. Of these, interferon (IFN) beta is the most prolifically prescribed treatment for patients with relapsing remitting forms of MS to decrease the frequency of clinical exacerbations and delay the accumulation of physical disability. There are currently two approved IFN beta-1a products: IFN beta-1a, 44 or 22 µg subcutaneously (sc) administered three times weekly (tiw; Rebif®), and IFN beta-1a, 30 µg intramuscularly administered once weekly (Avonex®). Despite the proven efficacy and tolerability of these approved products, IFN beta (like all therapeutically administered recombinant proteins) can be associated with the development of neutralizing antibodies (NAbs) (Durelli *et al.*, 2004). In the discipline of MS

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research, the impact of NAbs on clinical outcomes and the relative immunogenicity of IFN beta formulations remain hotly debated topics. Although the clinical relevance of NAbs remains unclear due to conflicting evidence (Antonelli *et al.*, 2005, Goodin *et al.*, 2002), long-term studies in patients with MS indicate that the development of persistent high-titer NAbs can reduce the clinical efficacy of IFN beta (IFNB Multiple Sclerosis Study Group, 1996, The PRISMS Study Group, 2001). However, treatment decisions based on NAb status are confounded by the fact that NAb-positive patients receiving high-dose, high-frequency IFN beta-1a may continue to experience similar efficacy compared with those patients receiving low-dose, low-frequency IFN beta-1a (Panitch *et al.*, 2002, Durelli *et al.*, 2002, Panitch, 2003, Goodin, 2004). Furthermore, because some patients with NAbs revert spontaneously to a NAb-negative status (Bellomi *et al.*, 2003, Giovannoni *et al.*, 2005, Sorensen *et al.*, 2005), it seems prudent to make treatment decisions based predominantly on clinical grounds (Antonelli *et al.*, 2005). Nevertheless, to reduce immunogenicity and improve injection tolerability, a new formulation of IFN beta-1a (Rebif® New Formulation; RNF) has been developed that is free from the addition of human serum albumin (HSA) and that does not require the use of fetal bovine serum during its production.

Indirect comparisons of the immunogenicity of IFN beta formulations must be treated cautiously due to differences in study methodology, variability in the sensitivity of assays employed by different studies, and intra-laboratory variability (Antonelli *et al.*, 2005). Importantly, comparisons fail to account for differences in route, dose and frequency of administration of IFN beta – all factors that are known to influence immunogenicity (Schellekens *et al.*, 2002). These limitations have prohibited the assessment of the relative immunogenicity of different formulations. To remove as many confounding variables as possible, we performed a direct comparison of the immunogenic potential of the new IFN beta-1a formulation, RNF, with the two commercially available formulations (Rebif® and Avonex®) using an identical dosing regimen and a single NAb assay in an *in vivo* murine model. In this model, all mice will eventually develop NAbs because recombinant IFN beta is a foreign, human protein. The immunogenic potential is, therefore, assessed based on time to development of NAbs and the resulting NAb titer.

MATERIALS AND METHODS

This study was performed using female BALB/c mice. Only females were included to prevent any form of aggressiveness known to have an effect on immune responses. Mice were housed in micro-isolator cages with *ad libitum* access to food and water. Animals were treated humanely in accordance with Italian regulations (D.Lvo 116/1992). A veterinary surgeon was present to check the health status of the animals to avoid physical injury, suffering and distress. All members of staff involved in the experiments were trained in working with mice. Animals received one of three formulations of IFN beta-1a (Avonex®, Rebif® or RNF) administered at a dose of 1 µg/mL sc tiw, or phosphate-buffered saline (control).

Serum samples were collected 72 hours after the third weekly administration of IFN beta on Days 7, 21, and 35 in our first, primarily exploratory, study (henceforth referred to as Study 1), and on Days 28, 42, 49, and 60 in a second follow-up study (Study 2). All samples were assayed for NAbs to IFN beta-1a using the virus-induced cytopathic effect assay, which is known to be sensitive to very low titers of antibody. Antibody titers were determined using a neutralization test against 10 IU of recombinant IFN beta-1a, as described previously (Antonelli *et al.*, 1998). Controls included titrations of the IFN preparations used in the assay, and titration of a reference standard antibody to IFN beta (NIH code G038-501-572).

Titers were quantified using the Kawade method, in which antiviral neutralization is expressed as a titer defined as the reciprocal of the serum dilution that reduces the IFN potency from 10 laboratory units (LU)/mL of IFN to 1 LU/mL (1 LU/mL is the level of IFN inducing 50% protection against the challenge virus in the assay) (Kawade, 1986, Grossberg *et al.*, 2001). Titers are expressed as ten-fold reduction units (t1/1). A sample with an order of dilution ≥10 was defined as NAb positive. The limit of detection of the assay is 10 TRU. NAb titers are expressed

in Log_{10} and represent the geometric mean (standard deviation) of two independent determinations. Mean values were compared using a *t*-test.

