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RESEARCH PAPER

Effect of Treatment Regimen on the Immunogenicity of Human Interferon Beta in Immune Tolerant Mice

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ABSTRACT

Purpose Interferon beta is commonly used as therapeutic in the first line of therapy for multiple sclerosis. However, depending on the product, it induces an antibody response in up to 60% of patients. This study evaluated the impact of therapy related factors like dose, route of administration and administration frequency on the immunogenicity of one of the originator interferon beta drugs (Betaferon®) in an immune tolerant transgenic mouse model.

Methods Immune tolerant transgenic mice received injections with Betaferon® via different routes, doses and injection frequencies. Anti-drug antibody (ADA) production was measured by ELISA to assess immunogenicity.

Results A single injection of Betaferon® was found to be sufficient for the induction of ADAs. The antibody titer was enhanced with increasing dose and treatment frequency. Among the tested administration routes, the intravenous route was the most immunogenic one, which is in contradiction with one of the dogma in immunogenicity research according to which subcutaneous administration is the most immunogenic route. Intramuscular, intraperitoneal and subcutaneous injections resulted in comparable immunogenicity.

Conclusion This study shows that treatment related factors affect significantly immunogenicity of Betaseron® and therefore substantiate the need for further studies on these factors in patients.

KEY WORDS interferon beta · immunogenicity · anti-drug antibodies · therapy related factors · animal study

ABBREVIATIONS

ADAs	anti-drug antibodies
ELISA	enzyme-linked immunosorbent assay
IM	intramuscular
IP	intraperitoneal
IV	intravenous
MS	multiple sclerosis
non-Tg	non-transgenic
PCR	polymerase chain reaction
PEG	poly(ethylene glycol)
rhIFNβ	recombinant human interferon beta
SC	subcutaneous
Tg	transgenic
TNFα	tumor necrosis factor α

INTRODUCTION

Recombinant human interferon beta (rhIFNβ) is an approved drug for the treatment of multiple sclerosis (MS). It improves the quality of patients' lives by reducing relapses and disease progression (1). However, rhIFNβ may lead to an antibody

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response that abolishes its therapeutic effect (2, 3). Of the rhIFNβ products of which the immunogenic properties are relatively well investigated, Betaferon® (rhIFNβ-1b) has been found to be the most immunogenic followed by Rebif® and Avonex® (rhIFNβ-1a) (4–7). Although aggregates are the main factors causing immunogenicity of different rhIFNβ products, other variables, including treatment factors, may also contribute to the development of anti-drug antibodies (ADAs) (4, 5, 8, 9). Nonetheless, clear evidence indicating which treatment variables increase the incidence of ADAs and magnitude of antibody response is lacking. The majority of clinical studies compare immunogenicity of different dosing, frequency of injections and route of administration, while different products are used. *Vice versa*, studies that compare immunogenicity of the different products, do not take into account that they have their distinct route/dose and frequency of administration. Moreover, the various studies are difficult to compare because of a lack of standardization of the ADAs assays (10, 11). As a result, conclusions on how treatment-related factors affect immunogenicity of rhIFNβ products are often contradictory (8, 12–15).

In this study we performed a series of experiments to evaluate to what extent the dose, frequency, and route of administration would affect immunogenicity of a single rhIFNβ product, Betaferon®. For this we used the immune tolerant transgenic mouse model developed by Hermeling *et al.* (6) which was later optimized by van Beers *et al.* (16). We found that all investigated treatment factors may significantly modify ADA production. To compare effect of treatment variables on the ADA production during immunogenicity reaction with classical immune response against foreign protein a non-transgenic mice were included in the study.

MATERIALS AND METHODS

Interferon Beta

Betaferon® (rhIFNβ-1b) was obtained from Schering (Berlin, Germany). Lyophilised powder containing 300 μg of rhIFNβ, 15 mg of human serum albumin (HSA) and 15 mg of mannitol was reconstituted in 1 ml of 10 mM sodium phosphate pH7.4, 137 mM sodium chloride (PBS). Before injection, Betaferon® was further diluted to desired concentrations in PBS (see sections below). rhIFNb-1a (Avonex® drug substance) was supplied by Biogen Idec and was formulated before use at a concentration of 270 μg/ml in 100 mM sodium phosphate pH7.2, 200 mM sodium chloride.

