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Research Paper

Development of a Transgenic Mouse Model Immune Tolerant for Human Interferon Beta

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Purpose. Therapeutic proteins may induce antibodies that inhibit their efficacy or have other serious biological effects. There is a great need for strategies to predict whether a certain formulation will induce an immune response. In principle, conventional animals develop an immune response against all human proteins no matter how they are formulated, which restricts their use. The aim of this study was to develop a mouse model immune tolerant for human interferon beta (hIFNβ).
Methods. A transgenic mouse model immune tolerant for hIFNβ was developed by making C57Bl/6 mice transgenic for the hIFNβ gene. To evaluate the model, both wild-type and transgenic mice were immunized with recombinant human interferon beta 1a (rhIFNβ-1a) and recombinant human interferon beta 1b (rhIFNβ-1b). Serum antibodies against rhIFNβ were detected by ELISA.
Results. The genetically modified mice were shown to be immune tolerant for mammalian cell–derived rhIFNβ-1a, which has a relative low immunogenicity in patients. However, *Escherichia coli*–derived rhIFNβ-1b, known to have a relatively high immunogenicity in patients, was shown not only to be immunogenic in the wild-type mice but could also break the immune tolerance of the genetically modified mice.
Conclusions. This animal model offers the possibility to study the many factors influencing the immunogenicity of hIFNβ and test new formulations before going into clinical trials. The model also provides the first evidence that the rhIFNβs differ in the immunological mechanisms responsible for the development of antibodies.

KEY WORDS: immune tolerance; immunogenicity; protein formulations; recombinant human interferon beta; transgenic mice.

INTRODUCTION

Immunogenicity of therapeutic proteins is attracting increasing interest from pharmaceutical companies and regulatory authorities. The possibility of predicting immunogenicity is becoming an important issue with the increasing awareness concerning the potential serious clinical consequences of antibody formation to administered proteins. In particular, the problems with a severe form of anemia associated with the induction of antibodies to epoetin alpha after treatment with recombinant epoetin alpha in patients with chronic renal failure have increased the concern of regulatory authorities about the immunogenicity of therapeutic proteins (1). Many factors influence the immunogenicity of these biopharmaceuticals (2). There are two different mechanisms by which therapeutic proteins can induce the formation of antibodies. When a nonhuman protein is administered, antibodies develop as a result of the classical immune response to foreign epitopes. The same mechanism is responsible for the induction of antibodies by human proteins in patients with an innate deficiency for the administered protein and thereby a lack of immune tolerance. However, in the majority of cases, human proteins are being administered to patients with a normal immune system, and the antibodies are the result of breaking immune tolerance (2). In general, binding antibodies (BAbs) are formed initially. This may be followed by neutralizing antibodies (NAbs). NAbs inhibit binding of the administered protein to its target and neutralize its biological actions (3). The biological significance of BAbs is still under debate. The antibodies and the therapeutic protein may form immunocomplexes that are rapidly cleared by the reticuloendothelial system, thus reducing the circulation time of the therapeutic protein and potentially diminishing its therapeutic effects.

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ABBREVIATIONS: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); BAb, binding antibody; CHO cells, Chinese hamster ovarian carcinoma cells; *E. coli*, *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; HSA, human serum albumin; IFNβ, interferon beta; MS, multiple sclerosis; NAb, neutralizing antibody; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; hIFNβ, human interferon beta; i.m., intramuscular; i.p., intra-peritoneally; i.v., intravenously; polyICLC, polyinosinic:polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose; rhIFNβ, recombinant human interferon beta; s.c., subcutaneous.

