7 Immunogenicity of Therapeutic Proteins

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INTRODUCTION

Proteins were first used as medicines at the end of the nineteenth century when antisera from animals were introduced for the treatment of serious infections, such as diphtheria and tetanus. However, because such antisera were loaded with proteins foreign to the patients' immune system, they often led to serious and sometimes even fatal side effects. In general, persons who had been treated had a warning in their passports or identification cards to alert physicians for a possible anaphylactic reaction after re-challenge with an antiserum.

In the 1920s porcine and bovine insulin products were introduced to treat diabetes. Many patients receiving these insulins developed antibodies neutralizing the protein (anti-drug antibodies, ADA). At first, this had been explained by the animal origin of the products. However, reduction of immunogenicity of these products following improvements in the production methods and increasing purity strongly indicated that the protein's animal origin was not the only factor leading to their immunogenicity. In the second half of the twentieth century, a number of human proteins from natural sources such as plasma derived clotting factors and growth hormone produced from pituary glands of cadavers was introduced into the clinic. These products were given mainly to children with an innate deficiency who therefore lacked the natural immune tolerance. Consequently, their immune response was also interpreted as a response to foreign proteins. The correlation between the factor VIII gene defects and level of deficiency with the immune response in hemophilia patients confirmed this explanation (Fakharzadeh and Kazazian Jr 2000). However, similar to the animal derived insulins, improved purification protocols led to reduced immunogenicity levels for some of the human protein products.

The true breakthrough in availability of therapeutic proteins occurred in the 1980s when recombinant DNA technologies allowed large-scale production of highly purified proteins. In 1982 human insulin was marketed as the first recombinant DNA derived protein for human use. Since then dozens of recombinant proteins have entered the market and some of these products, such as the interferons and the epoetins, are among the most widely used medicines in the world. However, although these proteins were developed as close copies of human endogenous proteins, nearly all of them induce ADA, sometimes even in a majority of patients (Table 7.1). Importantly, most of these products are used in patients who do not have an innate deficiency and thus one can assume that they developed immune tolerance to the protein.

The initial assumption was that the production by recombinant technology in non-human host cells and the downstream processing modified the proteins and the immunological response was the classical response to a foreign protein. However, in some cases circumventing B-cell tolerance might be the basis for the antibody response to human homologues. This phenomenon is not yet completely understood but appears to be different from the immune response against foreign antigens used in vaccines. On the other hand, for therapeutic monoclonal antibodies, there are usually determinants present in the molecule that are foreign to the recipient (see section "Issues Specifically Related to Monoclonal Antibodies").

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Therapeutic protein	Immunogenicity rate (%)	Reference
Growth hormone	7–22	Rougeot et al. (1991)
Factor VIII	3–35	Oldenburg et al. (2015)
Insulin	14–44	Fineberg et al. (2007)
Interferon beta	2–47	Bertolotto et al. (2004)
Monoclonal antibodies	0–89	Harding et al. (2010)
Interleukin-2	20–100	Prümmer (1997)

Table 7.1 ■ Non-exhaustive list of recombinant proteins showing immune reactions upon administration

The clinical manifestations of both types of reaction are very different. The typical vaccine-like response to foreign proteins occurs within days to weeks. Often a single injection is sufficient to induce a substantial ADA response. In general, high levels of neutralizing antibodies are induced and a re-challenge leads to a booster reaction, indicating a memory response.

However, the development of an immune response against certain recombinant human proteins may require months, sometimes years of chronic treatment. Moreover, secreted antibodies often do not neutralize the injected therapeutic protein and sometimes even do not manifest any apparent clinical effects. Additionally, in some cases these ADA, especially when produced in low or moderate amounts, tend to disappear shortly after the treatment has been stopped and sometimes even during treatment (Perini et al. 2001). Also, this response does not appear to generate immunological memory. In patients treated with recombinant human interferon β (rhIFNβ) or recombinant human growth hormone, the therapy often can be paused to allow the ADA levels to decline and then restarted without boosting of ADA titers (Schellekens and Casadevall 2004; Perini et al. 2001).

In contrast, the ADA response against therapeutic monoclonal antibodies such as adalimumab and infliximab follows a more classical pattern (Brandse et al. 2016; Bartelds et al. 2011), with ADA detected as early as 2 weeks after starting the treatment. Moreover, the vast majority of these ADA is neutralizing (van Schie et al. 2015a, 2017); and intermittent infliximab treatment is associated with more immunogenicity (Han and Cohen 2004).

THE IMMUNE RESPONSE

The therapeutic proteins currently available cover the whole spectrum, from completely foreign (e.g., bacteria-derived asparaginase) to completely human (e.g., recombinant human interferon α-2b (rhIFNα-2b)) as well as unnatural proteins, such as fusion proteins (e.g., etanercept), truncated antibodies (e.g., Fab fragments, nanobodies) and PEGylated proteins (e.g., PEGylated rhIFNα with PEG defined as polyoxyethylene glycol).