Animals

Heterozygote transgenic C57Bl/6 mice carrying the human interferon beta (hIFNβ) gene were crossed with wildtype FVB/N mice (Janvier, BioServices,Uden, The Netherlands). The genotype of the offspring (F1) was determined using PCR showing the presence or absence of the hIFNβ gene in chromosomal DNA isolated from ear tissue. Both PCR positive (transgenic) and PCR negative (non-transgenic) littermates of 6–10 weeks of age were used for the experiments. Mice were housed in standard perspex cages and given access to food (Hope Farms, Woerden, The Netherlands) and water (acidified) *ad libitum*. All testing was in accordance with the European guidelines on animal experiments.

Experimental Setup

Experiment 1: Number and Frequency of Injections

Both non-transgenic (non-Tg) and transgenic (Tg) mice were divided into 4 experimental groups which were administered either 1, 2, 3, or 4 intraperitoneal (IP) injections of Betaferon® ($n=6$ per group, dose: 5 μg/injection). The injections were given on consecutive days starting at day 1. Blood was collected before injections on day 0, 8 and 15. On day 21, the mice were sacrificed by cervical dislocation preceded by blood collection via heart puncture under isoflurane anesthesia.

In addition, non-Tg and Tg mice were subjected to either 1 or 2 IP injections of Betaferon® per week for 6 weeks ($n=6$ per group, dose 5 μg/injection). Animals receiving one injection per week were treated on days 1, 8, 16, 22, 29, and 36. Mice given two injections per week were additionally treated on days 3, 11, 18, 25, 32, and 39. Blood was collected by cheek puncture before injections (day 0) and every week during the first 3 immunization weeks (days 7, 14, and 21). At 6 weeks after the first injection (day 42), the mice were sacrificed by cervical dislocation preceded by blood collection via heart puncture under isoflurane anesthesia.

Plasma was isolated by spinning down the blood (3000 g at 4°C for 10 min) and was stored at −20°C until further analysis.

Experiment 2: Route of Administration

Both non-Tg and Tg mice were injected with Betaferon® for 3 weeks ($n=12$ per group, dose 5 μg/injection), on days 1 to 5, 8 to 12, and 15 to 18 via the IP, subcutaneous (SC, neck), intramuscular (IM, top part of hind legs) or intravenous route (IV, tail vein). Blood was collected by cheek puncture before (day 0) and during the treatment weeks (days 5, 8, 12, and 15). To prevent interference of ADA-drug complexes, blood was collected before injections. On day 19 the mice were sacrificed by cervical dislocation preceded by blood collection via heart puncture under isoflurane anesthesia.

Plasma was isolated and stored as described above.

Experiment 3: Administration Dose

Tg and non-Tg animals ($n=10$ per group) received 5 IP injections of Betaferon® per week for 3 weeks (days 1 to 5,

8 to 12, and 15 to 19). They were treated with a dose of either 0.1 μg, 0.5 μg, 1 μg, 2.5 μg, 5 μg or 20 μg Betaferon® per injection. Blood was collected by cheek puncture before the start of injections (day 0) and twice a week during the 3 week treatment (days 5, 8, 12, 15, and 19). During the treatment weeks, blood was collected before injections to prevent interference of ADA-drug complexes.

Plasma was isolated and stored as described above.