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Interferon beta (IFNβ) is a cytokine mainly produced by macrophages, epithelial cells, and fibroblasts. It is a regulatory protein with anti-inflammatory, antitumor, antiviral, and cell-growth regulatory effects (4,5). Chronic administration of human IFNβ (hIFNβ) is an effective treatment in relapsing-remitting multiple sclerosis (MS) patients (5). Three recombinant hIFNβ (rhIFNβ) formulations are currently on the market for the treatment of MS: Betaseron/Betaferon (rhIFNβ-1b; Betaseron, Berlex Laboratories, Montville, NJ, USA; Betaferon, Schering, Berlin, Germany), Avonex (rhIFNβ-1a; Biogen-Idec, Cambridge, MA, USA), and Rebif (rhIFNβ-1a; Serono, Inc., Rockland, MA, USA). Betaseron/Betaferon was the first formulation to come on the market (1994 in the United States and 1995 in Europe). Avonex followed in 1996 and 1997 in the United States and Europe, respectively. Rebif was the last one to be marketed (1998 in Europe and 2002 in the USA) (6). RhIFNβ-1b is produced in *Escherichia coli* (*E. coli*) cells and is nonglycosylated, has no N-terminal methionine, and Cys-17 is mutated to Ser-17 to reduce misfolding during the denaturation-renaturation step in downstream processing. RhIFNβ-1a is produced in CHO cells, is glycosylated, and has the same amino acid sequence as natural hIFNβ.

As is the case with almost all other therapeutic proteins, rhIFNβ can induce antibodies after a prolonged period of treatment. Antibodies formed after administration of rhIFNβ-1a fully cross-react with rhIFNβ-1b and vice versa (7,8). The immunogenicity of the three marketed products differs because of differences in structure, formulation and other factors [for an overview, see (2)], such as the dose and route of administration (see Table I). The difference in the administered dose between rhIFNβ-1a and rhIFNβ-1b is due to their distinct biological activity [200–270 MIU/mg and 32 MIU/mg, respectively (4)]. According to Runkel *et al.* (9), the difference in biological activity is related to the degree of glycosylation. Lack of glycosylation was correlated with an increase in aggregation of rhIFNβ-1b due to disulfide interchange. The three cysteines are normally inside the protein (10), and deglycosylation may cause disulfide interchange, which may make a large fraction of the protein to denature (9). Mark *et al.* have shown that rhIFNβ aggregates have a reduced biological activity (10).

The incidence of NAb development in patients is higher with rhIFNβ-1b than with rhIFNβ-1a (4,11), and among the rhIFNβ-1a formulations higher with Rebif than with Avonex (6,12). It is likely that the high immunogenicity observed with Betaseron (6,13) is caused by breaking of immune tolerance due to the presence of soluble aggregates in this product. Betaseron contains, based on size-exclusion chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), 60% noncovalently stabilized aggregates (9). It is less clear what underlies the immunogenicity of rhIFNβ-1a, particularly Rebif, where the percentage of patients developing NAbs has been reported to be as high as 58% (14).

These differences in immunogenicity were established in clinical studies. The availability of preclinical tests for predicting the immunogenicity of therapeutic proteins would save time and money and minimize patient risks. Immune tolerant transgenic animal models are important tools to develop safer therapeutic proteins. Transgenic mice have already been used to study the mechanisms of tolerance against self-proteins (15) and to study the immunogenicity of three therapeutic proteins: various forms of insulin (16), interferon alpha-2a (17), and mutated human tissue plasminogen activator (18). These models were primarily used to study the presence of new epitopes in modified proteins. The aim of the current study was to develop a transgenic mouse model tolerant for hIFNβ to enable study of the immunogenicity of new rhIFNβ formulations, to serve as a tool for quality control, and to elucidate the immunological mechanisms resulting in antibody induction.

MATERIALS AND METHODS

Animals

Wild-type (C57Bl/6) mice were obtained from Charles River Laboratories (Maastricht, The Netherlands). Food

Table I. Excipients, Route, Dose, and Frequency of Administration of the Three rhIFNβ Formulations Currently on the Market[a]

Formulation	Type	Form	Excipients	Route	Dose	Frequency
Betaseron/ Betaferon	rhIFNβ-1b	Lyophilized powder	HSA, di- and mono-basic sodium phosphate, sodium chloride, final pH 7.2	s.c.	250 μg	Every other day
Avonex	rhIFNβ-1a	Lyophilized powder	HSA, di- and monobasic sodium phosphate, sodium chloride, final pH 7.2	i.m.	30 μg	Once weekly
		Ready-to-use syringe	Arginine HCl, polysorbate 20, sodium acetate, glacial acetic acid, final pH 4.8	i.m.	30 μg	Once weekly
Rebif	rhIFNβ-1a	Ready-to-use syringe	Mannitol, HSA, sodium acetate, acetic acid, sodium hydroxide, final pH 3.8	s.c.	22 μg or 44 μg	Three times per week

HSA, human serum albumin; s.c., subcutaneous; i.m., intramuscular.
[a] Adapted from ref. (6).