RESULTS

In total, 48 mice were used: 24 in the first study and 24 in the second study (Table 1). Sufficient serum was collected to evaluate NAb activity in all 24 mice in Study 1. However, in Study 2, the serum quantity was insufficient for titration for two mice in the Avonex® group, and two mice in the Rebif® group.

NAbs were not detected in the control groups of mice in either study at any time point. In Study 1, NAbs were not detected in the serum of any mouse at Day 7 in any of the treatment groups (Figure 1). By Day 21, however, one mouse in the RNF group had NAbs, compared with three mice in the Rebif® group and four mice in the Avonex® group. At Day 35, all mice in the active treatment

TABLE 1 - *Numbers of mice included in each treatment group in the two study.*

Treatment group	*Study 1*	*Study 2*
Avonex®	6	6
Rebif®	6	6
RNF (Rebif® New Formulation)	6	6
Control (phosphate-buffered saline)	6	6

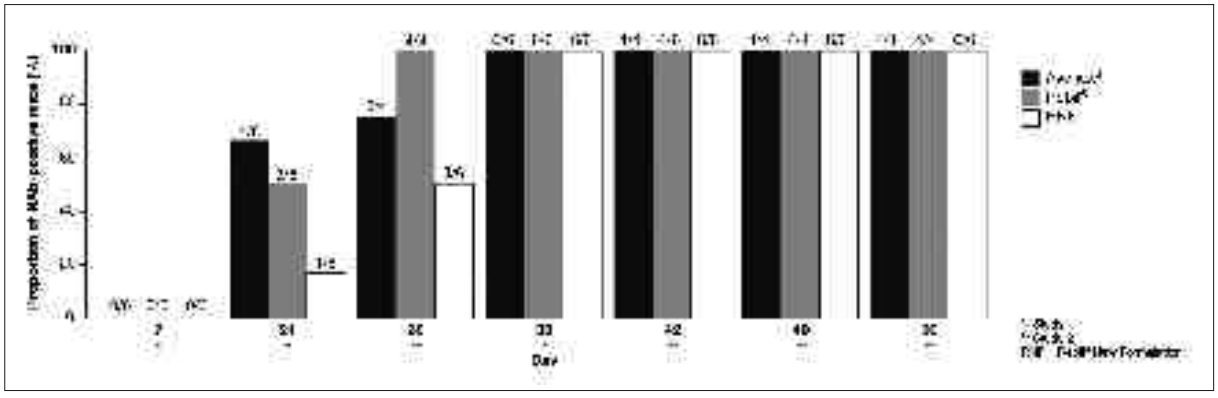

FIGURE 1 - *Incidence of seroconversion to a positive neutralizing antibody (NAb) status.*

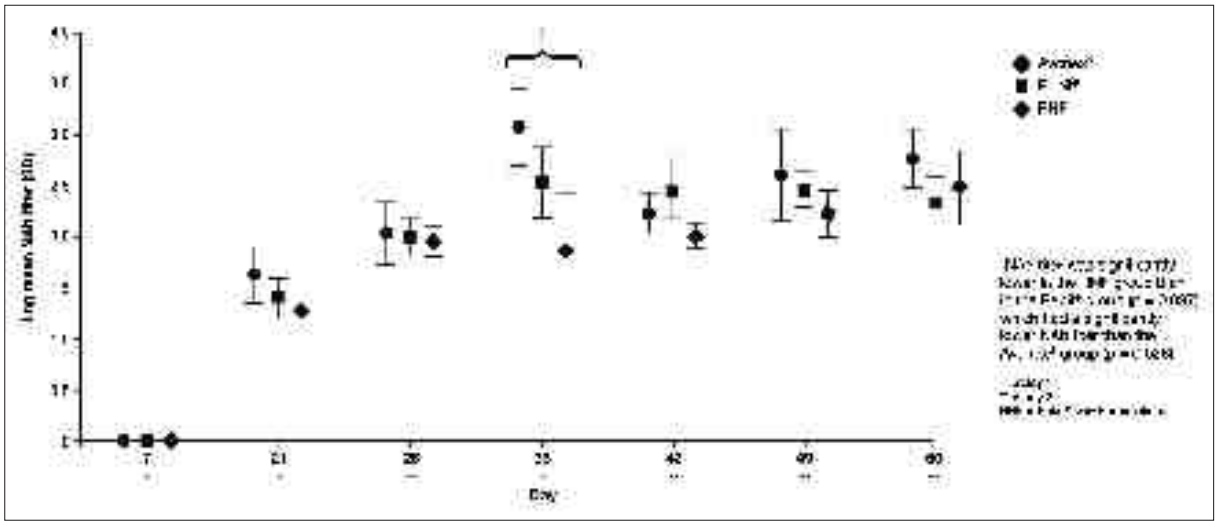

FIGURE 2 - *Log mean (SD) neutralizing antibody (NAb) titer of positive samples.*

groups had developed NAbs. In Study 2, three of the six mice in the RNF group had developed NAbs at Day 28 compared with three of the four mice in the Avonex® group and all four mice in the Rebif® group (Figure 1). Consistent with Study 1, all mice in all treatment groups had developed NAbs by Day 42 in Study 2.