Antibody Assay

ADAs' titers were measured by a modified direct sandwich ELISA described previously (6), using rhIFNb-1a (Avonex®) to coat ELISA plates and peroxidise labeled anti-mouse IgG (Invitrogen, Bleiswijk, The Netherlands) as detecting antibodies, and TMB [3,3',5,5;-TetraMethylBenzidine] (Invitrogen, Bleiswijk, The Netherlands) as substrate. 100-fold diluted plasma was screened and defined positive if the background corrected absorbance values were ten times higher than the average absorbance of the pre-treatment sera. The titer of ADA-positive plasma was determined by plotting the absorbance values at 450 nm (OD450) of a serial dilution against log dilution. The plots were fitted to a sigmoidal dose–response curve using GraphPad Prism version 4.00 (GraphPad Software, San Diego CA, USA). The reciprocal of the dilution of the EC50 value was considered the titer of the serum. Since ADAs' levels of the animals in *experiment 1* were considered positive using the cutoff as described before, but were too low to calculate titers as described, OD450 values using a dilution of 1:100 were used for calculations.

Statistical Analysis

Before determining statistical differences in antibody titers all data were tested for normal distribution. Because antibody titers were not normally distributed, non-parametric Mann–Whitney or Kruskal-Wallis tests were used to assess statistical differences between groups. All calculations were performed using SPSS 16.0 software and a p value$\leq$0.05 was considered significant.

RESULTS

Experiment 1: Number and Frequency of Injections

In order to assess the minimal number of injections necessary to induce ADA formation, animals were injected with Betaferon® one, two, three, or four times on consecutive days. As shown in Fig. 1, a single injection was sufficient to evoke an ADA

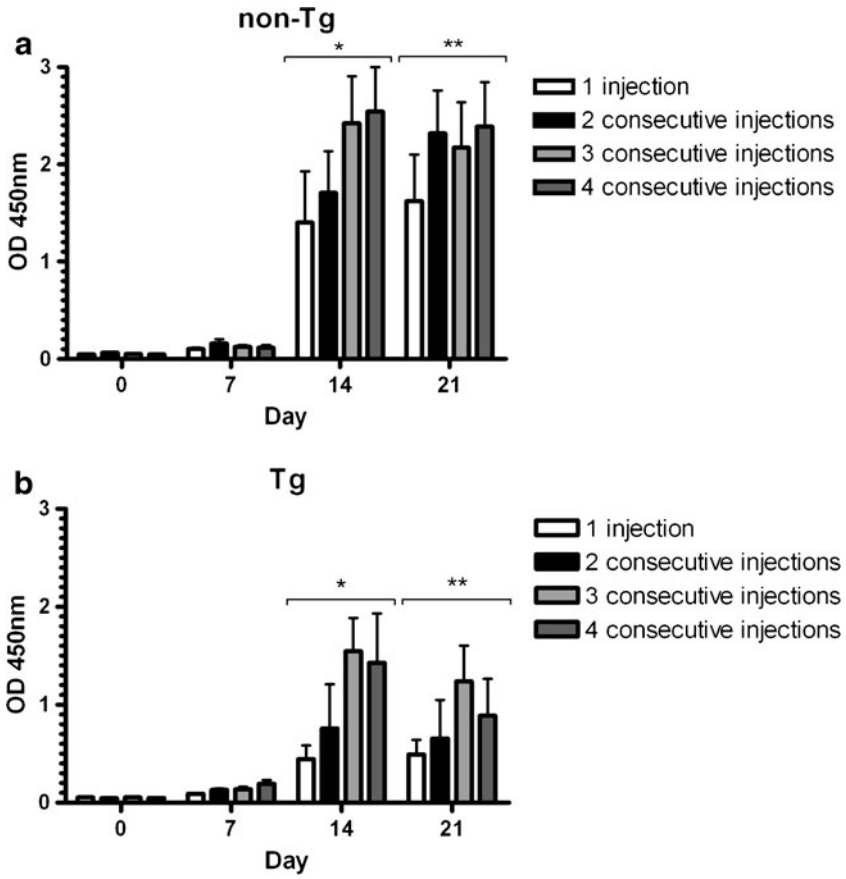

Fig. 1 Anti-rhIFNβ antibody levels in non-Tg (**a**) and Tg (**b**) mice after 1, 2, 3, and 4 consecutive IP injections (dose 5 μg per injection). Bars represent average OD 450 nm for 6 animals and corresponding SEM. A higher number of injections did not result in significantly higher ADA production in either Tg or non-Tg animals. However, ADA levels were significantly higher in non-Tg than Tg animals on day 14 ($p = 0.008$*) and day 21 ($p < 0.001$**).

