

# 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<sup>®</sup>/Erypo<sup>®</sup> and NeoRecormon<sup>®</sup>. In 2001 an upsurge of pure red cell aplasia (PRCA) associated with the subcutaneous (s.c.) administration of an epoetin formulation (Eprex<sup>®</sup>/Erypo<sup>®</sup>) was noted. In the formulation of Eprex<sup>®</sup>/Erypo<sup>®</sup>, human serum albumin (HSA) was replaced by Tween 80 (0.03 % (w/v)) in 1998. Micelles were identified in the Eprex<sup>®</sup>/Erypo<sup>®</sup> 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 (hIFN $\beta$ ). 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 hIFN $\beta$ -1a (rhIFN $\beta$ -1a). A rhIFN $\beta$ -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 rhIFN $\beta$ -1a to develop by a different mechanism than antibodies in patients

using rhIFN $\beta$ -1 $\beta$ .

**Chapter 5** describes the physico-chemical characteristics and immunogenicity of recombinant human interferon alpha2b (rhIFN $\alpha$ 2b) formulations containing different concentrations of Tween 20 or Tween 80. As observed for epoetin formulations (see chapter 3), a small fraction of rhIFN $\alpha$ 2b coeluted with micelles of the surfactant during GPC analysis. The surfactants did not induce detectable structural changes in rhIFN $\alpha$ 2b. There was no difference in immunogenicity between the different formulations.

**Chapter 6** describes the controlled degradation of rhIFN $\alpha$ 2b 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 hIFN $\alpha$ 2. Not all aggregates induced an immune response. The immunogenicity of rhIFN $\alpha$ 2b 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 rhIFN $\alpha$ 2b 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 rhIFN $\alpha$ 2b 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.

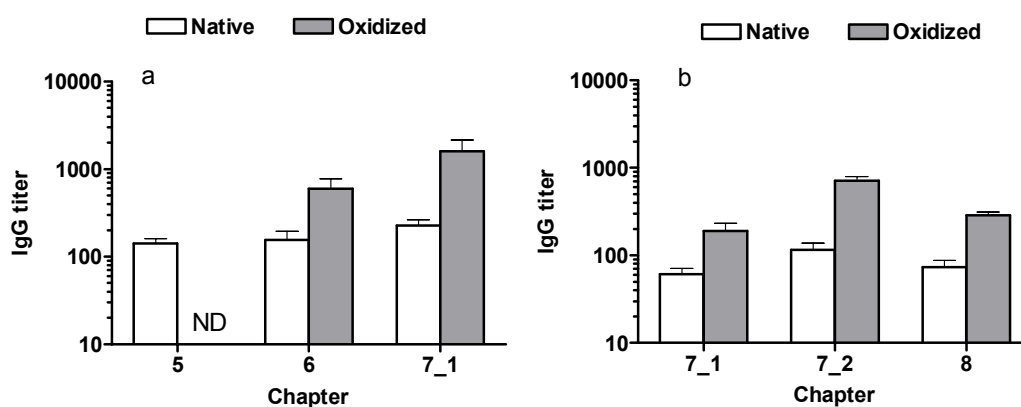
**Chapter 8** describes an accelerated stability study performed at 50 °C of rhIFN $\alpha$ 2b solutions kept at three different pH conditions (4.0, 7.2 and 9.0). In all solutions aggregates were found. The solutions stored at the 'extreme' pH conditions showed largely unfolded protein. Moreover, the 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 rhIFN $\alpha$ 2b, 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.

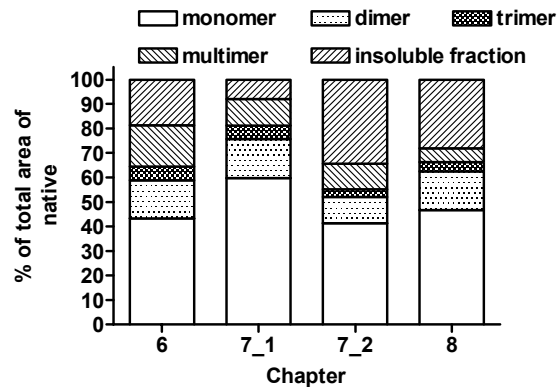
## Summarizing results of the animal models

In most in vivo studies described in this thesis native and metal catalyzed oxidized rhIFN $\alpha$ 2b 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 rhIFN $\alpha$ 2b (figure 1). Native rhIFN $\alpha$ 2b never induced antibodies, detectable by ELISA, in the transgenic mice, which confirms the existence of immune tolerance to native rhIFN $\alpha$ 2b. In chapter 6 it was shown that the tolerance was specific for rhIFN $\alpha$ 2b and not due to a general immune suppression.