(Hope Farms, Woerden, The Netherlands) and water (acidified) were available *ad libitum*. All animal experiments were approved by the appropriate national and institutional animal ethical committees. Also, the mandatory licenses from the appropriate national authorities for producing transgenic animals and handling genetically modified organisms were obtained.

Construction of Transgenic Mice

A 3.1-kb *Aat*II-*Xho*I DNA fragment encoding hIFNβ behind the mouse IFNβ promoter was excised from PF370, a plasmid derivative of pDOI5 expressing hIFNβ. The DNA fragment was microinjected into fertilized ova of mice. The offspring were analyzed for the hIFNβ gene in their chromosomal DNA by polymerase chain reaction (PCR). Positive offspring was used for further breeding.

Human Interferon Beta Production

In order to become immune tolerant for hIFNβ, the mice should express hIFNβ protein. This was tested by injecting PCR positive and negative mice polyICLC. PolyICLC is synthetic double-stranded RNA complexed with polylysine and carboxymethylcellulose (19). It is an inducer of IFNβ, because it mimics viral RNA (19,20). Because the hIFNβ gene is behind the mouse IFNβ promoter, polyICLC should induce not only murine but also hIFNβ if the transgene is active.

Mice received 1 mg/kg polyICLC intravenously (i.v.). Before and 6 h after the administration of polyICLC, blood was taken from the vena saphena. The presence of hIFNβ in the serum was analyzed by an ELISA specific for hIFNβ.

Immune Tolerance

Unformulated Avonex-rhIFNβ-1a (Avonex-rhIFNβ-1a in 100 mM sodium phosphate, 200 mM sodium chloride, pH 7.2) was supplied by Biogen-Idec Inc. To test whether the transgenic mice were immune tolerant, two groups of 5 wild-type mice and two groups of 5 transgenic mice were injected with 5 or 10 μg rhIFNβ-1a intraperitoneally (i.p.) on 5 days per week for 3 weeks. Blood was collected from the vena saphena before treatment, weekly during treatment, and 3 days after the last injection. Sera were stored at −20°C prior to antibody testing.

To show that the immune tolerance was specific and not based on a general immune suppression, Avonex-rhIFNβ-1a with human serum albumin (HSA) was injected in 5 wild-type mice and 5 transgenic mice with the same injection schedule as described above, and anti-HSA antibody formation was monitored. Also, the immunogenicity of Betaseron was tested. For this purpose, 5 wild-type and 5 transgenic mice were injected with 5 μg Betaseron using the same immunization schedule as described above.

ELISA for Determination of Antibodies Against rhIFNβ or HSA

Microlon 96-well plates (Greiner, Alphen aan de Rijn, The Netherlands) were incubated overnight with 100 μl rhIFNβ-1a or HSA (2 μg/ml) per well at 4°C. Then the wells were drained and washed 2 times with 300 μl wash buffer [0.05% Tween 20 in phosphate-buffered saline (PBS)]. After washing, the wells were carefully tapped dry on a tissue. Wells were blocked by incubating with 300 μl 1% bovine serum albumin in PBS for 1 h at room temperature while the plates were shaken. The plates were drained and washed 3 times with 300 μl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. After the addition of serum dilutions to the wells, the plates were incubated for 1 h at room temperature while gently shaken. The plates were washed 3 times with 300 μl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Peroxidase labeled anti-mouse IgG (Sigma, Zwijndrecht, The Netherlands) was added to the wells, and the plates were incubated for 1 h at room temperature while gently shaken. Plates were drained and washed 5 times with 300 μl wash buffer and once with 300 μl PBS. After the last wash, wells were carefully tapped dry on a tissue. ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)]-substrate (Roche, Almere, The Netherlands) was added, and absorbance was recorded after 30 min of incubation with a Novapath microplate reader (Biorad, Veenendaal, The Netherlands) at a wavelength of 415 nm and a reference wavelength of 490 nm. During all incubation steps, the plates were covered.

Sera were arbitrarily defined positive if the absorbance value of the 1:100 dilution of the sera minus the background was 10 times higher than the average absorbance value of the pretreatment sera minus the background.