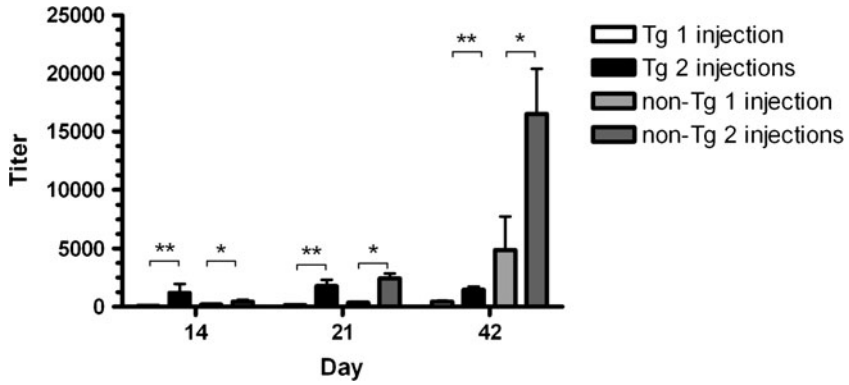

Fig. 2 Anti-rhIFNβ antibody titers in animals injected once or twice per week for 6 weeks with Betaferon. Bars show average titer of anti-rhIFNβ IgG and corresponding SEM ($n = 6$). Two instead of 1 injections per week significantly increased ADA production (Tg $p < 0.001$**, non- Tg $p = 0.009$*).

response in both the Tg and non-Tg animals. However, for both types of animals, the ADA production was much lower than in other experiments in which treatment lasted for at least 3 weeks (instead of at most 4 days). Therefore OD450 values were used as a measuret of ADA production. For all treatment groups the OD450 values in the non-Tg mice were significantly higher on days 14 and 21 compared to the Tg animals ($p = 0.008$ at day 14 and $p < 0.001$ at day 21). Increasing the number of injections did not results in significantly higher OD450 levels in the Tg mice ($p = 0.412$) or in the non-Tg ($p = 0.765$).