Throughout Study 1, NAb titers were lowest in the RNF group and highest in the Avonex® group (Figure 2). At Day 35, NAb titer was significantly lower in the RNF group (1.91 $t_{1/10}$ ± 0.55) than the Rebif® group (2.55 $t_{1/10}$ ± 0.35; p = 0.037), which had a significantly lower NAb titer than the Avonex® group (3.10 $t_{1/10}$ ± 0.38; p = 0.026). In Study 2, NAb titers were lowest in the RNF group at all time points, except for Day 60, but there were no statistically significant between-group differences (Figure 2).

DISCUSSION

To the authors' knowledge, this was the first direct comparative study to assess the immunogenicity of commercial formulations of IFN beta-1a used in the treatment of patients with MS using identical dosing regimens. The approach allows an objective and direct assessment of the relative immunogenicity of different IFN beta formulations.

We expected that all of the mice would eventually develop NAbs to IFN beta because these recombinant proteins are designed to be analogous to human proteins, not mouse proteins. However, there was a marked difference in the speed at which NAbs developed slower in the RNF group than either of the other two IFN beta-1a formulations. NAb titers confirmed these results if one considers the time of development. The lowest level of NAbs was seen in the mice given RNF. Indeed, at the end of Study 1 (Day 35), the log mean NAb titer was significantly lower in the RNF group than both the Rebif® and the Avonex® groups. Due to the low numbers of mice, significance was only reached at this time point in Study 1; however, NAb titers were consistently lowest in the RNF group.

Rebif® was reformulated to HSA-free RNF, and the final formulation was identified using state-of-the-art technologies with the express aim of minimizing immunogenicity and improving tolerability. Our study in a mouse model suggests that this aim has been achieved on a gram-for-gram basis RNF was less immunogenic than Rebif® and Avonex®. Among other process improvements that were implemented during the development of RNF, the removal of HSA may be a key factor accounting for the relatively low immunogenicity of this formulation. Other studies have shown that aggregates of HSA and IFN can form (Braun *et al.*, 1997, Hochuli, 1997); these aggregates are known to significantly increase immunogenicity compared with IFN monomers (Braun *et al.*, 1997, Schellekens, 2002). The removal of HSA from the RNF formulation will have precluded this possibility, and thus decreased aggregation could be the reason for the apparent lower immunogenicity of RNF. When the possibility of HSA-associated aggregate formation was eliminated for another IFN beta-1a product by reformulating it without HSA, patients with relapsing MS showed a low level of immunogenicity (Phillips *et al.*, 2004).

There are two different immunological mechanisms by which therapeutic proteins can induce the formation of antibodies. The classical immune response occurs as a result of administration of foreign epitopes, while the antibodies formed when non-foreign proteins are administered are the result of breaking immune tolerance (Hermeling *et al.*, 2004). The results of a recent study using a transgenic mouse model immune tolerant for IFNs suggested that in patients administered IFN beta-1a, antibodies are induced by the classical immune response, whereas in those administered IFN beta-1b, antibody formation is likely to be as a result of breaking immune tolerance (Hermeling *et al.*, 2005). Therefore, by using BALB/c mice, which will develop a classical immune response against the foreign antigen (IFN beta-1a), it is hoped that the results obtained in our study are predictive of the response to IFN beta-1a seen in patients.

It should be emphasized that the conditions used in our model do not directly reflect the route of administration and dosing regimen used in clinical practice in patients with MS. Like Rebif®, RNF was developed for administration at 44 µg sc tiw and, therefore, in clinical practice, these formulations will be administered at a higher dose and higher frequency than assessed in our

study, which will intrinsically result in exposure to a greater amount of recombinant protein. Also, it is difficult to separate the relative impact of dose and frequency of administration on immunogenicity (Sorensen *et al.*, 2005). These factors are likely to have the greatest influence on the development of NAbs when IFN beta is administered as indicated in the clinical setting. This, coupled with the fact that results were obtained using an *in vivo* model, means that caution should be exercised when interpreting the results of this study of RNF and Rebif® versus Avonex®. The major finding, however, is the apparent reduction in the relative immunogenicity for RNF compared with Rebif®. If this improvement can be extrapolated to the clinical setting, the new HSA-free formulation marks an important step towards combining the superior efficacy of a high-dose, high-frequency treatment with a lower potential for NAb development, which may result in still greater benefits for patients.

The results of this animal study, which employed an identical dosing regimen for all three IFN formulations tested, indicate that, on a gram-for-gram basis, RNF appears less immunogenic than the current formulation of Rebif® and Avonex®. A Phase IIIb clinical study (protocol 25632) is ongoing to investigate the safety and immunogenicity profile of RNF in patients with MS.

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