Structural aspects of the immunogenicity of therapeutic proteins

transgenic animals as predictors for breaking immune tolerance

Suzanne Hermeling

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S. Hermeling
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Structural aspects of the immunogenicity of therapeutic proteins

transgenic animals as predictors for breaking immune tolerance

Structurele aspecten van de immunogeniciteit van therapeutische eiwitten
transgene dieren als voorspellers voor het breken van immuuntolerantie
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Suzanne Hermeling
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Promotor: Prof. dr. D.J.A. Crommelin

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dr. H. Schellekens

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"Te weten wat men weet en te weten wat men niet weet, dat is kennis"

Confucius

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Chapter 1

General introduction

Introduction

The introduction of recombinant DNA techniques enabled the large scale production of copies of human proteins. These proteins are used for the treatment of several serious and life threatening diseases. Examples are given in table 1. Despite the similarities with endogenous proteins for which patients are immune tolerant, some patients develop antibodies against these therapeutic proteins. This immune response usually has two stages: patients will first develop binding antibodies (BAbs) which may be followed by the formation of neutralizing antibodies (NAbs). Whereas BAbs usually do not have biological consequences, NAbs bind to the protein at its active site, which may result in a decrease of efficacy. Sometimes the NAbs also recognize the endogenous protein, which can lead to serious complications (1, 2).

A number of factors affect the immune response seen in patients (3). Protein structure is one of these. Protein structure can be influenced by the production and purification methods, the formulation, the storage and handling of the protein. Although some clinical data are available, it is still largely unknown what structural properties of the protein are responsible for the immune response. When these could be established, protein formulations could be developed with minimal immunogenicity based on structural analyses only. Since there are also no validated animal models predicting immunogenicity in patients, presently the only possibility to evaluate whether a formulation will induce antibodies is to run clinical studies. Such studies have risks, are time consuming and expensive.

This thesis focuses on the relation between protein structure and immunogenicity with an emphasis on formulation effects. Moreover, the development and validation of animal models to predict immunogenicity are discussed.

Formulating therapeutic proteins

Most therapeutic proteins have to be administered parentally because of their poor bioavailability when administered via non-parenteral routes. They are marketed either as freeze dried formulations, which need to be dissolved with solvent, or as ready-to-use liquid formulations. Patients prefer the ready-to-use liquid formulations. During storage and shipment the protein can degrade chemically, e.g. by oxidation and deamidation, and/or physically, e.g. by adsorption, unfolding and aggregation. Freeze dried proteins are in general more stable than ready-to-use formulations. However, during drying and

reconstitution damage can occur to the protein, leading to immunogenicity. Different compounds, e.g. sugars, surfactants and amino acids, are used in formulations to prevent damage and degradation. Formulation design is an important part of product development, since each protein requires a unique set of excipients to guarantee a sufficient shelf-life under specified conditions (excellent articles have been written about this (4-9)).

Antibody induction by therapeutic proteins

Since most therapeutic proteins are copies of endogenous proteins, the immune system should consider these as self-proteins, eliciting no immune response. However, sometimes antibodies are induced. These antibodies can be induced via two mechanisms: a classical immune response by T-cells recognizing peptides on antigen presenting cells (APC) as foreign and activating B-cells into producing antibodies. The second mechanism by which antibodies can be formed is by breaking immune tolerance against self-antigens (10). Both mechanisms will be discussed in more detail.

Table 1 Some proteins reported to be immunogenic[1].

Type of protein	Protein
Hormones	Insulin Growth hormone
Cytokines	Interferon alpha Interferon beta Interleukin 2, 3 and 12
Enzymes	Factor VIII DNase Tissue plasminogen activator
Antibodies	Anti-CD3 (murine antibody) Anti-Her2 (humanized antibody) Anti-IgE (humanized antibody) Anti-Respiratory Sunctial Virus (humanized antibody) Anti-IL-2 receptor (humanized antibody)
Growth factors	G-CSF GM-CSF Erythropoietin Thrombopoietin

[1] adapted from (11) and (12).

The immune system

The immune system is designed to attack and eliminate foreign compounds invading the body. It can be divided into an innate immune system and an adaptive immune system. The innate immune system is not specific and reacts to all intruders. The adaptive immune system is antigen specific and mediated by antibodies. It is regulated mainly by B-cells and T-cells. B-cells originate from the bone marrow and recognize the spatial conformation of antigens. T-cells are produced in the thymus and recognize linear epitopes of the protein in combination with major histocompatibility complex (MHC)-molecules on the surface of APC.

The classical immune response

When an antigen enters the body, the APC will take it up, digest it and present peptides derived of the antigen in the groove of MHC-molecules on their surface. T-cells will recognize the peptide as foreign and will signal B-cells to proliferate and produce antibodies (figure 1). The antibodies will bind to the antigen and activate the complement system and macrophages, parts of the innate immune system, which will destroy and remove the antigens.

Tolerance

Early during embryonic life the developing lymphocytes are only surrounded by self-antigens. During their development in the thymus T-cells become tolerant to these self-antigens. Autoreactive T-cells which escape this mechanism and reach the periphery become tolerized or anergic when antigens are presented by cells lacking co-stimulatory molecules. The same mechanisms keep autoreactive B-cells from proliferating and producing antibodies.

Anergic autoreactive B-cells only start to produce antibodies when the auto-antigen is accompanied by a danger signal. One of these danger signals is the crosslinking of multiple B-cell receptors (figure 2) (13). Repetitive ordered antigen was shown to induce a 30-fold higher titer as compared to monomeric antigen (14). High repetitiveness of proteins naturally only occurs in infectious agents, which may explain the vigorous response of the immune system to these type of structures (13, 15, 16). For instance, the highly repetitive glycoprotein on the surface of vesicular stomatitis virus allows a very efficient crosslinking of B-cell receptors that is able to break the tolerance of transgenic mice (13). It has been shown that conjugation of a self-protein to papillomavirus virus-like

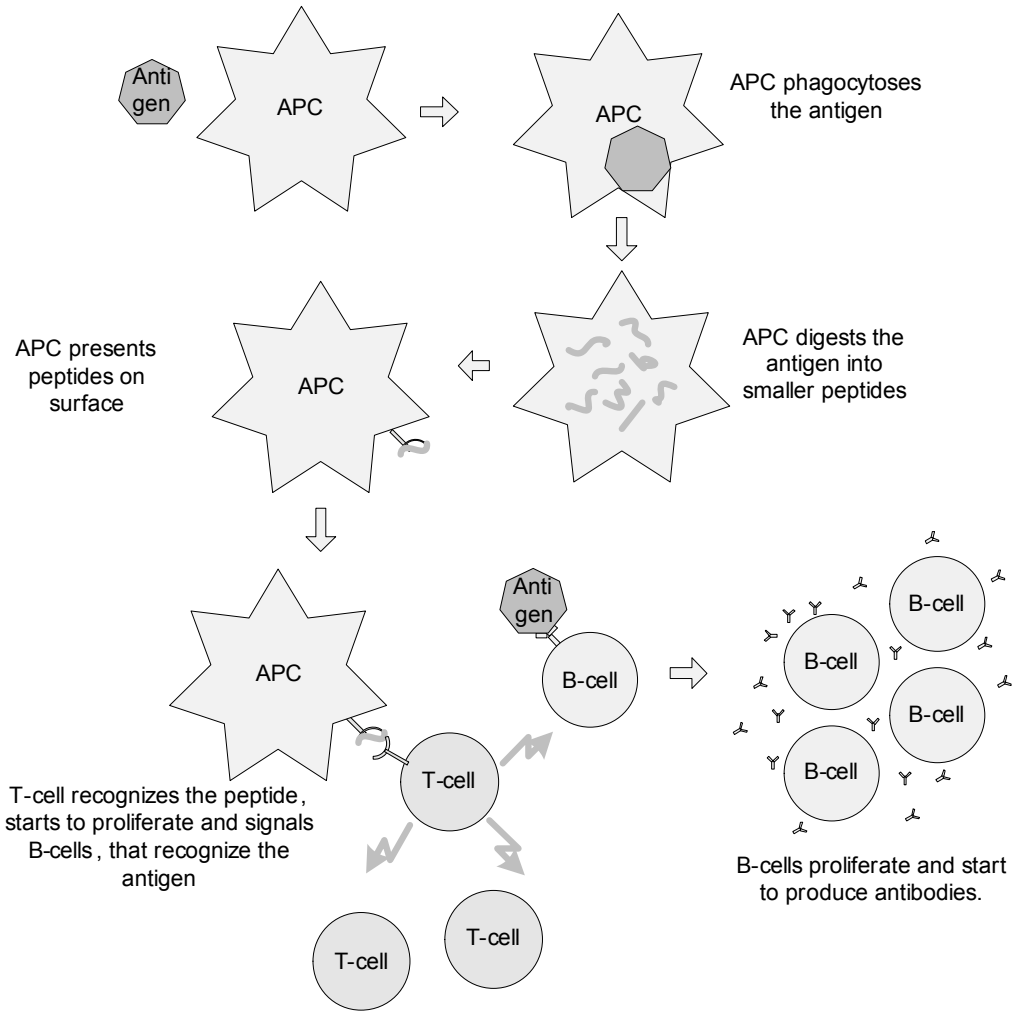

Figure 1 *Schematic overview of a classical immune response. The APC will phagocytose the antigen, digest it and present it on its MHC-molecules on the surface. A T-cell will recognize the peptide in combination with the MHC. The T-cell starts to proliferate and interacts with B-cells that recognize the antigen. The interaction of the T-cell with the B-cell will activate the B-cell. The activated B-cell starts to proliferate and produces antibodies. APC: antigen presenting cell.*

particles with a highly repetitive protein structure can give a strong IgG response against this self-protein (16-18).

Factors influencing the immune response

The factors known to influence the immunogenicity of therapeutic proteins are summarized in figure 3. They can be divided into structural properties of the protein and other factors. As mentioned before, protein formulations are susceptible to degradation. Degradation products can be immunogenic, e.g.

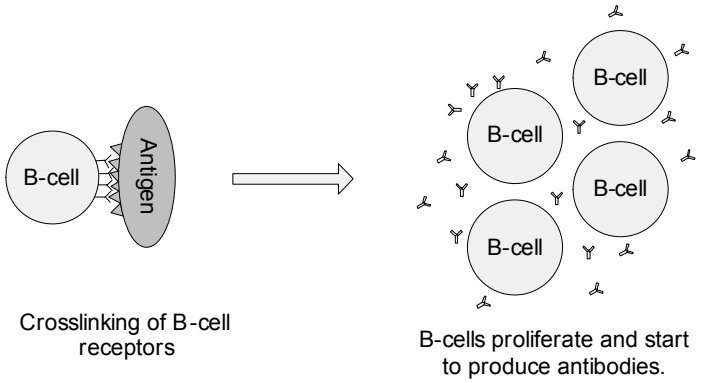

Figure 2 *Schematic representation of a possible mechanism of breaking of B-cell tolerance. The antigen contains multiple epitopes that crosslink B-cell receptors. The crosslinking of the receptors is seen as a danger signal by the B-cells. Because of the danger signal the B-cells will proliferate and start to produce antibodies, even against self-proteins.*

because of new epitopes, or because of multimeric antigen presentation, e.g. in aggregates. To prevent degradation, excipients are added. However, sometimes the stabilizer can contribute to the immunogenicity, as was shown for human serum albumin (HSA) in rhIFNα2a formulations (19). The route of administration is known to have an influence on the immunogenicity. Subcutaneous (s.c.) administration of rhIFNα2a gave the highest immune responses, while intravenous (i.v.) and intramuscular (i.m.) administration of rhIFNα2a were less immunogenic (20, 21). Aggregates are one of the most studied factors known to influence the immunogenicity of therapeutic proteins (19, 22-28). The reason why aggregates break tolerance could be because of multimeric antigen presentation, as well as conformational changes. Other aspects will be discussed in more detail in chapter 6.

Next to the factors related to the product, also patient characteristics can have an influence on the immunogenicity (3, 29-31) .

The influence of structural properties of the protein and the influence of the route of administration on the immunogenicity will be discussed in more detail in the following chapters.

Assays

Most of the data published about the immunogenicity of therapeutic proteins is obtained from clinical and post marketing studies. Comparing data from different studies, however, is a problem, because there is no standardization in the assays or the reporting of antibody analyses. For

instance, some studies only mention the number of patients producing antibodies, while others report the average antibody titers. Several methods for the determination of BAbs in patient sera are available, e.g. enzyme-linked immunosorbent assays (ELISA), surface plasmon resonance (SPR) analyses and radioimmunoassays (RIA). The assay format will influence the outcome of the results. For determining the concentration of neutralizing antibodies a bioassay has to be performed.

Therapeutic proteins

As mentioned before, nearly all therapeutic proteins are immunogenic, even if they are homologues of human proteins. Table 1 shows some proteins with reported immunogenicity. The clinical use, characteristics and immunogenicity of recombinant human erythropoietin (epoetin), recombinant human interferon beta (rhIFNβ) and recombinant human interferon alpha2

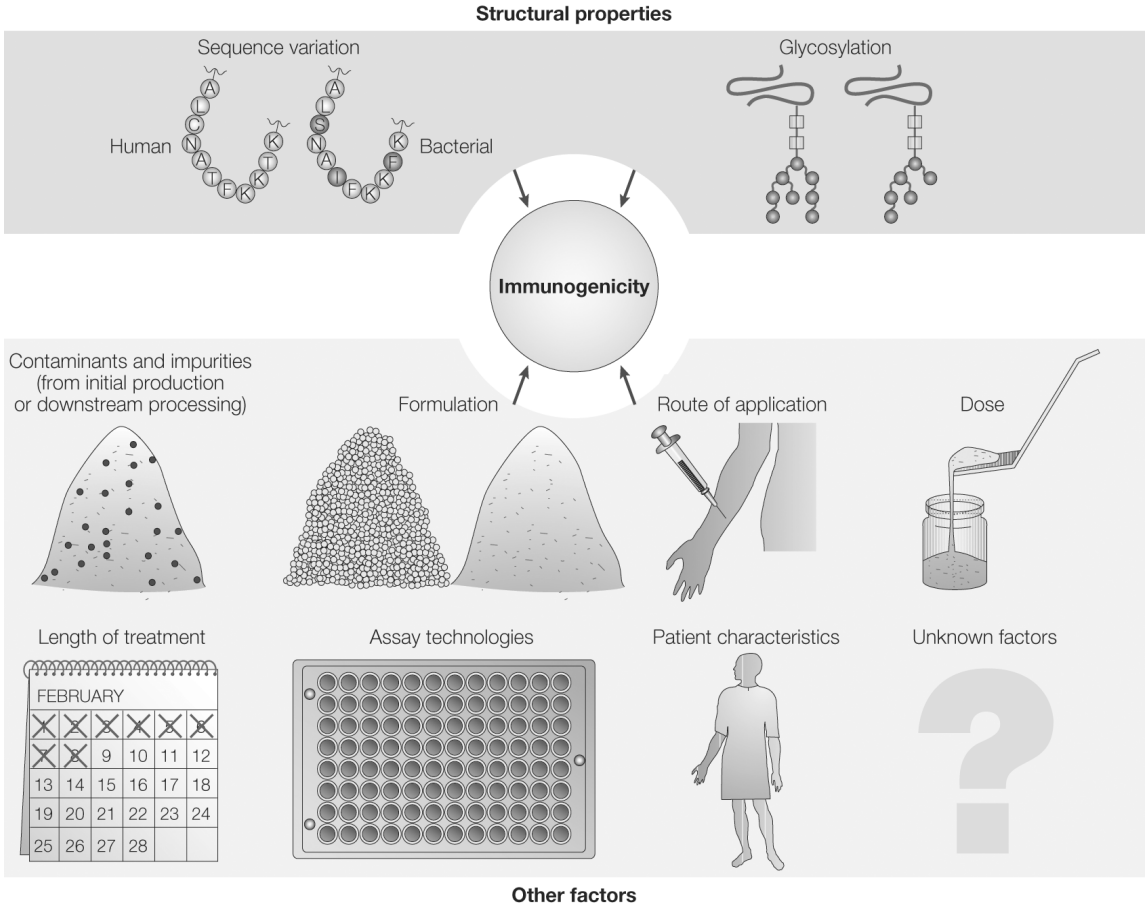

Figure 3 *Some of the factors that influence the immune response seen in patients. Taken from (3).*

(rhIFNα2) will be discussed in more detail below, since these proteins are used in this thesis.

Epoetin

Erythropoietin is a protein mainly produced in the kidneys. It stimulates the production of erythrocytes. Epoetin's primary indication is anemia associated with chronic renal failure. It is also given to other patients suffering from anemia (e.g. cancer patients).

Three forms of epoetin are commercially available, epoetin alfa, beta and darbepoetin alfa, which is a hyperglycosylated analog (32). They have a common primary sequence of 165 amino acids, identical to that of human erythropoietin, but their glycosylation pattern differs (33). Also under natural conditions different glycoforms are produced which differ in biological activities (34, 35).

In 1997 the first patient was described who developed antibodies against epoetin and as a result developed pure red cell aplasia (PRCA) (36). PRCA is a relatively rare severe form of progressive anemia. Antibody mediated PRCA is caused by antibodies cross-reacting with endogenous erythropoietin (37). In 2002 Casadevall reported 13 patients with antibody-mediated PRCA, which all occurred after 1998 (1). The route of administration of epoetin appeared in all cases to be s.c. and in the majority of cases Eprex® was involved, a product mainly distributed in Europe. Later the observation by Casadevall was extended by others and about 225 cases of PRCA have been described associated with the s.c. use of Eprex in patients with chronic renal failure (32). All cases occurred after 1998 when the formulation of Eprex® was changed: HSA was replaced by glycine and Tween 80. This reformulation process probably led to the sudden increase of PRCA cases.

After changing the route of administration for Eprex® from s.c. to i.v. administration the number of PRCA cases rapidly declined (37). Several theories have been postulated that might explain the increased number of PRCA cases. Leachates from the rubber plungers of pre-filled syringes (38), the formation of micelles (39) or handling and storage issues (37). None of these theories have been proven.

RhIFNβ

Interferon beta is a cytokine mainly produced by macrophages and epithelial and fibroblast cells. It is an endogenous protein with anti-inflammatory, anti-tumor, antiviral and cell-growth regulatory effects (40, 41). RhIFNβ has

been established as a treatment for relapsing-remitting multiple sclerosis (RRMS) patients (41). Natural hIFNβ is a glycosylated protein which contains 166 amino acids and has an apparent molecular weight upon SDS-PAGE analysis of approximately 25 kDa (42). It contains 1 disulfide bond between Cys-31 and Cys-141, 1 free cysteine at position 17 and a single N-linked carbohydrate at Asn-80 (42).

Three rhIFNβ formulations are available for the treatment of RRMS: rhIFNβ-1b (Betaseron®, Berlex Laboratories, Montville, New Jersey, USA; Betaferon®, Schering, Berlin, Germany), rhIFNβ-1a-Avonex (Biogen-Idec, Cambridge, Massachusetts, USA) and rhIFNβ-1a-Rebif (Serono, Geneva, Switzerland).

RhIFNβ-1a is produced in CHO-cells, is glycosylated and has an amino acid sequence that is similar to the endogenous hIFNβ. RhIFNβ-1b is produced in E. coli and therefore non-glycosylated. Moreover, Cys-17 is mutated to Ser-17 and the N-terminal methionine is deleted.

Patients using either one of the rhIFNβ formulations develop NAbs, which cause a reduction in clinical efficacy (43-46). Most rhIFNβ-Abs are of the IgG-type (46, 47). The NAbs are usually detectable after the first 6 to 12 months of the treatment (43, 46), but the negative clinical effects do not appear until after 18-24 months after the start of the treatment (48).

The number of patients developing NAbs is higher with rhIFNβ-1b than with rhIFNβ-1a (40, 49), with more patients developing NAbs with Rebif® treatment than with Avonex® treatment (43, 50). The increased immunogenicity of rhIFNβ-1b compared to rhIFNβ-1a is probably due to aggregated protein present in the rhIFNβ-1b formulation. The aggregation occurs because rhIFNβ-1b does not contain sugar chains to keep the protein in solution (51).

RhIFNα2

RhIFNα2 is a protein used in the treatment of a variety of malignancies and viral diseases (52). It inhibits viral replication, increases class I MHC expression, stimulates Th1-cells and inhibits proliferation of many cell types (12). There are 2 different forms of rhIFNα2 (rhIFNα2a and rhIFNα2b, which only differ in 1 amino acid) on the market. Both of them are also available as a PEGylated form (table 2). RhIFNα2 is produced in E. coli cells, which implies that the protein is non-glycosylated, while natural hIFNα2 is O-glycosylated (53).

RhIFNα2a induces higher levels of NAbs at a higher incidence than rhIFNα2b. Antibodies of patients receiving rhIFNα2a fully cross-react with

rhIFNα2b and vice versa (54). A decrease in immunogenicity of rhIFNα2a formulations was seen when the production, storage and handling conditions improved. A refrigerated lyophilizate without HSA was less immunogenic than products that contained HSA and were stored at room/ambient temperature (24).

To date most of the rhIFNα2 formulations that are prescribed to patients contain a PEGylated form, which is more effective than the non-PEGylated forms (55-57). PEGylated interferons can be administered less frequently. Also a reduced immunogenicity is claimed for the PEGylated products (11).

Table 2 Characteristics[1] of currently marketed rhIFNα2 products.

Product	Type	Appearance	Excipients[2]	Route of administration[3]
Intron A®	rhIFNα2b	lyophilized powder	glycine, dibasic sodium phosphate, monobasic sodium phosphate, HSA	i.m., s.c., i.v.
		multidose liquid	sodium chloride, dibasic sodium phosphate, monobasic sodium phosphate, edetate disodium, polysorbate 80, m-cresol	i.m., s.c.
PEG-Intron®	PEGylated rhIFNα2b	lyophilized powder	dibasic sodium phosphate, monobasic sodium phosphate, sucrose, polysorbate 80	s.c.
Roferon A®	rhIFNα2a	liquid	sodium chloride, polysorbate 80, benzyl alcohol, ammonium acetate	s.c., i.m.
		ready-to-use syringe	sodium chloride, polysorbate 80, benzyl alcohol, ammonium acetate	s.c.
Pegasys®	PEGylated rhIFNα2a	liquid	sodium chloride, polysorbate 80, benzyl alcohol, sodium acetate, acetic acid	s.c.
		ready-to-use syringe	sodium chloride, polysorbate 80, benzyl alcohol, sodium acetate, acetic acid	s.c.

[1] Taken from patient information leaflets
[2] HSA: Human serum albumin
[3] i.m.: intramuscular; s.c.: subcutaneous; i.v.: intravenous

Aim and outline of this thesis

The aim of this thesis is to correlate structural changes in proteins with changes in immunogenicity. The structural changes in proteins may be caused by the formulation of the protein.

In **chapter 2** a summary of the available literature about relationships between structural properties of therapeutic proteins and their immunogenicity is presented. Moreover, the use of predictive models is discussed herein. **Chapter 3** describes the characterization of two formulations of recombinant human erythropoietin (epoetin), Eprex® (epoetin alfa) and NeoRecormon® (epoetin beta). Differences between the formulations with respect to the extent of micelle-associated epoetin are investigated and the potential impact of these differences on the immunogenicity is discussed. In **chapter 4** the development and validation of a transgenic mouse model immune tolerant for hIFNβ is described. The transgenic mice are shown to be immune tolerant for the human protein. Moreover, the tolerance is shown to be broken by a rhIFNβ formulation (Betaseron®) that is known to be immunogenic in patients. **Chapters 5 through 8** deal with the characterization and immunogenicity of rhIFNα2b in wildtype mice and in transgenic mice, immune tolerant for hIFNα2. In **chapter 5** rhIFNα2b formulations containing surfactants as excipients are analyzed, to investigate whether the protein, like epoetin, can associate with micelles and whether this affects its immunogenicity. In **chapter 6** rhIFNα2b is degraded in a controlled way to evaluate the relation between structure and immunogenicity of oxidized and aggregated forms of rhIFNα2b. **Chapter 7** shows the influence of the route of administration on the immunogenicity of highly aggregated rhIFNα2b. Moreover, the sensitivity of the model to the dose of aggregated protein is investigated. In **chapter 8** degradation products of rhIFNα2b are produced by incubation at different pH conditions and elevated temperature, and studied for their immunogenicity. **Chapter 9** summarizes the results and conclusions. Furthermore the prospects of the work are discussed, particularly the potential use of transgenic animals for predicting the immunogenicity of therapeutic proteins in patients.

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Chapter 2

Structure-immunogenicity relationships of therapeutic proteins

Suzanne Hermeling, Daan J.A. Crommelin, Huub Schellekens, Wim Jiskoot

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Abstract

As more recombinant human proteins become available on the market the incidence of immunogenicity problems is rising. The antibodies formed against a therapeutic protein can result in serious clinical effects, such as loss of efficacy and neutralization of the endogenous protein with essential biological functions. Here we review the literature on the relations between the immunogenicity of the therapeutic proteins and their structural properties. The mechanisms by which protein therapeutics can induce antibodies as well as the models used to study immunogenicity are discussed. Examples of how the chemical structure (including amino acid sequence, glycosylation and PEGylation) can influence the incidence and level of antibody formation are given. Moreover, it is shown that physical degradation (especially aggregation) of the proteins as well as chemical decomposition (e.g. oxidation) may enhance the immune response. To what extent the presence of degradation products in protein formulations influences their immunogenicity still needs further investigation. Immunization of transgenic animals, tolerant for the human protein, with well-defined, artificially prepared degradation products of therapeutic proteins may shed more light on the structure-immunogenicity relationships of recombinant human proteins.

Introduction

Nowadays proteins are widely used as therapeutics. When the first proteins became available for therapeutic use (in the 1920s insulin was the first therapeutic protein introduced (1)), concerns were raised about their immunogenicity. Initially, proteins from animal origin (e.g. bovine and porcine insulin) were used. The non-human origin of these proteins was believed to be the reason for their immunogenicity. Later on, proteins purified from human tissue or sera were introduced. Surprisingly, these products also proved immunogenic. These products, such as factor VIII and growth hormone, were given mainly to children with an innate deficiency. The immunogenicity was explained by their lack of immune tolerance. Also, the impurities in these products were considered to be an important reason for the immunogenicity.

The introduction of recombinant DNA techniques enabled the large-scale production of highly purified proteins identical or nearly identical to the endogenous proteins. It was hoped that this would reduce the immune responses to the proteins. Currently, more than 60 recombinant human proteins (including recombinant human(ized) monoclonal antibodies) are available for therapeutic purposes in the European Union (2). Although in general, the level and incidence of immune responses against recombinant human proteins are low and variable, most of these products have been shown to be immunogenic. In some cases the immunogenicity of therapeutic proteins leads to serious problems.

Immunogenicity of recombinant human proteins can have a number of clinical consequences. Binding antibodies may influence the pharmacokinetics of the proteins (1, 3). High neutralizing antibody levels may result in more serious consequences, such as inhibition of the therapeutic effect or the neutralization of essential endogenous proteins. An example is the antibody formation reported after the long term subcutaneous administration of recombinant human erythropoietin (epoetin) in patients with chronic renal failure: the antibodies also neutralize endogenous erythropoietin, which results in pure red cell aplasia (4-6).

Table 1 shows that several factors can influence the immunogenicity of therapeutic proteins in humans. This review only focuses on the formation of antibodies after administration of recombinant human proteins to man in relation to the structural properties of the protein. Monoclonal antibodies will not be included in this review, because they are at best humanized and thus not completely human (2). First, the probable mechanisms of the immune responses are discussed, followed by the factors influencing the observed antibody reponse. The models used for the prediction of immunogenicity will

Table 1 Factors influencing the immunogenicity of therapeutic proteins in humans.

Factors directly affecting the immune response	Factors affecting the measured immune response
Protein structure	Timing and frequency of sampling
Immune modulatory effect of the protein	Assay methods
Formulation	Expression of titers
Contaminants and impurities	
Route, dose, frequency, and duration of administration	
MHC genotype of the patient	
Associated disease and concomitant therapy	

Adapted from (7, 8).

be evaluated. Next, the protein structure in relation to immunogenicity will be dealt with. In the final section formulation aspects are considered.

Mechanisms of immunogenicity

There are two basic immunological mechanisms by which therapeutic proteins induce antibodies in humans. Proteins carrying foreign epitopes like the proteins from microbial origin (e.g. streptokinase) induce a classical immune reaction which involves the presentation of the epitope by antigen presenting cells. These cells activate B-cells and T-helper cells resulting in antibody formation and in the induction of memory cells, leading to an enhanced reaction upon rechallenge.

The other mechanism by which recombinant human proteins induce antibodies is based on the breaking of immune tolerance to self-antigens (9). This tolerance is based on the elimination of the immune cells reacting with self-antigen in the thymus during early development of the individual. This central mechanism of tolerance induction only concerns self-antigens which are present in sufficient amounts in the thymus. Some B-cells may escape elimination in the thymus. Peripheral mechanisms, however, keep the B-cells directed to self-antigens under control. These B-cells may be eliminated by apoptosis when they meet their antigen. Also receptor editing has been described as a mechanism to make these cells harmless. The most likely

peripheral mechanism is the induction of functional anergy in these B-cells. Apparently these cells are not stimulated to produce antibodies by the circulating levels of endogenous proteins such as insulin, interferon and erythropoietin. In mice transgenic for human insulin, low levels of insulin silence the B-cells, although a few cells may escape silencing and produce antibodies. This silencing mechanism may act through antigen-antibody complexes, which react with the low affinity IgG receptor Fcγ2b on B-cells comparable with the mechanism by which anti-rhesus prophylaxis induces tolerance to RhD (10).

Anergic B-cells only start to produce significant amounts of antibodies after receiving a second signal or "danger" signal from T-helper cells. Bacterial endotoxins which react with Toll receptors may provide such a danger signal. This explains the production of antibodies to self-antigen associated with LPS. Also, CpG motifs present in DNA can trigger an immune reaction to self-antigens. This T-cell dependent activation may be weak. When self-antigens are coupled with foreign Th epitopes only a weak IgM response is induced, unless multiple high doses of antigen are given together with immune adjuvants.

The most potent way to induce high levels of IgG independent of T-cell help is to present the self-antigen arrayed on viruses and viral-like particles (VLP). The spacing of epitopes with a distance of 5-10 nm is unique to microbial antigens and the immune system has apparently learned to react vigorously to this type of antigen presentation. Self-antigens conjugated with papilloma VLP evoked a strong antibody response to the self-antigens (11). It is the density of the exposed self-antigens on the VLPs that determines the level of the effect. VLP pentamers had the same effect as complete particles.

Apart from the self-antigen in viral-like arrays other mechanisms exist which lead to the formation of antibodies to self-antigens:

1. Modification of the molecules, which gives antibodies cross-reacting with the unmodified self-antigens (T-cell responses are in general specific for the modified antigens).
2. Different allotypes for the gene coding for the product. This has been shown in mice with different allotypes for IL-2. When immunized the mice produced antibodies which reacted with all forms of IL-2. However, adjuvant was used to immunize the mice (12).
3. Binding to a non-self-antigen. This has been shown for DNA bound to T-antigen from polyomavirus (13).

Predictive models for immunogenicity

As we will see later, physico-chemical characterisation of the therapeutic proteins will not completely predict their immunogenicity. In vivo studies on immunogenicity of recombinant human proteins in conventional animals have limited value because these proteins, in general, are foreign in animals and will induce a classical immune response. These animals only provide the opportunity to study the relative immunogenicity of different protein formulations. The best models are transgenic animals, immune tolerant for the human protein, in which breaking of immune tolerance can be studied. This has been done by Ottesen et al (14), Palleroni et al (15) and Stewart et al (16) for recombinant human insulin, recombinant human interferon alpha2 (rhIFNα2) and recombinant human-tissue plasminogen activator (rhtPA), respectively.

Stewart et al studied the effect of the addition of an adjuvant on the immunogenicity of (mutated) rhtPA in transgenic mice (16). They immunized transgenic mice with unaltered rhtPA and with a mutant containing one amino acid substitution (rhtPA(E275)). Mice were immunized with these proteins with or without adjuvant. The results, summarized in table 2, clearly show that the adjuvant did not increase the reaction to the "self-protein" but increased the immune response against the altered protein in the transgenic mice. Adjuvant only stimulated the classical immune response in this model.

Most of the in vivo immunogenicity studies of therapeutic proteins have been done with mice, either wildtype or transgenic. Zwickl et al (17) performed studies in rhesus monkeys. They claim that the relative immunogenicity of various forms of human growth hormone (hGH) as predicted by the monkey model was confirmed in extensive clinical trials. However, according to the authors the monkey model might not be suitable for every protein.

Table 2 Effect of adjuvant on immunogenicity of rhtPA and mutant.

	Immunogen			
	rhtPA		rhtPA(E275)	
	Without adjuvant	With adjuvant	Without adjuvant	With adjuvant
Control mice	40/42	12/12	79/80	12/12
Transgenic mice	0/21	0/8	8/44	4/8

Mice were immunized with rhtPA of rhtPA(E275) (with or without Freund's adjuvant). The numbers refer to the number of mice making antibodies against the immunogen. The sera were analyzed using a standard radioimmunoprecipitation assay using polyethylene glycol to precipitate the antibodies. After (16).

The in vivo analysis of the immunogenicity may be complicated, if the therapeutic protein has immune modulatory effects which may interfere with antibody production (e.g. interferons). Braun et al studied the immunogenicity of rhIFNα2 in transgenic mice, tolerant for hIFNα2. Because of the species specificity of IFNα2, they injected recombinant murine IFNα2 (rmIFNα2) or polyIC (an interferon inducer) together with rhIFNα2 to mimic its immune modulatory effects (18). In transgenic mice the concurrent treatment did not break the tolerance towards rhIFNα2 monomers. In wildtype mice antibody titers to rhIFNα2 increased due to the co-injection of rmIFNα2 or polyIC (18).

Protein structure and immunogenicity

Factors influencing immunogenicity

Comparison of immune responses induced by different formulations can become complex due to the issues stated in table 1. Besides factors affecting the intrinsic response, assay variations can largely influence the measured immune response. Not only the methods used to determine antibody levels vary but also the ways the results are reported. For instance, in ELISAs the procedure used to coat the plate with antigens is of crucial importance. Depending on the chosen procedure antibodies directed against either native or non-native epitopes will be measured (19). In patient studies some groups report average antibody levels but no percentages of patients with antibodies, while other groups only mention percentages of patients with antibodies. So, standardization and validation of the assays and data presentation (e.g. level of antibodies and number of responders) is crucial.

Protein structure is one of the factors that can affect the immune reponse in humans (see table 1). Proteins are complex molecules so that a small change at a particular site may result in a major change in the overall properties. For instance, oxidation of a few amino acids of rhIFNα2 may lead to aggregation of the molecule (20). In general, it is very difficult to relate a particular change in protein structure to a change in immunogenicity, as will be discussed in the sections below.

Amino acid sequence

Divergence of the primary structure from the human counterpart explains why therapeutic proteins of animal sources are immunogenic. An example is insulin. The differences in primary structure between human, bovine and porcine insulin are depicted in figure 1. Porcine insulin induced slightly higher

A-chain	1	11	21
Human	giveqcctsi	cslyqlenyc	n
Bovine	giveqcc**a**s**v**	cslyqlenyc	n
Porcine	giveqcctsi	cslyqlenyc	n

B-chain	1	11	21
Human	fvnqhlcgsh	lvealylvcg	ergffytpkt
Bovine	fvnqhlcgsh	lvealylvcg	ergffytpk**a**
Porcine	fvnqhlcgsh	lvealylvcg	ergffytpk**a**

Figure 1 *The amino acid sequences of human, bovine and porcine insulin. Differences with the human sequence are depicted in bold (after (14)). Disulfide bridges between A7Cys - B7Cys and A20Cys - B19Cys connect the A and B-chain (21).*

antibody levels in patients than human insulin (1, 14, 22, 23). Even trace amounts of bovine insulin in porcine insulin preparations increased the immune response (22). Remarkably, deletion of A19Tyr from recombinant human insulin resulted in a decrease of antibody titers to 2.2 % as compared to the native form, whereas the molecule lost 1/3 of its receptor binding activity (24). This deletion results in a small conformational change and an increase in hydrophobicity, as measured by RP-HPLC. Changes in the A-chain of insulin have more effect on the immunogenicity than changes in the B-chain (14), which is not surprising as the A-chain is located at the outside of the insulin hexamers.