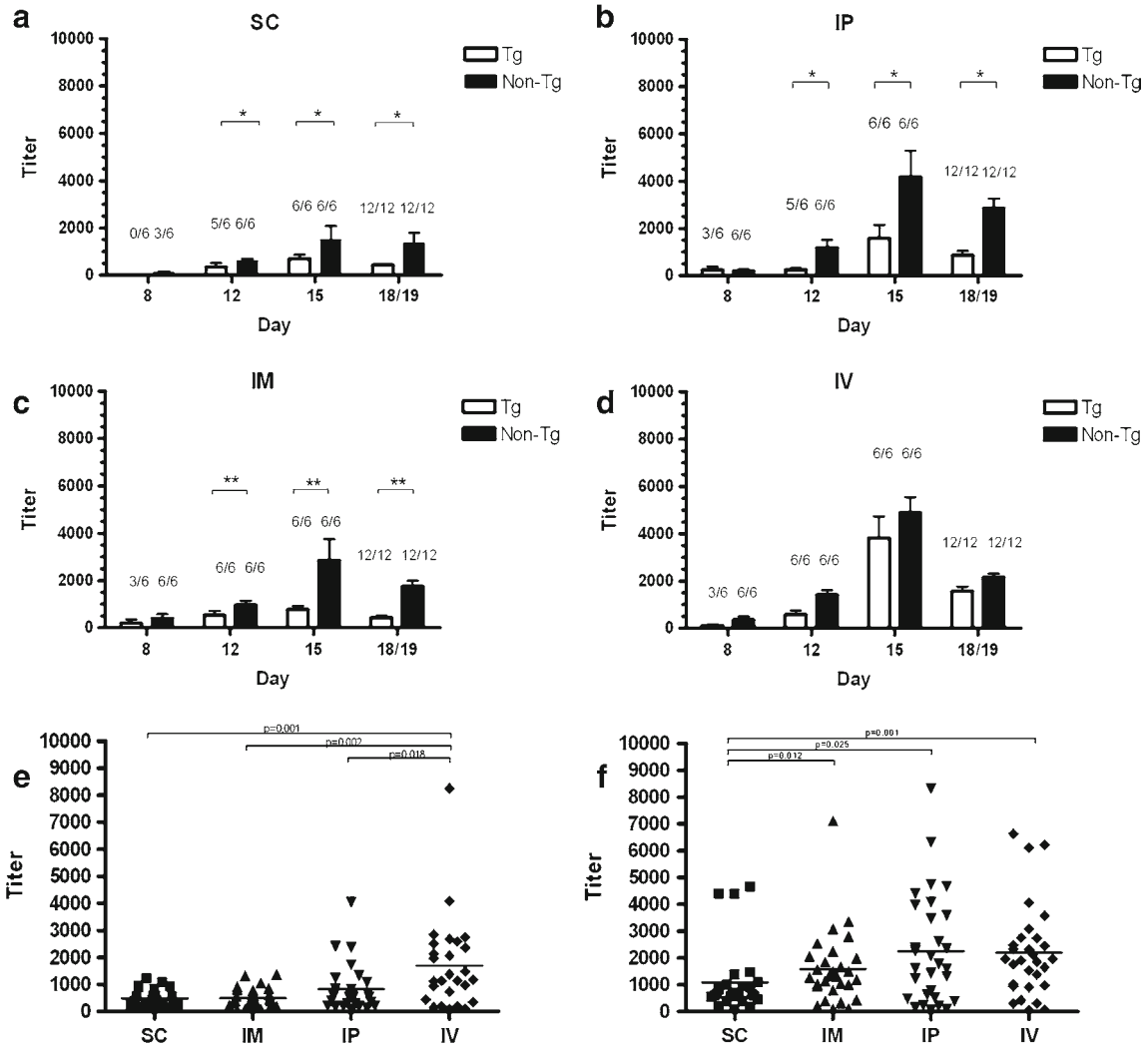

Fig. 3 Effect of route of administration on ADA response. Development of ADAs over time in animals injected SC (**a**), IM (**b**), IP (**c**), and IV (**d**). Bars show averages of titer of anti-rhIFNβ total IgG with corresponding SEM. Antibody responses in Tg mice were statistically lower compared to non-Tg mice for SC, IM, and IP administration (SC and IP $p < 0.05$; IM $p < 0.001$). Comparison of ADA production within the treatment period in Tg (**e**) and non-Tg (**f**) animals.

In the second part of this experiment, the impact of the frequency of injection on ADA production was tested. Tg and non-Tg mice received either one or two IP injections of Betaferon® per week for a total time period of 6 weeks. The two injections per week schedule resulted in higher ADA levels compared to one injection per week in both Tg and non-Tg mice (Fig. 2) with $p<0.001$ and $p=0.009$, respectively. Interestingly, ADA titers in Tg animals remained almost unchanged and relatively low during the treatment period, whereas in non-Tg mice production of ADAs seemed to intensify over time. On day 42 the ADA titers in non-Tg mice were 6–10 times higher than on day 21 ($p=0.017$).

Experiment 2: Route of Administration

As shown in Fig. 3, ADA production strongly depended upon the route of administration and the effect of administration route differed between Tg and non-Tg mice. For the non-Tg mice the least immunogenic route was SC administration, resulting in the lowest ADA titers (SC *vs* IM $p=0.012$, SC *vs* IP $p=0.025$, SC *vs* IV $p=0.001$). In addition, it appears that this route resulted in the lowest number of ADA-positive non-Tg animals during the treatment period. Administration of Betaseron® via the IV route, which is commonly believed to be the least immunogenic one ((17, 18)), resulted in ADA titers in non-Tg mice that were comparable to those induced via the IM and IP routes of administration ($p=0.133$ and $p=0.848$ respectively).