To determine the antibody titer of the positive sera, the sera were serially diluted, and the absorbance values were plotted against log dilution. Curves were fitted with GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA). The reciprocal of the dilution of the EC_{50} value was taken as the titer of the serum. A standard anti-hIFNβ serum was added to each plate, and the average log titer value was 2.630 with an estimated variation coefficient of 4% ($n = 12$).

RESULTS

Production and Characterization of Transgenic Mice

Two founders (strains 71 and 72) containing the intact interferon transgene were identified and bred for further experiments. Strains 71 and 72 were both tested for the inducibility of hIFNβ. Only strain 72 produced hIFNβ protein. The hIFNβ levels in the transgenic mice after polyICLC injection (168 ± 33 ng/ml) were significantly different from the hIFNβ levels before treatment (9.7 ± 2.8 ng/ml) (paired t test; $p = 0.0044$).

Immune Tolerance

Wild-type and transgenic mice were injected daily i.p. with 5 μg or 10 μg rhIFNβ-1a. The antibody titers of the wild-type mice are shown in Fig. 1. For the wild-type mice, rhIFNβ is a foreign protein and antibodies were formed, as expected. Although the mice receiving 5 μg produce an earlier response, the anti-IFNβ antibody levels after 21 days in the

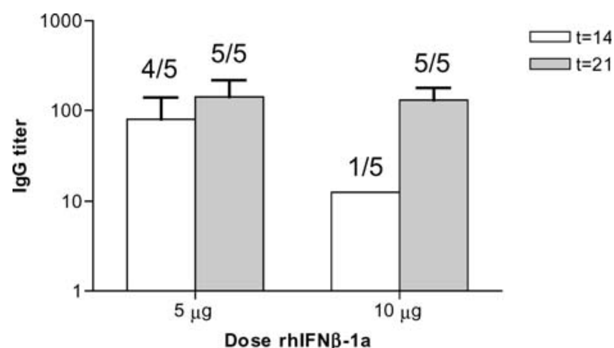

Fig. 1. Results of the ELISA after administration of 5 μg or 10 μg rhIFNβ-1a daily, i.p. Shown are the average (+SEM) antibody titers in the sera of positive wild-type mice. Values above bars represent the number of positive mice out of total mice.

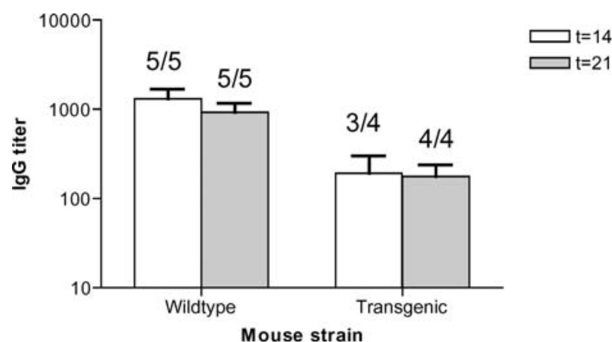

Fig. 3. Antibody titers against Betaseron in sera of wild-type and transgenic mice. Mice received daily 5 μg Betaseron i.p. on 5 consecutive days for 3 weeks. Shown are the average titers of positive mice (+SEM). Values above the bars represent the number of positive mice per total mice. One of the transgenic mice died during the experiment because of handling.

low-dose and high-dose groups were not significantly different (unpaired t test; $p = 0.8941$).

The transgenic mice did not develop antibodies against rhIFNβ-1a (Fig. 2), indicating that these mice are immune tolerant for rhIFNβ.

To test the immunogenicity of a marketed formulation, wild-type and transgenic mice were injected daily i.p. with 5 μg Betaseron. Sera were analyzed for the presence of antibodies against rhIFNβ. Figure 3 shows the average titers of the wild-type and transgenic mice. The wild-type mice show high antibody levels against rhIFNβ-1b with significantly higher titers than the wild-type mice against rhIFNβ-1a (unpaired t test; $p = 0.0159$), consistent with the observation in MS patients that rhIFNβ-1b is more immunogenic than rhIFNβ-1a. In addition, the immune tolerant transgenic mice show an immune response to the injected protein, indicating that Betaseron is able to break the immune tolerance against hIFNβ of the transgenic mice.