RhIFNα2 exists in different subtypes (see table 3). RhIFNα2a and rhIFNα2c both differ 1 amino acid from rhIFNα2b. The majority of the human population expresses the gene encoding rhIFNα2b (15). Differences in immunogenicity seen in patients could not be related to differences in primary structure. Antibodies from patients treated with either rhIFNα2 showed complete cross-reactivity (15). Human growth hormone was available in three different forms: purified out of human pituitaries (pit-hGH), recombinant human growth hormone with an extra methionine (met-rhGH) and natural sequence recombinant human growth hormone (humatrope). Pit-hGH and humatrope had the same primary structure while met-hGH had one extra N-terminal methionine residue. Differences in immunogenicity were shown but could not be related to the methionine residue (17, 22). More detailed studies are needed to explain the differences in immunogenicity.

RhtPA was mutated by one amino acid (R275 -> E275). Transgenic mice that were tolerant for htPA were immunized with the mutant and with the non-altered form. The altered protein elicited antibodies in a significantly higher percentage of transgenic mice than the non-altered protein (see table 2) (16).

Table 3 Amino acid differences between different rhIFNα2 variants.

Variant	AA23	AA34
rhIFNα2a	Lys	His
rhIFNα2b	Arg	His
rhIFNα2c	Arg	Arg

RhIFNα2a and rhIFNα2c both differ from rhIFNα2b with 1 amino acid (15, 25).

The impact of a mutation on the immunogenicity is dependent on the position and the type of amino acid involved. For rhtPA a positively charged amino acid was changed into a negatively charged one. The question arises if a more subtle change (e.g. R275 -> K275, both positively charged amino acids) also would give a different immune response.

As the changes in the primary structure reported are diverse and different test systems have been used (patients, monkeys, transgenic mice and non-transgenic mice), it is very difficult to draw any general conclusions about the influences of primary structure on the immunogenicity.

Glycosylation

Several therapeutic proteins are glycosylated. Glycosylation is one of the most common posttranslational modifications. Glycosylation may differ by sequence, chain length and position of linkage to the polypeptide chain and of branching sites (26). Glycosylation is species- and cell-specific. Moreover, the culture conditions of production cells have direct effects on N-linked glycosylation patterns (27). Thus, recombinant human glycoproteins will never have exactly the same glycosylation patterns as their endogenous counterparts.

The sugars of glycoproteins have various functions (listed in table 4). Below we will give some examples to illustrate the effect of the glycosylation profile on the immunogenicity of glycoproteins. Other posttranslational modifications (e.g. phosphorylation, acetylation, methylation) may also influence the immunogenicity, but published data are lacking.

Four out of 13 patients who received yeast-derived recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) formed antibodies to this product. The cross-reactivity of the antibodies with other forms of rhGM-CSF was tested (see also figure 2). Since the antibodies had the same reactivity with E. coli derived rhGM-CSF (no glycosylation), the authors concluded that the antibodies could not be directed against the sugars. The antibodies showed no cross-reactivity with CHO-cell derived rhGM-CSF

Table 4 Functions of glycoprotein glycans.

Type	Function
Physico-chemical	Modify solubility, electrical charge, mass, size and viscosity in solution Control protein folding Stabilize protein conformation Confer thermal stability and protection against proteolysis
Biological	Regulate intracellular trafficking and localization Determine circulation half-life Modify immunological properties Modulate activity Act as cell surface receptors for lectins, antibodies, toxins etc. Participate in cell-cell interactions

After (26).

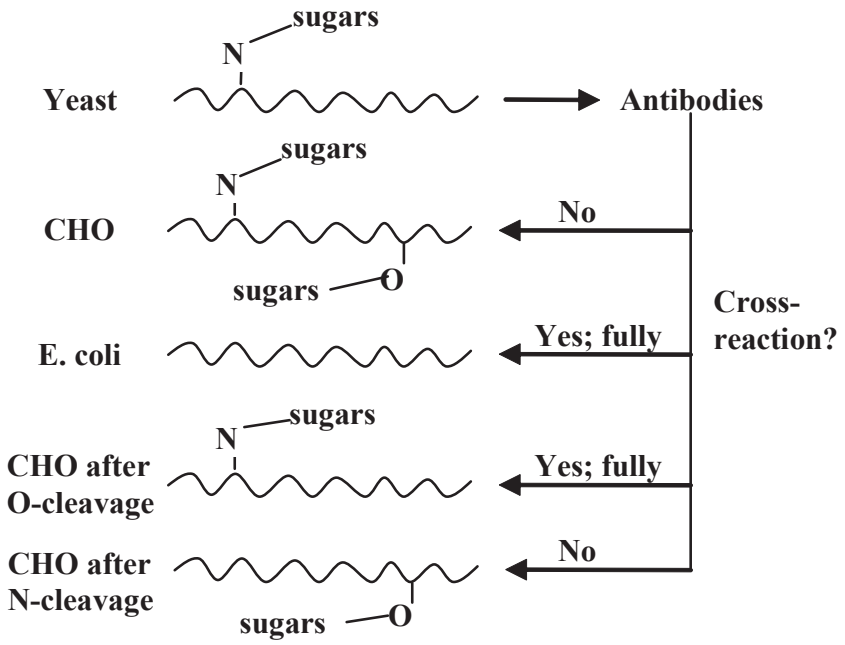

Figure 2 *Schematic representation of the cross-reactivity between antibodies against differently glycosylated forms of rhGM-CSF. RhGM-CSF, produced in yeast, induced antibodies in 4 out of 13 patients. The antibodies were fully cross-reactive with rhGM-CSF produced in E. coli. The antibodies did not recognize the mammalian rhGM-CSF (produced in CHO-cells). When the mammalian form was O-cleaved the antibodies did fully recognize the rhGM-CSF. This means that the antibodies are directed against a portion of the amino acid backbone protected by O-glycosylation in the native product.*

(O-linked and N-linked sugars). When the CHO-cell derived rhGM-CSF was N-cleaved cross-reactivity remained absent. However, when the CHO-cell derived rhGM-CSF was O-cleaved the cross-reactivity was completely regained, irrespective of the presence of N-linked sugars. It was concluded that the antibodies were directed against a part of the peptide backbone that is protected by the O-linked glycosylation in the endogenous protein. Only three patients received unglycosylated rhGM-CSF (produced in E. coli) and although none of them developed antibodies, definite conclusions can not be drawn about the immunogenicity of E. coli derived rhGM-CSF (28).

Naturally occurring hIFNα2 is O-glycosylated at Thr106 (29). No significant antigenic differences between glycosylated natural and non-glycosylated rhIFNα2 proteins have been identified (25). So, glycosylation probably does not affect the immunogenicity of hIFNα2.

Recombinant human interferon beta (non-glycosylated) expressed in E. coli (E. coli-rhIFNβ), and CHO-cells (CHO-rhIFNβ, glycosylated) as well as (non-recombinant human, glycosylated) interferon beta obtained from human fibroblasts (hIFNβ) were compared for their immunogenicity. The three forms possess equal anti-viral activities and a mouse monoclonal anti-hIFNβ antibody had the same affinity for the three variants. However, the anti-viral activity of CHO-rhIFNβ was neutralized more effectively by mouse monoclonal anti-hIFNβ than were hIFNβ or E. coli-rhIFNβ, which may be explained by glycosylation differences. Furthermore, during the production process of E. coli-rhIFNβ the protein is extracted from the bacteria with a strong detergent (while CHO-rhIFNβ and hIFNβ are secreted in the medium). This could result in a conformational change that might explain the different neutralization activity of the mouse monoclonal anti-hIFNβ (30).

In the above examples only cross-reactivity of an antibody with glycosylation variants was tested. Clearly, sugar chains are able to mask antigenic sites in vitro. Whether this has implications for the immunogenicity in vivo remains to be seen. A complicating factor when comparing the cross-reactivity of antibodies against recombinant proteins produced in different species is that the glycans of species differ. So, when the antibodies are directed against the glycans, cross-reactivity may never be seen.

PEGylation

Chemical conjugation of proteins with polyethylene glycol (PEG, a non-toxic water-soluble polymer) reduces their immunogenicity (31). As PEGylated proteins have a prolonged circulating time (32), PEGylation has the potential to reduce the immune response as a result of less frequent administration

schemes. In addition, it may shield immunogenic sites, like glycosylation does. In most cases PEGylation does not lead to conformational changes of the polypeptide chain (31). Important issues regarding protein activity and immunogenicity are the choice of the PEG chain length, the conjugation method, the number of PEG chains per protein molecule and the PEGylation sites (33).

Conjugates of rhIFNα2a with PEG of various molecular weights and structures (branched or linear) decreased the immunogenicity in comparison to non-PEGylated rhIFNα2a. In addition, one of the PEGylated forms showed no immunogenicity in mice (34, 35).

The immunogenicity of mono-PEGylated recombinant human insulin with distinct sites of substitution and varying molecular weights of the PEG was studied in mice (36). All forms, irrespective of molecular weight or site of conjugation, reduced the level of circulating insulin-specific IgG antibodies 10-1000 fold.

The heterogeneity in the length of the PEG-chain and the site where the PEG is attached make it difficult to establish structure-immunogenicity relationships. In our opinion, the best place to attach the PEG-chain would be around possible antigenic epitopes, if this does not result in decreased activity of the protein.

Physical degradation

Proteins have complex three-dimensional structures. Physical degradation processes such as unfolding, misfolding and aggregation of the protein can result in an immune response. Physical degradation of the protein can occur during the production and purification but can also result from improper formulation, storage or handling conditions. The choice of the formulation and the dosage form requires extensive research to guarantee the physical stability of the protein and thus minimize immunogenicity.

Especially aggregates have shown to increase the immunogenicity of various therapeutic proteins, which might be explained by their multiple-epitope character (cf. the viral-like arrays described earlier) and/or to conformational changes of the individual aggregated protein molecules.

For instance, aggregates of insulin can lead to antibody formation (1). Evidence exists that insulin aggregates possess antigenic sites that are absent in monomers. Further research is needed to determine whether these aggregates promote the formation of antibodies against insulin monomers (37).

Aggregates were present in rhIFNα2a formulations containing human serum albumin (HSA) stored for a prolonged period at 25 °C. Not only

rhIFNα2-rhIFNα2 aggregates, but also HSA-rhIFNα2 aggregates were present, which might have contributed to the observed immunogenicity (7, 15, 20). The effect of different rhIFNα2 aggregates on the immunogenicity was studied systematically in mice. Not only rhIFNα2-rhIFNα2 aggregates isolated from an expired bulk solution, were injected intraperitoneally (i.p.) in wildtype and transgenic mice, tolerant for human IFNα2, but also rhIFNα2-rhIFNα2, rhIFNα2-HSA and rhIFNα2-MSA (murine serum albumin) aggregates, made with glutaraldehyde. As controls rhIFNα2 monomers or a mixture of either HSA/rhIFNα2 or MSA/rhIFNα2 were injected. The aggregates (either one) induced antibody formation in both conventional and transgenic mice. In the transgenic mice the rhIFNα2-rhIFNα2 aggregates and albumin (human and murine)-rhIFNα2 aggregates could break an existing tolerance towards rhIFNα2 monomers, in contrast with non-aggregated rhIFNα2 formulations with or without albumin (18).

The presence of protein aggregates in formulations of (non-recombinant) hGH has been correlated with an increased frequency of immune responses. Three distinct patterns of antibody formation in patients were observed when different preparations of hGH were used. The patients were divided in three groups according to antibody formation (see table 5). The first group of patients developed antibodies that were persistent irrespective of the length and type of hGH therapy. The second group of patients developed antibodies that disappeared after switching to a different preparation of hGH or when the therapy was discontinued. The third group developed no antibodies. The first group had received a preparation of hGH that contained 50-70 % aggregates, the second group had received a preparation with less than 5 % aggregated hGH and the third group had received different types of hGH (either the 50-70 % aggregated, less than 5 % aggregated or monomeric), as determined after the study. It was concluded that the formation of antibodies was dependent on both the level of aggregates present and patient characteristics (38).

Table 5 Role of aggregated hGH and patient features in immunogenicity of (non-recombinant) hGH formulations.

Group	Antibodies	Number of patients	Formulation
1	Persistent	11	50-70 % aggregated
2	Transient	18	< 5 % aggregated
3	No antibodies	33	different types

Patients were divided in groups according to antibody formation. See text for details. Adapted from (38).

Aggregates are a decisive factor for an antibody response. Aggregates not only increase the classical immune response in conventional animals but also break immune tolerance in transgenic animals, made tolerant for the therapeutic protein.

Chemical degradation

Oxidation and deamidation, sometimes followed by isomerisation, are the major causes of chemical degradation of proteins, e.g. during storage or handling. One of the impurities found in rhIFNα2a formulations because of improper storage was an oxidized form (20). The oxidation sites were not determined. The oxidation product was isolated and compared with non-oxidized rhIFNα2a for its immunogenicity in wildtype mice. The oxidized form was more immunogenic than the non-oxidized rhIFNα2a (7, 20).

Deamidation mainly occurs at asparagine residues. In this reaction the asparagine residue is, via a succinimide intermediate, converted into an aspartate or an iso-aspartate residue (39-41). Also, glutamine residues can undergo deamidation resulting in glutamate residues. The rate of deamidation is dependent on the type of nearby amino acids and on the conformation (40, 42). Not much is known about the immunogenicity of deamidated proteins. Chen et al showed that antibodies can be raised specifically against the succinimide derivative of peptides, showing weak cross-reactivity with the parent peptide (43).

Concluding remarks/discussion

Structural changes in proteins may affect their immunogenicity. The presence of grafts (sugar or PEG chains) on the polypeptide chain may decrease their immunogenicity. There are no reports about the introduction of antigenic sites due to these additions. The most important recognized structural change known to increase the immune response is aggregation. Even small amounts of aggregates may be sufficient to elicit an immune response. The aggregation either reveals new epitopes recognized as non-self or leads to the spacing of the epitopes known to break self tolerance. Aggregates occur in different sizes and the proteins in the aggregates can have a native or non-native conformation. Folding probably has a great influence on immunogenicity since the misfolded protein may present different epitopes than the native protein. The effect of non-native conformers present in protein formulations on the immunogenicity is still unclear and needs to be investigated further.

To what extent do the antibodies induced by structural variants of a protein cross-react with the native protein? If the immune response is directed towards an epitope specific for the variant, the only clinical consequence may be the loss of effect of the therapeutic protein involved. However, when the immune response is directed towards an epitope present in the endogenous protein, the antibodies induced may also neutralize this endogenous protein, and serious clinical consequences may be anticipated.

The formulation of proteins plays an important role in the occurence of immunogenicity. Excipients in the formulation added to stabilize the protein may affect its immunogenicity. The excipients could change the presentation of the protein to the immune system, i.e. in a spacing of epitopes known to be a strong stimulus for the immune system. Lyophilization of HSA-containing rhIFNα2a formulations and storage at room temperature induced aggregates (HSA-rhIFNα2a and rhIFNα2a aggregates). Removal of the HSA and storage in a refrigerator led to less aggregation and diminished immunogenicity (7).

Still little is known about the relationship between changes in the structure of therapeutic proteins and their immunogenicity. The development of new and improvement of current analytical techniques will help in identifying impurities and non-native conformers in protein formulations. With the advent of transgenic animals tolerant for human proteins, systematic studies on the correlation between conformational and chemical changes and immunogenicity can be conducted. Well-defined degradation products can be prepared and their immunogenicity can be compared to that of native proteins. It is unlikely that one can completely predict the immunogenicity in patients by using transgenic animal models. However, such models will at least be useful to screen formulations or structural variants for their immunogenicity, and can therefore be valuable during the development of new therapeutic protein formulations, as well as for the establishment of proper storage and handling conditions.

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Chapter 3

Micelle-associated protein in epoetin formulations: a risk factor for immunogenicity?

Suzanne Hermeling, Huub Schellekens, Daan J.A. Crommelin, Wim Jiskoot

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Abstract

Purpose. An upsurge of pure red cel aplasia (PRCA) cases associated with subcutaneous treatment with epoetin alpha has been reported. A formulation change introduced in 1998 is suspected to be the reason for the induction of antibodies which also neutralize the native protein. The aim of this study was to detect the mechanism by which the new formulation may induce these antibodies.

Methods. Formulations of epoetin were subjected to gel permeation chromatography with UV detection and the fractions analyzed by an immunoassay for the presence of epoetin.

Results. The chromatograms showed that Eprex®/Erypo® contained micelles of Tween 80. A minute amount of epoetin (0.008-0.033 % of the total epoetin content) coeluted with the micelles, as evidenced by ELISA. When 0.03 % (w/v) Tween 80, corresponding to the concentration in the formulation, was added to the elution medium, the percentage of epoetin eluting before the main peak was 0.68 %.

Conclusions. Eprex®/Erypo® contains micelle-associated epoetin which may be a risk factor for the development of antibodies against epoetin.

Introduction

Over 80 recombinant human proteins are currently on the European market (1), several of which have been shown to induce antibody formation in patients (2, 3). There are many factors that contribute to the immunogenicity of therapeutic proteins, such as the route of administration, patient characteristics, dose, and duration of therapy. Moreover, the formulation and the structure of the protein may influence the immune response (2, 4). For instance therapeutic protein formulations containing protein aggregates are more immunogenic than formulations without aggregates (5-8).

Immunogenicity of recombinant human proteins can have serious clinical consequences. A good example is the antibody formation reported during treatment with recombinant human erythropoietin (epoetin). The formed antibodies not only block the therapeutic effect, but also neutralize the endogenous protein, resulting in pure red cell aplasia (PRCA) (9-12). The patients become transfusion dependent, some for prolonged periods of time. The majority of the reported PRCA cases occurred after 1998 in European patients who had received epoetin alpha subcutaneously (s.c.) in a new formulation. Only a few cases were reported to be associated with epoetin beta.

Endogenous erythropoietin as well as epoetin are heavily glycosylated: the molecular mass of the peptidic part is 18.2 kDa; the sugars increase the molecular mass to 30.4 kDa. Erythropoietin, epoetin alpha and epoetin beta differ slightly in their glycosylation pattern. However, it has been shown that the antibodies formed in the patients are not directed against the sugar chains of the protein (10, 12). Therefore, glycosylation differences are not expected to play a key role in the observed immunogenicity problem. Instead, differences in the formulation are more likely to influence the immunogenicity of epoetin. Table 1 illustrates that substantial differences exist in the composition of epoetin alpha (Erypo®, Eprex®) and epoetin beta (NeoRecormon®) formulations.

In this study we analyzed formulations of epoetin alpha and beta for potential interactions between the protein and excipients. It is shown that the epoetin alpha formulation contains small quantities of micelle-associated epoetin. This micellar form of epoetin may be an important risk factor for the development of antibodies in patients.

Table 1 Qualitative composition of Eprex®/Erypo® and NeoRecormon®.

Eprex®/Erypo®	NeoRecormon®	
Epoetin alpha	Epoetin beta	Glycine
Glycine	Urea	Leucine
Tween 80	Tween 20	Isoleucine
Sodium chloride	Sodium chloride	Threonine
Sodium phosphate	Sodium phosphate	Glutamic acid
Sodium citrate	Calcium chloride	Phenylalanine

Materials and methods

Epoetin formulations

Erypo® (Ortho Biotech, Neuss, Germany), Eprex® (Ortho Biotech, Tilburg, The Netherlands) and NeoRecormon® (Roche, Mannheim, Germany; Mijdrecht, The Netherlands) were used throughout the study. For all experiments ready-to-use syringes were used (see table 2). Eprex® and Erypo® contain the same excipients (see table 1), and were considered to be equivalent.

Gel permeation chromatography (GPC)

A Superdex 200 HR 10/30 column (Amersham, Roosendaal, The Netherlands) was used for GPC analyses. The mobile phase consisted of 50 mM sodium phosphate buffer, pH 6.8, and 300 mM sodium chloride (unless stated otherwise) and was passed through a 0.45-µm filter prior to use. The mobile phase was delivered to the column at a flow rate of 0.50 ml/min by a Waters 600 controller equipped with an autosampler (model 717, Waters). Chromatograms were recorded with a photodiode array detector (model 996, Waters). The column was calibrated by analyzing protein standards obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) with known molecular weights (i.e. thyroglobulin, bovine serum albumin, ovalbumin, α-chymotrypsin and myoglobin).

Samples of Erypo®, Eprex® and NeoRecormon® (1500 IU/sample) were applied on the GPC column without dilution or any other pre-treatment. In addition, Eprex® spiked with 0.03 % (w/v) Tween 80 (Serva, Heidelberg, Germany), NeoRecormon® spiked with 0.01 % (w/v) Tween 20 (Roche, Mannheim, Germany), and aqueous solutions of Tween 80 and Tween 20 were analyzed. In a separate experiment, Eprex® and NeoRecormon® were analyzed

by GPC after addition of 0.03% (w/v) Tween 80 or 0.01 % (w/v) Tween 20, respectively, to the mobile phase.

Fractions (0.25 ml) were collected from 10 until 35 minutes after injection and analyzed for the presence of epoetin by an immunoassay (see below).

Immunoassay

Human erythropoietin-specific immunoassays obtained from Roche (Penzberg, Germany) or Quantikine IVD (R&D Systems, Abingdon, UK) were used for analysis of the GPC fractions, according to the manufacturer's recommendations.

Roche Immunoassay

A biotinylated anti-epoetin antibody solution (125 µl) was added to a streptavidin coated microplate. The plate was incubated for one hour at room temperature under constant orbital shaking. Contents were discarded and the wells were washed three times with 300 µl of wash buffer (40 mM potassium phosphate buffer, pH 7.2; 0.1 % (w/v) Tween 20). After the last wash the wells were carefully tapped dry on a tissue. One hundred microliters of sample buffer (supplied by the manufacturer) and 20 µl of each fraction was added to a well. The plate was incubated for one hour at room temperature under constant orbital shaking. Contents were discarded and the wells were washed three times with 300 µl of wash buffer. After the last wash the wells were carefully tapped dry on a tissue. A solution of rabbit polyclonal anti-epoetin antibodies conjugated to horseradish peroxidase was added to each well and the plate was incubated on an orbital shaker at room temperature for two hours. Contents were discarded and the wells were washed three times with 300 µl of wash buffer. After the last wash the plate was tapped dry on a tissue. One hundred microliters of substrate solution was added to the wells and after incubation for 20 minutes the absorbance was read on a Novapath microplate reader (Biorad, Veenendaal, The Netherlands) at a wavelength of 405 nm and a reference wavelength of 490 nm.

To determine the epoetin concentration in the fractions, a calibration curve (made of the corresponding formulation analyzed by GPC) was included in the immunoassay. During all incubation steps the plate was covered with adhesive tape. Tween 80 and Tween 20 did not interfere in the analyses.

Table 2 Results of GPC-immunoassays of epoetin formulations tested.

Product (batch nr.)	Addition to mobile phase	Concentration	Epoetin in peak 2[1, 2]	
			1st run	2nd run
Erypo® (02DS14T)	none	4000 IU/0.4 ml	0.031 %	-[3]
Eprex® (02CS05T)	none	4000 IU/0.4 ml	0.008 %	0.014 %
Eprex® (02GS05T)	none	4000 IU/0.4 ml	0.033 %	0.033 %
NeoRecormon® (MH6410602)	none	3000 IU/0.3 ml	ND[4]	ND
NeoRecormon® (MH6459408)	none	4000 IU/0.3 ml	ND	ND
NeoRecormon® (MH6602301)	none	4000 IU/0.3 ml	ND	ND
Eprex® (02CS05T)	0.03 % Tween 80	4000 IU/0.4 ml	0.64 %	0.72 %
NeoRecormon® (MH6410602)	0.01 % Tween 20	3000 IU/0.3 ml	0.051 %	0.052 %

[1] for the position of peak 2 see figure 1.
[2] percentage of amount applied to the GPC column.
[3] -: not determined.
[4] ND: not detectable.

Quantikine IVD Immunoassay

One hundred microliters of assay diluent (supplied by the manufacturer) was added to the wells of a microplate coated with a murine monoclonal antibody against epoetin. Subsequently, 80 µl of dilution buffer (supplied by the manufacturer) and 20 µl of each fraction was added to a well. The plate was incubated for one hour at room temperature under constant orbital shaking. Contents were discarded and the wells were carefully tapped dry on a tissue. A solution of rabbit polyclonal anti-epoetin antibodies conjugated to horseradish peroxidase was added to each well and the plate was incubated on an orbital shaker at room temperature for one hour. Contents were discarded and the wells were washed four times with 400 µl of the supplied wash buffer. After the last wash the plate was tapped dry on a tissue. Two hundred microliters of substrate solution was added to each well. After incubation for 20 – 25 minutes 100 µl of stop solution was added to all wells and the absorbance was read on a Novapath microplate reader at a wavelength of 450 nm and a reference wavelength of 595 nm.

Results

Three batches of Eprex®/Erypo® and three batches of NeoRecormon® were tested in duplicate. Representative GPC profiles of Eprex®/Erypo® and NeoRecormon® are shown in figure 1A-B. Both Eprex®/Erypo® and NeoRecormon® batches showed a main peak corresponding to free epoetin (peak 3 in figure 1A-B). The most striking difference between the GPC profiles of Eprex®/Erypo® and NeoRecormon® is the presence of two overlapping peaks (peak 1 and 2 in figure 1A) with a shorter retention time than the free epoetin peak (peak 3) for all Eprex®/Erypo® batches tested. These two peaks (with apparent molecular weights of 670 kDa and 255 kDa) are indicative of high molecular-weight material present in the Eprex®/Erypo® batches. None of the NeoRecormon® batches showed peaks eluting before the free epoetin peak. Another difference between Eprex®/Erypo® and NeoRecormon® was the area of peak 4 (eluting at or near the total column volume, see figure 1A-B), which was much smaller for Eprex®/Erypo® as compared to NeoRecormon®. This is due to the larger number of low-molecular-weight excipients in the latter

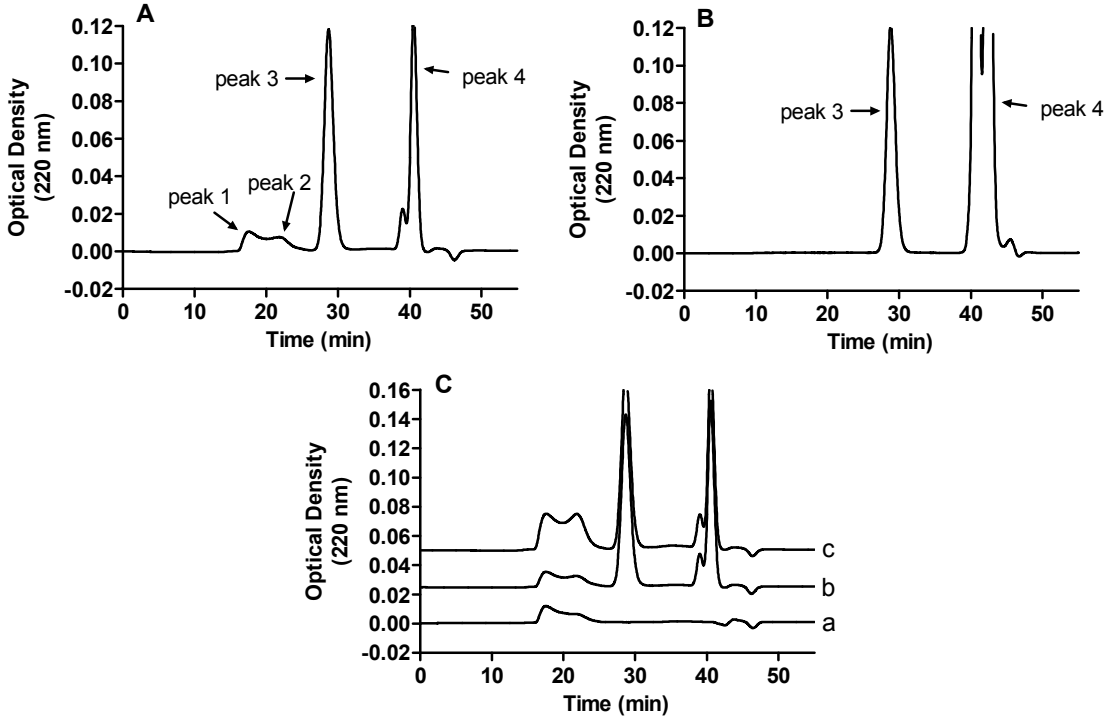

Figure 1 *GPC profiles obtained with a mobile phase consisting of 50 mM sodium phosphate buffer, pH 6.8; 300 mM sodium chloride. (A) Eprex®. (B) NeoRecormon®. Peak 1 has an apparent molecular weight of 670 kDa, peak 2 has an apparent molecular weight of 255 kDa and peak 3 has an apparent molecular weight of 55 kDa; peak 4 contains low molecular-weight material. (C) 0.03 % (w/v) Tween 80 (a), Eprex® (b) and Eprex® spiked with 0.03 % (w/v) Tween 80 (c). Different baseline offsets are represented for sake of clarity.*

formulation that show UV-absorbance (see table 1).

The appearance of the high-molecular-weight peaks in the Eprex®/Erypo® batches was thought to be due to the presence of Tween 80 micelles and/or the presence of epoetin oligomers. Both possibilities were investigated. Eprex®/Erypo® contains 0.03 % (w/v) Tween 80 (approximately twenty times the critical micelle concentration (CMC) (13)) and NeoRecormon® contains 0.01 % (w/v) Tween 20 (approximately 1.5 times the CMC (13)). When an aqueous solution of 0.03 % (w/v) Tween 80 was injected onto the GPC column, the chromatogram showed two overlapping peaks with similar retention times and comparable peak shapes and areas as peaks 1 and 2 of Eprex®/Erypo® (see figure 1C). At lower concentrations of Tween 80 (below the CMC), these peaks where absent (data not shown). Moreover, figure 1C shows that the size of these peaks was about doubled when Eprex® was spiked with 0.03 % (w/v) Tween 80. These data show that Eprex®/Erypo® indeed contains micelles of Tween 80. When NeoRecormon® was spiked with 0.01 % (w/v) Tween 20 no differences between the spiked and non-spiked GPC profile could be observed. Micelles of Tween 20 could only be detected when concentrations of 0.05 % (w/v) or higher were injected onto the column (data not shown).

To investigate the possible presence of epoetin in the high-molecular-weight fractions, the GPC fractions were analyzed by immunoassay. Figure 2 shows the results of one batch of Erypo® and one batch of NeoRecormon®, both of which are representative for all Eprex®/Erypo® and NeoRecormon® batches tested. Besides the main peak 3, Erypo® contained a small amount of epoetin coeluting with micellar Tween 80, i.e. at a position corresponding to peak 2 (for the position of the peaks see figure 1A). The amount of epoetin in peak 2 of Eprex®/Erypo® varied among batches and was calculated to be between

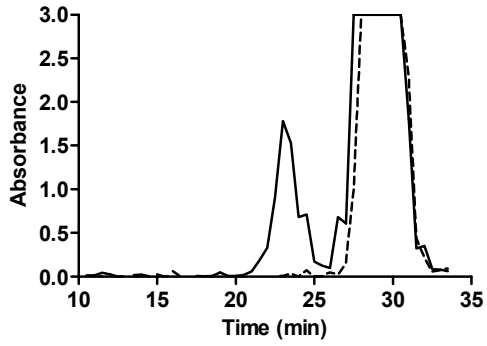

Figure 2 *Results of the immunoassay of Eprex® (solid line) and NeoRecormon® (dashed line) showing the epoetin activity in the fractions. The time reflects the time after injection. The fractions were collected during the GPC analyses with a mobile phase consisting of 50 mM sodium phosphate buffer, pH 6.8; 300 mM sodium chloride. The maximum absorbance of the microplate reader was 3.*

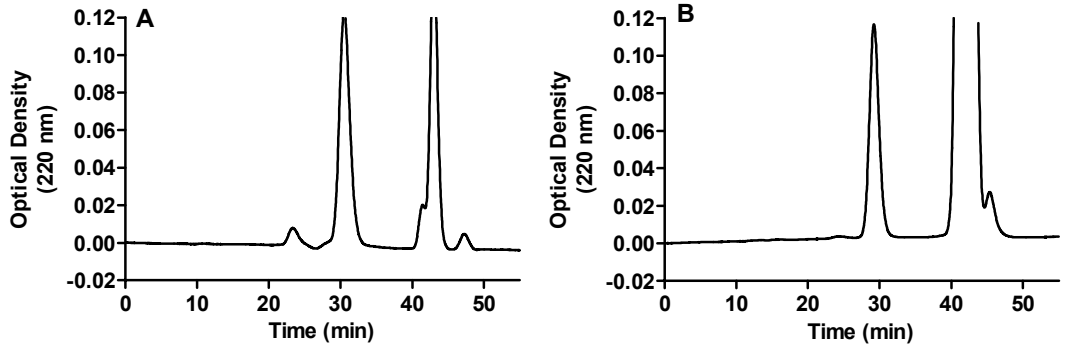

Figure 3 *GPC profile of Eprex® with a mobile phase consisting of 50 mM sodium phosphate buffer, pH 6.8; 300 mM sodium chloride; 0.03% (w/v) Tween 80 (A) and NeoRecormon® with a mobile phase consisting of 50mM sodium phosphate buffer, pH 6.8; 300 mM sodium chloride; 0.01 % (w/v) Tween 20 (B). The typical micellar peak as seen in figure 1 of Eprex®/Erypo® is no longer visible in panel A.*

0.008 % and 0.033 % of the total dose analyzed (see table 2). This extremely small amount hardly contributes to the optical density at 220 nm, which explains why the peak shapes and areas of the GPC profile of 0.03 % (w/v) Tween 80 were virtually the same as compared to those of peaks 1 and 2 of Eprex®/Erypo® batches (figure 1C). For all NeoRecormon® batches, epoetin was only detected in peak 3 (figure 2).

From these results it can be concluded that Eprex®/Erypo® batches contain Tween 80 micelles and a small amount of epoetin coeluting with the micelles. Neither micelles nor oligomeric epoetin were detected in NeoRecormon® batches. However, micelles are dynamic systems: surfactant monomers free in solution are in equilibrium with micellar surfactant. During the GPC procedure the (large) micelles will continuously be separated from the free (monomeric) surfactant molecules, which will shift the equilibrium between micellar surfactant and free surfactant to the monomers free in solution. If the epoetin molecules coeluting with the Tween 80 micelles were solubilized in the micelles, the amount of micellar epoetin present in the formulations would be underestimated when using the above GPC procedure. Therefore, we also analyzed Eprex® and NeoRecormon® after adding 0.03 % (w/v) Tween 80 and 0.01 % (w/v) Tween 20 to the mobile phase, respectively. Hereby the micelle-monomer equilibrium during the GPC run will be kept constant, which will result in GPC/immunoassay profiles that better represent the contents of the syringes. The chromatograms of the GPC analyses in the presence of Tween are shown in figure 3. For Eprex® a peak eluting at a position similar to that of peak 2 (cf. figure 1A) in front of the main epoetin peak was observed. Since Tween 80 is not detected when using this procedure (because the Tween 80 concentration in the sample equals that of the mobile phase), the peak

probably reflects epoetin. This was confirmed by immunoassay, which showed that the amount of epoetin in these fractions (corresponding to the position of peak 2) had increased from 0.008-0.033 % to approximately 0.68 % (see also table 2). For NeoRecormon® a very small peak eluted in front of the main epoetin peak (see figure 3). This peak contained epoetin (0.052 % of total amount applied to the column).