In the Tg animals, the SC route also seemed to be the least immunogenic, but the difference in ADA titers between SC/IM and SC/IP routes was not significant ($p=0.377$ and $p=0.335$, respectively). However, the onset of the ADAs response in the Tg animals seemed to be slower for the SC-treated mice compared to the animals injected via the IM or IP route. Surprisingly, the administration route leading to the highest ADA production in the Tg mice was IV (IV *vs* SC $p=0.001$, IV *vs* IM $p=0.002$, and IV *vs* IP $p=0.018$). Injection of

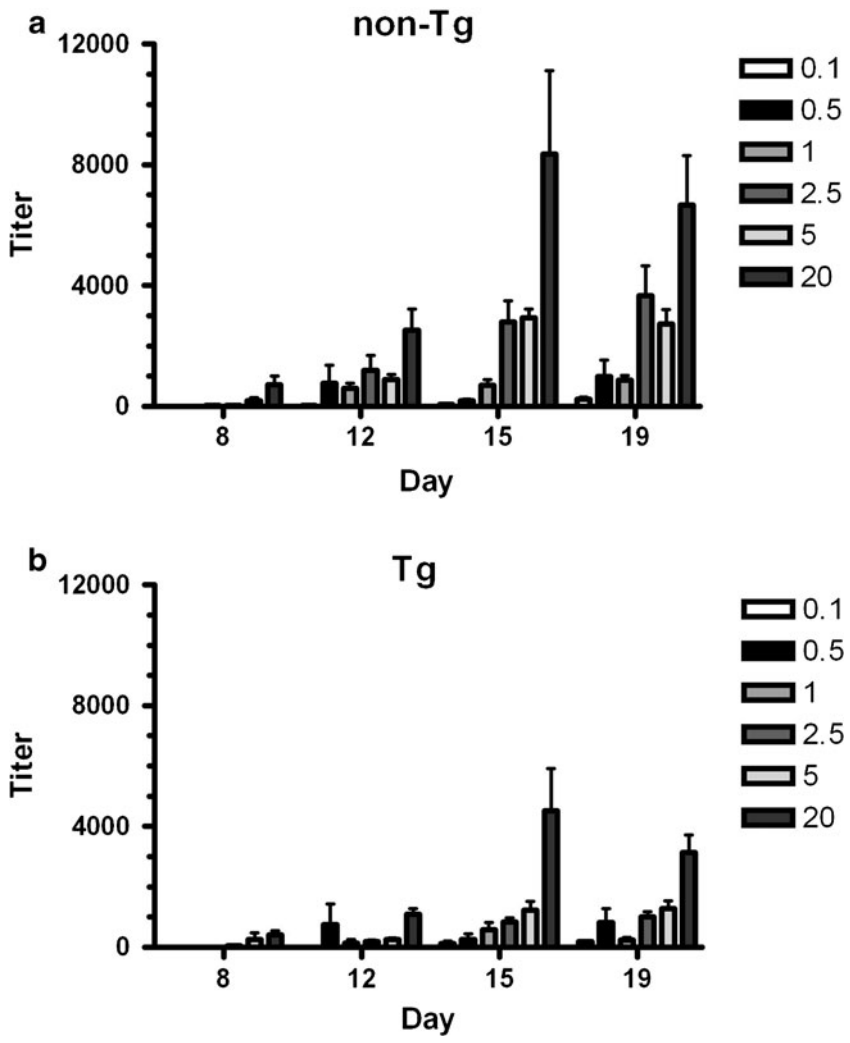

Fig. 4 Formation of anti-rhIFNβ antibodies in Tg (**a**) and non-Tg (**b**) animals injected with 0.1, 0.5, 1, 2.5, 5 and 20 μg Betaferon®. Bars represent average titer of ADA-positive animals and corresponding SEM.

Betaseron® via IP and IM routes resulted in similar ADA titers ($p=0.692$).

