Development of ADA Against Recombinant Human Interferon Beta in Immune Tolerant Mice Requires Rapid Recruitment of CD4$^+$ T Cells, Induces Formation of Germinal Centers but Lacks Susceptibility for (Most) Adjuvants

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ABSTRACT: Immunological processes leading to formation of antidrug antibodies (Abs) against recombinant human proteins remain poorly understood. Animal and clinical studies revealed that immunogenicity shares both T-cell-dependent (requirement of CD4$^+$ T cells, isotype switching) and T-cell-independent (involvement of Marginal Zone B cells, apparent lack of memory) characteristics. We used immune tolerant mice to study the mechanism underlying immunogenicity in more detail. We found that CD4$^+$ T cells were crucial at early stages of Ab responses against rhIFNβ. In addition, we found a similar number of germinal centers (GCs) in spleen after rhIFNβ treatment as after treatment with a foreign protein. However, neither Ab titers nor the number of GCs was increased by adsorption of rhIFNβ on aluminum hydroxide. Therefore, we tested the effect of several immune adjuvants in a follow-up study. We found that only conjugation of rhIFNβ to a carrier protein (cholera toxin subunit B) was effective in boosting Ab titers. However, these conjugates failed to trigger rhIFNβ specific memory formation. Our findings show that early events of the immunogenicity reaction to self-proteins are CD4$^+$ T-cell dependent. Nevertheless, despite those similarities, immunogenicity of human proteins is clearly not a classical CD4$^+$ T-cell-dependent response. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:396–406, 2015
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INTRODUCTION

The introduction of therapeutic proteins into clinical practice was a breakthrough in the therapies of many severe diseases such as diabetes, hemophilia, and multiple sclerosis (MS). Development of recombinant technology allowed large-scale production of highly purified proteins with sequences identical to endogenous counterparts. Unfortunately, the therapy outcome is often affected by the production of antidrug antibodies (ADA).[1–3] ADA can neutralize the biological activity of the drug, resulting in a decreased efficacy of the therapy. Moreover, in rare cases, ADA can also cross-react with endogenous proteins leading to severe, even life-threating, side effects.[4,5] Although many factors have been linked to an increased risk of immunogenicity, the immunological processes leading to ADA development are poorly understood. That lack of knowledge hampers efforts aiming at production of nonimmunogenic therapeutic proteins.

Two distinct mechanisms may lead to the production of ADA. The classical immune response against foreign proteins triggers B cells to produce antibodies (Abs) via a CD4$^+$ T-cell-dependent (TD) mechanism, which is characterized by (i) isotype class switching, (ii) affinity maturation of secreted Abs, as well as (iii) the formation of germinal centers (GCs) in lymph nodes and spleens. In these GCs, maturation and differentiation of B cells occur.[6–9] Moreover, immune responses against TD antigens can be easily enhanced by multiple adjuvants.

The second mechanism is a T-cell-independent (TI) immune response resulting in a direct activation of B lymphocytes by antigen without the involvement of CD4$^+$ T cells. A TI immune response is usually triggered by bacterial molecules such as CpG, LPS (TI antigens type I) or polysaccharides (TI antigens type II). In contrast to the TD response, a TI Ab response is mainly restricted to the IgM isotype, involves marginal zone (MZ) B-cells, typically leads to no or a reduced GC formation and usually lacks the formation of immunological memory.[10,11] TI responses usually, in contrast to TD reaction, cannot be easily enhanced by adjuvants.

Although the rhIFNβ has been used for the treatment of MS for over 20 years, the immune processes leading to ADA formation remain poorly understood. Both clinical studies as well as our studies in immune tolerant mice revealed an immune response with both TD and TI characteristics. Depending

Abbreviations used: Ab, antibody; ADA, antidrug antibodies; CFA, complete Freund's adjuvant; CTB, cholera toxin subunit B; GCs, germinal centers; HSA, human serum albumin; IFA, incomplete Freund's adjuvant; i.p., intraperitoneal; MS, multiple sclerosis; MZ, marginal zone; PBS, phosphate-buffered saline; PNA, peanut agglutinin; TD, T cell/thymus dependent; tg, transgenic; TI, T cell/thymus independent; TMB, 3,3′,5,5′-tetramethylbenzidine; rhIFNβ, recombinant human interferon beta.

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on the product, rhIFNβ may induce ADA in up to 90% of patients and these Abs interfere with rhIFNβ efficacy.[12–15] The differences in frequency of ADA induced by distinct rhIFNβ products originates most likely from different aggregate content of these products. Betaferon® containing the highest number of aggregates has been found to be the most immunogenic in several clinical studies as well as in immune tolerant mice.[13,16,17] Nevertheless, the production of ADA against different rhIFNβ products shares common features between these products suggesting that the same immunological processes are involved in ADA production against all rhIFNβ drugs. Moreover, similar characteristics of ADA formation have been found in both clinical studies and in experiments with immune tolerant mice. For example, IgG has been identified as the main ADA isotype in both patients and mice.[16,18–20] Also, in immune tolerant mice $CD4^+$ T cells were shown to be vital for ADA response, suggesting a TD mechanism underlying immunogenicity of rhIFNβ.[19] However, other clinical and mouse data have shown features typical for a TI response such as an apparent lack of immunological memory and the involvement of MZ B cells.[17,19,20]