Discussion

We investigated whether differences between Eprex®/Erypo® and NeoRecormon® formulations could be found that might be correlated with their immunogenicity observed in patients. The formulations of Eprex® and NeoRecormon® are substantially different (table 1). On the Swissmedic website (http://www.swissmedic.ch/) it is reported that the estimated incidence of antibody positive PRCA cases per 10,000 patient years for patients treated exclusively with Eprex® was 0.03 (1989-1997; the period before the change in the formulation of Eprex®) and 1.24 (1998-2002). For patients treated exclusively with NeoRecormon® these values are 0.1 (1990-1997) and 0.14 (1998-2002). All cases (Eprex® and NeoRecormon®) occured after s.c. administration. Considering the possible role of multimeric antigen presentation in breaking the immune tolerance against endogenous proteins (14), we focused on the differences in type and concentration of surfactants. Eprex®/Erypo® contains a Tween 80 concentration (0.03 % (w/v)) far above its CMC, NeoRecormon® Tween 20 at a concentration (0.01 % (w/v)) only slightly above its CMC.

We clearly showed that Eprex®/Erypo® not only contains monomeric epoetin, but also an amount of the protein eluting in high-molecular-weight fractions on GPC. For NeoRecormon® minute amounts of epoetin eluted before the main peak only after addition of Tween 20 to the eluent. At least two possibilities have to be considered that might explain the presence of epoetin in the high-molecular-weight fractions: A) epoetin oligomers coincidentally coeluting with Tween 80 micelles; B) oligomers or monomers solubilized in or attached to Tween 80 micelles. The first option seems unlikely, because the addition of Tween to the mobile phase led to an increase in the amount of epoetin in peak 2 (see table 2).

The second explanation assumes that the Tween 80 micelles contained several epoetin molecules. The average apparent molecular weight of the fractions containing micelle-associated epoetin was ca. 255 kDa. Thus at least a few epoetin molecules can be present in one micelle. This could lead to

increased immunogenicity as a result of the presence of multiple epitopes exposed on the micellar surface. The immune system reacts vigorously to multimeric forms of epitopes: B-cells of the immune system respond independently of T-helper cells if identical multimeric antigen epitopes are recognized (14). When several epoetin molecules are attached to micelles, identical multimeric epitopes are present that may prompt the B-cells of the immune system to make antibodies. These antibodies would also recognize the endogenous erythropoietin.

It cannot be excluded that mixed micelles of Tween 80 and (monomeric or oligomeric) epoetin contains partly unfolded or misfolded epoetin. Nevertheless, as epoetin in the micelle fractions was recognized by antibodies in the immunoassay, these fractions at least contain preserved epitopes that are also present on native epoetin. Confirming the protein structure of micelle associated epoetin (e.g. with spectroscopic techniques) is an extremely difficult task, because the epoetin content in these fractions is very small relative to the (at least 100-fold) excess of free epoetin.

It is possible that after s.c. administration of Eprex®/Erypo® the small fraction of micellar epoetin is responsible for the induction of antibodies observed in some patients. After s.c. injection, the micelles may initially stay intact and encounter immune cells before they are diluted by body fluids. No cases have been reported associated with intravenous administration. It may be that the micelles disappear rapidly due to the immediate dilution of Tween 80 below its CMC in the blood stream. For NeoRecormon® the Tween 20 concentration in the formulation is very close to the CMC. Therefore, upon s.c. injection, rapid dilution by interstitial fluids makes exposure of multimeric forms to the immune system highly unlikely.

We recognize that the presence of micelle-associated epoetin as a risk factor for immunogenicity in patients at this moment remains hypothetical. Follow-up studies in which the micelle-associated epoetin is tested in animal models may shed light on the correlation between the formulation of epoetin and its immunogenicity. If such a correlation can be confirmed, ways to improve the formulation of epoetin should be studied. Lowering the surfactant concentration seems obvious, but other adjustments of the formulation might be necessary to guarantee both a sufficient shelf-life and the absence of immunogenicity of epoetin.

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Chapter 4

Development of a transgenic mouse model immune tolerant for human interferon beta

Suzanne Hermeling, Wim Jiskoot, Daan J.A. Crommelin,
Claus Bornæs[1], Huub Schellekens

[1] Department of Biology and Pharmacology, Maxygen ApS, Denmark

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Abstract

Purpose. Therapeutic proteins may induce antibodies which 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 wildtype 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, E. coli derived rhIFNβ-1b, known to have a relatively high immunogenicity in patients, was shown not only to be immunogenic in the wildtype 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 rhIFNβ's differ in the immunological mechanisms responsible for the development of antibodies.

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 following 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 non-human 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 which are rapidly cleared by the reticulo-endothelial system, thus reducing the circulation time of the therapeutic protein and potentially diminishing its therapeutic effects.

Interferon beta (IFNβ) is a cytokine mainly produced by macrophages, epithelial cells and fibroblasts. It is a regulatory protein with anti-inflammatory, anti-tumor, anti-viral and cell-growth regulatory effects (4, 5). Chronic administration of human IFNβ (hIFNβ) is an effective treatment in relapsing-remitting multiple sclerosis (RRMS) patients (5). Three recombinant hIFNβ (rhIFNβ) formulations are currently on the market for the treatment of RRMS: Betaseron®/Betaferon® (rhIFNβ-1b; Betaseron®, Berlex Laboratories, Montville, New Jersey, USA; Betaferon®, Schering, Berlin, Germany), Avonex® (rhIFNβ-1a; Biogen Idec Inc., Cambridge, Massachusetts, USA) and Rebif® (rhIFNβ-1a; Serono Inc., Rockland, Massachusetts, USA). Betaseron®/Betaferon® was the first formulation to come on the market (1994 in the USA and 1995 in Europe). Avonex® followed in 1996 and 1997 in the USA 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 E. coli cells and is non-glycosylated, has no N-terminal methionine and Cys-17 is mutated to Ser-17 to reduce misfolding during the denaturation-renaturation step in down-stream 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 1). 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 with 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

Table 1 Excipients, route, dose and frequency of administration of the three rhIFNβ formulations currently on the market[1].

Formulation	Type	Form	Excipients[2]	Route[3]	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	arginineHCl, polysorbate 20, sodium acetate, glacial acetic acid, sodium hydroxide, final pH 3.8	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

[1] Adapted from (6).
[2] HSA: human serum albumin
[3] s.c.: subcutaneous; i.m.: intramuscular

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 SDS-PAGE, 60 % non-covalently 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 alpha2a (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 present study was to develop a transgenic mouse model tolerant for hIFNβ to enable studying 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

Wildtype (C57Bl/6) mice were obtained from Charles River laboratories. Food (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 ovums of mice. The offspring were analyzed for the hIFNβ gene in their chromosomal DNA by a 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 with polyICLC. PolyICLC is synthetic doublestranded RNA complexed with polylysine and carboxymethylcellulose (19). It is an inducer of IFNβ, because it mimics viral RNA (19, 20). Since the hIFNβ gene is behind the mouse IFNβ promoter, polyICLC should induce not only murine but also human IFNβ if the transgene is active.

Mice received 1 mg/kg polyICLC intravenously (i.v.). Before and 6 hours after the administration of polyICLC blood was collected 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 five wildtype mice and two groups of five transgenic mice were injected with 5 or 10 μg rhIFNβ-1a intraperitoneally (i.p.) on five days per week for three weeks. Blood was collected from the vena saphena before treatment, weekly during treatment and three 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 five wildtype mice and five 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 five wildtype and five 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 twice with 300 µl wash buffer (0.05 % (w/v) 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 % (w/v) bovine serum albumin in PBS for one hour at room temperature, while the plates were shaken. The plates were drained and washed three 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 one hour at room temperature while gently shaken. The plates were washed three times with 300 µl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Peroxidase labeled goat anti mouse IgG (Sigma, Zwijndrecht, The Netherlands) was added to the wells and the plates were incubated for one hour at room temperature while gently shaking. Plates were drained and washed five 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 minutes of incubation, on 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 ten 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, California, USA). The reciprocal of the dilution of the EC50 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

Wildtype and transgenic mice were injected daily i.p. with 5 μg or 10 μg rhIFNβ-1a. The antibody titers of the wildtype mice are shown in figure 1. For the wildtype mice rhIFNβ is a foreign protein and antibodies were formed, as expected. Although the mice receiving 5 μg produced an earlier response, the anti-IFNβ antibody levels after 21 days in the 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 (figure 2), indicating that these mice are immune tolerant for rhIFNβ.

To test the immunogenicity of a marketed formulation, wildtype 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 wildtype and transgenic mice. The wildtype mice showed high antibody levels against rhIFNβ-1b with significantly higher titers than the wildtype 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 showed 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 wildtype and transgenic mice. The wildtype and transgenic mice showed similar antibody titers (unpaired t-test; P=0.4974), indicating that the immune system of the

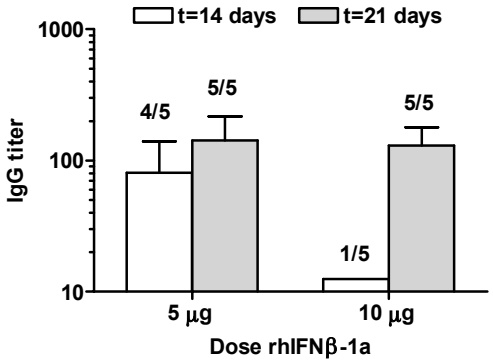

Figure 1 *Results of the ELISA after administration of 5 μg or 10 μg rhIFNβ-1a i.p. on five consecutive days for three weeks. Shown are the average (+ SEM) antibody titers in the sera of positive wildtype mice. Values above bars represent the number of positive mice out of total mice.*

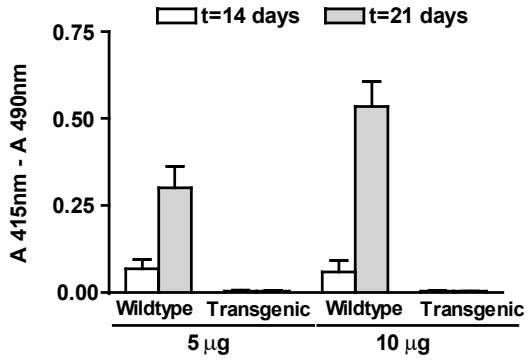

Figure 2 *Results of the ELISA after administration of 5 μg or 10 μg rhIFNβ-1a i.p. on five consecutive days for three weeks. Shown are the average (+ SEM) absorbance values of the 1:100 dilution of the sera of wildtype and transgenic mice.*

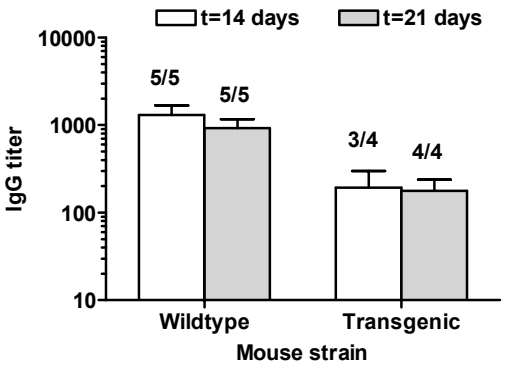

Figure 3 *Antibody titers against Betaseron® in sera of wildtype and transgenic mice. Mice received 5 µg Betaseron® i.p. on five consecutive days for three weeks. Shown are the average (+ SEM) titers of positive mice. Values above the bars represent the number of positive mice per total mice. One of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

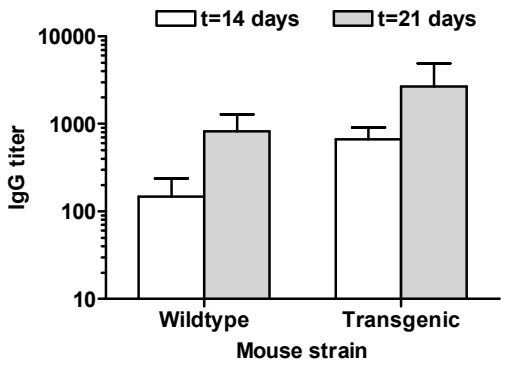

Figure 4 *Anti-HSA titers in wildtype and transgenic mice. Mice received unformulated rhIFNβ-1a with HSA i.p. on five consecutive days for three weeks. Shown are the average (+ SEM) titers. All mice were positive.*

transgenic mice – as compared with the wildtype 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. Since 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 immunogenicity 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 a first step towards a way to predict whether a hIFNβ formulation can break immune tolerance in patients, before it is 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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Chapter 5

The effect of polysorbate 20 and 80 on the structure and immunogenicity in wildtype and transgenic mice of recombinant human interferon alpha2b

Suzanne Hermeling, Daan J.A. Crommelin, Huub Schellekens, Wim Jiskoot

Abstract

Purpose. Many formulations of therapeutic proteins contain surfactants to prevent aggregation and adsorption. Interactions with a surfactant may lead to a conformational change in the protein or insertion of the protein in surfactant micelles which both can cause immunogenicity. In this study we investigated the effect of polysorbate 20 and 80 at concentrations above the critical micelle concentration (CMC) on the structure and immunogenicity of recombinant human interferon alpha2b (rhIFNα2b).

Methods. Solutions of rhIFNα2b (0.5 mg/ml) with or without polysorbate 20 or 80 were prepared. Protein surfactant interactions were monitored with gel permeation chromatography (GPC) followed by ELISA, and with circular dichroism and fluorescence spectroscopy. Reversed-phase HPLC was used to measure the chemical integrity of the protein. The immunogenicity of the formulations was tested in wildtype mice and in transgenic mice, immune tolerant for hIFNα2. Anti-rhIFNα2b serum antibodies were measured by ELISA (IgG) and surface plasmon resonance (SPR) analysis (total Ig).

Results. When the rhIFNα2b solution contained a high concentration (ca. 20x CMC) polysorbate 20 or 80, a small fraction of rhIFNα2b coeluted with polysorbate micelles on the GPC column. None of the formulations showed structural or chemical changes in rhIFNα2b. All formulations induced the same level of anti-rhIFNα2b antibodies in wildtype mice. All formulations showed a very weak immune response in the transgenic mice, as detected by SPR but not by ELISA.

Conclusion. Polysorbate micelles do not measurably affect the rhIFNα2b structure nor do they enhance the immunogenicity of the protein.

Introduction

Many marketed biotherapeutics are lyophilized for stability reasons, whereas liquid formulations are preferred because of the ease of use for the patients (1). However, in liquid formulations proteins are more sensitive to chemical and physical degradation. Chemical degradation refers to changes in the primary structure such as oxidation, deamidation, hydrolysis etc., while physical degradation refers to changes in higher-order structures of the protein such as denaturation, adsorption to surfaces and aggregation (2). All these changes can increase the immunogenicity and decrease the activity of the formulation (3). Especially aggregates are considered to play a key role in inducing an immune response (4-10).

Surfactants are used in protein formulations to inhibit aggregation, precipitation and adsorption to surfaces (2). Roferon-A®, Pegasys® and (PEG-)Intron® are (PEGylated) recombinant human interferon alpha2 (rhIFNα2) formulations currently on the market. They contain polysorbate 80 at a concentration well above its critical micelle concentration (CMC) (0.0015 % (w/v) in 50 mM sodium chloride) (11).

Aggregation is the main cause of immunogenicity of therapeutic proteins. However, after substitution of human serum albumin by polysorbate 80 in the formulation of Eprex® a number of patients formed antibodies against the recombinant protein (epoetin). The antibodies cross-reacted with endogenous erythropoietin resulting in pure red cell aplasia (PRCA) (12). We showed that a small fraction of epoetin molecules in this new formulation was associated with micelles of polysorbate 80 (13). This may have led to an increased immune response due to multimeric antigen presentation (14-17).

We studied whether rhIFNα2b also interacts with polysorbate and whether such an interaction leads to changes in the protein structure and/or an increased immune response. Therefore, rhIFNα2b solutions containing different amounts of polysorbate 20 or 80 were prepared and characterized physico-chemically. The immunogenicity of the formulations was tested in wildtype mice and in transgenic mice immune tolerant for hIFNα2 (4).

Materials and methods

Preparation of formulations

Two and a half ml of rhIFNα2b in 40 mM sodium phosphate buffer (PB), 50 mM sodium chloride, pH 7.2 (a gift from AlfaWassermann, Bologna, Italy) was added to a PD-10 desalting column (Amersham, Roosendaal, The

Netherlands) equilibrated with 20 mM PB, pH 7.4. The flow-through was discarded and the protein was eluted with 3.5 ml 20 mM PB, pH 7.4. After elution the protein concentration was determined by measuring the absorbance at 278.5 nm (extinction coefficient (0.1 %; 1 cm; 278.5 nm) = 1.06 (18)).

Four formulations were prepared in 20 mM PB, pH 7.4. As excipients the formulations contained mannitol (40 mg/ml; Sigma, Zwijndrecht, The Netherlands), sucrose (10 mg/ml; Riedel de Haën, Seelze, Germany) and various concentrations of polysorbate (table 1). Stock solutions of the surfactants (3 % (w/v)) were prepared in 20 mM PB, pH 7.4. For each formulation, the appropriate amount of the surfactant stock solution was added to the buffer containing the excipients, and as a last step the protein was added to a final concentration of 0.5 mg/ml. Formulations were stored at 4 °C.

Interaction of surfactant with protein

All dilutions were made with the corresponding formulation buffer.

Gel permeation chromatography (GPC)

The formulations were analyzed with a Superdex 200 10/300 GL column (Amersham). The mobile phase, 50 mM PB, pH 7.2, and 200 mM sodium chloride was passed through a 0.2-µm filter prior to use and delivered to the column at a flow rate of 0.5 ml/min by a Waters 2695 controller equipped with an autosampler. Chromatograms were recorded with a photodiode array detector (model 2996, Waters). Fractions of the eluate (0.25 ml) were collected between 15 and 35 minutes after application of the sample (150 µl; 0.5 mg/ml rhIFNα2b). The fractions were analyzed by ELISA for the presence of rhIFNα2b as described below.

ELISA for rhIFNα2b

Microlon 96-well plates (Greiner, Alphen aan de Rijn, The Netherlands) were incubated with 100 µl of a monoclonal antibody against rhIFNα2b (diluted 1:2500; Pierce, Etten-Leur, The Netherlands) in 10 mM sodium phosphate buffered saline (PBS) for one hour. The wells were drained and washed four times with 300 µl wash buffer (0.1 % (w/v) polysorbate 20 in PBS). After each wash the plates were tapped dry on a tissue. Wells were blocked with 200 µl 2 % (w/v) bovine serum albumin (BSA) in PBS for one hour. The wells were drained and washed four times with 300 µl wash buffer.

After each wash the plates were tapped dry on a tissue. Standards and fractions (50 µl) were diluted in the wells in duplicate with an equal volume of 2 % (w/v) BSA in PBS. Plates were incubated for one hour. The wells were drained and washed four times with 300 µl wash buffer. After each wash the plates were tapped dry on a tissue. Wells were incubated with a polyclonal rabbit antiserum against rhIFNα2b for one hour. The wells were drained and washed four times with 300 µl wash buffer. After each wash the plates were tapped dry on a tissue. Peroxidase labeled goat anti-rabbit IgG (Sigma) was added to the wells and the plates were incubated for one hour. The wells were drained and washed four times with 300 µl wash buffer and twice with 300 µl PBS. After each wash the plates were tapped dry on a tissue. ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) substrate (Roche, Almere, The Netherlands) was added and the absorbance was recorded after 30 minutes on a Novapath microplate reader (Biorad, Veenendaal, The Netherlands) at a wavelength of 415 nm and a reference wavelength of 490 nm. All incubation steps were performed in covered plates at room temperature with constant orbital shaking.

The presence of polysorbate 20 or 80 in the fractions did not interfere with the results. A serial dilution of a rhIFNα2b standard was included on each plate to calculate the amount of protein present in the samples.

Analysis of protein conformation

Circular dichroism (CD) spectroscopy

CD spectra were recorded from 260-195 nm at 25 °C in quartz cuvettes (path length 0.5 mm) with a dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, Georgia, USA). The subtractive double-grating monochromator was equipped with a fixed disk, holographic gratings (2400 lines/mm, blaze wavelength 230 nm), and 1.24-mm slits. The protein concentration was 200 µg/ml. Each measurement was the average of at least ten repeated scans (step resolution 1 nm, 1 s each step) from which the spectrum of the corresponding blank formulation was subtracted. The measured CD signals were converted to delta molar extinction based on a mean residual weight of 117. The curves were smoothed with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, California, USA), by taking the weighted average of nine points.

Fluorescence spectroscopy

Fluorescence emission spectra (300 - 450 nm; 1-nm steps) were measured in quartz cuvettes (path length 1 cm) on a Fluorolog III fluorimeter. Excitation was at 295 nm and slits were set at 5 nm. The formulations (50 μg/ml) were measured at 25 °C with stirring. Integration time per data point was 0.1 s and the average of ten scans was taken. The spectrum of the appropriate blank formulation was subtracted.

Reversed phase HPLC (RP-HPLC)

RP-HPLC was performed to measure the chemical integrity of rhIFNα2b. The method was adapted from the European Pharmacopeia (EP) (19). A ProSphere C18 (300 Å; 5 μm) in combination with an All-guard C18 guard column (Alltech, Breda, The Netherlands) was used. The column was equilibrated with the starting conditions (44 % (w/w) acetonitrile in 0.2 % (v/v) trifluoroacetic acid in water) for at least 30 minutes. After application of the sample (50 μl; 100 μg/ml) a gradient according to the EP was applied to the column, except that after 30 minutes the starting conditions were applied again. The mobile phase was delivered to the column at a flow rate of 1.0 ml/min by a Waters 2695 controller equipped with an integrated autosampler. Chromatograms were recorded with a Waters 2475 multiwavelength fluorescence detector. The excitation and emission wavelengths were set at 295 and 344 nm, respectively.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The gels consisted of a separating gel containing 15 % (w/v) acrylamide and 0.1 % (w/v) SDS, and a stacking gel containing 5 % (w/v) acrylamide and 0.1 % (w/v) SDS. Gels of 0.75 mm thickness were run under reducing (sample buffer containing 5 % (v/v) β-mercaptoethanol) and non-reducing conditions at 200 V at room temperature. The electrophoresis buffer consisted of 25 mM tris (hydroxymethyl) aminomethane, 192 mM glycine and 0.1 % (w/v) SDS. Gel electrophoresis was performed with a Biorad Protean III system (Biorad). Samples analyzed under reducing conditions were boiled for five minutes before application onto the gel. A low range molecular weight standard (Biorad) was included on the gel. The protein bands were visualized with silver staining (Biorad).

Immunogenicity

Animal experiment

The animal experiment was approved by the Institutional Ethical Committee. Wildtype (FVB/N) mice were obtained from Charles River laboratories. Transgenic mice, immune tolerant for hIFNα2, were bred at the Central Laboratory Animal Institute. Food (Hope Farms, Woerden, The Netherlands) and water (acidified) were available ad libitum.

Mice (five per group) were injected intraperitoneally (i.p.) with 10 μg protein on days 0-4, 7-11 and 14-18. Blood was collected from the vena saphena on days 0, 7 and 14 prior to the administration of protein and on day 21. Blood samples were incubated on ice for two hours. Sera were collected after centrifugation and stored at -20 °C.

Serum analysis by ELISA

Sera were analyzed by ELISA for the presence of antibodies against rhIFNα2b. Microlon 96-well plates were incubated with 100 μl rhIFNα2b (2 μg/ml) per well for one hour. Subsequently the wells were drained and washed four times with 300 μl wash buffer. After washing the wells were carefully tapped dry on a tissue. Wells were blocked by incubating with 200 μl 2 % (w/v) BSA in PBS for one hour. The plates were drained and washed four times with 300 μl wash buffer. After each wash, wells were carefully tapped dry on a tissue. Serum dilutions (1:100 in 2 % (w/v) BSA) were added to the wells and the plates were incubated for one hour. The plates were washed four times with 300 μl wash buffer. After each wash, wells were carefully tapped dry on a tissue. Peroxidase labeled goat anti-mouse IgG (Sigma) was added to the wells and the plates were incubated for one hour. Plates were drained and washed four times with 300 μl wash buffer and twice with 300 μl PBS. After each wash, wells were carefully tapped dry on a tissue. ABTS-substrate was added and absorbance was recorded after 30 minutes of incubation, on a Novapath microplate reader at a wavelength of 415 nm and a reference wavelength of 490 nm. All incubation steps were performed in covered plates at room temperature with constant orbital shaking.

Sera were arbitrarily defined positive when the absorbance value of the 1:100 dilution of the sera minus the background was three 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 added to the plates, coated with rhIFNα2b, in three-fold serial dilutions (starting from 1:10). The other steps of the ELISA procedure were as described above and the

absorbance values were plotted against log dilution. Curves were fitted with a sigmoidal curve (GraphPad Prism version 4.02 for Windows). The dilution needed to obtain 50 % of the maximum absorbance was taken as the titer of the serum. When the absorbance of the 1:10 sample was too low to obtain a good fit, the titer was set to be <10 and the sera were defined to be negative.

Serum analysis by surface plasmon resonance (SPR)

Sera of the wildtype and transgenic mice were analyzed by SPR on a Biacore 3000 (Biacore, Uppsala, Sweden). Native rhIFNα2b (10 μg/ml) was coupled with an amine coupling kit (Biacore) to flow cells 2 and 4 of a CM5 sensor chip (Biacore), according to the manufacturer's instructions. The same procedure was applied to flow cells 1 and 3 with buffer, instead of rhIFNα2b. The flow cells were washed with HBS-EP buffer (Biacore; 10 mM HEPES, pH 7.4, 0.15 M sodium chloride, 3 mM EDTA, 0.005 % (w/v) polysorbate 20). Sera were diluted with HBS-EP buffer containing 1 mg/ml carboxymethyl dextran (Fluka, Zwijndrecht, The Netherlands) and regeneration of the sensor chip was performed with 25 mM hydrochloric acid according to Takacs et al (20). Approximately the same amount of rhIFNα2b was immobilized on flow cells 2 (increase of 2505 relative response units (RU)) and 4 (increase of 2546 RU) of the sensor chip.

After the immobilization of rhIFNα2b, 250 μl of a polyclonal anti-rhIFNα2b rabbit serum (diluted 1:50) was injected on the sensor chip with a flow rate of 10 μl/min. After injection, the dissociation was followed for 1000 s. The surface of the sensor chip was regenerated with 10 μl 25 mM hydrochloric acid. This was repeated 20 times to check the stability of the surface of the sensor chip. The binding of the polyclonal anti-rhIFNα2b serum was stable for these 20 cycles, as was the baseline in flow cells 2 and 4 (data not shown).

The sera of individual mice were pooled per group and per time point. All pretreatment sera, diluted 1:1000, the sera of the wildtype mice at day 21, diluted 1:4000, 1:2000 and 1:1000, and the sera of the transgenic mice at day 21, diluted 1:1000, were injected on the sensor chip and their dissociation was followed for 1000 s. The sensorgrams obtained with flow cells 2 and 4 were corrected by subtracting the sensorgrams obtained with flow cells 1 and 3, respectively. The corrected RU at the start of the dissociation was taken as the level of binding of the sera. After each dissociation the sensor chip was regenerated with 10 μl 25 mM hydrochloric acid.

The dissociation constants of the sera at day 21 were calculated with BIAevaluation software version 4.1 (Biacore). The dissociation of antibodies from the antigen is independent of the concentration. Thus, the dissociation

constants of the three dilutions should have similar dissociation constants and were therefore averaged.

The amount of anti-rhIFNα2b IgG relative to the amount of anti-rhIFNα2b total Ig in the sera of timepoints 7, 14 and 21 days was determined by injecting 10 µl of the sera (diluted 1:100), followed by an injection of 10 µl of a polyclonal goat anti-mouse IgG solution (diluted 1:10). The surface of the sensor chip was regenerated with 10 µl 25 mM hydrochloric acid. The dissociation of the sera during this analysis was negligible.

Results

Interaction of surfactant with protein

GPC and ELISA

The chromatograms obtained with GPC showed in all formulations next to monomeric rhIFNα2b a small amount of dimeric rhIFNα2b (arrow in figure 1c). In formulations with high concentrations of polysorbate (20x CMC) a micelle peak is visible (arrows in figures 1a and b). The polysorbate 20 concentration in formulation B (see table 1 for the composition of the formulations) was too low to be detected as micelles, probably because of dilution of the surfactant on the column (13). The amounts of rhIFNα2b in the "micellar" fractions (range between the dotted lines in figure 1a), as determined by ELISA, are reported in table 1. Formulations A and B, which did not show micelles on GPC (figure 1), did not contain detectable rhIFNα2b in this range. For formulation C, approximately 0.11 % of the total amount of rhIFNα2b

Table 1 Surfactant concentrations and the amount of rhIFNα2b in the micellar fraction of the rhIFNα2b formulations used in this study[1].

	Surfactant	Concentration (% w/v)	Amount of rhIFNα2b in micellar fraction[2]
Formulation A	none	not applicable	0
Formulation B	polysorbate 20	0.03 (4x CMC)	0
Formulation C	polysorbate 20	0.15 (20x CMC)	0.11 %
Formulation D	polysorbate 80	0.03 (20x CMC)	0.14 %

[1] All formulations contained, next to the surfactant, rhIFNα2b (0.5 mg/ml), mannitol (40 mg/ml) and sucrose (10 mg/ml) in 20 mM PB, pH 7.4. The osmolarity of the formulations was 0.3 osm/l.
[2] Percentage of amount rhIFNα2b applied to the column.

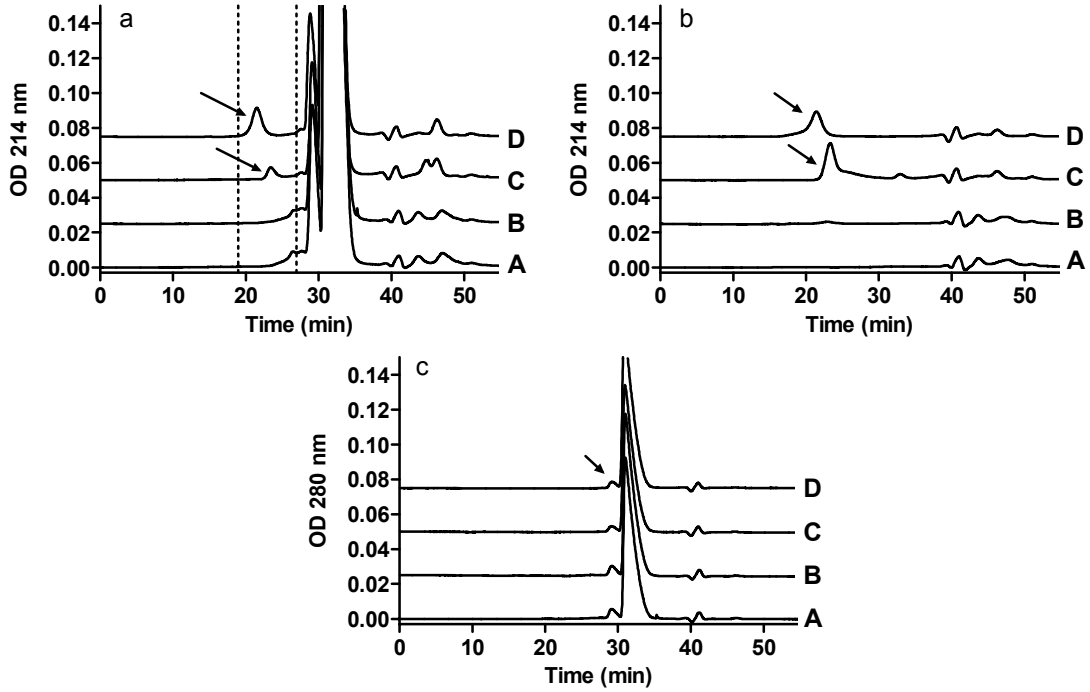

Figure 1 *GPC-chromatograms of the formulations A-D (a) and their placebos (b) (see table 1). Detection was performed at 214 nm (a, b) and 280 nm (c). Different baseline offsets are used for the sake of clarity. The arrows in panels a and b indicate the micelle peaks of polysorbate 20 and polysorbate 80. The dotted lines in panel a mark the fractions (eluting between 19-27 min) used to determine the amount of rhIFNα2b coeluting with micelles. In panel c, the arrow indicates the peak corresponding to dimeric rhIFNα2b.*

applied onto the column, coeluted with the micelles. For formulation D, approximately 0.14 % eluted in the micellar fraction.

Protein structure

Far-UV CD (figure 2a) and fluorescence emission measurements (figure 2b) showed minor differences in intensity between the four formulations, indicating that the polysorbates did not affect the gross secondary and tertiary protein structure, respectively. The far-UV CD spectra were typical for an α-helical protein, as expected for native rhIFNα2b. The fluorescence emission spectra of the formulations all showed a peak maximum at 337 nm. Second derivative fluorescence emission spectra, which can be used to detect subtle changes in tertiary structure (21), failed to show differences between the formulations (data not shown).

RP-HPLC showed a peak of oxidized protein (arrow in figure 3) eluting before the main peak of rhIFNα2b in all formulations (22). No differences between the four formulations were found. The minor peak indicated by # in

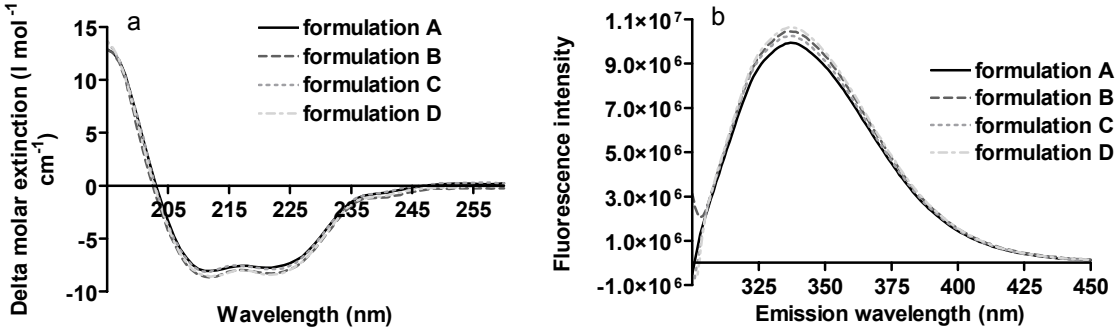

Figure 2 *Conformational analyses of the formulations A-D (see table 1). a: far-UV CD spectra; b: fluorescence emission spectra. The appropriate placebo signals were subtracted.*

figure 3 was not present in the chromatograms obtained with the placebo formulations. Since the peak was not visible with absorbance detection at 280 nm (results not shown), it either is not protein related or the amount of protein was below the detection limit of the absorbance detector at 280 nm.

The results of SDS-PAGE are shown in figure 4. All formulations contained a similar amount of covalent dimer. Formulations B and D showed a minor amount of high-molecular-weight (> 100 kDa) aggregates in the gel run under non-reducing conditions.