Metal catalyzed oxidized rhIFN $\alpha$ 2b 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 rhIFN $\alpha$ 2b to transgenic mice different antibody titers were

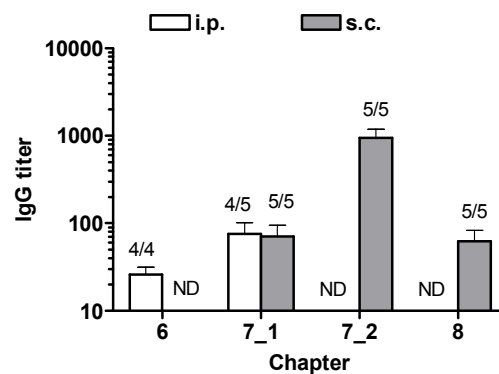


**Figure 1** Anti-rhIFN $\alpha$ 2b IgG titers observed in the different chapters of this thesis at 21 days after i.p. (a) or s.c. (b) administration of native or metal catalyzed oxidized rhIFN $\alpha$ 2b to wildtype mice. Mice received 10  $\mu$ g rhIFN $\alpha$ 2b on five consecutive days for three 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 rhIFN $\alpha$ 2b of chapter 7.



**Figure 2** Composition of metal catalyzed oxidized rhIFN $\alpha$ 2b 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 rhIFN $\alpha$ 2b:  $AUC_{peak}/AUC_{native,total} * 100 \%$ . The percentages of the insoluble fraction were calculated from the total peak area in GPC and the total peak area in GPC of native rhIFN $\alpha$ 2b:  $(AUC_{native,total} - AUC_{sample,total})/AUC_{native,total} * 100 \%$ . Chapter 7\_1 and 7\_2: first and second batch, respectively, of metal catalyzed oxidized rhIFN $\alpha$ 2b of chapter 7.

obtained with the different batches (figure 3). The batch of metal catalyzed oxidized rhIFN $\alpha$ 2b 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-rhIFN $\alpha$ 2b IgG titers reported in the different chapters of this thesis at 21 days after i.p. or s.c. administration of metal catalyzed oxidized rhIFN $\alpha$ 2b to transgenic mice. Mice received 10  $\mu$ g metal catalyzed oxidized rhIFN $\alpha$ 2b 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 rhIFN $\alpha$ 2b 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 rhIFN $\alpha$ 2b 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 rhIFN $\alpha$ 2b. Moreover, it should be tested whether the presence of native rhIFN $\alpha$ 2b 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 rhIFN $\alpha$ 2a 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 rhIFN $\alpha$ 2a molecules which increased the immunogenicity of the formulations.

In the late 1990s the Eprex<sup>®</sup> 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<sup>®</sup> 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<sup>®</sup> 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 rhIFN $\alpha$ 2b molecules coeluted, as observed with Eprex<sup>®</sup>, 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<sup>®</sup> 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<sup>®</sup> 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. rhIFN $\beta$ -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 rhIFN $\alpha$ 2b induced the highest response in the wildtype mice after i.p. administration; for native and boiled rhIFN $\alpha$ 2b 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 rhIFN $\alpha$ 2b.

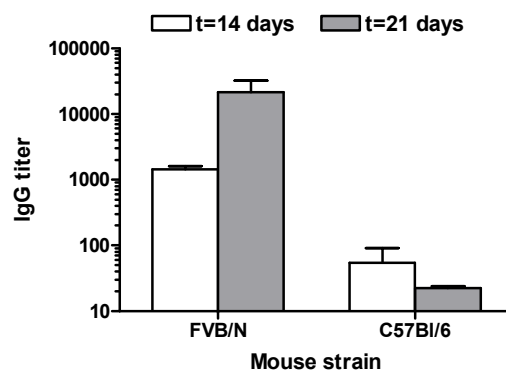
#### *Animal models to predict immune responses in patients*

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 rhIFN $\beta$ -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-rhIFN $\beta$ -1a IgG titers in FVB/N and C57Bl/6 mice after receiving 10  $\mu$ g rhIFN $\beta$ -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 immunodominant T-cell epitopes by a single amino acid change was reported to reduce the immunogenicity of rhIFN $\beta$ -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.