We used immune tolerant mice to study the immune mechanisms underlying immunogenicity of recombinant human interferon beta (rhIFNβ). These mice are, like patients, immune tolerant for rhIFNβ. Therefore, administration of rhIFNβ could to certain extent mimic the processes leading to immunogenicity in patients. As both patients and mice receive injections of a self-like protein, the basic immunological processes that take place (e.g., activation of $CD4^+$ T cells, GCs, immunological memory formation, etc.), should reflect the ADA production in both patients and mice.[21]

In this report, we describe a series of experiments designed to improve our understanding of the mechanisms underlying immunogenicity of rhIFNβ using immune tolerant mice. We aimed to determine at which time point during ADA formation $CD4^+$ T cells are most crucial and to assess the formation of GCs to explain the apparent lack of immunological memory found in our previous studies. Additionally, we studied the effect of adjuvants on immunogenicity of rhIFNβ in immune tolerant mice.

MATERIAL AND METHODS

Animals

Heterozygous transgenic (tg) C57Bl/6 mice carrying the human interferon beta (*hIFN*β) gene, originally developed in our institute, were bred at the Central Laboratory Animal Institute (Utrecht University, The Netherlands).[22] tg C57Bl/6 males were crossbred with non-transgenic (non-tg) FVB/N mice (Janvier; BioServices, Uden, The Netherlands).[16] The hIFNβ genotype of the offspring (F1) was determined by PCR of chromosomal DNA isolated from ear tissue. Both PCR positive (tg) and PCR negative (non-tg) littermates (males and females) of 6–10 weeks of age were used for the experiments. The non-tg mice were included in experiments as reference mice that develop an immune response via a TD mechanism. Mice were housed in standard perspex cages and given access to food (Hope Farms, Woerden, The Netherlands) and water (acidified) *ad libitum*. All experiments were performed according to Institutional Ethical Committee Regulations of the Utrecht University, the Netherlands.

Recombinant hIFNβ

Betaferon® (recombinant human IFNβ-1b, rhIFNβ-1b drug product) 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 pH 7.4, 137 mM sodium chloride (phosphate-buffered saline, PBS). Because of the lack of glycosylation, rhIFNβ-1b forms significant amounts of aggregates upon reconstitution.[23] Before injection, Betaferon® was further diluted to the desired concentration in PBS. RhIFNβ-1a drug substance was supplied by Biogen Idec (Cambridge, Massachusetts) and was formulated to a concentration of 270 µg/mL in 100 mM sodium phosphate pH 7.2, 200 mM sodium chloride. Before injections rhIFNβ-1a was diluted with PBS.

Animal Experiments

Anti $CD4^+$ T-Cell Treatment

Both tg and non-tg animals received intraperitoneal (i.p.) injections of 5 µg of Betaferon® in 100 µL of PBS for 3 weeks (on days 1–5, 8–12, and 15–19).

To study the involvement of $CD4^+$ T cells, mice received i.p. injections of 100 µg of anti-CD4 Ab GK1.5 (Bioceros, Utrecht, The Netherlands) in 100 µL of PBS on three consecutive days. Depletion was maintained by administration of an additional 100 µg of GK1.5 every 3–4 days until the end of the experiment. One group of tg and non-tg mice started GK1.5 treatment before Betaferon® treatment (day −4, $n = 13$), whereas other groups started GK 1.5 treatment after 2, 4, or 6 injections of Betaferon® (days 2, 4, or 8, $n = 10$). A control group without $CD4^+$ T-cell depletion received a corresponding volume of saline (100 µL) on the same days as the group that was depleted before the start of Betaferon® treatment (from day −4, $n = 6$). Depletion efficiency was assessed for each group on two or three days after initial GK 1.5 injections and every 6–7 days until the end of the experiment. At each time point, three mice per group were sacrificed, spleens isolated and checked for $CD4^+$ T-cell depletion by an PE anti $CD4^+$ Ab (clone RM4.4 eBiosciences, Vienna, Austria), which recognizes a different epitope than GK1.5, using a FACS Canto II flow cytometer (BD Biosciences, Breda, The Netherlands). The depletion efficiency was on average 80%.

Blood was collected via cheek puncture at different time points before the start of Betaferon® treatment (day 1), during the three treatment weeks (days 8, 12, and 19) and 1 week after treatment (day 26) in lithium heparin tubes (Greiner-bio-One, Alphen aan den Rijn, The Netherlands). On day 26, animals were sacrificed by decapitation after which blood was collected.

Plasma was isolated by spinning down the blood for 10 min at 3000*g* at 4°C and stored at −20°C until further assessment for anti-rhIFNβ Abs.

Formation of GCs

In order to study, the presence of GCs after treatment with therapeutic interferon beta, mice received rhIFNβ-1a drug substance. This rhIFNβ product does not contain HSA (in contrast to Betaferon®) and allows assessment of GCs specific for rhIFNβ.

tg and non-tg mice ($n = 16$) were treated with 5 µg of rhIFNβ-1a drug substance in 100 µL of PBS on days 1–5, 8–12, and 15–18. As controls tg and non-tg mice ($n = 8$) were treated with

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