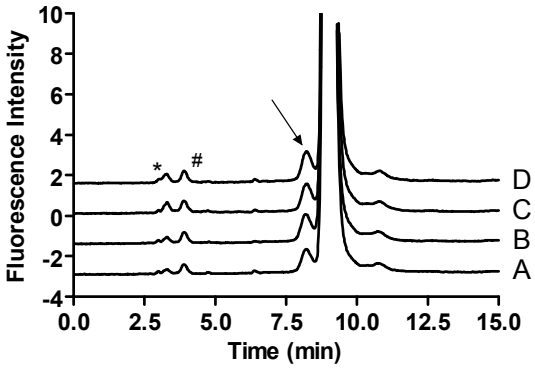

Figure 3 *RP-HPLC chromatograms of the formulations A-D (see table 1). Different baseline offsets are represented for the sake of clarity. The arrow indicates the peak representing oxidized rhIFNα2b. The peaks at 3 minutes (*) were also present in the placeboes.*

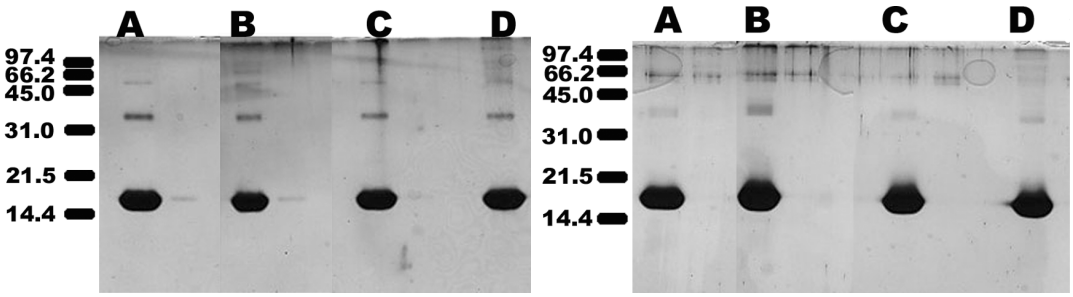

Figure 4 *SDS-PAGE gels run under non-reducing (left panel) and reducing conditions (right panel) of formulations A-D. The position of the bands of the molecular weight marker are indicated by their masses in kDa.*

Immunogenicity

ELISA

The anti-rhIFNα2b IgG titers of the sera of the wildtype mice are shown in figure 5. The antibody levels in the sera of the mice immunized with the four formulations, as determined by ELISA, were statistically the same (P = 0.1667; parametric ANOVA). None of the formulations was able to break the tolerance of the transgenic mice (IgG titers <10), as determined by ELISA.

SPR

The corrected sensorgrams of the pooled, 1000-fold diluted sera of the wildtype mice at 21 days are shown in figure 6a. The pretreatment sera did not show any binding. The dissociation constant of the serum from the wildtype mice treated with formulation A was significantly lower than the dissociation constants of the sera from the mice treated with the surfactant-containing formulations (P < 0.001; table 2). The dissociation constants of the sera from the transgenic mice were similar for the four formulations (P=0.2432; ANOVA) and approximately 4-5 fold higher than the dissociation constants of the sera from the wildtype mice (see table 2). The level of binding of the sera is shown in figure 6b. The level of binding of the sera from the wildtype mice was higher than the level of binding of the sera from the transgenic mice.

The ratio of the binding of anti-mouse IgG to the binding of total Ig in the sera of the wildtype mice at day 21 was approximately 1 (table 2), indicating that, on average, one anti-mouse IgG antibody binds to one anti-rhIFNα2b IgG antibody of the serum. For the sera of the transgenic mice at 21 days this ratio was approximately 3, except for the sera of the transgenic mice treated with formulation D, which showed a ratio of approximately 8 (table 2). At earlier timepoints the level of binding of anti-rhIFNα2b Ig was lower as compared to the level of binding of anti-rhIFNα2b Ig at day 21, both for the sera of the

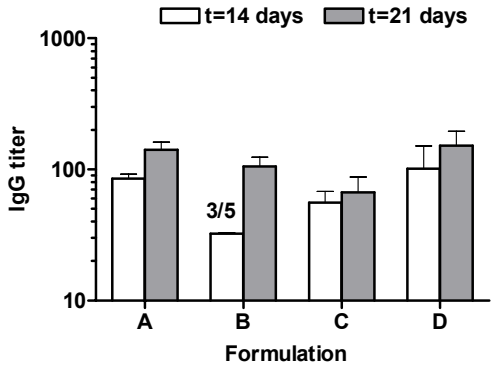

Figure 5 *Anti-rhIFNα2b IgG titers of the wildtype mice treated with formulation A, B, C or D (table 1). The bars represent average values (+ SEM) of positive mice. At t=14 days only three (out of five) mice receiving formulation B showed antibodies. All mice receiving the other formulations showed antibodies. At t=21 days all mice showed antibodies.*

wildtype mice, as well as for the sera of the transgenic mice. The ratio of anti-rhIFNα2b IgG over anti-rhIFNα2b Ig was higher at earlier timepoints as compared to the ratio at day 21, both for the sera of the wildtype mice, as well as for the sera of the transgenic mice (data not shown).

Discussion

We showed that formulations with high (i.e. ca. 20x the CMC) concentrations of polysorbate contain rhIFNα2b eluting in high-molecular weight fractions in GPC. Two possible explanations for these results are: a) aggregates coincidentally elute at the same retention time as the surfactant micelles; b) monomers or oligomers are solubilized in the surfactant micelles.

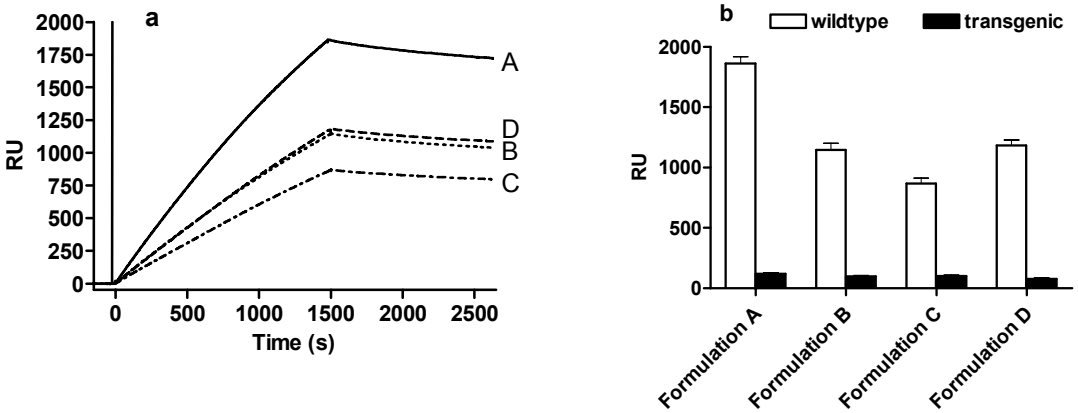

Figure 6 *The corrected sensorgrams (a) as obtained with the pooled sera (diluted 1:1000) at day 21 of the wildtype mice and the observed binding (b) of the pooled sera (diluted 1:1000) at day 21 of the wildtype and transgenic mice treated with formulation A, B, C or D. Bars represent the average (+ range) corrected signals of flow cells 2 and 4.*

Table 2 Characteristics of the sera of the mice treated with the rhIFNα2b formulations, as measured by SPR.

	Dissociation constants x 10^{-5} (1/s)[1]		Binding (RU)[4]		Ratio IgG/total Ig[5]	
Formulation	Wildtype mice[2]	Transgenic mice[3]	Wildtype mice	Transgenic mice	Wildtype mice	Transgenic mice
A	6.0 ± 0.2	34 ± 1	985 ± 23	33 ± 7	1.0 ± 0.01	3.0 ± 0.2
B	8.8 ± 0.2	41 ± 2	480 ± 8	33 ± 2	1.2 ± 0.01	2.7 ± 0.3
C	8.6 ± 0.3	41 ± 3	380 ± 15	20 ± 12	1.3 ± 0.01	3.3 ± 0.2
D	7.6 ± 0.1	40 ± 2	607 ± 21	10 ± 3	1.2 + 0.002	7.7 ± 1.0

[1] Dissociation constants of the antibodies in the pooled sera at t=21 days, as calculated by BIAevaluation software version 4.1.
[2] Values represent average values ± SEM, n=6.
[3] Values represent average ± range, n=2.
[4] Binding of the antibodies in the pooled sera at t=21 days diluted 100 fold. Values represent average ± range, n=2.
[5] Ratio of binding of goat anti-mouse IgG over binding of anti-rhIFNα2b Ig of the pooled sera at t=21 days diluted 100 fold. Values represent average ± range, n=2.

The first option is unlikely because the formulation without surfactants did not contain aggregates and surfactants are added to formulations to prevent aggregation (1). The amount of rhIFNα2b coeluting with the micellar fraction is most likely an underestimation of the micellar rhIFNα2b content, as was demonstrated for epoetin in chapter 3, because the GPC run leads to dilution of the surfactants and a decrease of the number of micelles while eluting on the GPC column.

SDS-PAGE showed that formulations B (with a low level of polysorbate 20) and D (with a high level of polysorbate 80) contained a minor amount of high-molecular-weight aggregates (> 100 kDa), which were not observed in the GPC analysis. These aggregates either failed to enter the GPC column or were missed by the ELISA. These aggregates, however, did not have an effect on the immunogenicity in our mouse models.

Binding of surfactant molecules to rhIFNα2b may lead to structural changes in the protein. In this study no differences in rhIFNα2b conformation were seen. Only GPC showed interaction between polysorbate and small amounts of rhIFNα2b. Either no changes in protein structure had occurred due to the interaction, or the techniques we used were not sensitive enough to detect such changes.

The antibody response in the wildtype mice evaluated by ELISA showed no significant differences between the formulations. SPR, however, showed that

antibodies of the mice treated with rhIFNα2b without surfactant dissociated slower than the antibodies of the mice treated with the polysorbate containing formulations. The difference in dissociation is rather small and the reason for this difference in characteristics of the sera is not known.

The ratio of binding of goat anti-mouse IgG over the binding of anti-rhIFNα2b Ig of the sera of the wildtype mice decreased in time. Since a polyclonal anti-mouse IgG antibody was used the ratio of binding of goat anti-mouse IgG over the binding of anti-rhIFNα2b Ig can become higher than one. It is unlikely that the decrease of the ratio in time is because the relative amount of anti-rhIFNα2b IgG in the sera at earlier timepoints is higher, since the first reaction in an immune response usually consists of IgM antibodies and class-switching to IgG occurs at later timepoints, after repeated contact. It is more likely that the higher ratios at earlier timepoints are due to the lower amount of anti-rhIFNα2b Ig bound to the immobilized rhIFNα2b at these timepoints. With a low number of bound anti-rhIFNα2b Ig there is more space between the bound antibodies. This will make binding of the anti-mouse IgG easier, resulting in a higher ratio of goat anti-mouse IgG binding over anti-rhIFNα2b Ig binding of the serum. This also explains why the ratio is higher in the transgenic mice, than in the wildtype mice. The high ratio observed with the serum of the transgenic mice treated with formulation D can also be explained by the low binding of anti-rhIFNα2b Ig in this serum to the immobilized rhIFNα2b.

The ELISA did not show any anti-rhIFNα2b antibodies in the sera of the transgenic mice. SPR, however, did show a weak binding when the sera were injected over the immobilized rhIFNα2b. Since the SPR signal increased when an anti-mouse IgG solution was injected after the injection of the sera, this weak binding was mediated, at least in part, by IgG antibodies. So, the difference between the ELISA data and the SPR results are primarily due to differences in sensitivity of the techniques (rather than by the fact that ELISA measures IgG only, whereas SPR measures total Ig). The low binding and fast dissociation of the antibodies, as was seen with SPR, is probably the reason why they were missed by the ELISA. Low-affinity antibodies will probably not give major complications for the patients, although affinity maturation can occur during prolonged treatment giving rise to higher affinity antibodies (23). It could be that after a prolonged treatment of the transgenic mice with rhIFNα2b an antibody response can be measured by the ELISA. This is also seen in patients: only after a prolonged treatment of rhIFNα2 anti-rhIFNα2 antibodies can be detected in their sera.

The significance of these results for the use of the transgenic mouse model in the prediction of immunogenicity in patients has to be investigated further, e.g.

by following the antibody levels during prolonged administration of rhIFNα2b to the transgenic mice. It should be noted that our studies were done with freshly prepared rhIFNα2b formulations. To establish whether polysorbates do have an effect on the structure and immunogenicity of rhIFNα2b after long-term storage, further studies are needed.

Conclusions

We have shown that rhIFNα2b molecules coeluted with micelles of polysorbate 20 and 80, if the surfactant concentration was well above the CMC. The surfactants did not induce major changes in the protein structure and did not enhance the antibody response against rhIFNα2b in wildtype mice and transgenic immune tolerant mice.

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Chapter 6

Structural characterization and immunogenicity in wildtype and immune tolerant mice of degraded recombinant human interferon alpha2b

Suzanne Hermeling, Liliana Aranha, J. Mirjam A. Damen[1], Monique Slijper[1], Huub Schellekens, Daan J.A. Crommelin, Wim Jiskoot

[1] Department of Biomolecular Mass Spectrometry, Utrecht Institute for Pharmaceutical Sciences (UIPS) and Bijvoet Center for Biomolecular research

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Abstract

Purpose. To study the influence of protein structure on the immunogenicity in wildtype and immune tolerant mice of well-characterized degradation products of recombinant human interferon alpha2b (rhIFNα2b).

Methods. RhIFNα2b was degraded by metal catalyzed oxidation (M), crosslinking with glutaraldehyde (G), oxidation with hydrogen peroxide (H) and incubation in a boiling water bath (B). The products were characterized with UV absorption, circular dichroism and fluorescence spectroscopy, gel permeation chromatography, reversed-phase HPLC, SDS-PAGE, Western blotting and mass spectrometry. The immunogenicity of the products was evaluated in wildtype mice and in transgenic mice immune tolerant for hIFNα2. Serum antibodies were detected by ELISA or surface plasmon resonance.

Results. M-rhIFNα2b contained covalently aggregated rhIFNα2b with three methionines partly oxidized to methionine sulfoxides. G-rhIFNα2b contained covalent aggregates and did not show changes in secondary structure. H-rhIFNα2b was only chemically changed with four partly oxidized methionines. B-rhIFNα2b was largely unfolded and heavily aggregated. Native (N) rhIFNα2b was immunogenic in the wildtype mice but not in the transgenic mice, showing that the latter were immune tolerant for rhIFNα2b. The anti-rhIFNα2b antibody levels in the wildtype mice depended on the degradation product: M-rhIFNα2b > H-rhIFNα2b ~ N-rhIFNα2b >> B-rhIFNα2b; G-rhIFNα2b did not induce anti-rhIFNα2b antibodies. In the transgenic mice, only M-rhIFNα2b could break the immune tolerance.

Conclusions. RhIFNα2b immunogenicity is related to its structural integrity. Moreover, the immunogenicity of aggregated rhIFNα2b depends on the structure and orientation of the constituent protein molecules and/or on the aggregate size.

Introduction

Nearly all therapeutic proteins induce antibodies. In many cases the incidence and the clinical consequences are limited, but the development of a number of promising new biopharmaceuticals has been stopped because of immunogenicity problems. The development of antibodies in patients may be associated with life-threatening side effects and limits the clinical use of biopharmaceuticals for the treatment of chronic and serious diseases for which there is no alternative effective medical treatment (1).

There are two immunological mechanisms by which these antibodies are induced. A classical, relatively fast immune reaction occurs after treatment with a therapeutic protein from non-human origin, e.g. streptokinase or asparaginase. Patients react to these foreign proteins as to vaccines. A second mechanism for the induction of antibodies is based on breaking B-cell tolerance, which in general takes many months. This may occur in patients who are treated with products which are homologues of human proteins. The exact mechanisms by which therapeutic proteins break tolerance is unknown, although impurities and aggregates are known to be important factors (2-8). Impurities may act as danger signals initiating a response to self-antigens and aggregates may present the self-antigens in a repetitive array form, which is supposed to activate B-cells without T-cell help (9-11).

Although several predictive approaches have been advocated, clinical trials are still the only way to establish the immunogenicity of protein drugs. Immune tolerant transgenic mice are the most useful tool in studying the immunogenicity of therapeutic proteins. They have been used to evaluate whether sequence variations of existing therapeutic proteins as insulin induced new epitopes (12) and to study the breaking of tolerance by aggregates (4).

We have extended these studies and have evaluated whether such models can also be used for more detailed studies on the relation between structure of the therapeutic proteins and the breaking of B-cell tolerance. As a model protein we have selected recombinant human interferon alpha2b (rhIFNα2b). Transgenic immune tolerant animals carrying the human IFNα2 gene are available (4). Moreover, this protein is used in the treatment of a variety of malignancies and viral diseases (13) and has a record of relative high immunogenicity (14). The induction of antibodies is associated with the loss of efficacy (14). Marketed rhIFNα2 products contain either rhIFNα2a or rhIFNα2b, which differ in one amino acid which does not affect immunogenicity (15). A review of the production and purification process has been published (5). Lyophilization of rhIFNα2b formulations containing human serum albumin and improper storage conditions have been shown to

lead to the formation of aggregates (5, 16). The level of anti-rhIFNα2 antibodies is related to the presence of aggregates (5, 15).

We present here data on the relation between the structure of rhIFNα2b, modified by different chemical and physical treatments, and its immunogenicity in wildtype and transgenic immune tolerant mice.

Materials and methods

Degradation of rhIFNα2b

RhIFNα2b (a gift from AlfaWassermann, Bologna, Italy) was degraded in four different ways. All samples were in 10 mM sodium phosphate buffer, pH 7.4 (PB).

To oxidize the rhIFNα2b it was treated with hydrogen peroxide (H_2O_2), according to the European Pharmacopeia (17), by incubating rhIFNα2b (1 mg/ml) with 0.005 % (v/v) H_2O_2 at 37 °C for 20 hours. The oxidation was stopped by the addition of 12 mg methionine per ml followed by incubation at room temperature for one hour.

The metal catalyzed oxidation was achieved by incubating rhIFNα2b (300 μg/ml) with 4 mM ascorbic acid and 0.04 mM $CuCl_2$ for three hours at room temperature according to Li et al (18). The reaction was stopped by adding 100 mM EDTA to a final concentration of 1 mM.

The rhIFNα2b was also crosslinked with glutaraldehyde according to Braun et al (4): incubation of rhIFNα2b (200 μg/ml) with 0.04 % (v/v) glutaraldehyde at room temperature for two minutes, followed by 20 hours incubation after adding sodium borohydride in a final concentration of 1.2 mM.

RhIFNα2b was also modified by incubating 300 μg/ml in a boiling waterbath for ten minutes.

The oxidized and glutaraldehyde samples were dialyzed against 10 mM PB before use. All samples were stored at -20 °C.

Protein concentration

Protein concentrations were measured with a modified Lowry method (19). Bovine serum albumin (BSA) (Sigma-Aldrich, Zwijndrecht, The Netherlands) was used as a standard.

Characterization of rhIFNα2b

Unless stated otherwise, all dilutions were in 10 mM PB and all analyses were performed without prior filtration.

UV spectroscopy

UV spectra (200-450 nm) of the samples (200 μg/ml) were recorded on a Perkin Elmer Lambda 2 UV/VIS spectrophotometer in quartz cuvettes with a pathlength of 1 cm. Ten mM PB was used as a blank.

Circular dichroism (CD) spectroscopy

CD spectra were recorded from 260-180 nm at 25 °C in 0.5-mm quartz cuvettes with a dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, Georgia, USA). The subtractive double-grating monochromator was equipped with a fixed disk, holographic gratings (2400 lines/mm, blaze wavelength 230 nm), and 1.24-mm slits. The protein concentration was 100 μg/ml. Each measurement was the average of at least ten repeated scans (step resolution 1 nm, 1 s each step) from which the corresponding buffer spectrum was subtracted. The CD signals were converted to delta molar extinction based on a mean residual weight of 117. The curves were smoothed with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, California, USA), by taking the weighted average of nine points.

Fluorescence spectroscopy

Fluorescence emission spectra (280 – 450 nm; 1-nm steps) of 55 μg/ml samples were measured in 1-cm quartz cuvettes in a Fluorolog III fluorimeter at 25 °C while stirring. Excitation was at 295 nm. Slits were set at 5 nm. Integration time per data point was 0.1 s and the average of ten scans was taken. The buffer signal was subtracted.

Dynamic light scattering (DLS)

Samples (200 μg/ml) were analyzed with DLS to obtain an average diameter of the particles (Z_{ave}) and their polydispersity at an angle of 90°. A Malvern CGS-3 apparatus equipped with He-Ne (633 nm) JDS Uniphase laser, an optical fiber based detector, and ALV/LSE-5003 correlator was used.

Gel permeation chromatography (GPC)

Samples (100 μg/ml) were analyzed with a Superdex 200 10/300 GL column (Amersham, Roosendaal, The Netherlands) using a mobile phase of 50 mM PB and 200 mM sodium chloride, filtered through a 0.2 μm filter prior to use, at a flow rate of 0.50 ml/min by a Waters 2695 controller equipped with an autosampler. Chromatograms were recorded with a photodiode array detector (model 2996, Waters). The column was calibrated by analyzing protein standards obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) with known molecular weights (i.e. thyroglobulin, BSA, ovalbumin, α-chymotrypsin and myoglobin).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The gels consisted of a separating gel containing 15 % (w/v) acrylamide and 0.1 % (w/v) SDS, and a stacking gel containing 5 % (w/v) acrylamide and 0.1 % (w/v) SDS. Gels of 0.75 mm thickness were run under reducing (sample buffer containing 5 % (v/v) β-mercaptoethanol) and non-reducing conditions at 200 V at room temperature. The electrophoresis buffer was 25 mM tris (hydroxymethyl) aminomethane, 192 mM glycine and 0.1 % (w/v) SDS. Gel electrophoresis was done with a Biorad Protean III system (Biorad, Veenendaal, The Netherlands). Samples analyzed under reducing conditions were boiled for five minutes before application to the gel. A low range molecular weight standard (Biorad) was included on the gel for determination of molecular weight.

Western blotting

SDS-PAGE gels were blotted onto a nitrocellulose sheet with a Scie-Plas semi-dry blotter (Scie-Plas, UK). Blots were blocked overnight at 4 °C with 1 % (w/v) non-fat milk powder in 0.005 % (w/v) Tween 20 in phosphate buffered saline (PBS) with constant orbital shaking. After washing with 0.005 % (w/v) Tween 20 in PBS and with water, the blots were incubated with polyclonal rabbit anti-rhIFNα2b serum in 0.1 % (w/v) non-fat milk powder in 0.005 % (w/v) Tween 20 in PBS for one hour at room temperature with constant orbital shaking. Blots were washed with 0.005 % (w/v) Tween 20 in PBS and with water. Blots were incubated with peroxidase labeled goat anti-rabbit IgG (Sigma) in 0.1 % (w/v) non-fat milk powder in 0.005 % (w/v) Tween 20 in PBS for one hour at room temperature with constant orbital shaking. Blots were washed with 0.005 % (w/v) Tween 20 in PBS and with water and incubated in a solution of 4-chloro-1-naphtol (Sigma) in methanol (15 % (v/v)),

water and H_2O_2 (0.015 % (v/v)). After color development the blots were stored overnight in the dark in water to increase the intensity of the bands.

Reversed-phase high pressure liquid chromatography (RP-HPLC)

A ProSphere C18 (300 Å; 5 μm) was used in combination with an All-Guard C18 guard column (Alltech, Breda, The Netherlands). The column was equilibrated with 45 % (w/w) acetonitrile in 0.2 % (v/w) trifluoroacetic acid (TFA) for at least 30 minutes. After application of the sample (50 μl, 100 μg/ml) a gradient of 45-50 % (w/w) acetonitrile in 0.2 % (v/w) TFA in 25 minutes was applied to the column. The mobile phase was delivered to the column at a flow rate of 1 ml/min by a Waters 2695 controller equipped with an integrated autosampler. Chromatograms were recorded with a photodiode array detector (model 2996, Waters).

Mass spectrometry

Mass spectrometric measurements were performed on an ESI-ToF (LCT) mass spectrometer (Micromass, UK) to obtain the total mass of the protein. The instrument was equipped with a Z-nano-electrospray source (Micromass) operating in the positive ionization mode. Nanoflow electrospray needles were made from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments, Sarasota, Florida, USA) on a P-97 puller (Sutter instruments, Novato, California, USA). The needles were coated with a thin gold layer using a Scancoat Six sputter coater (Edwards Laboratories, Milpitas, California, USA). To remove sodium ions, samples were dialyzed first overnight against 50 mM ammonium acetate.

To analyze and identify the oxidation sites in the oxidized rhIFNα2b samples matrix-assisted laser desorption ionization mass spectrometry (MALDI ToF-ToF, AB 4700 Proteomics analyzer, Applied Biosystems) was used. This instrument is equipped with a 200 Hz Nd:YAG laser operating at 355 nm. Experiments were performed in a reflectron positive ion mode using delayed extraction. Typically, 2250 shots per spectrum were acquired in the MS mode.

The oxidized samples and native rhIFNα2b were reduced with dithiothreitol (DTT) and treated with iodoacetamide to protect the cysteines. After iodoacetamide treatment the samples were dialyzed against 10 mM PB and digested with trypsin (Sigma) for 20 hours at 37 °C. The trypsin to protein ratio was 1 to 50 (w/w). The digested protein was mixed with 5 mg/ml α-cyano-4-hydroxycinnamic acid in 50 % (v/v) acetonitrile and 0.1 % (v/v) TFA

and spotted on the MALDI plate. The obtained mass spectra were compared with theoretical peptide masses obtained after theoretical digestion of the protein and oxidation of the methionines using the 'proteomics tools' utility of the ExPASy web site (20).

Immunogenicity

Animal experiment

The animal experiment was approved by the Institutional Ethical Committee. Wildtype (FVB/N) mice were obtained from Charles River laboratories. Transgenic mice were bred at the Central Laboratory Animal Institute. Food (Hope Farms, Woerden, The Netherlands) and water (acidified) were available ad libitum.

To test the immunogenicity of the samples wildtype or transgenic mice (five per group) were injected intraperitoneally (i.p.) with 10 μg protein on days 0-4, 7-11 and 14-18. Blood was collected from the vena saphena on days 0, 7, 14, before injection and on day 21. The blood samples were incubated on ice for two hours. Sera were collected after centrifugation and stored at -20 °C.

To make sure that the immune tolerance of the transgenic mice was specific for hIFNα2, five wildtype and five transgenic mice were immunized with 10 μg ovalbumin using the same injection protocol as described above. The presence of anti-ovalbumin antibodies in the serum was determined as described below.

Serum analysis by ELISA

Sera were analyzed by ELISA for the presence of antibodies against native rhIFNα2b and against the corresponding degraded sample, or against ovalbumin. Microlon 96-well plates (Greiner, Alphen aan de Rijn, The Netherlands) were incubated with 100 μl per well of a solution (2 μg/ml) of native rhIFNα2b, degraded rhIFNα2b, or ovalbumin for one hour at room temperature with constant orbital shaking. Then the wells were drained and washed four times with 300 μl wash buffer (0.1 % (w/v) Tween 20 in PBS). After washing the wells were carefully tapped dry on a tissue. Wells were blocked by incubating with 200 μl 2 % (w/v) BSA in PBS for one hour at room temperature with constant orbital shaking. The plates were drained and washed four times with 300 μl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Serum dilutions (1:100) were added to the wells and the plates were incubated for one hour at room temperature with constant orbital shaking. The plates were washed four times with 300 μl wash

buffer. After the last wash, wells were carefully tapped dry on a tissue. Peroxidase labeled goat anti-mouse IgG (Sigma) was added to the wells and the plates were incubated for one hour at room temperature with constant orbital shaking. Plates were drained and washed four times with 300 µl wash buffer and twice 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 minutes of incubation, on a Novapath microplate reader (Biorad) 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 when the absorbance value of the 1:100 dilution of the sera minus the background was three 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 added to the plates, coated with native rhIFNα2b or ovalbumin, in serial dilutions (starting from 1:10). The other steps of the ELISA procedure were as described above and the absorbance values were plotted against log dilution. Curves were fitted with a sigmoidal curve (GraphPad Prism version 4.02 for Windows). The dilution needed to obtain 50 % of the maximum absorbance was taken as the titer of the serum.

Serum analysis by surface plasmon resonance

The sera of the mice treated with boiled rhIFNα2b were analyzed by surface plasmon resonance (SPR) with a Biacore (Biacore, Uppsala, Sweden). Native and boiled rhIFNα2b were coupled via an amine coupling to two different flow cells of a CM5 sensor chip (Biacore). The flow cells were washed with HBS-EP buffer (10 mM HEPES, pH 7.4, 0.15 M sodium chloride, 3 mM EDTA, 0.005 % (w/v) Tween 20). After the coupling, 10 µl serum (diluted 10 fold with HBS-EP buffer) of the wildtype mice (pooled per time point) or of the transgenic mice (pooled per time point) were injected on the two different flow cells of the CM5 sensor chip. After the injection the amount of bound material was determined by measuring the increase in relative response units. Complete dissociation of the sera from the sensor chips was obtained by washing the flow cells with a 1.5 M glycine solution.

Results

Characterization of degraded rhIFNα2b

All samples were optically clear and colorless, without visible aggregation or precipitation. Only after repeated freeze-thawing, boiled rhIFNα2b showed visible precipitation.

UV spectroscopy

The UV spectra of proteins can give valuable information about the presence of aggregates in the solution. The metal catalyzed oxidized and boiled rhIFNα2b preparations showed significant optical densities at 350 nm (table 1) and above, which indicates the presence of aggregates (21). In boiled rhIFNα2b, metal catalyzed oxidized rhIFNα2b and glutaraldehyde treated rhIFNα2b a decrease in A_{280}/A_{260} ratio as compared to native rhIFNα2b was observed, also indicative for the presence of aggregates (21). The UV spectrum of H_2O_2 oxidized rhIFNα2b almost overlapped with native rhIFNα2b (data not shown).

Table 1 Characteristics of spectroscopy measurements of native and degraded rhIFNα2b.

	UV		CD	Fluorescence		
	Optical density		Δ ε	Maximum		Scatter peak
	350 nm	A_{280}/A_{260}	222 nm	λ (nm)	Intensity[1]	Intensity[1]
Native	5.4 x 10^{-4}	1.7	-6.2	337	9.8	2.8
Oxidized rhIFNα2b						
by metal catalysis	2.5 x 10^{-2}	1.2	-5.0	339	7.7	236
by H_2O_2	1.4 x 10^{-3}	1.8	-6.0	337	9.6	10
Aggregated rhIFNα2b						
by glutaraldehyde	3.3 x 10^{-3}	1.4	-6.6	338	8.5	56
by boiling	1.7 x 10^{-1}	1.0	ND[2]	344	15.3	225

[1] arbitrary units.
[2] not determined.

CD spectroscopy

Far-UV CD spectroscopy was used to determine the secondary structure of rhIFNα2b, which contains five α-helices (data not shown). Native rhIFNα2b gave a far-UV CD spectrum typical for an α-helical protein. Metal catalyzed oxidized rhIFNα2b showed a small reduction of the intensity of the entire spectrum (but not the shape), indicating a small decrease in α-helical content (table 1). Glutaraldehyde treated and H_2O_2 oxidized rhIFNα2b showed similar far-UV CD spectra as compared to native rhIFNα2b. A far-UV CD spectrum of boiled rhIFNα2b could not be obtained, due to the presence of precipitate in the solution.

Fluorescence spectroscopy

Fluorescence spectroscopy, when performed with an excitation wavelength of 295 nm, gives information about the environment of the two tryptophans in rhIFNα2b at positions 76 and 140. In addition, the scattering of the light (signal of 295 nm) provides information on the presence of particles or aggregates in the solution.

Native rhIFNα2b showed an emission maximum at 337 nm and little scattering (table 1). The emission spectrum of metal catalyzed oxidized rhIFNα2b was slightly red-shifted, indicating a more hydrophilic environment of at least one of the tryptophans. The substantial increase in scatter intensity indicates the presence of aggregates. Glutaraldehyde treated rhIFNα2b showed also a slightly red-shifted maximum and an increased scatter peak, but less than metal catalyzed oxidized rhIFNα2b. The emission spectrum of H_2O_2 oxidized rhIFNα2b had the same characteristics as that of native rhIFNα2b. The emission spectrum of boiled rhIFNα2b showed a large red-shift, indicating that the environment of the tryptophans had become more hydrophilic. The protein was, however, not completely unfolded, because in 6 M guanidine chloride the emission maximum was at 356 nm (data not shown). The high intensity of the scatter peak of boiled rhIFNα2b was about the same as that of metal catalyzed oxidized rhIFNα2b, pointing to extensive aggregation.

DLS

Only the metal catalyzed oxidized and boiled rhIFNα2b samples scattered enough light to analyze the particle size by DLS. In the metal catalyzed oxidized solution the average diameter of the particles was ca. 0.7 μm, with a high polydispersity index (0.84), indicating the presence of aggregates

heterogeneous in size. Boiled rhIFNα2b showed large (ca. 1-3 μm) heterodisperse aggregates.

GPC

GPC was used to analyze the samples for the presence of soluble aggregates (figure 1 and table 3). Native rhIFNα2b showed a peak of monomeric protein and a small dimer peak. Metal catalyzed oxidized rhIFNα2b contained clearly less monomer than native rhIFNα2b, whereas dimers and some trimers had been formed. Also a small, broad peak of larger aggregates was present in this sample. Glutaraldehyde treated rhIFNα2b showed a huge decrease in monomer content, with a concomitant increase in di-, tri- and tetramer content and some bigger oligomers. H_2O_2 oxidized rhIFNα2b showed a profile similar to that of native rhIFNα2b. Boiled rhIFNα2b did not show any peaks on GPC (data not shown), indicating that practically all protein molecules had formed insoluble aggregates.

SDS-PAGE

Formation of covalent aggregates was determined by comparing SDS-PAGE gels run under non-reducing and reducing conditions (figures 2a and b,

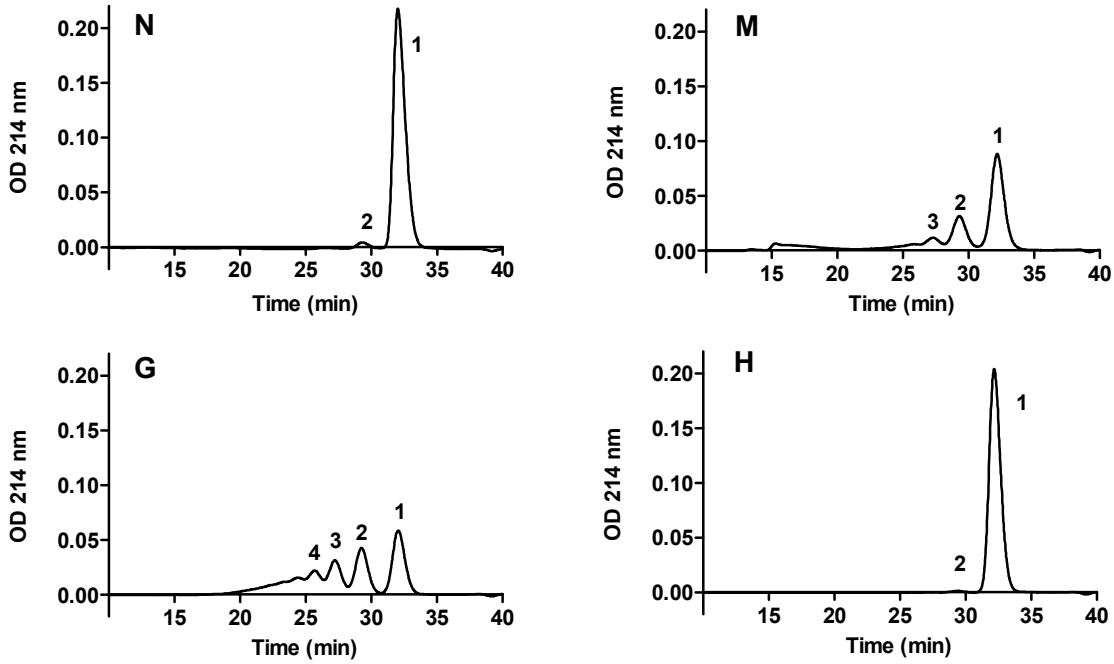

Figure 1 *Gel permeation chromatograms of native rhIFNα2b (N) and degraded samples. M: metal catalyzed oxidized rhIFNα2b, G: glutaraldehyde treated rhIFNα2b and H: H_2O_2 oxidized rhIFNα2b. Numbers correspond to: 1: monomer; 2: dimer; 3: trimer; 4: tetramer. Masses were confirmed with a calibration curve obtained as described in the materials and methods section.*

respectively). Native and H_2O_2 oxidized rhIFNα2b showed a monomer band and a small dimer band under non-reducing conditions. The dimer band was nearly absent under reducing conditions, indicating that the dimer formation was primarily mediated by disulfide bonds. Also the multimeric bands of metal catalyzed oxidized rhIFNα2b seen under non-reducing conditions were substantially less intense under reducing conditions, pointing to disulfide bonds forming the multimers. Both gels showed similarly intense bands of aggregated protein for glutaraldehyde treated rhIFNα2b. These aggregates are apparently covalently linked via (non-reducible) glutaraldehyde crosslinks. The non-reduced sample of boiled rhIFNα2b contained dimers and larger multimers formed through disulfide bridges, as they were almost absent after reduction of the samples.