Experiment 3: Administration Dose

Animals were treated with 1 of 6 different doses of Betaferon® in the range of 0.1 μg–20 μg per IP injection (5 injections per week for 3 weeks). As shown in Fig. 4 and in Table I, ADA production depended upon the dose administered. The higher the dose used, generally the higher the ADA production in both Tg ($p<0.01$) and non-Tg ($p<0.01$) mice. Moreover, increasing the dose resulted in a faster onset of ADA response and a higher number of ADA positive animals ($p=0.0028$ for Tg animals, $p=0.0167$ for non-Tg animals). In addition, as shown in Table I and Fig. 4, the onset of ADAs appeared to be similar in Tg and non-Tg mice. The overall ADA titers in non-Tg mice were significantly higher than in the Tg mice ($p<0.01$).

Table I Number of ADA Positive Animals Injected with Different Doses of Betaferon® at Successive Time Points

Dose [μg]	Time point [Day]	Tg responders[1] [Number/%]	Non-Tg responders[1] [Number/%]
0.1	8	0/0	0/0
	12	0/0	3/60
	15	2/40	2/40
	19	1/20	5/100
0.5	8	0/0	0/0
	12	2/40	3/60
	15	4/80	5/100
	19	5/100	5/100
1	8	0/0	1/20
	12	3/60	5/100
	15	5/100	5/100
	19	5/100	5/100
2.5	8	2/40	5/100
	12	5/100	5/100
	15	5/100	5/100
	19	5/100	5/100
5	8	3/60	5/100
	12	5/100	5/100
	15	5/100	5/100
	19	5/100	5/100
20	8	5/100	5/100
	12	5/100	5/100
	15	5/100	5/100
	19	5/100	5/100

[1)] The cutoff used to determine positivity was established by multiplying by a factor of 10 the average OD450 from screening Elisa before treatment with Betaferon®. Samples with OD450 > cutoff point were considered as positive and used for titer calculations

DISCUSSION

The treatment regimen used for the administration of rhIFNβ during MS therapy has been proposed to be one of the major factors influencing ADA production (17, 19). However, it is not known which treatment component, i.e. dose, frequency, or route of administration, is the most prominent in reducing or enhancing ADA production to rhIFNβ. Reports are often contradictory and therefore the impact of treatment related factors on ADA production is not clear (12, 13, 15).

The experiments described here show that different treatment-related factors affect the antibody response against rhIFNβ. We show that even a single administration of Betaferon® can lead to formation of ADAs in our immune tolerant mice. Interestingly, increasing the number of daily injections did not increase ADAs' levels significantly. This suggests that the magnitude of ADAs response might depend on the treatment duration and that a treatment duration limited to a maximum of 4 days is too short to strongly activate the immune system in the immune tolerant mice. In the second part of *experiment 1*, animals were given 1 or 2 injections of Betaferon® per week for 6 weeks. In non-Tg animals the ADAs' levels intensified over time, which suggests that injections given to these mice in the second and later weeks might have resulted in a booster effect as found after vaccine administration. In contrast, in Tg animals ADA titers from day 14 onward remained unchanged. The cumulative results from the two parts of *experiment 1* show that the probability of an immune response and magnitude of ADA production against Betaferon® increases with higher administration frequency and/or higher number of injections and longer treatment duration. However, in Tg animals the impact of treatment duration seems to be more complex than in non-Tg mice; longer treatment duration in the second part of *experiment 1* resulted in higher ADA production but a lack of an booster effect led to stable ADA titers over time.

These data partially correlate with clinical observations. We show here that in both Tg and non-Tg mice, even a single application of Betaferon® is sufficient to induce ADAs In contrast, in MS patients ADA production begins 3 to 12 months after the start of treatment (20). On the other hand, stable ADA levels found in Tg mice correspond to clinical data, where ADA titers have a tendency to persist unchanged for a long period of time or even show a tendency to decline (8, 20). Moreover, 2 injections of Betaferon® per week resulted in higher ADA production in both types of mice. Also in patients, products administered more frequently seem to be more immunogenic. Betaferon® and Rebif® which require administration 3–4 times per week are more immunogenic in terms of neutralizing antibodies than Avonex® which is injected once a week. However, more extensive studies in humans focusing only on one product are necessary to fully confirm the data presented here.