**References**

1. G. Schernthaner. Immunogenicity and allergenic potential of animal and human insulins. *Diabetes Care* 16 Suppl 3: 155-165 (1993).
2. R. M. Zinkernagel. Uncertainties - discrepancies in immunology. *Immunol Rev* 185: 103-125 (2002).
3. J. C. Ryff. Clinical investigation of the immunogenicity of interferon-alpha 2a. *J Interferon Cytokine Res* 17 Suppl 1: S29-33 (1997).
4. K. Boven, J. Knight, F. Bader, J. Rossert, K. U. Eckardt and N. Casadevall. Epoetin-associated pure red cell aplasia in patients with chronic kidney disease: solving the mystery. *Nephrol Dial Transplant* 20 Suppl 3: iii33-40 (2005).
5. A. P. Villalobos, S. R. Gunturi and G. A. Heavner. Interaction of polysorbate 80 with erythropoietin: a case study in protein-surfactant interactions. *Pharm Res* 22: 1186-1194 (2005).
6. T. A. Stewart, P. G. Hollingshead, S. L. Pitts, R. Chang, L. E. Martin and H. Oakley. Transgenic mice as a model to test the immunogenicity of proteins altered by site-specific mutagenesis. *Mol Biol Med* 6: 275-281 (1989).
7. P. Kontsek, H. Liptakova and E. Kontsekova. Immunogenicity of interferon-alpha 2 in therapy: structural and physiological aspects. *Acta Virol* 43: 63-70 (1999).
8. R. Prescott, H. Nakai, E. L. Saenko, I. Scharrer, I. M. Nilsson, J. E. Humphries, D. Hurst, G. Bray and D. Scandella. The inhibitor antibody response is more complex in hemophilia A patients than in most nonhemophiliacs with factor VIII autoantibodies. Recombinate and Kogenate Study Groups. *Blood* 89: 3663-3671 (1997).
9. E. Koren, L. A. Zuckerman and A. R. Mire-Sluis. Immune responses to therapeutic proteins in humans-clinical significance, assessment and prediction. *Curr Pharm Biotechnol* 3: 349-360 (2002).
10. A. Braun, L. Kwee, M. A. Labow and J. Alsenz. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. *Pharm Res* 14: 1472-1478 (1997).
11. C. M. Zwickl, K. S. Cocke, R. N. Tamura, L. M. Holzhausen, G. T. Brophy, P. H. Bick and D. Wierda. Comparison of the immunogenicity of recombinant and pituitary human growth hormone in rhesus monkeys. *Fundam Appl Toxicol* 16: 275-287 (1991).
12. P. U. Simioni, L. G. Fernandes, D. L. Gabriel and W. M. Tamashiro. Induction of systemic tolerance in normal but not in transgenic mice through continuous feeding of ovalbumin. *Scand J Immunol* 60: 257-266 (2004).
13. A. M. Faria, S. M. Ficker, E. Speziali, J. S. Menezes, B. Stransky, B. A. Verdolin, W. M. Lahmann, V. S. Rodrigues and N. M. Vaz. Aging and immunoglobulin isotype patterns in oral tolerance. *Braz J Med Biol Res* 31: 35-48 (1998).

14. P. von Wussow, D. Jakschies, M. Freund and H. Deicher. Humoral response to recombinant interferon-alpha 2b in patients receiving recombinant interferon-alpha 2b therapy. *J Interferon Res* 9 Suppl 1: S25-31 (1989).
15. V. P. Yeung, J. Chang, J. Miller, C. Barnett, M. Stickler and F. A. Harding. Elimination of an immunodominant CD4+ T cell epitope in human IFN-beta does not result in an in vivo response directed at the subdominant epitope. *J Immunol* 172: 6658-6665 (2004).
16. S. Saha, M. Bhasin and G. P. Raghava. Bcipep: A database of B-cell epitopes. *BMC Genomics* 6: 79 (2005).