Western blotting

The reactivity of the individual bands on SDS-PAGE (non-reducing conditions) with polyclonal anti-rhIFNα2b serum was tested by Western blotting (figure 2c). As expected, the monomer of native rhIFNα2b reacted with the polyclonal antiserum. This was also true for the single band of H_2O_2 oxidized rhIFNα2b. Aggregates that were present in metal catalyzed oxidized

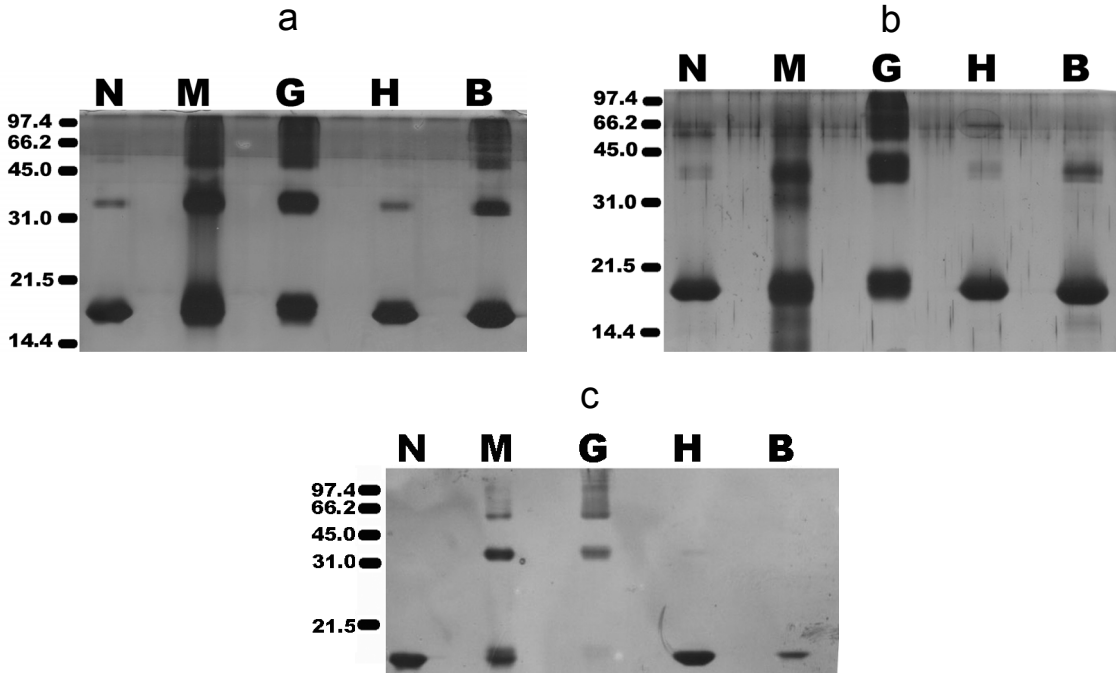

Figure 2 *SDS-PAGE gels (a: non-reducing and b: reducing) and Western blot of a gel run under non-reducing conditions (c) of native and degraded rhIFNα2b. Numbers on the left represent band positions (in kDa) of the molecular weight markers. Lane N = native rhIFNα2b; M = metal catalyzed oxidized rhIFNα2b; G = glutaraldehyde treated rhIFNα2b; H = H_2O_2 oxidized rhIFNα2b and B = boiled rhIFNα2b.*

rhIFNα2b and glutaraldehyde treated rhIFNα2b reacted with the antiserum as well. The dimers and larger aggregates in boiled rhIFNα2b did not react with the polyclonal antiserum. Possibly the antiserum did not recognize the aggregates or the amount of aggregates transferred onto the nitrocellulose sheet was too low to be detected by this method.

The monomer of glutaraldehyde treated rhIFNα2b did not react with the antiserum. Apparently the chemical treatment had not only crosslinked the protein molecules, but also destroyed the immuno-dominant epitopes in the remaining monomer.

RP-HPLC

Only native and H_2O_2 oxidized rhIFNα2b showed peaks in the RP-HPLC chromatogram (data not shown). Metal catalyzed oxidized, glutaraldehyde treated and boiled rhIFNα2b did not show any peaks, probably because the aggregated rhIFNα2b was captured by the guard column and the amount of non-aggregated rhIFNα2b was too low to be detected with this method. However, GPC showed that metal catalyzed oxidized rhIFNα2b contained 43 % of monomeric rhIFNα2b (table 3). Possible explanations for the apparent discrepancy between GPC and RP-HPLC are: (a) the solubility of metal catalyzed oxidized rhIFNα2b in the mobile phases used in RP-HPLC was lower than that of native rhIFNα2b; (b) the aqueous GPC solvent was capable of breaking apart non-covalent aggregates more easily than the organic RP-HPLC solvents; (c) a smaller amount of protein was applied on RP-HPLC than on GPC; (d) adsorption to the guard column present in RP-HPLC, or (e) a combination of the above possibilities.

Compared with native rhIFNα2b, H_2O_2 oxidized rhIFNα2b showed an extra peak with a shorter retention time, representing oxidized rhIFNα2b (5). Based on the peak areas, about 60 % of the protein was oxidized.

Mass spectrometry

In the electrospray mass spectrometer the protein becomes multiple charged, resulting in different m/z peaks in the mass spectrum (22). From this so-called charge envelope the mass of the sample can be calculated. The base peak of native rhIFNα2b had a mass of 19,266 Da (corresponding to the theoretical value of 19,269 Da (23)). The base peaks of both oxidized rhIFNα2b preparations corresponded to a mass of 19,282 Da. The increase of 16 mass units confirms the addition of 1 oxygen atom, indicating that oxidation had occurred. The peak corresponding to non-oxidized rhIFNα2b was also

Table 2 Ratios of oxidized to non-oxidized peptides determined by MALDI-ToF-ToF spectrometry.

Methionine at position	Sample		
	Native rhIFNα2b	Metal catalyzed oxidized rhIFNα2b	H_2O_2 oxidized rhIFNα2b
17 or 22[1]	0.09	0.40	1.28
17 and 22	0.01	0.10	0.19
60	0[2]	ND[3]	ND
112	0	ND	0
149	0.55	1.75	1.61

[1] Only peptides containing both methionines were detected. These peptides were detected with 1 (17 or 22) or 2 (17 and 22) methionines oxidized. It was not possible to distinguish between the two methionines.
[2] Only peaks corresponding to peptides containing the oxidized methionine were detected.
[3] ND: no peaks of peptides containing this methionine were detected, neither oxidized, nor non-oxidized.

visible, indicating that not all molecules were oxidized. No peaks in the mass-spectrum of glutaraldehyde treated rhIFNα2b could be obtained with this method; the sample was too heterogeneous in mass. Boiled rhIFNa2b had the same calculated mass as native rhIFNa2b. However, the charge envelope of the boiled sample contained more peaks and the average m/z ratio was shifted to lower m/z values, indicating that the boiled protein was unfolded (22). Aggregates were not detected with this method.

Table 2 shows the ratios of the peak intensities of peptides (obtained by tryptic digestion, see materials and methods) containing oxidized methionines over those of the corresponding non-oxidized peptides, as obtained with MALDI-ToF-ToF analysis. In native rhIFNα2b some oxidized peptides were detected. Methionines 17, 22 and 149 were shown to be oxidized in some of the protein molecules. In the oxidized samples, both metal catalyzed oxidized as well as H_2O_2 oxidized, the fraction of molecules containing these methionines oxidized had increased substantially as compared to native rhIFNα2b, as is clear from the higher peak ratios of oxidized/non-oxidized peptides (see table 2). Peaks corresponding to peptides containing methionine 60, oxidized or non-oxidized, could not be detected for both oxidized samples. In addition, for metal catalyzed oxidized rhIFNα2b no peaks corresponding to peptides containing methionine 112 could be detected. The mass spectra showed no evidence of oxidized amino acid residues other than the methionines.

With MALDI-ToF-ToF of the peptide digests more oxidation sites were

Table 3 Summarized physico-chemical characteristics of the rhIFNα2b samples.

	Soluble fraction[1]				Insol. fraction[2]	Size[3] (μm)	Oxidation[4]	Denaturation[5]	Destruction of epitopes[6]
	Monomers	Dimers	Trimers	Larger aggr.					
Native	98 %	2 %	0 %	0 %	0 %	-[7]	+	-	-
Oxidized rhIFNα2b									
by metal catalysis	43 %	16 %	6 %	17 %	19 %	0.7	+++	+	-
by H_2O_2	99 %	1 %	0 %	0 %	0 %	-	+++	-	-
Aggregated rhIFNα2b									
by glutaraldehyde	27 %	20 %	15 %	33 %	5 %	-	-	+	+
by boiling	0 %	0 %	0 %	0 %	100 %	1-3	-	+++	+

[1] Percentages were calculated based on GPC peak areas relative to the total peak area in GPC of native rhIFNα2b: $AUC_{peak}/AUC_{native,\ total}$ x 100 %.
[2] The insoluble fraction was defined as the fraction that was not recovered by GPC; percentages were calculated from the total peak area in GPC and the total peak area in GPC of native rhIFNα2b: $(AUC_{native,\ total}-AUC_{sample,\ total})/AUC_{native,\ total}$ x 100 %.
[3] Shown by DLS.
[4] Shown by MS and for H_2O_2 oxidized rhIFNα2b also by RP-HPLC.
[5] Shown by fluorescence and CD spectroscopy (for boiled rhIFNα2b only shown by fluorescence).
[6] Shown by Western blotting.
[7] Symbols used: -: not detectable; +: mild; +++: extensive.

found than with ESI-ToF of the intact polypeptide chain. Probably the peak intensity of oxidation products with more than one oxidation site was too low to be resolved by ESI-ToF.

Summary

The main characteristics of the degraded samples, including the estimated amount of aggregated species, are summarized in table 3.

Immunogenicity

Different immunization protocols (route of administration, dose, dosing schedule) were tested beforehand to optimize our animal model. We aimed for maximum and reproducible antibody levels for native rhIFNα2b in the wildtype mice. No matter which protocol was used, the transgenic mice never formed detectable antibodies against the native protein. This illustrates that they are immune tolerant for the native rhIFNα2b, indeed. These studies led to the immunization scheme mentioned in the materials and methods section.

To assure that the immune tolerance of the transgenic mice is specific for hIFNα2 and not due to a general immune suppression mechanism, we compared the antibody response against ovalbumin in the wildtype and the transgenic mice. Ovalbumin is foreign to both the wildtype and the transgenic mice, and should therefore elicit similar antibody responses. Figure 3 shows that the anti-ovalbumin IgG titers of the wildtype and the transgenic mice are comparable indeed, illustrating that the immune system of the transgenic mice is fully functional.

The anti-rhIFNα2b IgG titers of the wildtype mice immunized with native and degraded rhIFNα2b are shown in figure 4. Metal catalyzed oxidized and H_2O_2 oxidized rhIFNα2b induced antibodies to native rhIFNα2b in the

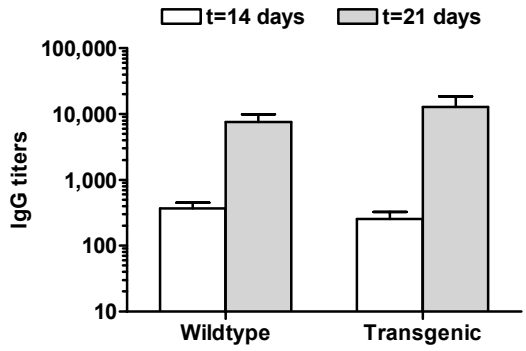

Figure 3 *Anti-ovalbumin IgG titers in sera of wildtype and transgenic mice. Values represent average (+ SEM) titers.*

wildtype mice.

Only one of the wildtype mice treated with the boiled product produced antibodies to native rhIFNα2b, while glutaraldehyde treated rhIFNα2b did not induce antibodies to native rhIFNα2b at all.

However, both the oxidized and glutaraldehyde treated rhIFNα2b preparations did induce antibodies in the wildtype mice to the respective modified material (figure 4b).

We failed to find an antibody response to native rhIFNα2b in the mice treated with boiled rhIFNα2b. To exclude the possibility that this lack of response was due to the inability to coat ELISA plates with boiled protein, we also analyzed the sera with SPR. With this technology we picked up a weak antigen-antibody reaction, although it proved more difficult to immobilize boiled than native rhIFNα2b to the SPR chip. The boiled sample may have contained some native rhIFNα2b, which was more efficiently coupled to the chip than boiled rhIFNα2b. Therefore, we could not conclude from these results whether the antibodies were anti-rhIFNα2b or anti-boiled rhIFNα2b.

Figure 5 shows the antibody response of the immune tolerant transgenic mice treated with the different rhIFNα2b's. Only metal catalyzed oxidized rhIFNα2b was able to break the immune tolerance in the transgenic mice. The other degraded samples did not induce an antibody response, neither to native rhIFNα2b (figure 5), nor to the corresponding samples (data not shown). Also SPR failed to show antibodies against native or boiled rhIFNα2b in the sera of transgenic mice treated with boiled rhIFNα2b.

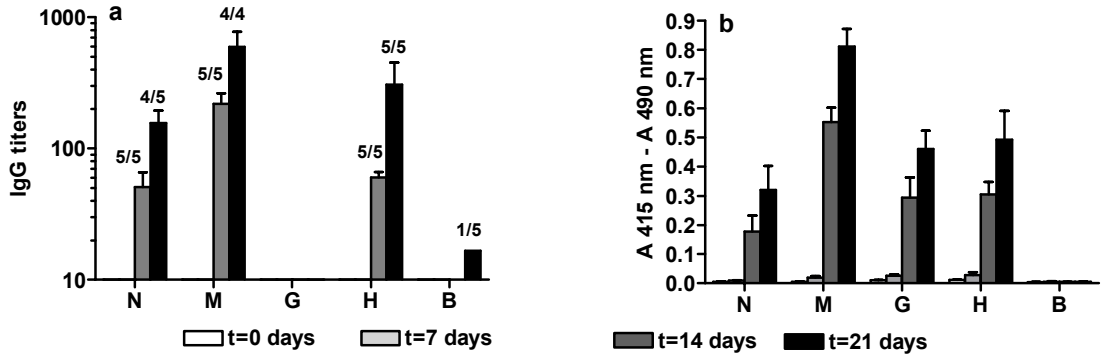

Figure 4 *Anti-rhIFNα2b IgG titers (a) and absorbance values of the 1:100 diluted (b) sera of wildtype mice treated with native (N) and degraded rhIFNα2b. The ELISA plate was coated with native rhIFNα2b (a) or the corresponding test samples (b). M: metal catalyzed oxidized rhIFNα2b; G: glutaraldehyde treated rhIFNα2b, H: H_2O_2 oxidized rhIFNα2b or B: boiled rhIFNα2b. The values represent average (+ SEM) titers of responding mice. The numbers above the bars (a) represent the number of responding mice out of the number of total mice. In the group mice receiving metal catalyzed oxidized rhIFNα2b one mouse died at day 14 because of handling.*

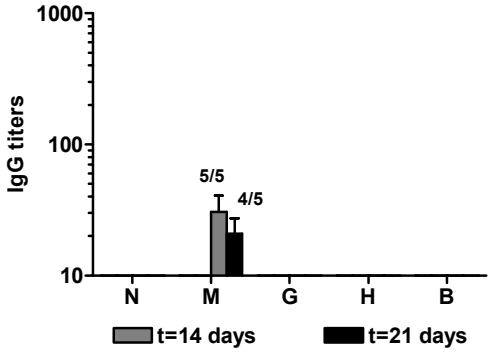

Figure 5 *Anti-rhIFNα2b IgG titers in sera of transgenic mice treated with native (N) and degraded rhIFNα2b. M: metal catalyzed oxidized rhIFNα2b; G: glutaraldehyde treated rhIFNα2b, H: H_2O_2 oxidized rhIFNα2b or B: boiled rhIFNα2b. The values represent average (+ SEM) titers of responding mice. The numbers above the bars represent the number of responding mice out of the number of total mice.*

Discussion

Aggregates in therapeutic protein formulations are known to be important factors in inducing an antibody response (4-8). The immunological mechanism of this antibody induction is not completely understood. Aggregation may lead to conformational changes and the presentation of new epitopes. Aggregation may also lead to the type of multimeric epitope presentation which can break immune tolerance. Somehow the B-cell receptor is capable of responding without T-cell help to epitope patterns meeting certain criteria, such as spacing of 5-10 nm between at least 10 repeating epitopes (10, 24-26).

For wildtype mice, rhIFNα2b is a foreign protein and it induces a classical immune response. All samples induced antibodies, although the level of antibodies induced by boiled rhIFNα2b was very low and antibodies were formed only in 1 mouse. The antibodies induced after immunization with glutaraldehyde treated rhIFNα2b did not cross-react with native rhIFNα2b. This modified protein had apparently obtained new epitopes, due to the crosslinking, which are not present in native rhIFNα2b, as confirmed by Western blotting (figure 2c). However, the aggregates present in glutaraldehyde treated rhIFNα2b still contained native epitopes, but the level might be too low to elicit an immune response.

The observed poor immunogenicity of boiled rhIFNα2b was not expected but may be related to the size of the aggregates. The number of epitopes exposed to the immune system is inversely related to the size of the particles. Also the large size of the aggregates may slow down the clearance from the peritoneal cavity or impair the uptake by phagocytic cells. It was shown before that liposomes (average size 130 nm) greatly decreased the clearance of

encapsulated drugs out of the peritoneal cavity as compared to the free drug (27). The same authors also showed that the clearance was even more retarded when the liposomes were aggregated via avidin/biotin. The size of boiled rhIFNα2b could be well in the range of the aggregated liposomes.

In the transgenic mice immune tolerant for hIFNα2, antibodies to this protein can be induced either by breaking tolerance or via the classical immune response. Metal catalyzed oxidized rhIFNα2b was the only preparation capable of inducing antibodies in the transgenic mice. It is unlikely that the oxidation per sé explains this immunogenicity, because H_2O_2 oxidized rhIFNα2b lacked this effect while both products showed the same oxidation sites. It is more likely that the aggregate formation accompanying the metal catalyzed oxidation of rhIFNα2b was responsible for breaking the tolerance. The mechanism by which metal catalyzed oxidation induces aggregation of rhIFNα2b is unknown, but aggregation has also been reported for metal catalyzed oxidation of relaxin (18). It should also be noted that H_2O_2 oxidized rhIFNα2b and native rhIFNα2b contain dimers and apparently multimeric aggregation is needed to break tolerance.

Glutaraldehyde treated rhIFNα2b contained aggregates that were not able to break tolerance in the transgenic mice. The antibody response in the wildtype mice showed this sample to contain new epitopes for which the transgenics lack tolerance. Nevertheless, the transgenic mice did not respond. A reason for this could be that T-cells recognized native linear epitopes, still present in the modified protein, for which the transgenic mice are immune tolerant. So the T-cells will not activate the B-cells into antibody production. In contrast with our results, Braun et al did show breaking of immune tolerance with glutaraldehyde induced aggregates (4). Reasons for this discrepancy could be differences in modification conditions, immunization protocol and/or route of administration.

Altogether, our data indicate that the dogma 'aggregation leads to immunogenicity of therapeutic proteins' should be taken with caution. The three different aggregated samples (obtained by metal catalyzed oxidation, by glutaraldehyde treatment, and by boiling), which differ both in size distribution and in protein structure, widely vary in immunogenicity: whereas aggregates obtained by metal catalyzed oxidation are able to break the immune tolerance, those obtained by glutaraldehyde treatment and boiling are not. If optimally spaced (5-10 nm) repetitive (more than 10) native (or native like) epitopes are the predominant mechanism by which aggregates would break immune tolerance (10, 24-26), only the metal catalyzed oxidation product apparently fulfills these criteria. Also, the large aggregates detected by DLS and/or the larger aggregates detected by GPC, rather than the smaller

oligomers observed by GPC, are most likely to be responsible for the increased immunogenicity of metal catalyzed oxidized rhIFNα2b. Besides the number and the orientation of epitopes, however, the structure of the protein molecules that form the aggregates may be crucial. Both boiled rhIFNα2b (loss of native conformation) and glutaraldehyde treated rhIFNα2b (major chemical modifications) may not sufficiently resemble native rhIFNα2b to break the immune tolerance. So, aggregates composed of native-like protein molecules may be more likely to break tolerance than aggregates consisting of denatured proteins. This would imply that preventing the formation of native-like aggregates should be a major task for protein formulation scientists in their search for non-immunogenic therapeutic protein formulations.

Conclusions

In this study we tried to correlate structure with immunogenicity and ability to break immune tolerance. We found that large aggregates of denatured protein are not necessarily more immunogenic than smaller aggregates composed of more "native-like" protein. It appears that both the structure of the constituent proteins and/or the size of the aggregates determine immunogenicity. We also found that oxidation in itself does not enhance immunogenicity. Moreover, we have shown that screening the immunogenicity of protein drugs should include assays for antibodies to the modifications present in the product.

Although the aggregates produced in this study may not fully represent the degradation products typically formed during production, storage or handling of rhIFNα2b formulations, with the present study we gained more insight into which structural changes affect the immunogenicity of rhIFNα2b. Clearly, more research is needed to clarify the relation between structure and immunogenicity of rhIFNα2b in particular and therapeutic proteins in general, to assess the minimum amount of modified proteins inducing antibody formation, and to evaluate the predictive value of immune tolerant transgenic mouse models for the immunogenicity of therapeutic proteins in patients.

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Chapter 7

Antibody response to aggregated recombinant human interferon alpha2b in wildtype and transgenic immune tolerant mice

Suzanne Hermeling, Huub Schellekens, Coen Maas[1], Martijn F.B.G. Gebbink[1], Daan J.A. Crommelin, Wim Jiskoot

[1] Thrombosis & Haemostasis Laboratory, University Medical Center Utrecht, Utrecht, The Netherlands

Abstract

Purpose. Transgenic immune tolerant mice can be used to study the immunogenicity of aggregated recombinant human interferon alpha2b (rhIFNα2b) preparations. The sensitivity of the mice for the level of aggregation was studied.

Methods. RhIFNα2b was aggregated by oxidation using metal catalysis. To obtain samples with different levels of aggregation, native rhIFNα2b was mixed with metal catalyzed oxidized rhIFNα2b at different ratios (1:0, 3:1, 1:1, 1:3 and 0:1 (v/v)). The preparations were characterized by UV and fluorescence spectroscopy, gel permeation chromatography, dynamic light scattering and SDS-PAGE. The immunogenicity of the preparations was evaluated in wildtype mice and transgenic mice, immune tolerant for hIFNα2. Anti-rhIFNα2b serum IgG was detected by ELISA.

Results. Oxidation of rhIFNα2b by metal catalysis resulted in partly aggregated protein (dimers, trimers, oligomers and insoluble protein) without denaturation. In wildtype mice at least 25 % of aggregated protein was needed to increase the immunogenicity after s.c. administration. The transgenic mice showed a dose dependent loss of tolerance starting at the level of 14 % of aggregates, the lowest amount tested in this study.

Conclusion. The immune response to rhIFNα2b formulations in wildtype mice and transgenic immune tolerant mice is dependent on the level of aggregation. Moreover, transgenic immune tolerant mice are more sensitive to aggregates than wildtype mice.

Introduction

Immunogenicity is being recognized as a potential serious problem for the use of therapeutic proteins. The incidence and level of antibody formation against therapeutic proteins is dependent on a number of factors (1, 2), including patient and protein characteristics, formulation and route of administration. The presence of aggregates is one of the main factors increasing the immune response to a therapeutic protein (3-7).

Prediction of immunogenicity of therapeutic proteins would be advantageous, but at present no fully validated predictive approaches are available. In conventional animals, in principle, all human proteins are foreign and will therefore induce a classical immune response. Mice that are transgenic for the gene of the human therapeutic protein share immune tolerance for this protein with humans. The results obtained with the transgenic mice will more closely resemble the patient situation, since the immunogenicity seen in patients is mostly due to breaking of tolerance.

Recombinant human interferon alpha2a/b (rhIFNα2) is a protein used for the treatment of a variety of malignancies and viral diseases (8). Aggregates of rhIFNα2a were shown to break the tolerance of transgenic mice, immune tolerant for hIFNα2 (9). In chapter 6 of this thesis some of the characteristics of rhIFNα2b aggregates breaking the tolerance in these transgenic mice were described. Aggregates consisting of rhIFNα2b with preserved conformation, obtained by metal catalyzed oxidation, were shown to break the tolerance of transgenic mice after intraperitoneal (i.p.) administration. The oxidation per sé was shown not to break tolerance, but rather the aggregates accompanying this oxidation (chapter 6).

The reason for the immunogenicity of aggregates was probably their repetitive antigen presentation, since a spacing of 5-10 nm between more than 10 repeating epitopes is a potent way to induce high levels of antibodies, even to self-proteins (10-13). Apparently, metal catalyzed oxidized rhIFNα2b met these requirements.

Heavily aggregated rhIFNα2b obtained by incubation in a boiling water bath, did not induce an antibody response in wildtype or transgenic mice. A possible explanation for this lack of immune response is that the large size of the particles slowed down the clearance from the peritoneal cavity or impaired the uptake by phagocytic cells.

The purpose of the present study was to gain more insight into the sensitivity of our mouse model to the aggregate level. Dependence on the route of administration was studied by comparing the immune response in wildtype and transgenic mice, immune tolerant for hIFNα2, after subcutaneous (s.c.)

and i.p. administration of aggregated rhIFNα2b. To determine the sensitivity of the mouse models to the level of aggregation, metal catalyzed oxidized rhIFNα2b was mixed with native rhIFNα2b at different ratios and evaluated for their level of aggregation and immunogenicity.

Materials and methods

Aggregation of rhIFNα2b

Aggregated rhIFNα2b was obtained by incubation in a boiling water bath or metal catalyzed oxidation as previously described (chapter 6). In short, rhIFNα2b (300 μg/ml in 10 mM sodium phosphate buffer (PB), pH 7.4; a gift from AlfaWassermann, Bologna,Italy) was either incubated in a boiling water bath for ten minutes or oxidized in the presence of metal ions and ascorbic acid for three hours at room temperature. The oxidation reaction was stopped by the addition of EDTA and the sample was dialyzed against 10 mM PB, pH 7.4.

To obtain preparations with different levels of aggregates unmodified rhIFNα2b was mixed with metal catalyzed oxidized rhIFNα2b in different ratios (preparations A: 1:0; B: 3:1; C: 1:1; D: 1:3 and E: 0:1). The total protein concentration was equal in all preparations.

Characterization

All dilutions were made in 10 mM PB, pH 7.4. Protein concentrations were determined with a modified Lowry method (14) with bovine serum albumin (BSA) (Sigma, Zwijndrecht, The Netherlands) as a standard.

UV-spectroscopy

UV spectra (200-450 nm) of the samples (100 μg/ml) were recorded with a Perkin Elmer Lambda 2 UV/VIS spectrophotometer in 1 cm quartz cuvettes.

Dynamic light scattering (DLS)

DLS was used to measure the hydrodynamic diameter of aggregated and native rhIFNα2b (100 μg/ml). A Malvern CGS-3 apparatus equipped with a He-Ne (633 nm) JDS Uniphase laser, an optical fiber based detector at an angle of 90°, and an ALV/LSE-5003 correlator was used. Data are expressed as the Z-averaged diameter and polydispersity index.

Gel permeation chromatography (GPC)

A Superdex 200 10/300 GL column (Amersham, Roosendaal, The Netherlands) was used to analyze the composition of the samples (100 μg/ml). The mobile phase consisted of 50 mM PB, pH 7.2, and 200 mM sodium chloride, was passed through a 0.2-μm filter and delivered to the column at a flow rate of 0.50 ml/min by a Waters 2695 controller equipped with an autosampler. Chromatograms were recorded with a multi wavelength fluorescence detector (model 2475, Waters) or a photodiode array detector (model 2996, Waters). The column was calibrated with protein standards obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The samples were filtered using a 0.2-μm cellulose acetate filter prior to analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The gels consisted of a separating gel containing 15 % (w/v) acrylamide and 0.1 % (w/v) SDS, and a stacking gel containing 5 % (w/v) acrylamide and 0.1 % (w/v) SDS. Gels of 0.75 mm thickness were run under reducing (sample buffer containing 5 % (v/v) β-mercaptoethanol) and non-reducing conditions at 200 V at room temperature. The electrophoresis buffer was 25 mM tris (hydroxymethyl) aminomethane, 192 mM glycine and 0.1 % (w/v) SDS. Gel electrophoresis was performed with a Biorad Protean III system (Biorad, Veenendaal, The Netherlands). Samples analyzed under reducing conditions were first boiled for five minutes. A low range molecular weight standard (Biorad) was included on the gels. Protein bands were visualized by silver staining (Biorad).

Fluorescence spectroscopy

Emission spectra (280 – 450 nm, 1-nm steps) were measured with a Fluorolog III fluorimeter at an excitation wavelength of 295 nm, with 5-nm slits. Preparations A-E (55 μg/ml) were measured at 25 °C and stirred during measurement. Integration time per data point was 0.1 s and the average of ten scans was taken. The buffer spectrum was subtracted. Second derivative spectra were calculated to study changes in tertiary structure, according to Kumar et al (15). The smoothed normalized spectra were derivatized twice and after each derivatization the spectra were smoothed. Smoothing was done by taking the average of 13 neighbouring points. All calculations were performed with GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, California, USA).

Immunogenicity

Animal experiments

The animal experiments were approved by the Institutional Ethical Committee. Wildtype (FVB/N) mice were obtained from Charles River laboratories. Transgenic mice were bred at the Central Laboratory Animal Institute. Food (Hope Farms, Woerden, The Netherlands) and water (acidified) were available ad libitum.

Groups of five mice received 10 μg of the different protein preparations i.p. or s.c. on days 0-4, 7-11 and 14-18. Blood was collected from the vena saphena on days 0, 7 and 14 just before injection of the interferon preparations and on day 21. The blood samples were incubated on ice for two hours. Sera were collected after centrifugation and stored at -20 °C.

Sera were analyzed for the presence of antibodies by an ELISA as described below. It was shown in chapter 6 that the tolerance of the transgenic mice was specific for hIFNα2 and not caused by a general immune suppression.

Antibody assay

Sera were analyzed by ELISA for antibodies against native rhIFNα2b. Microlon 96-well plates (Greiner, Alphen aan de Rijn, The Netherlands) were incubated with 100 μl native rhIFNα2b (2 μg/ml) per well for one hour. Then the wells were drained and washed four times with 300 μl wash buffer (0.1 % (w/v) Tween 20 in phosphate buffered saline (PBS)). After washing the wells were carefully tapped dry on a tissue. Wells were blocked by incubating with 200 μl 2 % (w/v) BSA in PBS for one hour. The wells were drained and washed twice with 300 μl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Sera (diluted 100 fold with 2 % (w/v) BSA in PBS) were added to the wells and the plates were incubated for one hour. The plates were washed four times with 300 μl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Peroxidase labeled goat anti-mouse IgG (Sigma) was added to the wells and the plates were incubated for one hour. Plates were drained and washed four times with 300 μl wash buffer and twice 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 the absorbance was recorded after 30 minutes on a Novapath microplate reader (Biorad) at 415 nm and a reference wavelength of 490 nm. All incubations were carried out in covered plates at room temperature with constant orbital shaking. Sera were arbitrarily defined positive if the absorbance of the 1:100 dilution minus the

background was three times higher than the average absorbance value of the pretreatment sera minus the background.

Antibody titers against native rhIFNα2b of the positive sera were determined by adding the sera in three-fold serial dilutions (starting from 1:10) to plates coated with native rhIFNα2b. The other steps of the ELISA procedure were as described above and the absorbance values were plotted against log dilution. Curves were fitted with a sigmoidal curve (GraphPad Prism version 4.02 for Windows). The dilution needed to obtain 50 % of the maximum absorbance was taken as the titer of the serum.

Results

Protein characterization

The characteristics of the samples were in line with the results shown in chapter 6. During the incubation of rhIFNα2b in the boiling water bath a visible precipitate was formed after seven minutes. Metal catalyzed oxidized rhIFNα2b and native rhIFNα2b solutions were clear. The mean particle size of boiled rhIFNα2b was in the micrometer range. The mean particle size of metal catalyzed oxidized rhIFNα2b was 0.4 μm with a rather high polydispersity index (0.76), indicating particles heterogenous in size. Native rhIFNα2b did not scatter enough light to obtain reliable results about the particle size. GPC showed that native rhIFNα2b contained a very small amount of dimers and that metal catalyzed oxidized rhIFNα2b contained monomers, dimers, trimers and oligomers. Metal catalyzed oxidized rhIFNα2b showed a small red-shift in fluorescence and the second derivative spectrum showed a slight increase in intensity around 325 nm (data not shown), indicating a subtle change in the protein tertiary structure (15). Metal catalyzed oxidized rhIFNα2b showed in the UV spectrum an increased optical density at wavelengths where native rhIFNα2b does not absorb light (> 320 nm)(data not shown), indicating the presence of aggregates. Previous studies showed that during the oxidation of rhIFNα2b by metal catalysis up to three methionines became partly oxidized (chapter 6).

Aggregation level

Native and oxidized rhIFNα2b were mixed to obtain preparations with different levels of aggregates. The aggregation level of the preparations as determined by GPC is shown in figure 1. Preparations B, C, and D consisted of varying amounts of insoluble protein, as well as soluble monomers, dimers,

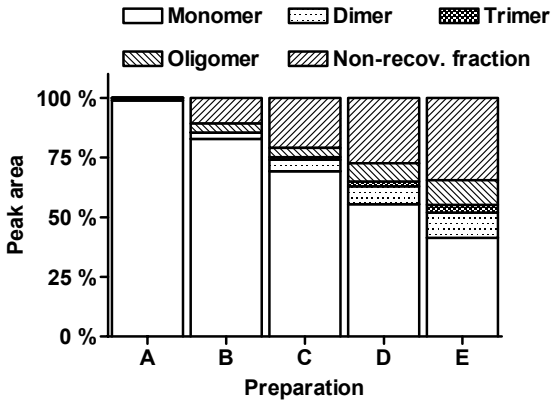

Figure 1 *Measured composition of the mixed preparations. Peak area percentages were calculated based on GPC areas relative to the total peak area in GPC of native rhIFNα2b: $AUC_{peak}/AUC_{native,total}$ * 100 %. The percentages of the non-recoverable (Non-recov.) fraction were calculated from the total peak area in GPC and the total peak area in GPC of native rhIFNα2b: $(AUC_{native,total} - AUC_{sample,total})/AUC_{native,total}$ * 100 %.*

trimers and larger aggregates (larger aggregates being oligomers larger than trimers plus the non-recoverable fraction). The compositions of the mixtures B, C and D measured by GPC, were close to the calculated compositions of the preparations based on measured compositions of unmixed preparations A and E (data not shown). In SDS-PAGE most of the multimers were only visible under non-reducing conditions (figure 2), indicating aggregates formed by reducible disulfide bonds, consistent with earlier results (cf. chapter 6).