The most commonly accepted hierarchy of the immunogenicity of administration routes places IV as the least and SC as the most immunogenic one. Moreover, this classification seems to be confirmed by pre-clinical and clinical data (21, 22). However, the finding that the IV route is highly immunogenic corresponds to at least two other reports on immunogenicity of biotherapeutics. An anti-TNFα antibody was found to induce higher antibody titers in cynomolgus monkeys when injected IV than after SC application (23). Similarly, a PEGylated form of Factor VIII was more immunogenic in haemophilia A mice when administrated IV (24). This study, together with these reports, shows that immunogenicity is dependent on the route of administration and that immunogenicity due to different routes might not always be in accordance with generally believed dogma in clinical practice (4, 17). Clinical testing in patients should therefore confirm which route of application is most safe, ideally for different therapeutic protein drugs.

Why the different routes of administration are more or less immunogenic will probably be a combination of product characteristics, such as the presence of aggregates, the microenvironment present at the site of injection, and kinetics of drug disposition. A higher aggregate content may result in prolonged exposure at the site of injection, giving more time for activation of local immune cells.

High immunogenicity due to SC administration is usually explained by an elongated exposure of the antigen to anigen-presenting cells, increasing the probability of their activation and subsequent stimulation of T- and B cells (25). The low immunogenicity of the SC route observed in our experiment might be the effect of a difference in skin-muscle connections in mice compared to humans, resulting in faster spreading of injected solution under the skin than in humans. That might lead to faster clearance of protein and as a consequence different activation of skin immune cells. However, the low immunogenicity of rhIFNβ after SC injection might be an intrinsic property of this protein. IM and IP administration may create a drug deposit inside the muscle or peritoneal cavity that slowly releases the protein. Slow release of protein from the injection site, together with close proximity to draining lymph nodes might explain relatively high immune response in non-Tg mice after administration of proteins via those routes. Since Betaferon® is a foreign protein for non-Tg animals it is expected to activate immune cells more efficiently than in Tg mice. Indeed, Tg animals developed very similar ADA titers after SC, IM and IP.

A high content of aggregates may be responsible for the high immunogenicity of Betaferon® after IV administration. Large aggregates present in Betaferon® may be easily recognized and taken up by splenic macrophages and therefore might lead to activation of other immune cells e.g. marginal zone B cells. Frequent injection of such structures may lead to sustained activation of those cells and therefore promotion of ADAs generation. However, due to a lack of convincing immunological data supporting this hypothesis, further studies are needed to investigate potentially higher activation of splenic macrophages by frequent injections of aggregated proteins, and the effect of the proximity of draining lymph nodes.

The high treatment dose used for Betaferon® (dose 250 μg/injection) compared to the dose given for Avonex® and Rebif® (30 μg/injection or 22–44 μg/injection, respectively), has been suggested as one of the reasons why the former is more immunogenic in patients. However, data from clinical studies do not always confirm that a higher dose of protein correlates with higher immunogenicity (4, 23). In our study, we show that the dose is an important factor affecting immunogenicity in our immune tolerant mice. The higher the dose, the more ADAs were produced by both Tg and non-Tg animals. Moreover, our results and clinical data suggest that the impact of dose on immunogenicity might be strongly protein dependent. More detailed human studies are necessary to fully understand the influence of dose on ADA production.

CONCLUSIONS

This report describes the results of a study focused on the impact of treatment-related factors on the immunogenicity of therapeutic rhIFNβ in immune tolerant mice, with the focus on the rhIFNβ-1b product; Betaferon®. We studied the effect of protein dose, number of injections, treatment frequency, and route of administration on ADA response, experiments which are difficult to do in patients. We found that all these factors may have a significant impact on the risk of ADA development. One has to keep in mind that direct translation of our results from animals to patients is not possible and the results shown in this report should be, as far as possible, confirmed in humans.

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