Figure 4 shows the anti-HSA antibody titers in wild-type and transgenic mice. The wild-type and transgenic mice showed similar antibody titers (unpaired t test; $p = 0.4974$), indicating that the immune system of the transgenic mice—as compared with the wild-type mice—is fully functional, except for the immune tolerance for hIFNβ.

DISCUSSION

The results show that the transgenic mice are immune tolerant for rhIFNβ-1a. By looking at the response to HSA, the immune tolerance was shown to be specific for hIFNβ and not based on a general immune suppression induced by the transgenic modification. Although the lack of standardization of antibody testing and differences in dosing and route of administration suggests that data from different clinical trials should be interpreted with caution, there is a consensus that Betaseron is more immunogenic than the CHO cell–derived products (3,8,21). The differences between Avonex and Betaseron in our model correlate qualitatively with the clinical data.

The results suggest, for the first time, that the mechanism of antibody induction in patients after rhIFNβ-1a administration (classical immune response) is different from the mechanism of antibody induction after rhIFNβ-1b administration (breaking of immune tolerance). Much more validation is necessary to establish how predictive the model is for the many factors influencing the immunogenicity of hIFNβ. Because Betaseron was formulated during the experiment and rhIFNβ-1a was unformulated, the differences seen in immunogenicity between Betaseron and rhIFNβ-1a could also be related to formulation aspects (2). The im-

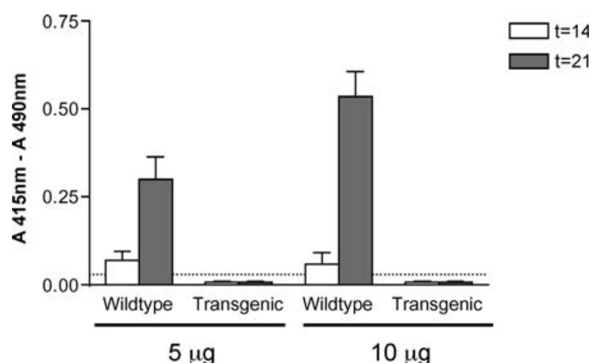

Fig. 2. Results of the ELISA after administration of 5 μg or 10 μg rhIFNβ-1a daily, i.p. Shown are the average (+SEM) absorbance values of the 1:100 dilution of the sera of wild-type and transgenic mice. The dotted line represents 10 times the average of the pretreatment values minus the background.

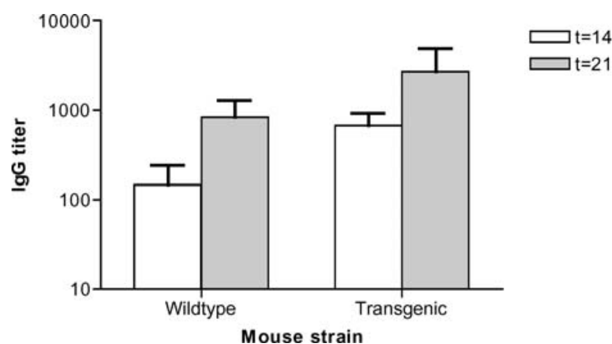

Fig. 4. Anti-HSA titers in wild-type and transgenic mice. Mice received daily unformulated rhIFNβ-1a with HSA i.p. on 5 consecutive days for 3 weeks. Shown are the average titers (+SEM). All mice were positive.

munogenicity of Betaseron seen in patients has been associated with the presence of aggregates in the formulation (21). This is probably also the reason for the immunogenicity seen in the transgenic mice. Another explanation could be that the HSA, which is present in Betaseron, has an adjuvant effect. However, we have indications that HSA may decrease, rather than increases, the immune response against rhIFNβ (unpublished results).

In this article, we have described the development of a transgenic mouse model immune tolerant for hIFNβ. This model is the first step toward a way to predict whether a hIFNβ formulation can break immune tolerance in patients before the formulation is actually tested in clinical trials. The model can also be useful to study the mechanism of breaking of immune tolerance and to evaluate treatment options in antibody-positive patients. Further research is needed to test the full potential and the restrictions of the model to predict the immunogenicity in patients.

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