Immunogenicity

All wildtype mice treated with boiled, metal catalyzed oxidized and native rhIFNα2b showed antibodies cross-reacting with native rhIFNα2b at days 14 and 21 (data not shown), indicating that all three samples were able to provoke an immune reaction. As expected, native rhIFNα2b did not induce antibodies in the transgenic mice, neither after s.c. nor after i.p. administration (table 1). The transgenic mice also showed a high immune tolerance to boiled rhIFNα2b. Administration of metal catalyzed oxidized rhIFNα2b resulted in antibodies after both routes of administration. After s.c. administration antibodies were induced in more mice than after i.p. administration (table 1). Because of the better reproducibility and earlier onset of antibody production after s.c. administration, this route was chosen to test the sensitivity of the mouse models to the dose of aggregated protein. Therefore, the mice were immunized with mixed preparations of native rhIFNα2b and metal catalyzed oxidized rhIFNα2b.

In the wildtype mice all mixed preparations led to the formation of

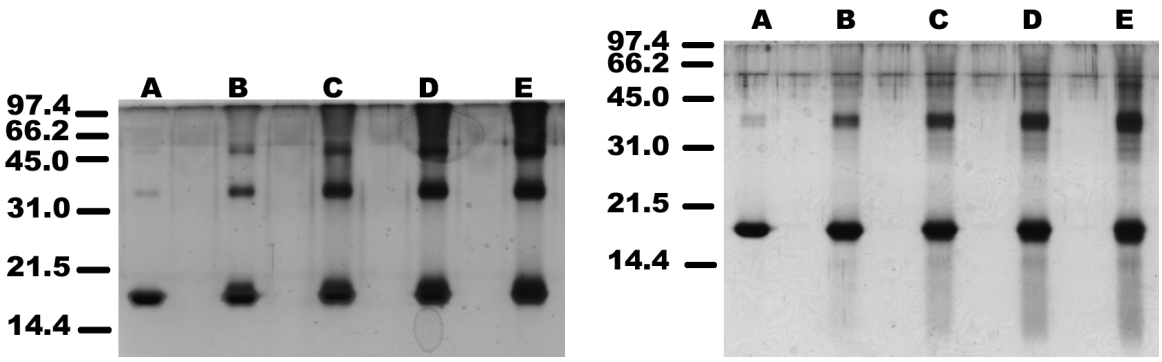

Figure 2 *Silver stained SDS-PAGE gels of the mixed preparations (A-E). The left picture represents the gel run under non-reducing conditions, while the right picture represents the gel run under reducing conditions. The bands and numbers on the left represent the positions of the molecular weight markers in kDa.*

antibodies (figure 3a). However, preparations C, D and E induced significantly higher antibody titers as compared to native rhIFNα2b (preparation A). Since the aggregates in metal catalyzed oxidized rhIFNα2b were the reason for the increased immunogenicity in the wildtype mice and the breaking of tolerance of the transgenic mice (cf. chapter 6), it can be said that at least 2.5 μg of aggregated protein (i.e. soluble oligomers and insoluble aggregates) per injection was needed to elevate the immune response in the wildtype mice as compared to native rhIFNα2b. Increasing the aggregate content per injection did not elevate the immune response.

The transgenic mice were immune tolerant for native rhIFNα2b (figure 3b). All preparations containing metal catalyzed oxidized rhIFNα2b were able to break this tolerance and induced antibodies. The antibody response depended on the level of aggregation. With the lowest amount of aggregates (1.4 μg/injection) only two out of 5 mice produced antibodies. When the amount of aggregates was increased, not only the number of mice producing antibodies, but also the level of antibodies increased.

Discussion

We showed that the onset of the immune response in the transgenic mice after administration of metal catalyzed oxidized rhIFNα2b depends on the administration route. This difference may be related to differences in B-cell type. B1-cells, mainly located in the peritoneal cavity, produce low affinity antibodies. B2-cells are present throughout the body and produce high affinity antibodies (16). High affinity antibodies are easier to detect in an ELISA than

Table 1 Percentage of positive transgenic mice (five mice per group) after s.c. and i.p. administration of native, boiled and metal catalyzed oxidized rhIFNα2b.

	s.c.		i.p.	
	14 days	21 days	14 days	21 days
Native rhIFNα2b	0	0	0	0
Boiled rhIFNα2b	0	20	0	0
Metal catalyzed oxidized rhIFNα2b	100	100	40	80

low affinity antibodies.

We have also demonstrated that the immune response induced by rhIFNα2b preparations is dependent on the level of aggregation. In the wildtype mice an increased immune response, as compared to native rhIFNα2b, was seen when 2.5 µg (25 %) of the injected protein was present as aggregates (i.e. oligomers and insoluble aggregates). To break the tolerance in transgenic mice less aggregates were necessary (1.4 µg per injection).

A number of important questions remain. Do the transgenic and immune tolerant mice react to a relative or absolute amount of aggregates? Or, in other words, is the immunogenic effect of 250 µg with 1 % aggregates and 25 µg with 10 % aggregates similar? And can the increased immunogenicity in wildtype

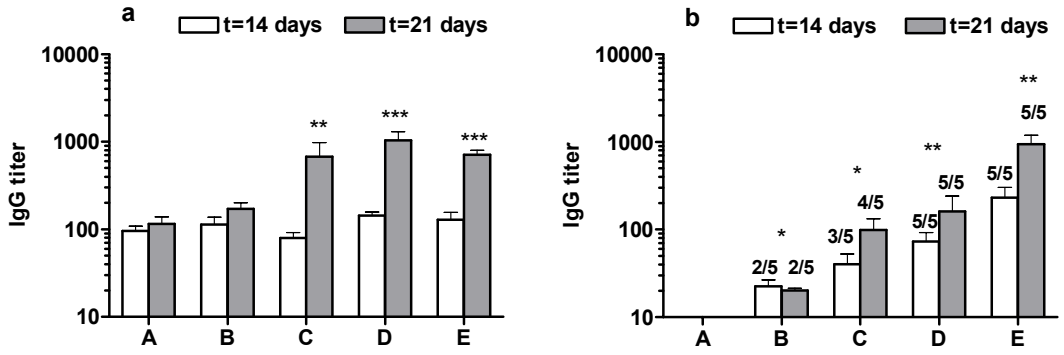

Figure 3 *Anti-rhIFNα2b IgG titers obtained after immunization of wildtype (a) and transgenic (b) mice. The values represent average titers (+SEM) of positive mice. The numbers above the bars (b) represent the number of positive mice out of total mice. All wildtype mice were positive. Statistical analysis of the wildtype mice was done with a Tukey-Kramer parametric ANOVA. This was not possible for the transgenic mice since not all mice were positive. Kaplan-Meier survival analysis was performed on the number of positive transgenic mice per group. The titers of the sera of the transgenic mice that received preparation B may be inaccurate, since the data points covered only the first part of the sigmoidal OD-log(dilution) curve. * P<0.05, ** P<0.01, *** P<0.001 as compared to preparation A.*

mice of aggregated rhIFNα2b also be seen when higher amounts of native material are injected? This has to be investigated in separate experiments.

The sensitivity for aggregates we have found in these models concern rhIFNα2b. The relevance of these data for other proteins needs to be determined. Our results can not answer the question of how much aggregates should be allowed in therapeutic proteins. This may well be product specific (17). The results obtained in this study show a clear trend: the more aggregates present, the more immunogenic the formulations.

Marketed products differ widely in their aggregate content. RhIFNα2 formulations contain less than 1 % (0.04 - 0.24 µg per administered dose) in an aggregated (dimers and multimers) form (18). In Betaseron®, a recombinant human interferon beta formulation, 60 % of the protein (150 µg per administered dose) is in the high molecular weight fraction (>600 kDa) (19).

In our experiments we did not define the lowest amount of aggregates capable of breaking tolerance. So, we do not know whether the low amount of aggregated protein in rhIFNα2 formulations would be immunogenic in the transgenic mice. In chapter 4 of this thesis it was shown that the amount of aggregates in Betaseron® formulations were immunogenic in the transgenic animal model, immune tolerant for human interferon beta.

The maximum level of aggregates acceptable in protein formulations is difficult to define. Not only because it is not known which aggregated component is responsible for the increased immunogenicity, but also because the immune response is dependent on many factors such as (among others) the dose, frequency of injections, route of administration, type of protein, species and animal strain. To investigate which aggregated fraction is responsible for the increased immune reponse in the wildtype mice and/or breaking of tolerance in the transgenic mice the different aggregated fractions (monomer, dimer, trimer, oligomers, insoluble fraction) should be isolated and their structure and immunogenicity in transgenic and wildtype mice determined.

Conclusions

The antibody response against rhIFNα2b in the wildtype mice and immune tolerant transgenic mice is dependent on the level of aggregation. Since breaking of tolerance is the likely mechanism responsible for the appearance of antibodies in patients, the transgenic mouse model is more relevant for the prediction of immunogenicity in humans than the wildtype animal model. Moreover, we showed that the transgenic mouse is more sensitive for aggregates than the wildtype.

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Chapter 8

Structure and immunogenicity in wildtype and immune tolerant mice of recombinant human interferon alpha2b aged under accelerated conditions

Suzanne Hermeling, Huub Schellekens, Daan J.A. Crommelin, Wim Jiskoot

Abstract

Purpose. To study the influence of aging at different pH and elevated temperature conditions on the structure and immunogenicity of recombinant human interferon alpha2b (rhIFNα2b).

Methods. RhIFNα2b solutions of pH 4.0 and 9.0 were stored at 50 °C for 1 week and of pH 7.2 for 2 weeks. After storage the solutions were characterized by UV and fluorescence spectroscopy, gel permeation chromatography, dynamic light scattering, SDS-PAGE and Western blotting. The immunogenicity of the samples was tested in wildtype and transgenic mice, immune tolerant for hIFNα2. Anti-rhIFNα2b serum antibodies were measured by ELISA.

Results. Storing the protein at pH 4.0 or 9.0 at 50 °C resulted in largely unfolded and aggregated protein with loss of native epitopes. Storage at pH 7.2 at 50 °C also resulted in aggregation, but much less changes in the tertiary structure and no loss of native epitopes occurred. All treated samples induced higher antibody titers in the wildtype mice than native rhIFNα2b. They were also able to induce antibodies in the transgenic mice, although to a different extent. The sample stored at pH 9.0, which had lost most of its native structure, was able to break tolerance in less transgenic mice than the samples stored at pH 4.0 and 7.2.

Conclusion. Aggregates with native-like structures are more immunogenic than aggregates with unfolded protein structures.

Introduction

Nearly all therapeutic proteins induce antibodies in patients. The consequences are mostly limited, although sometimes serious complications can arise (1, 2). The antibodies can be induced via two mechanisms: 1) a classical immune response when proteins of non-human origin are administered, or 2) breaking of tolerance. The mechanism of breaking tolerance is not exactly known, although aggregates and other impurities are important factors (3-9). Aggregates may act as danger signals initiating a response to self-antigens and/or aggregates may present the self-antigens in a repetitive array form, which is supposed to activate B-cells without T-cell help (10-12).

In chapters 5-7 it was shown that transgenic mice immune tolerant for human interferon alpha2 (hIFNα2) can be used to test the immunogenicity of recombinant hIFNα2b (rhIFNα2b) preparations. In chapter 6 the immunogenicity of structural variants of rhIFNα2b obtained by boiling, glutaraldehyde treatment, or forced oxidation by hydrogen peroxide or metal catalysis was studied. Although the degradation products obtained may not represent those that are typically formed during production, storage and handling of rhIFNα2b formulations, these studies provided useful insights into the relationships between protein structure and immunogenicity. In particular, it was demonstrated that not all aggregates of rhIFNα2b can break the tolerance of the transgenic mice.

To rapidly screen the stability of formulated proteins, accelerated stability studies under stress conditions, such as extreme pH and temperature, are often performed. Although these studies are not always predictive for long term stability (13), the degradation products obtained are probably more relevant than artificially obtained structural variants (such as the degraded rhIFNα2b products reported in chapter 6).

At intermediate pH (4.5-7.5) mostly deamidation and oxidation take place, whereas hydrolysis and disulfide scrambling predominantly occur at lower pH and higher pH, respectively. Conformational changes/unfolding may occur at any pH (14-17), especially at extreme (high or low) temperatures.

In this chapter we investigated the immunogenicity of rhIFNα2b aged at different pHs (4, 7.2 and 9) and elevated temperature (50 ^{o}C). For comparison, metal catalyzed oxidized rhIFNα2b (shown before to be able to break immune tolerance) was prepared. The products were physicochemically characterized and their immunogenicity was tested in wildtype and transgenic, immune tolerant mice.

Materials and methods

RhIFNα2b was a gift from AlfaWassermann, Bologna, Italy. Protein solutions (300 μg/ml) were prepared of pH 4.0 (10 mM sodium acetate buffer), pH 7.2 (10 mM sodium phosphate buffer (PB)) and pH 9.0 (10 mM tris (hydroxymethyl) aminomethane). The ionic strength was adjusted to 0.14 M with sodium chloride. Preliminary tests showed that after two weeks of incubation at pH 7.2 and 50 °C, the protein was partly aggregated. However, at pH 4.0 and 9.0, hardly any protein was detectable by gel permeation chromatography (GPC). Therefore, the solutions were incubated at 50 °C for one week (pH 4.0 and 9.0) or two weeks (pH 7.2). After incubation, the solutions were dialyzed against 10 mM PB, pH 7.4 and stored at -20 °C.

Metal catalyzed oxidized rhIFNα2b was prepared as described previously (chapter 6) and used as a positive control in the immunogenicity study. In short, rhIFNα2b (300 μg/ml in 10 mM PB, pH 7.4) was incubated in the presence of metal ions and ascorbic acid for three hours at room temperature. The reaction was stopped by the addition of EDTA. The sample was dialyzed against 10 mM PB, pH 7.4 and stored at -20 °C.

Characterization

Unless stated otherwise, all dilutions were made in 10 mM PB, pH 7.4. Protein concentrations were determined with a modified Lowry method (18) using bovine serum albumin (BSA) (Sigma, Zwijndrecht, The Netherlands) as a standard.

UV-spectroscopy

UV spectra (200-450 nm) of the samples (100 μg/ml) were recorded on a Perkin Elmer Lambda 2 UV/VIS spectrophotometer in 1-cm quartz cuvettes. Ten mM PB, pH 7.4 was used as a blank.

GPC

GPC of the samples (100 μg/ml) was performed using a Superdex 200 10/300 GL column (Amersham, Roosendaal, The Netherlands). The mobile phase consisted of 50 mM PB pH 7.2, and 200 mM sodium chloride, was passed through a 0.2-μm filter prior to use and was delivered to the column at a flow rate of 0.50 ml/min by a Waters 2695 controller equipped with an autosampler. Chromatograms were recorded with a photodiode array detector

(model 2996, Waters). The column was calibrated by analyzing protein standards obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The samples were passed through a 0.2 μm-cellulose acetate filter prior to analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The gels consisted of a separating gel containing 15 % (w/v) acrylamide and 0.1 % (w/v) SDS, and a stacking gel containing 5 % (w/v) acrylamide and 0.1 % (w/v) SDS. Gels of 0.75 mm thickness were run under reducing (sample buffer containing 5 % (v/v) β-mercaptoethanol) and non-reducing conditions at 200 V at room temperature. The electrophoresis buffer was 25 mM tris (hydroxymethyl) aminomethane, 192 mM glycine and 0.1 % (w/v) SDS. Gel electrophoresis was performed with a Biorad Protean III system (Biorad, Veenendaal, The Netherlands). Samples analyzed under reducing conditions were boiled for five minutes before application onto the gel. A low range molecular weight standard (Biorad) was included on the gel. The protein bands were visualized by silver staining (Biorad).

Dynamic light scattering (DLS)

Samples (100 μg/ml) were analyzed with DLS to obtain an average diameter of the particles (Z_{ave}) and a polydispersity index (PDI), which is a measure for the heterogeneity of the sample, at an angle of 90°. A Malvern CGS-3 apparatus equipped with a He-Ne (633 nm) JDS Uniphase laser, an optical fiber based detector, and an ALV/LSE-5003 correlator was used.

Fluorescence spectroscopy

Emission spectra of the samples (300 - 450 nm, 1-nm steps; 330 - 345 nm, 0.1-nm steps) and emission spectra of native rhIFNα2b in the presence of increasing amounts of guanidine hydrochloride (300 - 450 nm, 0.5-nm steps) were measured on a Fluorolog III fluorimeter. Excitation was at 295 nm. Slits were set at 5 nm. Samples (protein concentration: 55 μg/ml) were measured at 25 °C and stirred during measurement. Integration time per data point was 0.1 s and the average of ten scans was taken. The buffer spectrum was subtracted.

Second derivative spectra were calculated to study changes in tertiary structure, according to Kumar et al (19). Smoothed normalized spectra were derivatized twice and after each derivatization the spectra were smoothed. Smoothing was performed by taking the average of 13 neighbouring points,

with a simplified least squares procedure as described by Savitzky and Golay (20). All calculations were performed with GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, California, USA).

Epitope analysis

Western blotting

SDS-PAGE gels were blotted onto a nitrocellulose sheet with a Scie-Plas semi-dry blotter (Scie-Plas, UK). Blots were blocked with 1 % (w/v) non-fat milk powder in 0.005 % (w/v) Tween 20 in phosphate buffered saline (PBS) for one hour at room temperature with constant orbital shaking. After washing with 0.005 % (w/v) Tween 20 in PBS and with water, the blots were incubated with a polyclonal rabbit anti-rhIFNα2b serum in 0.1 % (w/v) non-fat milk powder in 0.005 % (w/v) Tween 20 in PBS for one hour at room temperature. Blots were washed with 0.005 % (w/v) Tween 20 in PBS and with water. Blots were incubated with peroxidase labeled goat anti-rabbit IgG (Sigma) in 0.1 % (w/v) non fat milk powder in 0.005 % (w/v) Tween 20 in PBS for one hour at room temperature. Blots were washed with 0.005 % (w/v) Tween 20 in PBS and with water and incubated in a solution of 4-chloro-1-naphtol (Sigma) in methanol (15 % (v/v)), water and H_2O_2 (0.015 % (v/v)). After color development the blots were stored overnight in the dark in water to increase the intensity of the bands.

ELISA for rhIFNα2b

Microlon 96-well plates (Greiner, Alphen aan de Rijn, The Netherlands) were coated with 100 µl of a monoclonal antibody against rhIFNα2b (diluted 1:2500; Pierce, Etten-Leur, The Netherlands) in 10 mM PBS for one hour. The wells were drained and washed four times with 300 µl wash buffer (0.1 % (w/v) Tween 20 in PBS). After each wash the plates were tapped dry on a tissue. Wells were blocked with 200 µl 2 % (w/v) BSA in PBS for one hour. The wells were drained and washed twice with 300 µl wash buffer. After each wash the plates were tapped dry on a tissue. Samples were added in two-fold serial dilutions (starting at 200 ng/ml) to the wells, containing 2 % (w/v) BSA in PBS. Plates were incubated for one hour. The wells were drained and washed four times with 300 µl wash buffer. After each wash the plates were tapped dry on a tissue. Wells were incubated with a polyclonal rabbit antiserum against rhIFNα2b for one hour. The wells were drained and washed four times with 300 µl wash buffer. After each wash the plates were tapped dry on a tissue.

Peroxidase labeled goat anti-rabbit IgG was added to the wells and the plates were incubated for one hour. The wells were drained and washed four times with 300 µl wash buffer and twice times with 300 µl PBS. After each wash the plates were tapped dry on a tissue. ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) substrate (Roche, Almere, The Netherlands) was added and the absorbance was recorded after 30 minutes on a Novapath microplate reader (Biorad) at a wavelength of 415 nm and a reference wavelength of 490 nm. All incubation steps were performed at room temperature with constant orbital shaking. During all incubation steps the plates were covered. The absorbance values were plotted against log protein concentration of the samples.

Immunogenicity

Animal experiment

The animal experiment was approved by the Institutional Ethical Committee. Wildtype (FVB/N) mice were obtained from Charles River laboratories. Transgenic mice were bred at the Central Laboratory Animal Institute. Food (Hope Farms, Woerden, The Netherlands) and water (acidified) were available ad libitum.

Mice (five per group) were injected subcutaneously with 10 µg protein on days 0-4, 7-11 and 14-18 and blood was collected from the vena saphena on days 0, 7 and 14 (each time before injection) and on day 21. Native and metal catalyzed oxidized rhIFNa2b were included in the animal experiment as negative and positive controls, respectively. The blood samples were incubated on ice for two hours. Sera were collected after centrifugation and stored at -20 °C. Sera were analyzed for the presence of anti-rhIFNα2b antibodies as described below.

ELISA for anti-rhIFNα2b antibodies

Microlon 96-well plates were incubated with 100 µl native rhIFNα2b (2 µg/ml) per well for one hour. Then the wells were drained and washed four times with 300 µl wash buffer. After washing the wells were carefully tapped dry on a tissue. Wells were blocked by incubating with 200 µl 2 % (w/v) BSA in PBS for one hour. The wells were drained and washed twice times with 300 µl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Sera (diluted 100 fold with 2% (w/v) BSA in PBS) were added to the wells and the plates were incubated for one hour. The plates were washed four times with

300 µl wash buffer. After the last wash, wells were carefully tapped dry on a tissue. Peroxidase labeled goat anti-mouse IgG (Sigma) was added to the wells and the plates were incubated for one hour. Plates were drained and washed four times with 300 µl wash buffer and twice with 300 µl PBS. After the last wash, wells were carefully tapped dry on a tissue. ABTS was added and the absorbance was recorded after 30 minutes of incubation, on a Novapath microplate reader at a wavelength of 415 nm and a reference wavelength of 490 nm. All incubations were performed in covered plates at room temperature with constant orbital shaking.

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

Antibody titers against native rhIFNα2b of the positive sera were determined by adding sera in three-fold serial dilutions (starting from 1:10) to the wells. The other steps of the ELISA procedure were as described above and the absorbance values were plotted against log dilution. Curves were fitted with a sigmoidal curve (GraphPad Prism version 4.02 for Windows). The dilution needed to obtain 50 % of the maximum absorbance was taken as the titer of the serum.

Results

Characterization

After the incubation at 50 °C all three samples had turned turbid. Moreover, the sample incubated at pH 4.0 showed a white precipitate. The pH of the samples at the end of the incubation was similar (± 0.1) to the starting pH. Native and metal catalyzed oxidized rhIFNα2b showed clear, colorless solutions.

UV-spectroscopy

The aged samples showed a decrease in A_{280}/A_{260}, as well as an increased optical density at wavelengths where rhIFNα2b does not absorb light (>320 nm) (figure 1), indicative of the presence of aggregates (21). Also metal catalyzed oxidized rhIFNα2b showed a slightly increased optical density above 320 nm and a decrease in A_{280}/A_{260} as compared to native rhIFNα2b, indicating the presence of aggregates in this solution.

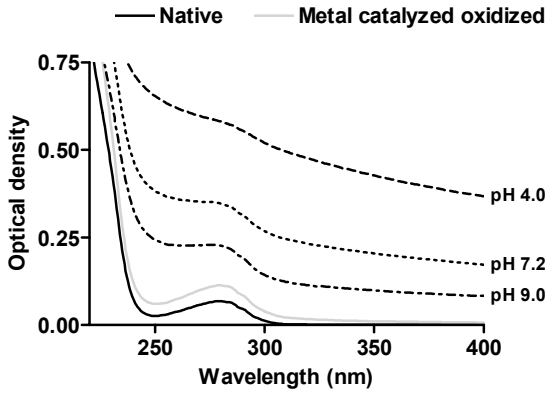

Figure 1 *UV spectra of native and metal catalyzed oxidized rhIFNα2b and of the samples incubated at 50 °C and different pHs.*

GPC

GPC was used to analyze the samples for the presence of soluble aggregates (figure 2). Native rhIFNα2b contained mostly monomeric protein and a small dimer peak. Metal catalyzed oxidized rhIFNα2b contained clearly less monomer, more dimers, and additionally trimers and higher molecular weight products were formed (figure 2A). The samples incubated at pH 4.0 and 7.2 showed only a small amount of monomeric rhIFNα2b (figure 2B). The sample incubated at pH 9.0 did not show any major protein peak at all (figure 2B). Differences between the total peak area of the samples and the total peak area of native rhIFNα2b can be due to either insoluble aggregates or hydrolysis of the protein. This is refered to as the non-recoverable fraction in table 2.

SDS-PAGE

Formation of covalent aggregates or fragments was analyzed by comparing SDS-PAGE gels run under non-reducing and reducing conditions (figures 3A and B, respectively). Aggregates were visible in all samples, except native

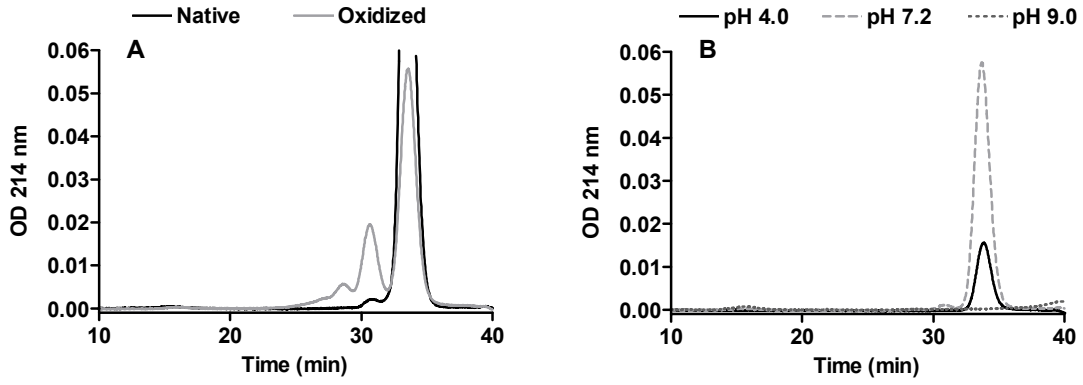

Figure 2 *GPC chromatograms of native and metal catalyzed oxidized rhIFNα2b (A) and the aged samples (B).*

rhIFNα2b. Most of the aggregates were covalently linked via disulfide bonds. The samples incubated at pH 7.2 and 9.0 showed precipitate not able to enter the stacking gel, as was visible during the electrophoresis of the gel run under non-reducing conditions**.** This did not occur in the gel run under reducing conditions, indicative of large covalent aggregates. Metal catalyzed oxidized rhIFNα2b showed a higher amount of dimers and less larger aggregates than the samples incubated at pH 7.2 and 9.0. The sample incubated at pH 4.0 showed only a small amount of aggregates. Moreover, some hydrolysis was observed in this sample.

DLS

Native rhIFNα2b did not scatter enough light to obtain reliable results about its particle size. The other samples showed diameters between approximately 0.4 and 2 μm with relatively high polydispersity indices (table 2), indicating the presence of aggregates heterogeneous in size. The sample incubated at pH 4.0 contained visible particles, which sedimented to the bottom of the measuring tube and were therefore missed by the DLS analysis.

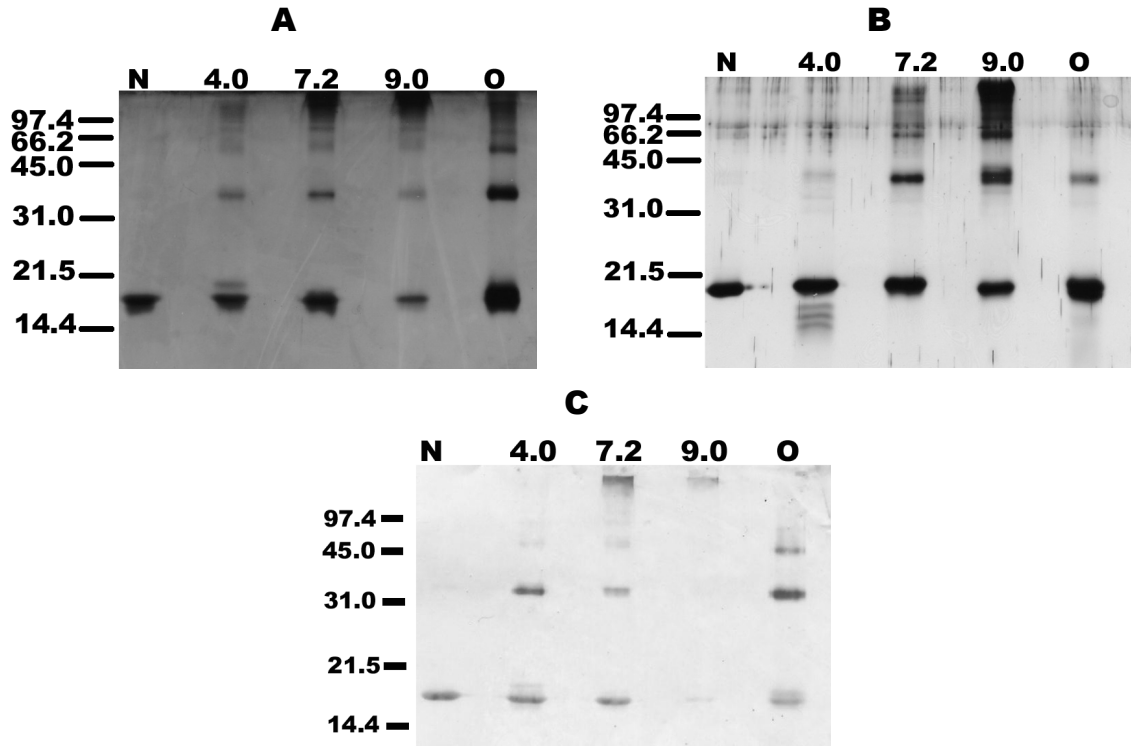

Figure 3 *SDS-PAGE (A: non-reducing, B: reducing) and Western blot of an SDS-PAGE gel run under non-reducing conditions (C) of native rhIFNα2b (N), the aged samples incubated at the different pHs and metal catalyzed oxidized rhIFNα2b (O). Numbers on the left represent band positions (in kDa) of the molecular weight markers.*

Fluorescence

Fluorescence spectroscopy at an excitation wavelength of 295 nm gives information about the environment of the two tryptophans in rhIFNα2b at positions 76 and 140. The emission spectra are shown in figure 4A and the peak maxima listed in table 1. Metal catalyzed oxidized rhIFNα2b and the samples incubated at pH 4.0 and 9.0 showed a red-shift in fluorescence maximum, indicating that in these samples at least one of the tryptophans was in a more hydrophilic environment as compared to native rhIFNα2b. The spectrum obtained with the sample incubated at pH 9.0 showed extreme light scattering. The shift shown by the samples incubated at pH 4.0 and 9.0 was large, indicative of either a non-native protein structure with incomplete unfolding or a large fraction of (partly) unfolded protein. RhIFNα2b in 5 M guanidine chloride (completely unfolded) has an emission maximum at 356 nm. The sample incubated at pH 7.2 did not show a shift in emission maximum. Kumar et al showed that small changes in second derivative emission spectra around 325 nm, with similar intensities around 335 nm, are indicative of small changes in tertiary structure of the protein, even when the emission spectra do not show a shift in peak maximum (19). The second derivative spectra of native, metal catalyzed oxidized rhIFNα2b and the sample incubated at pH 7.2 are shown in figure 4B. For comparison, the second derivative spectra of rhIFNα2b with increasing concentrations of guanidine hydrochloride are shown in figure 4C. Guanidine hydrochloride is a chaotropic agent, known to unfold proteins.

The structure of rhIFNα2b in less than 3.4 M guanidine hydrochloride was unchanged, as compared to rhIFNα2b without guanidine hydrochloride. Starting at 3.4 M, the protein unfolded with increasing concentrations of guanidine hydrochloride, as can be seen by the increase of the second derivative spectrum around 325 nm, while the intensity around 337 nm remained constant. Eventually, a complete loss of the peak around 325 nm occurred (figure 4C), indicating that the two tryptophans are in similar,

Table 1 Maxima of the emission spectra (330-345 nm).

Sample	Intensity[1]	λ_{max} (nm)
Native	7.3	337.1
pH 4.0	10	340.9
pH 7.2	8.0	337.2
pH 9.0	5.2	344.2
Metal catalyzed oxidized	6.8	338.1

[1] Arbitrary unit.

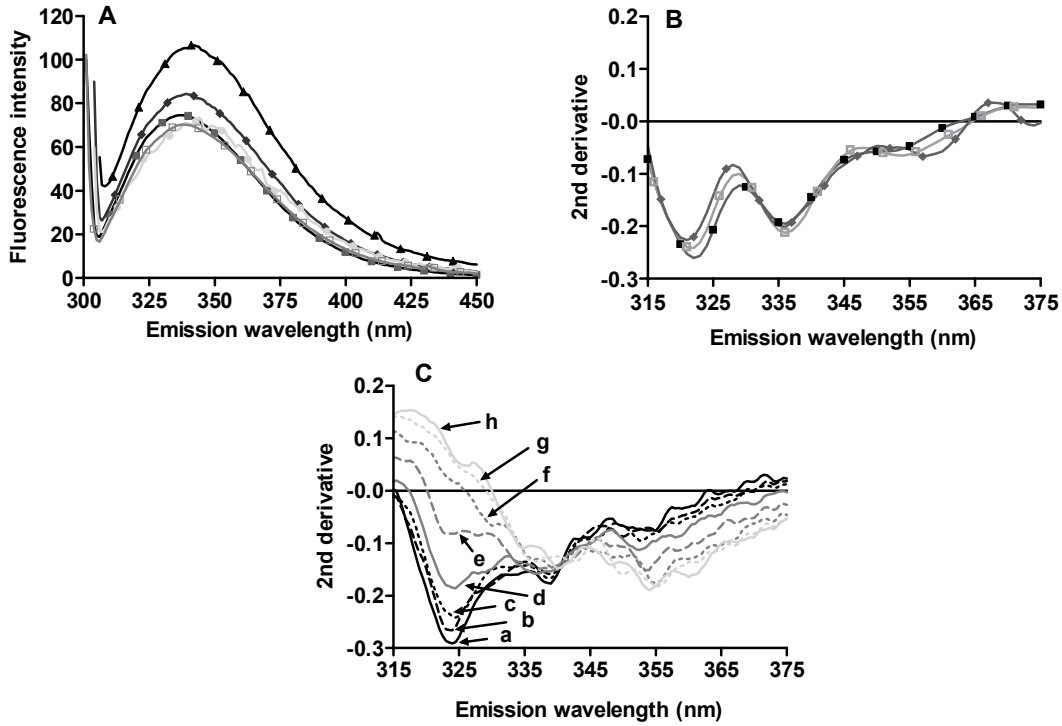

Figure 4 *Emission spectra (A) and second derivative emission spectra (B) of native rhIFNα2b (closed squares), aged samples incubated at pH 4.0 (triangles), pH 7.2 (diamonds) and pH 9.0 (circles) and metal catalyzed oxidized rhIFNα2b (open squares). Second derivative spectra (C) of rhIFNα2b in guanidine hydrochloride (GnHCl). a: 0 M, b: 3.4 M, c: 3.6 M, d: 3.8 M, e: 4.0 M, f: 4.2 M, g: 4.4 M and h: 4.6 M GnHCl. RhIFNα2b in GnHCl below 3.4 M GnHCl gave identical spectra as rhIFNα2b in 0 M GnHCl. RhIFNα2b in GnHCl above 4.6 M GnHCl gave identical spectra as rhIFNα2b in 4.6 M GnHCl. The fluorescence intensity is an arbitrary unit.*

relatively hydrophilic environments (19). When the concentration of guanidine hydrochloride was further increased, the second derivative spectrum showed differences in the region 350 -375 nm, yielding spectra that resembled the spectrum of N-acetyl-L-tryptophanamide (NATA) in water (19).

The second derivative spectrum of metal catalyzed oxidized rhIFNα2b showed an intensity around 325 nm in between that of the reference spectra of rhIFNα2b in 3.2 M and 3.4 M guanidine hydrochloride. The intensity around 325 nm in the second derivative spectrum of the sample incubated at pH 7.2 was near that of the reference spectrum of rhIFNα2b in 3.6 M guanidine hydrochloride. This indicates that both samples, metal catalyzed oxidized rhIFNα2b and the sample incubated at pH 7.2, contain protein with slightly altered tertiary structures, or a small fraction of (partly) unfolded protein in the presence of excess 'native' protein as compared to native rhIFNα2b.

Epitope analysis

Western blot

The reactivity of the bands in the SDS-PAGE gel with a polyclonal anti-rhIFNα2b serum is shown in figure 3C. The monomers of all samples reacted with the polyclonal antiserum, although faintly for the pH 9.0 sample. The dimers as well as the aggregates present in the samples incubated at pH 7.2 and 9.0 also reacted with the polyclonal antiserum.

ELISA

The reactivity of the samples with a monoclonal antibody was also tested in an ELISA, which avoids the denaturation step needed for SDS-PAGE and subsequent Western blot. The sample incubated at pH 9.0 did not react in the ELISA, even the most concentrated dilution showed an absorbance value near the baseline. This indicates the loss of the epitope recognized by the monoclonal antibody, or the loss of epitopes recognized by the polyclonal antiserum. The absorbance values obtained with a concentration of 25 ng/ml were within the linear part of the dose-response curve for all samples, except for the sample incubated at pH 9.0. This allows the differences in the absorbance to be explained by a difference in antigenicity (table 2). There is no measurable difference in the binding of the sample incubated at pH 7.2 and the metal catalyzed oxidized sample compared to native rhIFNα2b, indicating that the major (immunodominant) epitopes were preserved. The samples incubated at pH 4.0 and pH 9.0, however, showed drastically reduced reactivities, indicating loss of epitopes.

Summary

The main characteristics of the samples as determined by the analyses discussed above are summarized in table 2. The samples incubated at pH 4.0 and 7.2 showed partly aggregated protein. The sample incubated at pH 4.0 was partly denatured and had lost about 60 % of its native epitopes. The sample incubated at pH 7.2 showed a slight change in tertiary structure and retained most of its native epitopes. The sample incubated at pH 9.0 showed a highly aggregated sample, with a complete loss of native epitopes. Metal catalyzed oxidized rhIFNα2b showed the same characteristics as described in chapter 6: partly aggregated, a slightly changed tertiary structure and preservation of native epitopes.

Table 2 Summarized physicochemical characteristics of the rhIFNα2b samples.

Sample	DLS		GPC					Denaturation[3]	Native epitopes[4]
	Z_{ave} (μm)	PDI	Monomer[1]	Dimer[1]	Trimer[1]	Multimer[1]	Non-recov. fraction[2]		
Native	ND[5]	ND	99 %	1 %	ND	ND	ND	-[6]	100 ± 27 %
pH 4.0	0.40	0.16	13 %	ND	ND	2 %	86 %	++	41 ± 9 %
pH 7.2	0.41	0.32	47 %	1 %	ND	3 %	49 %	+	90 ± 2 %
pH 9.0	2.2	0.36	ND	ND	ND	2 %	98 %	++	1.4 ± 0 %
Oxidized	0.38	0.75	47 %	16 %	4 %	6 %	28 %	+	104 ± 11 %

[1] Percentages were calculated based on GPC peak areas relative to the total peak area in GPC of native rhIFNα2b: $AUC_{peak}/AUC_{native, total}$ x 100 %.
[2] Non-recoverable fraction: percentages were calculated from the total peak area in GPC and the total peak area in GPC of native rhIFNα2b: $(AUC_{native, total}-AUC_{sample, total})/AUC_{native, total}$ x 100 %.
[3] Shown by fluorescence.
[4] Shown by ELISA: percentages were calculated based on the absorbance in the ELISA for native rhIFNα2b. Values represent average ± range. n=2.
[5] ND: not detectable.
[6] Explanation of used symbols: -: not detected; +: mild; ++: extensive.

Immunogenicity

Native rhIFNα2b induced antibodies in the wildtype mice (figure 5), as was expected. The degraded samples and metal catalyzed oxidized rhIFNα2b control showed an increased immune response as compared to native rhIFNα2b in the wildtype mice at day 21.

All samples, except native rhIFNα2b were able to break the tolerance of transgenic mice although the levels of antibody production differed (figure 5): Metal catalyzed oxidized rhIFNα2b was the most potent sample, whereas the sample incubated at pH 9.0 was the least capable of breaking tolerance.

Discussion

In this study we have stored rhIFNα2b without excipients under stress conditions, i.e elevated temperature and different pHs. The solutions were incubated below the melting temperature of rhIFNα2b, which is around 55 - 60 °C, under all conditions tested (22). Aggregation was observed in all samples, probably due to unfolding of the protein, which has been reported before to be caused by acidic and alkaline conditions and/or elevated temperatures (15-17, 22-24). When the protein unfolds, hydrophobic surfaces become exposed and their interaction may lead to aggregation. Disulfide scrambling, which is most favorable at moderate to high pH (25), could also explain the aggregate formation of the sample incubated at pH 9.0.

Unfolding of protein in the samples stored at pH 4.0 and 9.0 was shown by

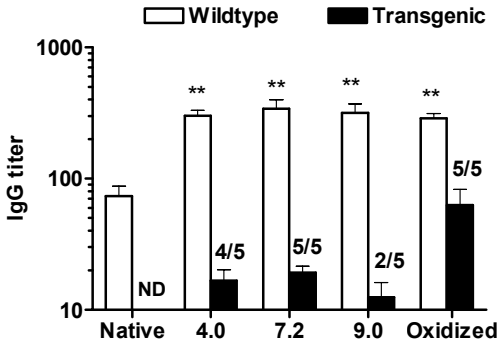

Figure 5 *Anti-rhIFNα2b IgG titers at t=21 days of the wildtype and transgenic mice after administration of native (Native) rhIFNα2b, aged samples incubated at pH 4.0 (4.0), pH 7.2 (7.2) or pH 9.0 (9.0) and metal catalyzed oxidized rhIFNα2b (Oxidized). The values represent average titers (+SEM) of positive mice. All wildtype mice were positive for antibodies. The numbers above the bars represent the number of positive mice out of total mice. **: P<0.01 as compared to native rhIFNα2b (Tukey-Kramer multiple comparisons test). No statistical test could be performed to compare the titers of the transgenic mice, since not all mice showed antibodies. ND: not detectable*

the largely red-shifted fluorescence spectra and the lower reactivity with the monoclonal antibody and polyclonal antiserum. The sample incubated at pH 7.2 only showed a slight change in tertiary structure (second derivative fluorescence, and complete recognition by the monoclonal antibody and/or polyclonal antiserum), which might explain the low level of aggregation observed in this sample.

Besides physical degradation also chemical degradation could have occurred (e.g. deamidation, disulfide bond formation, oxidation and hydrolysis). Hydrolysis predominantly occurs at mild acidic conditions (14, 15), which explains the hydrolysis seen for the sample incubated at pH 4.0.

No further chemical analyses of the samples were pursued. Complete chemical analysis of the samples would not be easy, since the samples have a high level of aggregation and heterogeneity.

All samples, except native rhIFNα2b, contained aggregates known to be important in inducing an antibody response (5-9). Aggregation and chemical degradation may lead to conformational changes and the presentation of new epitopes. Aggregation may also lead to the type of multimeric epitope presentation that can break immune tolerance. The B-cell receptor is capable of responding without T-cell help to epitope patterns meeting certain criteria, such as spacing of 5-10 nm (11, 26-28). All samples, except native rhIFNα2b, were able to break tolerance in transgenic mice. The degraded samples were less immunogenic in the transgenic mice than metal catalyzed oxidized rhIFNα2b. The immunogenicity of the degraded samples seemed not to be related to the level of aggregation of the samples, but to the presence of native like protein in the aggregates. This was in line with previous results (chapter 6). Considering repetitive antigen presentation as an explanation for breaking tolerance, the low immunogenicity of the sample incubated at pH 9.0 can be explained by the destruction of native epitopes in this sample. Also boiled rhIFNα2b showed a correlation between denaturation and lack of capacity to break tolerance (chapters 6 and 7). The samples stored at pH 4.0 and 7.2 showed aggregation but also preservation of native epitopes (table 2), which may explain their higher immunogenicity in the transgenic mice compared with the pH 9.0 sample.

In the wildtype mice all degraded samples (including metal catalyzed oxidized rhIFNα2b) were more immunogenic than native rhIFNα2b. Although the transgenic mice showed clear differences in the immunogenicity of the different degraded samples (including metal catalyzed oxidized rhIFNα2b), all degraded samples (including metal catalyzed oxidized rhIFNα2b) showed the same level of immunogenicity in the wildtype mice. This discrepancy between the transgenic mice and the wildtype mice can be explained by the different

mechanisms that play a role in the antibody formation. In the wildtype mice a classical immune response takes place, while in the transgenic mice breaking of tolerance leads to the formation of antibodies. In a classical immune response the antigen is taken up by antigen presenting cells (APC), digested and peptides of the protein are presented on the surface of the APC in combination with major histocompatability complex (MHC) class II molecules. T-cells will recognize the peptides in combination with the MHC molecule and activate B-cells into making antibodies. The mechanism of breaking of tolerance is not exactly known, but as previously mentioned, multimeric epitope presentation is considered to be important. So in the wildtype mice linear epitopes are most important in inducing an immune response, while in the transgenic mice repeated conformational epitopes seem to be important. The aggregates in the degraded samples might act as a danger signal, recruiting APC to the injection site, which might explain the increased immunogenicity of the degraded samples as compared to native rhIFNα2b in the wildtype mice.

It is not clear from these studies which fraction of the samples (e.g. monomer, dimer, trimer, multimer and insoluble aggregates) is responsible for the increased immunogenicity seen in the wildtype mice and the transgenic mice. This has to be investigated further by isolating the different fractions. After isolation, the fractions have to be characterized and their immunogenicity tested in wildtype and transgenic mice.

In this study extreme conditions were used and hardly any to no native protein at all was left in the samples incubated at pH 4.0 and 9.0. More subtle changes will occur if the protein is stored for shorter incubation periods, or at improper storage conditions, e.g. at room temperature instead of the required refrigerator temperature. Therefore, it would be interesting to study the kinetics of the degradation process of rhIFNα2b under the conditions used in this study (i.e. low and high pH and high temperature) and the relation between degree of degradation and immunogenicity more quantitatively.

Conclusions

In this study we investigated the stability of rhIFNα2b at elevated temperatures and acidic and alkaline conditions. The immunogenicity of the degradation products was studied in wildtype and transgenic immune tolerant mice. Native-like aggregates seem to be the main factor responsible for the increased immunogenicity in wildtype and transgenic mice.

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Chapter 9

Summary and perspectives

Summary

Animal antisera were the first proteins introduced in medicine more than a century ago, followed in the 1920s by insulin from porcine and bovine origin (1). This first generation of therapeutic proteins proved to be immunogenic which is expected from foreign proteins. The next generation of therapeutic proteins consisted of proteins such as growth hormone and clotting factors purified from human tissue or plasma. These therapeutic proteins were immunogenic as well. These products were mainly administered to patients with an innate deficiency and therefore a lack of immune tolerance. Today, most proteins used in medicine are made by recombinant DNA technologies. Many of these proteins are copies of human proteins and are administered to patients with an uncompromised immune system. But, the majority of these proteins still induces antibodies.

Many factors are known to influence the immunogenicity, but their interplay is highly complex and it is still difficult to predict the occurrence of immune reactions in patient populations and, even more so, in individual patients.

A number of the uncertainties regarding the immunogenicity of therapeutic proteins and the conflicting results from clinical trials or post marketing surveillance studies are caused by the lack of standardization of the antibody assays. Each laboratory has its own in-house method to determine the antibody levels in patient sera making it nearly impossible to compare data. The need for fully validated assays is clearly there and initiatives are being taken to meet this demand, e.g. for interferon beta where an EMEA (European Medicines Agency) supported study group is working out the experimental details.

There is a great need for a validated strategy to predict the immunogenicity of a therapeutic protein before the start of clinical trials or before a batch of a therapeutic protein with a marketing authorization is introduced into the market. The in vitro characterization methods and animal models may help to identify factors playing a role in immunogenicity or to compare the relative immunogenicity of different products, but no absolute predictors of antibody induction have been identified until now.

Therefore, it was the aim of this thesis to contribute to the development of a generic strategy to predict the immunogenicity of therapeutic proteins in clinical settings. Different conformations of proteins were created and physico-chemically characterized. Their immunogenicity was assessed in wildtype and immune tolerant transgenic mice. Also, the effect of formulation excipients on protein structure was studied and attempts were made to correlate structural properties of the protein with immunogenicity. Finally, a start was made to evaluate the value of the animal models, wildtype and transgenic immune

tolerant mice, in predicting the immune response in patients.

Chapter 2 is a literature survey of the structural factors influencing the immunogenicity of therapeutic proteins. The classical immune response and breaking of tolerance are discussed as the two main mechanisms for induction of antibodies. The main animal models available to study immunogenicity of therapeutic proteins before going into clinical trials are wildtype mice, non-human primates and immune tolerant transgenic mice. Physical degradation (especially aggregation) of the proteins, as well as chemical modifications (e.g. oxidation) are considered main structural factors in inducing an immune response.

Chapter 3 is a report of the characterization of two recombinant erythropoietin (epoetin) formulations, Eprex®/Erypo® and NeoRecormon®. In 2001 an upsurge of pure red cell aplasia (PRCA) associated with the subcutaneous (s.c.) administration of an epoetin formulation (Eprex®/Erypo®) was noted. In the formulation of Eprex®/Erypo®, human serum albumin (HSA) was replaced by Tween 80 (0.03 % (w/v)) in 1998. Micelles were identified in the Eprex®/Erypo® formulations which coeluted with a small amount of epoetin of a GPC-column. Since the amount of epoetin in the micellar fraction increased upon addition of Tween 80, solubilization of epoetin in the micelles seemed likely. Several epoetin molecules per micelle might lead to multimeric antigen presentation known to be able to break tolerance (2).

Chapter 4 describes the development of a transgenic mouse model immune tolerant for human interferon beta (hIFNβ). To study the immunogenicity of therapeutic proteins conventional animal models have limited value, since all human proteins will induce an immune response in these animals. They can be used, however, to compare the immunogenicity of different proteins and protein formulations. Transgenic mice, immune tolerant for a human protein, seem to be a promising model for studying the immunogenicity of human therapeutic proteins, because they share immune tolerance with patients. The transgenic mouse model developed, was shown to be immune tolerant for recombinant hIFNβ-1a (rhIFNβ-1a). A rhIFNβ-1b formulation known to induce antibodies in 90 % of the patients, was shown to be able to break the tolerance of the transgenic mice, indicating that, under these conditions, the results in the animal model correlated with the outcome in humans. Moreover, the results indicated the antibodies in patients using rhIFNβ-1a to develop by a different mechanism than antibodies in patients

using rhIFNb-1β.

Chapter 5 describes the physico-chemical characteristics and immunogenicity of recombinant human interferon alpha2b (rhIFNα2b) formulations containing different concentrations of Tween 20 or Tween 80. As observed for epoetin formulations (see chapter 3), a small fraction of rhIFNα2b coeluted with micelles of the surfactant during GPC analysis. The surfactants did not induce detectable structural changes in rhIFNα2b. There was no difference in immunogenicity between the different formulations.

Chapter 6 describes the controlled degradation of rhIFNα2b to investigate structure-immunogenicity relationships. The degraded products were characterized with several physico-chemical techniques and their immunogenicity was tested in wildtype and transgenic mice, immune tolerant for hIFNα2. Not all aggregates induced an immune response. The immunogenicity of rhIFNα2b aggregates was dependent on their size and the structure of the protein in the aggregates. Oxidation in itself did not lead to an increased immune response. Moreover, the results showed that it is important to include assays for the antibody response against the altered protein when evaluating immunogenicity, since the presence of antibodies against the altered protein might effect the efficacy and/or clearance of the protein.

In **Chapter 7** parameters influencing the sensitivity of the transgenic animal model are reported. The antibody response after s.c. administration of aggregated, native-like rhIFNα2b was higher than after intraperitoneal (i.p.) administration. Therefore, s.c. administration was used to determine the antibody response against formulations with different levels of aggregates. A formulation containing 14 % of aggregated protein was able to induce serum antibodies that cross-reacted with native rhIFNα2b in the transgenic mice immune tolerant to the non-aggregated protein, while in wildtype mice at least 25 % of aggregated protein was needed to enhance the immune response as compared to the non-aggregated proteins.

Chapter 8 describes an accelerated stability study performed at 50 °C of rhIFNα2b solutions kept at three different pH conditions (4.0, 7.2 and 9.0). In all solutions aggregates were found. The solutions stored at the 'extreme' pH conditions showed largely unfolded protein. Moreover, the solution stored at pH 4.0 showed some hydrolysis. All three solutions showed an increase in immune response in wildtype mice, as compared to native rhIFNα2b, and they were able to break the tolerance in transgenic mice. It was shown that the more

native-like the structure of the protein in the aggregated solution was, the more immunogenic the solution was in transgenic mice.

Summarizing results of the animal models

In most in vivo studies described in this thesis native and metal catalyzed oxidized rhIFNα2b were used as negative and positive controls, respectively. Similar antibody levels in wildtype mice were obtained in each experiment both after i.p. and s.c. administration of native rhIFNα2b (figure 1). Native rhIFNα2b never induced antibodies, detectable by ELISA, in the transgenic mice, which confirms the existence of immune tolerance to native rhIFNα2b. In chapter 6 it was shown that the tolerance was specific for rhIFNα2b and not due to a general immune suppression.

Metal catalyzed oxidized rhIFNα2b was prepared freshly before the start of each experiment and each batch differed in its composition of aggregated protein (figure 2). Despite the differences in composition, the antibody levels after i.p. administration in wildtype mice were similar in both experiments (figure 1a). S.c. administration of these batches in wildtype mice resulted in different antibody titers per batch (figure 1b), indicating that this route of administration was more suitable to compare the immunogenicity of test solutions with small differences in composition. The antibody titers in the transgenic mice after i.p. administration could not be statistically compared, because not all mice were positive (figure 3). After s.c. administration of metal catalyzed oxidized rhIFNα2b to transgenic mice different antibody titers were

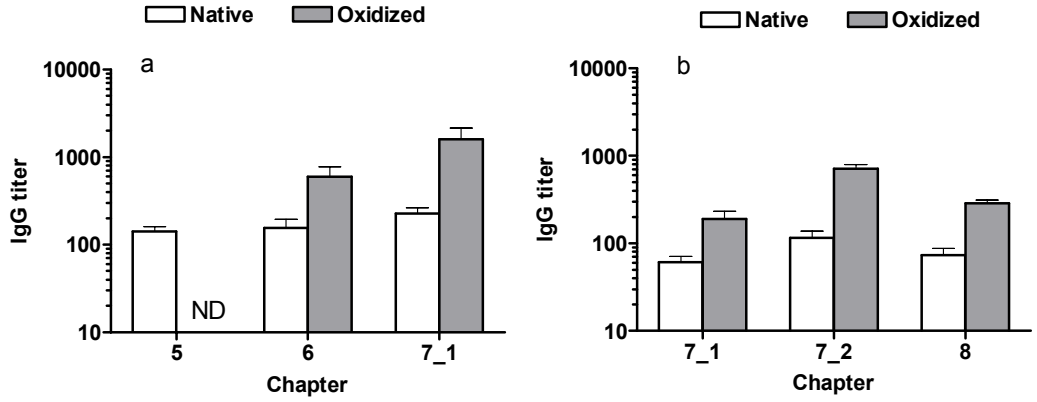

Figure 1 *Anti-rhIFNα2b IgG titers observed in the different chapters of this thesis at 21 days after i.p. (a) or s.c. (b) administration of native or metal catalyzed oxidized rhIFNα2b to wildtype mice. Mice received 10 µg rhIFNα2b on five consecutive days for three weeks. Bars represent average (+ SEM) titers (n=5). ND: not done. Chapter 7_1 and 7_2: first and second batch, respectively, of metal catalyzed oxidized rhIFNα2b of chapter 7.*

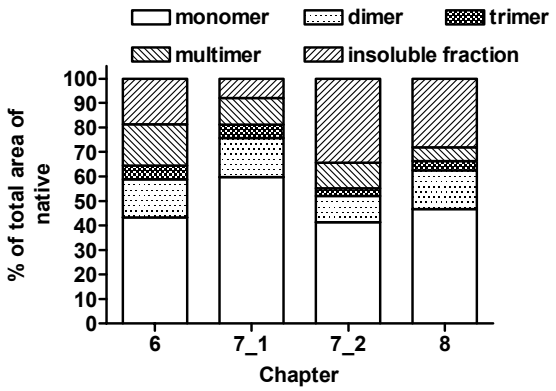

Figure 2 *Composition of metal catalyzed oxidized rhIFNα2b used in the in vivo experiments reported in the different chapters of this thesis, as determined by GPC. Peak area percentages were calculated based on GPC areas relative to the total peak area in GPC of native rhIFNα2b: $AUC_{peak}/AUC_{native,total}$ * 100 %. The precentages of the insoluble fraction were calculated from the total peak area in GPC and the total peak area in GPC of native rhIFNα2b: $(AUC_{native,total} - AUC_{sample,total})/AUC_{native,total}$ * 100 %. Chapter 7_1 and 7_2: first and second batch, respectively, of metal catalyzed oxidized rhIFNα2b of chapter 7.*

obtained with the different batches (figure 3). The batch of metal catalyzed oxidized rhIFNα2b with the highest amount of insoluble fraction (i.e. fraction non-recoverable by GPC) induced the highest antibody titers in the wildtype and transgenic mice, after s.c. administration (the batch used in chapter 7_2, see figures 2 and 3). This limited set of data suggests that the insoluble fraction plays a major role in the induction of antibodies.

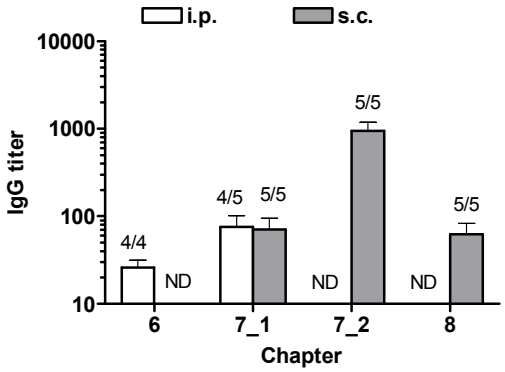

Figure 3 *Anti-rhIFNα2b IgG titers reported in the different chapters of this thesis at 21 days after i.p. or s.c. administration of metal catalyzed oxidized rhIFNα2b to transgenic mice. Mice received 10 µg metal catalyzed oxidized rhIFNα2b on five consecutive days for three weeks. Bars represent average titers of positive mice (+ SEM). Numbers above the bars represent positive mice out of total mice. ND: not done. Chapter 7_1 and 7_2: first and second batch, respectively, of metal catalyzed oxidized rhIFNα2b of chapter 7.*

Perspectives

The measured immune response increased both in wildtype and transgenic mice, when the fraction of insoluble aggregates increased. Furthermore, in several chapters it was noticed that the less native-like the protein structure was, the lower the measured antibody response cross-reacting with native rhIFNα2b in the transgenic mice was. Thus, the protein inside the insoluble aggregates should have a native-like structure to break the tolerance of the transgenic mice. In the wildtype mice the presence of aggregates, irrespective of the structure of the protein inside the aggregates, increased the immune response. Further investigation is necessary to investigate which fraction of the degraded protein species is responsible for the increased immune response in wildtype and transgenic mice. The fractions (e.g. monomer, dimer, trimer, insoluble fraction) should be isolated and characterized in detail. The immunogenicity of each fraction should be compared to the immunogenicity of native rhIFNα2b. Moreover, it should be tested whether the presence of native rhIFNα2b has an influence on the immunogenicity of the fractions.

Protein formulation and immunogenicity

Proteins in solution are susceptible to degradation. To prevent degradation they are usually formulated with several excipients. One reason to use formulation excipients is to ensure sufficient shelf-life, at least 2 years. Since degraded protein species can be a risk factor for immunogenicity, excipients can play a role in minimizing the immunogenicity of protein formulations. However, sometimes the excipients can become part of the immunogenicity problem. For instance, liquid rhIFNα2a formulations with HSA induced antibodies in more patients than a HSA-free liquid formulation (3). Both formulations were stored at 4 °C. HSA formed aggregates with the rhIFNα2a molecules which increased the immunogenicity of the formulations.

In the late 1990s the Eprex® formulations (cf. chapter 3) were changed: HSA was replaced by glycine and Tween 80. This change made the formulation more immunogenic, causing an increase in PRCA cases and the question was: what is the reason? In chapter 3 of this thesis the analysis of Eprex® formulations was described. It was shown that the formulation contained micelles of Tween 80 and that epoetin molecules coeluted with Tween 80 micelles of a GPC column, suggesting an interaction between the protein molecules and the Tween 80 micelles. This interaction could have led to multimeric antigen presentation. Another possible explanation for the increased immunogenicity of Eprex® is the presence of leachates. These

leachates were suggested to be extracted from the uncoated rubber stoppers by Tween 80 (4). Villalobos et al claim that it has been proven that the leachates from the uncoated rubber stoppers are the reason for the increased number of PRCA cases (5). These leachates would act as adjuvant (4). But, adjuvants increase an immune response rather than induce it (6). This means that the leachates could have increased the immune response, but could not have been the underlying reason for the induction of the immune response. The same group also state that the epoetin we detected in the micellar fractions (cf. chapter 3) was in fact dimeric epoetin that coincidentally had the same retention time on the GPC column as Tween 80 micelles. However, as reported in chapter 3, we excluded that possibility by showing that the fraction of coeluting epo increased with the Tween 80 concentration in the mobile phase. As described in chapter 3, micelles are dynamic systems and during the GPC run the micelles will continuously be separated from the free (monomeric) surfactant molecules, which will shift the equilibrium between micellar surfactant and free surfactant to the monomers free in solution. This means that if epoetin molecules are solubilized in Tween 80 micelles the amount of epoetin in the micellar fraction would be underestimated with the described GPC method. Therefore Tween 80 was added to the mobile phase, to keep the micelle-monomer equilibrium constant during the GPC run. An increase in epoetin coeluting with the Tween 80 micelles was seen in this case. This result favors the hypothesis that epoetin molecules are solubilized in Tween 80 micelles, since an increase would not have been seen with dimeric epoetin.

In chapter 5 of this thesis it was shown that rhIFNα2b molecules coeluted, as observed with Eprex®, with micelles of Tween 80 and Tween 20 of a GPC column. The animal studies performed with these formulations did not show a difference in antibody titers in wildtype mice, as compared to a formulation without surfactant. None of the formulations was able to break the tolerance of the transgenic mice.

With all these results in mind it is difficult, if not impossible, to pinpoint one specific cause for the increased number of PRCA cases after the formulation change. Probably a combination of factors played a role. Fortunately the problem seems to be solved, since the number of PRCA cases decreased after all precautions taken, such as emphasizing strict adherence to storage and handling procedures, a contraindication for s.c. administration of Eprex® in the European Union and coating of the rubber stoppers.

Another issue left for discussion and study concerns the question whether animal or clinical immunogenicity studies could have ever picked up the increased immunogenicity of the Eprex® formulations considering the low incidence (~ 50 cases / 100000 patient-years) (4)?

A lesson that can be learned from the above example is that after formulation changes a close monitoring of the performance of a protein drug product is required.

Immunization scheme: the lab situation versus the human situation

In patients breaking of tolerance by therapeutic proteins usually requires a treatment period of 6-12 months. The immunization scheme used in this thesis (daily injections for 3 weeks) could be too short for measuring this type of immune response as is seen in patients receiving, e.g. rhIFNβ-1a treatment. Another difference between the results obtained in the studies in this thesis and the data seen in patients concerns the number of individual responders. The antibody response of patients to interferons varies from a few percent to a majority. In the animal studies, all wildtype and most transgenic mice produced antibodies against the altered protein. Several reasons may be responsible for this discrepancy. First of all, in contrast to patients, the mice used, were genetically identical. This may lead to a more consistent response in the mouse population than in the genetically more variable patient population. Secondly, the mice were injected simultaneously with identical preparations with a high content of altered products associated with immunogenicity. Patients receive products from different batches and ages with varying, usually very small, amounts of modified proteins. It would therefore be relevant to evaluate the immune response in wildtype and transgenic mice treated with small amounts of aggregated or oxidized proteins for a prolonged period of time. Thirdly, patient characteristics have an influence on the immune response as well. The genetic background and the disease status of the patient are known to be of influence (7). It was shown for factor VIII that antibodies are induced more frequently in patients with large deletions or non sense mutations in their factor VIII genes (8). Cancer patients may have a weakened immune system, while patients with auto immune diseases have an activated immune system (9), which will influence the immune response. These influences cannot be included in the transgenic mouse model. The transgenic mice will, however, still be able to identify poor quality batches of protein formulations before they are released for the market.

Sometimes, it is suggested to use adjuvants in studies on the immunogenicity of proteins to increase antibody formation. The use of adjuvants to increase the immune response should be discouraged. First of all, adjuvants are not capable of breaking tolerance in immune tolerant transgenic mice (6). Secondly, adjuvants may denature proteins and therefore add epitopes. This means that adjuvants are not to be used when structural variants are investigated. Thirdly,

adjuvants may change the 'fate' of the therapeutic protein in the body: there may be interactions with different cells (e.g. dendritic cells), other routes of elimination (e.g. lymphatic transport may become more important) and the residence time at the site of injection may be prolonged. If higher immune responses are desired, increasing the frequency of injections or the length of the treatment are better options (10).

In chapter 7 it was shown that the route of administration that induced the highest or most reproducible immune response differed per (degraded) protein species. Metal catalyzed oxidized rhIFNα2b induced the highest response in the wildtype mice after i.p. administration; for native and boiled rhIFNα2b both i.p. and s.c. administration induced the same level of immune response in the wildtype mice. The best schedule in wildtype mice did not necessarily translate to transgenic mice, as was seen for metal catalyzed oxidized rhIFNα2b.

Animal models to predict immune responses in patients

Breaking (B-cell) tolerance is the basic immunological mechanism by which human therapeutic proteins induce antibodies. In wildtype mice human proteins are foreign and will, in principle, always induce an immune response. The results obtained in these animals cannot absolutely predict the immunogenicity of a human therapeutic protein, but can be useful to compare the relative immunogenicity as experiments in this thesis show. Non-human primates show a high degree of homology with and immune tolerance for human proteins and have been used to study the induction of antibodies and their biological effects by a number of products such as growth hormone (11). However, also non-human primates are not immune tolerant for all human proteins, cannot completely predict immunogenicity in patients and ethical and economical considerations limit their use especially for long-term studies. In this thesis the advantages and limitations of transgenic mice expressing the human protein are shown. The advantages include that the classical immune response as well as breaking of tolerance can be tested, since the transgenic mice are immune tolerant for the human protein and will therefore mimic the human response more than wildtype mice or even non-human primates. However, caution has to be taken to interpret the results obtained with the transgenic animals as absolute answers to what might happen in humans.

The level of immune response can depend on the mouse strain used to study the immunogenicity. Mouse strains differ in their MHC haplotypes, which influences their immune response. FVB/N mice, e.g. showed a stronger immune response against rhIFNβ-1a than C57Bl/6 mice (figure 4). Therefore,

it is important to test whether the wildtype mouse strain, from which the transgenics are to be derived, develops antibodies against the studied protein before making the transgenic animals.

It is also possible to make mice tolerant by chronically administering the antigen in large quantities (12). The mice have to be tested individually for their immune tolerance prior to the experiments. This is time and material consuming. In addition, these mice differ in their degree of immune tolerance (13). The construction of immune tolerant transgenic mice does not have these drawbacks and is preferred if a lot of immunogenicity testing needs to be done.

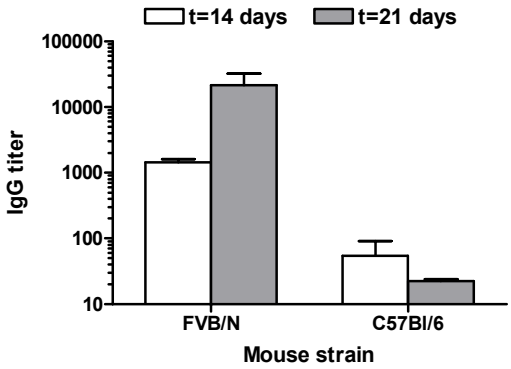

Figure 4 *Anti-rhIFNβ-1a IgG titers in FVB/N and C57Bl/6 mice after receiving 10 µg rhIFNβ-1a i.p. for three weeks on five consecutive days per week. Values represent average (+ SEM) titers (n=5).*

Antibody assay strategy

As already mentioned in chapter 1 of this thesis, different assay formats can be used to test for antibodies. Each assay has its specific properties and applications. In all our experiments the same assays to test for antibodies were used to be able to directly compare results. To gain an insight into all aspects of immunogenicity, a combination of assays is necessary. Usually, the first screening of antibodies is performed with an ELISA or other type of binding assay. These assays are easy to perform and many sera can be tested simultaneously. Because a screening assay is optimized for sensitivity which reduces specificity, positive assays need to be confirmed. Such confirmations can be evaluating the reduction of reactivity by adding the protein to the serum or by a binding assay based on another principle than the original test. The sera confirmed positive for binding antibodies can be screened further for neutralizing antibodies with a bioassay. Neutralizing antibodies are considered to be responsible for biological effects as reduction of efficacy or cross neutralization of endogenous proteins.

Further analysis of the antibodies by evaluating their isotype or affinity may

be important (14). Although isotyping is possible in an ELISA, it is easier to perform in a surface plasmon resonance (SPR) assay. This assay format can also give information about the affinity of antibodies. Another advantage of the SPR technology is its capability to detect low affinity antibodies. The washing steps necessary during the ELISA procedure may remove low affinity antibodies.

Prediction of immunogenicity: alternatives to transgenic animals

In addition to the methods discussed in this thesis, other strategies are advocated to predict immunogenicity such as prediction of epitopes based on amino acid sequence analysis or MHC binding. Modifications in immuno dominant T-cell epitopes by a single amino acid change was reported to reduce the immunogenicity of rhIFNβ-1b in BALB/cByJ mice (15). Analyses of B-cell epitopes are also ongoing, but are more difficult because conformational epitopes are included (16). It is important to realize that these types of analyses concern the immunogenicity of foreign proteins in mice and help to reduce the classical vaccination type of immune response. However, the majority of immune reactions induced by therapeutic proteins are based on breaking (B-cell) tolerance which is not very well understood. Transgenic immune tolerant animals provide excellent models to study the mechanism of breaking (B-cell) tolerance in more detail.

The alternative approaches, mentioned above, miss the effects of conformational variability in proteins. In the section of 'animal models' the pros and cons of other animal based approaches have been briefly discussed.

In conclusion, there are a number of good reasons to favor immunogenicity testing in wildtype mice and immune tolerant transgenic mice during the development phase of a therapeutic protein at present. The tests in wildtype mice will serve as a control to see if the therapeutic protein is capable of initiating an immune response at all. The results in the transgenic mice will tell whether the formulation is capable of breaking immune tolerance. Postmarketing surveillance of patients to check for antibodies is necessary since the animal experiments will never fully predict the immunogenicity.

Proper analytical profiling of the protein (formulations) is essential to gain insight into the structural background of the immunogenic reactions of recombinant human proteins. Eventually, this may lead to situations where analytical characterization of protein formulations will offer a substitute for animal experiments to test immunogenicity.

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Appendix 1

Nederlandse samenvatting

Samenvatting

Meer dan een eeuw geleden werden antisera van dieren als eerste eiwitten geïntroduceerd in de geneeskunde. Dit werd in de jaren 20 van de twintigste eeuw gevolgd door insuline van varkens of van runderen. Deze eerste generatie van therapeutische eiwitten was immunogeen, maar dat was ook te verwachten, aangezien het lichaamsvreemde eiwitten waren. De volgende generatie therapeutische eiwitten bestond uit eiwitten, zoals groeihormoon en stollingsfactoren, gezuiverd uit humaan weefsel of bloedplasma. Deze therapeutische eiwitten waren ook immunogeen. Deze producten werden voornamelijk toegediend aan patiënten die, vanwege een aangeboren afwijking, niet immuuntolerant waren voor het product. Vandaag de dag worden de meeste eiwitten, die in de geneeskunde gebruikt worden, gemaakt met behulp van recombinante DNA technieken. Veel van deze eiwitten zijn kopieën van humane eiwitten en worden toegediend aan patiënten met een functionerend immuunsysteem. De meerderheid van deze eiwitten induceert echter nog steeds antistoffen.

De immunogeniciteit wordt beïnvloed door vele factoren en hun samenspel is erg complex vandaar dat het nog steeds erg moeilijk is om een immuunrespons in patiëntenpopulaties, laat staan in een individu, te voorspellen.

Een aantal van de onzekerheden aangaande de immunogeniciteit van therapeutische eiwitten en de tegensprekende resultaten van klinische onderzoeken of post-marketingtoezicht wordt veroorzaakt door het gebrek aan standaardisatie van de antistofbepalingen. Elk laboratorium heeft zijn eigen methode om de antistoftiter in het serum van patiënten te bepalen. Dit maakt het bijna onmogelijk om data te vergelijken. Er is een duidelijke behoefte aan volledig gestandaardiseerde bepalingen. Er zijn initiatieven genomen om aan deze vraag te voldoen, zoals bijvoorbeeld voor interferon-bèta, waarvoor de EMEA (Europese medicijnen commissie) het initiatief heeft genomen.

Er bestaat grote behoefte aan een gevalideerde strategie om de immunogeniciteit van een therapeutisch eiwit te voorspellen, alvorens klinische studies worden gestart of wanneer een batch van een therapeutisch eiwit dat al op de markt is, wordt vrijgegeven voor de markt. De in vitro karakteriseringsmethoden en diermodellen bieden hulp ter identificatie van factoren welke een rol spelen in de immunogeniciteit, of ter vergelijking van de relatieve immunogeniciteit van verschillende producten. Er is op dit moment echter geen volledig betrouwbare methode beschikbaar om de inductie van antistoffen in patiënten te voorspellen.

Het was dan ook het doel van dit proefschrift om een bijdrage te leveren aan

de ontwikkeling van een strategie om de immunogeniciteit van therapeutische eiwitten te voorspellen. Hiertoe was de structuur van eiwitten op verschillende manieren veranderd en fysisch-chemisch gekarakteriseerd. De immunogeniciteit werd bepaald in wildtype en immuuntolerante transgene muizen. Tevens werd het effect van formuleringshulpstoffen op de eiwitstructuur bestudeerd en zijn er pogingen gedaan om de structurele eigenschappen van het eiwit te correleren aan immunogeniciteit. Tenslotte is een begin gemaakt met het evalueren van de diermodellen (wildtype en transgene, immuuntolerante muizen) om de immuunrespons in patiënten te voorspellen.

Hoofdstuk 2 is een literatuuroverzicht van de structurele factoren die de immunogeniciteit van therapeutische eiwitten beïnvloeden. De klassieke immuunrespons en het breken van immuuntolerantie zijn besproken als de twee belangrijkste mechanismen voor het opwekken van antistoffen. De belangrijkste diermodellen, die beschikbaar zijn om de immunogeniciteit van therapeutische eiwitten te bestuderen voordat klinische studies worden uitgevoerd, zijn wildtype muizen, niet-humane primaten en immuuntolerante, transgene muizen. Fysische degradatie (met name aggregatie) en chemische modificaties (bijv. oxidatie) van de eiwitten worden gezien als de belangrijkste structurele factoren in het opwekken van een immuunrespons.

Hoofdstuk 3 is een verslag van de karakterisering van twee recombinante Erytropoëtine (epoëtine) formuleringen: Eprex®/Erypo® en NeoRecormon®. In 2001 werd er een grote toename van “pure red cell aplasia” (PRCA), gerelateerd aan de onderhuidse toediening van een epoëtineformulering (Eprex®/Erypo®), waargenomen. In 1998 werd het humane serum albumine (HSA) in de formulering van Eprex®/Erypo® vervangen door Tween 80 (0.03 % (w/v)). Er werd aangetoond dat in de Eprex®/Erypo® formulering micellen aanwezig waren, welke op een GPC kolom co-elueerden met een kleine hoeveelheid epoëtine. Aangezien de hoeveelheid epoëtine in de micellaire fractie toenam na toevoeging van Tween 80 aan de mobiele fase, lijkt het aannemelijk dat de epoëtine gesolubiliseerd was in de micellen. Meerdere epoëtinemoleculen per micel kunnen leiden tot multimere-antigenpresentatie, welke erom bekend staat tolerantie te kunnen doorbreken.

Hoofdstuk 4 beschrijft de ontwikkeling van een transgeen muismodel immuuntolerant voor humaan interferon-bèta (hIFNβ). Conventionele diermodellen hebben beperkte waarde om de immunogeniciteit van therapeutische eiwitten te bestuderen, aangezien alle humane eiwitten een

immuunrespons in deze dieren zullen opwekken. Ze kunnen echter wel gebruikt worden om de immunogeniciteit van verschillende eiwitten en formuleringen te vergelijken. Transgene muizen, immuuntolerant voor een humaan eiwit, zijn veelbelovende modellen om de immunogeniciteit van humane therapeutische eiwitten te bestuderen, aangezien ze, net als patiënten, immuuntolerant zijn. Er werd aangetoond dat het transgene muismodel dat ontwikkeld was, immuuntolerant is voor recombinant hIFNβ-1a (rhIFNβ-1a). Een formulering van rhIFNβ-1b, die in 90 % van de patiënten antistoffen induceert, was in staat de tolerantie van de transgene muizen te doorbreken. Dit was een aanwijzing dat onder deze omstandigheden de resultaten in het diermodel correleerden met de resultaten in mensen. Tevens was dit een aanwijzing dat de antistoffen in patiënten die rhIFNβ-1a gebruiken via een ander mechanisme geïnduceerd worden dan de antistoffen in patiënten die rhIFNβ-1b gebruiken.

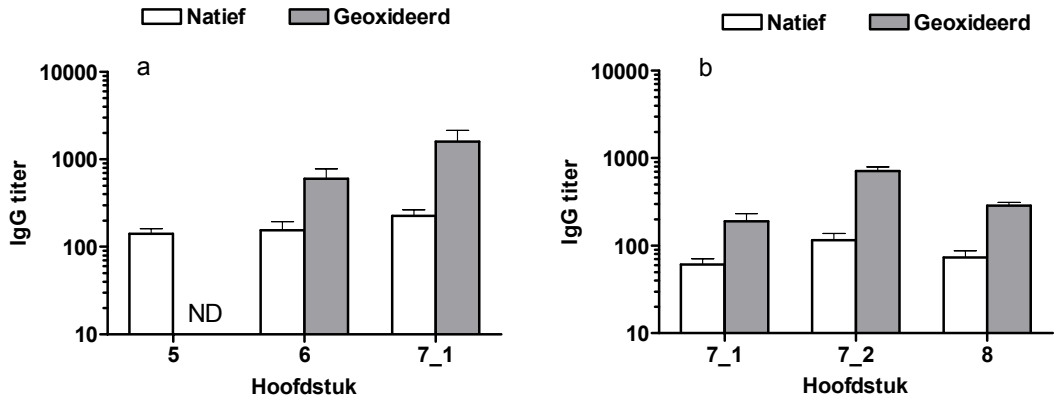
Hoofdstuk 5 beschrijft de fysisch-chemische karakterisering en immunogeniciteit van recombinant interferon-alfa2b (rhIFNα2b) formuleringen met verschillende hoeveelheden Tween 20 of Tween 80. Net zoals bij epoëtine formuleringen (hoofdstuk 3), co-elueerde een klein deel van het rhIFNα2b met micellen van de oppervlakte-actieve stof bij GPC-analyse. De oppervlakte-actieve stoffen zorgden niet voor aantoonbare structurele veranderingen in rhIFNα2b. Ook was er geen verschil in immunogeniciteit tussen de verschillende formuleringen.

Hoofdstuk 6 beschrijft de gecontroleerde degradatie van rhIFNα2b ter bestudering van structuur-immunogeniciteitsrelaties. De gedegradeerde producten werden gekarakteriseerd met verschillende fysisch-chemische technieken en de immunogeniciteit werd getest in wildtype en transgene muizen, immuuntolerant voor hIFNα2. Niet alle aggregaten induceerden een immuunrespons. De immunogeniciteit van de rhIFNα2b aggregaten was afhankelijk van de grootte van de aggregaten en/of van de structuur van de eiwitmoleculen welke de aggregaten vormden. Oxidatie alleen leidde niet tot een verhoogde immuunrespons. Tevens is aangetoond dat het belangrijk is om testen voor de detectie van antistoffen gericht tegen het veranderde eiwit toe te voegen wanneer de immunogeniciteit van een product wordt bepaald. De aanwezigheid van antistoffen gericht tegen het veranderde product zouden namelijk de effectiviteit en/of klaring van het eiwit kunnen beïnvloeden.

In **hoofdstuk 7** worden parameters besproken die de gevoeligheid van het transgene diermodel beïnvloeden. De antistofrespons na onderhuidse

toediening van geaggregeerd, natief lijkend rhIFNα2b was groter dan na toediening in de buikholte. Onderhuidse toediening werd daarom gebruikt om de antistofrespons gericht tegen formuleringen met verschillende hoeveelheden aggregaten te bestuderen. In de transgene muizen, immuuntolerant voor het niet-geaggregeerde eiwit, was een formulering met 14 % geaggregeerd eiwit in staat om antistoffen, die kruisreageerden met natief rhIFNα2b, te induceren. In de wildtype muizen was minstens 25 % geaggregeerd eiwit nodig om de immuunrespons te verhogen ten opzichte van niet-geaggregeerd eiwit.

Hoofdstuk 8 beschrijft een studie waarbij rhIFNα2b oplossingen van drie verschillende pH's (4,0; 7,2 en 9,0) bij 50 °C werden bewaard ter verkrijging van eiwitdegradatieproducten. In alle oplossingen werden aggregaten gevormd. De oplossingen die bewaard waren bij de extreme pH's bevatten grotendeels ontvouwen eiwit. De oplossing bewaard bij pH 4,0 toonde ook enige hydrolyse. Alle drie de oplossingen lieten, ten opzichte van het natieve rhIFNα2b, een verhoogde immuunrespons zien in de wildtype muizen. Tevens waren alle oplossingen in staat de tolerantie van de transgene muizen te doorbreken. Er werd aangetoond dat hoe meer de structuur van het eiwit in de geaggregeerde oplossing op natief rhIFNα2b leek, des te immunogener de oplossing was in de transgene muizen.

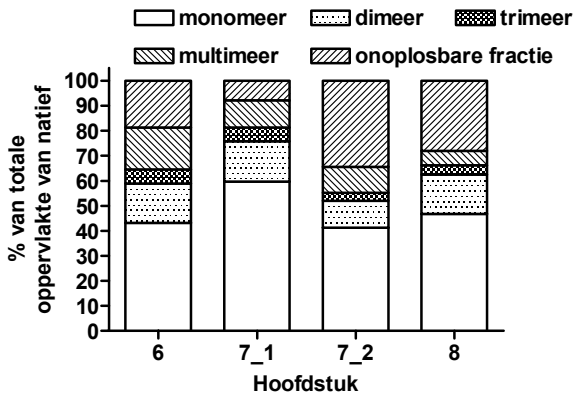


Figuur 1 *Anti-rhIFNα2b IgG titers in de wildtype muizen, 21 dagen na toediening in de buikholte (a) of onderhuidse toediening (b) van natief of metaal-gekatalyseerd geoxideerd rhIFNα2b verkregen in de verschillende hoofdstukken van dit proefschrift. De muizen kregen 10 µg rhIFNα2b op vijf achtereenvolgende dagen toegediend gedurende drie weken. Staven tonen gemiddelde titers (+ SEM) (n=5). ND: niet uitgevoerd. Hoofdstuk 7_1 en 7_2: respectievelijk eerste en tweede batch metaal-gekatalyseerd geoxideerd rhIFNα2b van hoofdstuk 7.*

Samenvattende resultaten van het diermodel

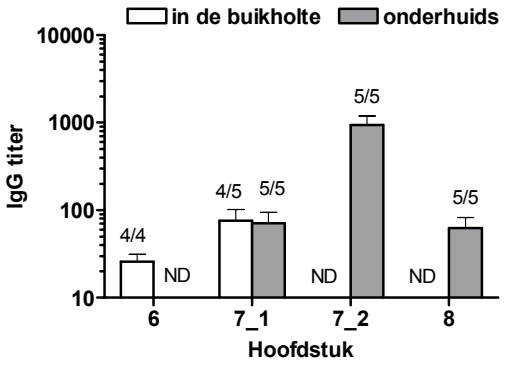
In de meeste in-vivo studies werden natief en metaal-gekatalyseerd-geoxideerd rhIFNα2b gebruikt als respectievelijk negatieve en positieve controle. In de wildtype muizen werden in alle experimenten vergelijkbare antistoftiters verkregen, zowel na toediening in de buikholte als na onderhuidse toediening van natief rhIFNα2b (figuur 1). Natief rhIFNα2b induceerde nooit antistoffen, detecteerbaar met een ELISA, in de transgene muizen. Dit bevestigt dat de transgene muizen immuuntolerant zijn voor natief rhIFNα2b. In hoofdstuk 6 was aangetoond dat de tolerantie specifiek was voor rhIFNα2b en niet te wijten was aan een algehele immuunsuppressie.

Metaal-gekatalyseerd-geoxideerd rhIFNα2b werd voor de aanvang van ieder experiment vers gemaakt. Elke batch verschilde in zijn samenstelling van geaggregeerd eiwit (figuur 2). Ondanks deze verschillen waren de antistoftiters na toediening in de buikholte van de wildtype muizen statistisch vergelijkbaar tussen beide experimenten (figuur 1a). Onderhuidse toediening van deze batches aan wildtype muizen resulteerde in statistisch verschillende antistoftiters per batch (figuur 1b). Dit is een indicatie dat de onderhuidse toediening beter geschikt is om de immunogeniciteit van oplossingen met kleine verschillen in samenstelling te vergelijken. De antistoftiters na toediening in de buikholte van de transgene muizen konden statistisch niet vergeleken worden, omdat niet alle muizen antistoffen hadden (figuur 3). Na



Figuur 2 *Samenstelling van metaal-gekatalyseerd-geoxideerd rhIFNα2b dat gebruikt is in de in-vivo experimenten beschreven in de verschillende hoofdstukken van dit proefschrift, zoals bepaald met GPC. Percentages piekoppervlakte werden berekend op basis van piekoppervlakte gerelateerd aan de totale oppervlakte van natief rhIFNα2b:* $AUC_{piek}/AUC_{natief,totaal}$ ** 100 %.*
De percentages van de onoplosbare fractie werden berekend op basis van de totale oppervlakte in GPC en de totale oppervlakte van natief rhIFNα2b in GPC: $(AUC_{natief,totaal}-AUC_{sample,totaal})/AUC_{natief,totaal}$ ** 100 %.*
Hoofdstuk 7_1 en 7_2: respectievelijk eerste en tweede batch metaal-gekatalyseerd-geoxideerd rhIFNα2b van hoofdstuk 7.

onderhuidse toediening van metaal-gekatalyseerd-geoxideerd rhIFNα2b aan transgene muizen werden verschillende antistoftiters verkregen met de verschillende batches (figuur 3). De batch metaal-gekatalyseerd-geoxideerd rhIFNα2b met de grootste onoplosbare fractie induceerde de hoogste antistoftiters in de wildtype en transgene muizen na onderhuidse toediening (de batch gebruikt in hoofdstuk 7_2, zie figuur 2 en 3). Deze, weliswaar beperkte hoeveelheid, gegevens suggereren dat de onoplosbare fractie een grote rol speelt in de inductie van antistoffen.



Figuur 3 *Anti-rhIFNα2b IgG titers in de transgene muizen op dag 21 na toediening in de buikholte of onderhuidse toediening van metaal-gekatalyseerd-geoxideerd rhIFNα2b, zoals gerapporteerd in de verschillende hoofdstukken van dit proefschrift. Muizen kregen 10 µg metaal-gekatalyseerd-geoxideerd rhIFNα2b op vijf achtereenvolgende dagen toegediend gedurende drie weken. Staven tonen gemiddelde titers (+ SEM). De getallen boven de staven geven het aantal positieve muizen per totaal aantal muizen aan. ND: niet uitgevoerd. Hoofdstuk 7_1 en 7_2: respectievelijk eerste en tweede batch metaal-gekatalyseerd-geoxideerd rhIFNα2b van hoofdstuk 7.*

Samenvattend heeft dit onderzoek aangetoond dat een aantal goede redenen bestaat, om de immunogeniciteit in wildtype en transgene, immuuntolerante muizen tijdens de ontwikkelingsfase van een therapeutisch eiwit te testen. De testen in wildtype muizen dienen ter controle of het therapeutisch eiwit überhaupt in staat is een immuunrespons op te wekken. De resultaten in de transgene muizen laten zien of een formulering in staat is de immuuntolerantie te doorbreken. Post-marketingtoezicht op de aanwezigheid van antistoffen in patiënten blijft echter nog steeds noodzakelijk, omdat de dierstudies de immunogeniciteit nooit volledig zullen voorspellen.

Appendix 2

Dankwoord

Dankwoord

Eindelijk is het dan zover. Het is tijd om het dankwoord te schrijven. Op de eerste plaats wil ik de personen bedanken die het voor mij mogelijk hebben gemaakt om dit boekje te mogen schrijven: Wim Hennink, Wim Jiskoot en Daan Crommelin. Wim en Wim, na de avonturen in mijn eerste AIO-project hebben jullie me vlak voor kerstmis 2001 fantastisch opgevangen. Ook het gestelde vertrouwen in mij, door me het huidige project aan te bieden, heb ik ontzettend gewaardeerd. Daan, bedankt voor de ondersteuning tijdens de besprekingen in het eerste project en het gestelde vertrouwen door mijn promoter te zijn bij dit project. Hans de Cock en Jan Tommassen van de vakgroep Moleculaire microbiologie van de faculteit Biologie hebben me tijdens mijn eerste AIO-project geholpen met de bacteriële werkzaamheden. Jan, bedankt dat je tijd wilde vrijmaken om mijn resultaten te bespreken en uit te leggen aan iedereen van Biofarmacie. Hans, jij hebt me de beginselen van de recombinant-DNA technieken bijgebracht. Het isoleren, knippen en ligeren van DNA en het transformeren en laten groeien van bacteriën was volkomen nieuw voor mij. Helaas lukte het niet om het eiwit tot expressie te brengen, maar ik heb er ontzettend veel van geleerd, wat me prima van pas is gekomen bij mijn tweede project. Bedankt!

I want to thank AlfaWasserman for supplying the recombinant interferon alpha2b solutions which made the experiments described in this thesis possible. I especially would like to thank Paolo, Lucia and Giuseppe from AlfaWassermann. During my three weeks stay at AlfaWassermann, Paolo helped me the first week with performing activity assays and learned me how to work with cells. Lucia helped me the other two weeks with all kinds of different experiments. The discussions with Giuseppe made a lot clear to me about the characteristics of the protein. Lucia and Giuseppe, thank you very much for your hospitality during the three weeks. Not only in the lab, but also outside the lab. I really enjoyed the evenings at your place, together with Giovanni and Franscesca.

The transgenic interferon alpha2b mice were supplied by professor Pestka. Thank your very much for this. Without your help we would not have been able to do all the experiments we did.

Verder wil ik mijn directe begeleiders Wim Jiskoot en Huub Schellekens en mijn promoter Daan Crommelin bedanken voor alle hulp tijdens de afgelopen 3,5 jaar. Zonder alle besprekingen met hun was de inhoud van dit boekje niet zo geworden als hij nu is. Vooral alle kritische vragen, opmerkingen en discussies hebben het boekje gemaakt tot wat het nu is. Huub, bedankt voor het regelen van de samenwerkingen met alle bedrijven. Dit heeft hoofdstuk 3 en

4 opgeleverd. Ook was ik altijd verbaasd hoe jij mijn teksten wist te corrigeren, waarbij er wel nog dezelfde boodschap stond, maar dan in de helft van de woorden, dat scheelt een hoop aan drukkosten. Wim, bedankt voor alle discussies tijdens onze wekelijkse werkbesprekingen. De dingen die ik ietwat wilde verbloemen doorzag je altijd meteen. Mede door de kritische vragen werd ik gedwongen dieper over de data na te denken dan dat ik in eerste instantie zou doen. Ook je kritische kijk op eerste, tweede, derde etc. versies van manuscripten heb ik erg gewaardeerd, hoewel ik misschien soms het tegenovergesteld liet blijken. Daan, als promoter spraken we elkaar alleen op de twee-maandelijkse besprekingen, waarbij vaak veel data in 1 keer te bespreken was. Toch wist jij altijd te zorgen dat er ook over het boekje en publicaties werd nagedacht.

Tijdens dit project was ik in dienst van het GDL. Hoewel ik toch de meeste tijd te vinden was in het Went-gebouw, heb ik met een aantal mensen van het GDL veel samengewerkt, die ik via dit dankwoord persoonlijk wil bedanken. Alle dierproeven die in het boekje beschreven staan heb ik (gelukkig) niet allemaal alleen hoeven te doen. Voor alle experimenten waren transgene muizen nodig, welke werden gefokt in de SPF afdeling van het GDL. In het begin werd dit gecoördineerd door Sabine Versteeg, naderhand is dit overgenomen door Kitty Hermans. Het verplaatsen van muizen van de SPF afdeling naar de “open afdeling” werd altijd zeer snel uitgevoerd, ook als ik de muizen binnen twee dagen nodig had. Dames allebei hartelijk bedankt voor alle goede zorgen voor mijn muisjes.

Het injecteren en bloed afnemen van de muizen op de afdeling Klein Wit heb ik ook niet zelf gedaan. Dit werd in de eerste plaats geregeld door Anja van der Sar. Anja, jij hebt mij geleerd om bloed af te nemen bij de muizen, zodat we het lange tijd iedere maandagochtend om 10.30 uur samen deden. Tevens heb je geprobeerd me het injecteren van de muizen te leren, maar helaas ging dat niet altijd even goed. Bedankt voor alle goede zorgen, de dagelijkse injecties en de maandagochtend bloedtapuurtjes! Gelukkig zijn we in de loop van het project wel wat sneller geworden met bloedtappen. Ik vond de maandagochtenden altijd erg gezellig. Sorry dat het injecteren voor mij echt een nachtmerrie was.

De laatste paar maanden had Sabine Versteeg de bloedtap-taken en injectie-taken van Anja grotendeels overgenomen. Sabine ook jij natuurlijk van harte bedankt voor de goede zorgen voor mijn muisjes, het injecteren en de gezellige maandagochtenden!

Helma, Joyce, Tamara en Toon bedankt voor het inspringen tijdens het bloedtappen als Anja of Sabine geen tijd hadden. En verder natuurlijk iedereen van het GDL die ik vergeten ben: Bedankt!

Zoals al gezegd was ik de meeste tijd in het Went-gebouw te vinden bij de

vakgroep Biofarmacie en Farmaceutische technologie. Ook hier wil ik een aantal mensen persoonlijk noemen. Tijdens mijn AIO-periode deelde ik het lab samen met Nataša Jovanović. Nataša, thank you very much for the nice company in the lab. You were always the person who listened when I was having a bad day or was depressed. You also are very good in the theory behind the experiments, which helped me a lot in understanding my results. We also had a lot of protein related (and other) discussions with Marc on the lab.

Marc bedankt voor alle hulp tijdens mijn project. Ook als je voor de eerste keer met een techniek te maken kreeg wist je altijd door logisch na te denken zinnige dingen te zeggen over de resultaten. Ik kon altijd naar je toe stappen met vragen. Dit was ook prima te doen tijdens de ProFormA besprekingen. Deze waren altijd prima geregeld. Als er niemand problemen of vragen had, wist jij altijd wel een onderwerp aan te kaarten waar we toch een uur lang over konden discussiëren.

Theo, bedankt dat je al die GPC experimenten inclusief ELISAs hebt willen doen voor me. Als echte chemicus viel het niet mee om dit soort experimenten te doen, maar je inzet was fantastisch! Sommige mensen waren niet blij met het droogslaan van de ELISA-platen, maar gelukkig trok je je daar niks van aan.

Mies, jij hebt verder geen experimenten voor mij hoeven doen. Toch stond je altijd klaar als er weer eens iets niet klopte bij een apparaat. Meestal was dat de HPLC die weer eens een keer niet deed wat ik wilde. Bedankt!

Frits, de experimenten op de Biacore zijn verwerkt in hoofdstuk 5. Het is niet veel tekst, maar het meten heeft des te meer tijd gekost, aangezien er wat problemen waren in het begin. Bedankt voor alle hulp tijdens de Biacore experimenten en het bespreken van de resultaten.

Tijdens mij AIO-periode heb ik ook samengewerkt met een aantal studenten. De eerste was Liliana. Liliana, you were a very nice student to work with. You understood the things you did and thanks to you chapter 6 has become what it is. Thank you very much for all the work!

After Liliana, Carla came for 6 months. Carla, you performed two projects in the period you were present in our lab. Unfortunately none of them made it into this booklet, but the experiments you did were very helpful in the progress of my work and also of others. Thank you very much!

Laura was vooral aangenomen voor de ondersteuning tijdens de dierexperimenten. Laura, je inzet was bewonderenswaardig. Bedankt voor het testen van de vele staartjes en het uitvoeren van de vele ELISAs. Hierdoor had ik tijd over voor andere experimenten.

Georgina was de laatste student die ik heb mogen begeleiden. Georgina, you came during the final year of my project. Because of this I was not always

available for you especially after I moved to the seventh floor to concentrate more on my writing. Despite this you always knew what experiments to perform. Most of your experiments you performed at the department of Mass Spectrometry. Your results are part of chapter 6. Thank you very much for everything you did. At this point I would also like to thank Monique and Mirjam from the department of Mass Spectrometry for the supervision of Georgina.

Zoals al gezegd ben ik de laatste maanden verhuisd naar de 7e verdieping om een rustige schrijfplek te hebben. Marieke volgde niet lange tijd daarna. Marieke, ik vind het erg knap hoe jij binnen (zeer) korte tijd hebt geregeld dat je toch nog dit jaar kon promoveren. Hierdoor was er redelijk wat stress volgens mij, maar dat was niet te merken aan je. Er was gelukkig ook nog tijd om te pog-gen (proefschrift ontwijkend gedrag) tussen het lay-out gestoei met Word. Het is en blijft een eigenwijs programma. Ik vond het erg gezellig en dat kwam niet alleen door al je versieringen op de kamer. Heel veel succes en plezier in San Fransisco!

Natuurlijk wil ik iedereen van de vakgroep Biofarmacie en Farmaceutische technologie bedanken voor de gezellig koffiepauzes, lunchpauzes, borrels, bbqs, labuitjes, ladiesdiners etc. Dit heeft er mede voor gezorgd dat ik altijd met veel plezier aan deze periode zal terugdenken.

Na alle mensen van de werkvloer bedankt te hebben, ik hoop dat ik niemand vergeten ben, wordt het tijd om naar de persoonlijke sfeer te gaan. Myrra en Esther bedankt dat jullie mijn paranymf willen zijn. Esther, ik zal proberen alle vragen zelf te beantwoorden tijdens de verdediging. Myrra, jouw boekje zal ook een pracht exemplaar worden.

Ronald, bedankt voor het maken van de omslag. Je kende me niet en wist niet wat ik deed, maar na een uitleg, die blijkbaar redelijk duidelijk was, heb jij het muizenplaatje getekend wat nu op de voorkant staat. Hij is super geworden!

Verder wil ik natuurlijk al mijn vrienden bedanken voor de etentjes, verjaardagen, uitjes, bioscoopbezoekjes etc.

Pap en mam, eindelijk is het dan zover, wat later dan gepland, maar jullie dochter gaat toch promoveren. Bedankt voor alle luisterende oren en adviezen, vooral toen het niet zo lekker liep met mijn eerste project. Mark en Monique hebben hier enorm in geholpen tijdens onze vakantie in Oostenrijk.

Monique, ik hoop dat het in het Nederlands mag, maar volgens mij moet dat ondertussen wel kunnen. Dank je wel voor je adviezen en luisterende oren in Oostenrijk. Je enthousiasme om de promotie te mogen bijwonen werkt aanstekelijk, vooral de vergelijking met een huwelijk is erg leuk. Mark, als ik het vragenrondje van 45 minuten doorsta is er dan eindelijk die prijsuitreiking.

Pap, mam, Mark en Monique, jullie snapten niet veel van de artikels die ik jullie opstuurde, maar er werd toch een poging gedaan om de grote lijnen te volgen. Het enthousiasme van de rest van de familie (ooms en tantes, neven en nichten) tijdens het toeleven naar 25 november werkt erg aanstekelijk en we zullen er dan ook met zijn allen een super leuke dag van maken.

Natuurlijk heb ik de belangrijkste persoon tot het laatst bewaard. Raymond/Deef, ik leerde jou kennen toen ik nog student was, waarna ik vrijwel meteen naar Amerika vertrok voor een half jaar. Toen ik AIO werd zijn we gaan samenwonen, terwijl je wist dat ik redelijk veel tijd aan mijn werk zou gaan besteden. Maar dat zou maar 4 jaar duren. Helaas heeft het bij mij wat langer geduurd, maar jij hebt mij altijd gesteund in al mijn beslissingen, ook toen ik besloot te stoppen en aan een nieuw project (van weer 4 jaar) te beginnen. Het gooide onze plannen wat in de war, maar je hebt nooit geklaagd. Gelukkig hebben we toch dat huis kunnen kopen wat we graag wilden. Bedankt voor alle begrip, luisterende oren, de vele avondetentjes die klaar stonden als ik thuiskwam en adviezen. Hopelijk wordt het nu allemaal wat rustiger en kunnen we meer tijd aan elkaar besteden. En volgend jaar hebben we samen een groot feest!

Familie, vrienden, (ex)collega's

Iedereen bedankt

Appendix 3

Curriculum vitae

Curriculum vitae

Suzanne Hermeling was born on April 16th, 1976 in Heerlen. In 1994 she finished high school (VWO) at the "St. Janscollege" in Hoensbroek. In that year she started her study of pharmacy at the faculty of Pharmaceutical Sciences at Utrecht University. During her study she visited the University of Maryland Pharmacy School in Baltimore (USA) for a research project of 6 months, entitled "A molecular strategy for achieving brain drug delivery". She graduated from Utrecht University in 1998. In 1999 she started working at the department of Pharmaceutics at Utrecht University on a project entitled "Prodrug targeting to the intestinal peptide carrier". This was in cooperation with the department of Molecular Microbiology of the faculty of Biology at Utrecht University. In 2002 she started her PhD project under the supervision of dr. W. Jiskoot, dr. H. Schellekens and prof. dr. D.J.A. Crommelin. The results of this project are described in this thesis. Since November 2005 she is working at OctoPlus Development B.V.

Appendix 4

List of publications

List of publications

S. Hermeling, H. Schellekens, D.J.A. Crommelin, W. Jiskoot. Micelle-associated protein in epoetin formulations: a risk factor for immunogenicity? *Pharmaceutical Research, 20* (2003), 1903-1907

S. Hermeling, D.J.A. Crommelin, H. Schellekens, W. Jiskoot. Structure-immunogenicity relationships of therapeutic proteins. *Pharmaceutical Research, 21* (2004), 897-903.

S. Hermeling, W. Jiskoot, D.J.A. Crommelin, C. Bornæs, H. Schellekens. Development of a transgenic mouse model immune tolerant for human interferon beta. *Pharmaceutical Research, 22* (2005), 847-851.

S. Hermeling, L. Aranha, J.M.A. Damen, M. Slijper, H. Schellekens, D.J.A. Crommelin, W. Jiskoot. Structural characterization and immunogenicity in wildtype and immune tolerant mice of degraded recombinant human interferon alpha2b. *Accepted for publication in Pharmaceutical Research (2005).*

S. Hermeling, D.J.A. Crommelin, H. Schellekens, W. Jiskoot. Immunogenicity of therapeutic proteins. In S. C. Gad (editor), *Handbook of Pharmaceutical Biotechnology*, Vol. IV, Handbook Series, John Wiley & Sons, Inc., Hoboken, NJ. *In preparation*.

S. Hermeling, W. Jiskoot, D.J.A. Crommelin, H. Schellekens. Letter to the editor: Interaction of polysorbate 80 with erythropoietin: a case study in protein-surfactant interactions. *Submitted for publication.*

S. Hermeling, H. Schellekens, C. Maas, M.F.B.G. Gebbink, D.J.A. Crommelin, W. Jiskoot. Antibody response to aggregated human interferon alpha2b in wildtype and transgenic immune tolerant mice. *Manuscript in preparation.*

Appendix 5

Abbreviations

Abbreviations

A_{280}	absorbance at 280 nm
Abs	antibodies
ABTS	2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	analysis of variance
APC	antigen presenting cells
AUC	area under the curve
BAb	binding antibody
BSA	bovine serum albumin
CD	circular dichroism
CHO-cells	Chinese hamster ovarian carcinoma cells
CHO-rhIFNβ	recombinant human interferon beta expressed in CHO-cells
CMC	critical micelle concentration
CpG	cytosin-guanosin dinucleotides
DLS	dynamic light scattering
DTT	dithiothreitol
E. coli	Escherichia coli
E. coli-rhIFNβ	recombinant human interferon beta expressed in E. coli
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMEA	European Medicines Agency
EP	European Pharmacopeia
Epoetin	recombinant human erythropoietin
ESI-ToF	electrospray ionization time of flight
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
GnHCl	guanidine hydrochloride
GPC	gel permeation chromatography
HBS-EP	HEPES buffered saline containing EDTA and polysorbate 20
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGH	human growth hormone
hIFNα2	human interferon alpha2
hIFNβ	human interferon beta
HSA	human serum albumin
Humatrope	natural sequence recombinant human growth hormone
Ig	immunoglobulin
IgG	immunoglobulin G

IgM	immunoglobulin M
IL-2	interleukin 2
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IFNβ	interferon beta
LPS	lipopolysaccharide
MALDI-ToF-ToF	matrix-assisted laser desorption ionization time of flight time of flight
met-rhGH	recombinant human growth hormone with an extra methionine
MHC	major histocompatibility complex
MS	mass spectrometry
MSA	murine serum albumin
NAb	neutralizing antibody
NATA	n-acetyl-L-tryptophanamide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PB	sodium phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	polydispersity index
PEG	polyethylene glycol
pit-hGH	human growth hormone purified out of human pituitaries
polyIC	polyinosinic:polycytidylic acid
polyICLC	polyIC complexed with poly-L-lysine and carboxymethylcellulose
PRCA	pure red cell aplasia
rhGM-CSF	recombinant human granulocyte macrophage colony stimulating factor
rhIFNα2	recombinant human interferon alpha2
rhIFNβ	recombinant human interferon beta
rhtPA	recombinant human-tissue plasminogen activator
RIA	radioimmunoassay
rmIFNα2	recombinant murine interferon alpha2
RP-HPLC	reversed phase high pressure liquid chromatography
RRMS	relapsing-remitting multiple sclerosis
RU	response units
s.c.	subcutaneous

SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SPR	surface plasmon resonance
TFA	trifluoroacetic acid
UV	ultraviolet
VLP	viral-like particles