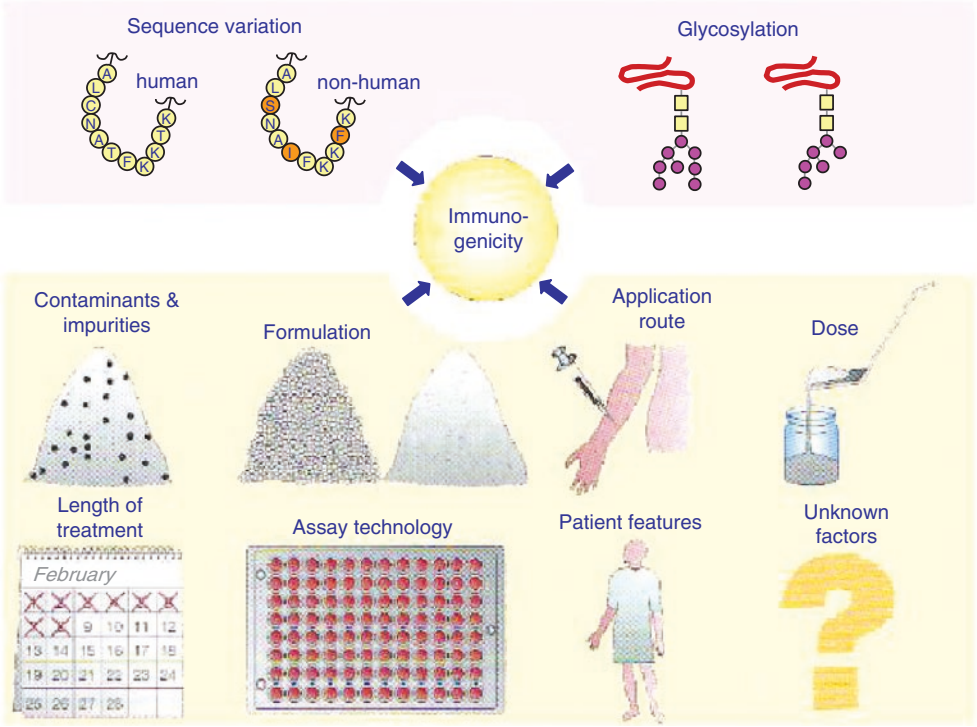
Foreign proteins elicit antibodies by the classical pathway, which includes ingestion and cleaving of the proteins into peptides by macrophages and dendritic cells, presentation of peptides by the MHC (major histocompatibility complex)-II system and activation of B-cells, and affinity maturation and isotype switching of the B-cells by helper $CD4^+$ T-cells. Furthermore, they induce memory B-cells (see Chap. 14 for details).

It is much less clear how B-cell tolerance is circumvented. One of the theories to explain antibody formation against a self(-like) therapeutic protein was that aggregated protein induced exclusively T-cell independent B-cell activation via crosslinking of B-cell receptors by protein aggregates resembling bacterial or viral structures (Moore and Leppert 1980; Bachmann et al. 1993). Such structures present repetitive epitopes in a highly regular pattern, allowing rapid recognition as foreign substance and inducing a response without engaging $CD4^+$ T-cells. However, since this mechanism was first proposed, numerous preclinical and clinical observations have indicated involvement of $CD4^+$ T-cells in the antibody response against therapeutic self(-like) proteins. During $CD4^+$ T-cell independent responses, mostly low-affinity IgMs are produced, but in patients and in animal models mostly IgGs have been observed. Efficient isotype switching is one of the hallmarks of a $CD4^+$ T-cell response. However, production of non-neutralizing antibodies in part of the patients suggests impaired affinity maturation.

Another indication of a $CD4^+$ T-cell dependent mechanism is the observation that certain HLA alleles correlate with a higher probability of ADA formation against, e.g., rhIFNβ, epoetin and anti-TNF (tumor necrosis factor) mAb (Barbosa et al. 2006; Moss et al. 2013; Praditpornsilpa et al. 2009; Fijal et al. 2008). Moreover, in several animal models $CD4^+$ T-cell depletion resulted in almost complete abolishment of antibody production (reviewed by Jiskoot et al. 2016). Nevertheless, full explanations for immunogenicity occurrence in only part of the patients receiving the protein, the frequent formation of non-neutralizing antibodies, and the apparent lack of immunological memory are still missing.

FACTORS INFLUENCING ANTIBODY FORMATION TO THERAPEUTIC PROTEINS

Figure 7.1 depicts the different factors that influence immunogenicity (Schellekens 2002a; Hermeling et al. 2004). These factors will be discussed below.



Schellekens, Nature Reviews Drug Discovery, 2002

Figure 7.1 ■ Factors influencing immunogenicity

■ Structural Factors

The similarity of a therapeutic protein's sequence to that of the human counterpart is one of the factors determining the probability of an ADA response against the therapeutic protein. However, the degree of non-self that is necessary to induce a vaccine-type response is highly dependent on the protein involved and the site of the divergence from the natural sequence of the endogenous protein. For instance, single mutations in inulin can lead to a new epitope and an ADA response, whereas other mutations have no influence at all; and consensus IFNα, in which more than 10% of the amino acids diverge from the nearest naturally occurring IFNα subtype, is not more immunogenic than the IFNα-2 homologue. Moreover, some unnatural proteins, such as etanercept, are relatively poorly immunogenic.

Glycosylation is another important structural factor for the immunogenicity of therapeutic proteins. There is little evidence that modified glycosylation, e.g., by expressing human glycoproteins in plant cells or other non-human eukaryotic hosts, may induce an immune response (Singh 2011). However, the level of glycosylation has a clear effect. For instance, rhIFNβ produced in *E. coli* (non-glycosylated) is much more immunogenic than rhIFNβ produced in mammalian cells. This may be explained by the higher hydrophobicity, causing higher aggregation levels in the non-glycosylated *E. coli*-derived product.

Futhermore, in certain populations, pre-existing IgE antibodies to non-human glycans present on cetuximab resulted in severe anaphylactic reactions (Chung et al. 2008).

■ Impurities

Impurities are considered to be important risk factors for the immunogenicity of therapeutic protein products. Among the clinically relevant impurities, protein aggregates have received most attention; the presence of aggregates is widely accepted as one of the most important risk factors for immunogenicity. Aggregation may be triggered by a variety of factors, such as thermal stress, pH shift, agitation, freeze-thawing and UV irradiation. Importantly, aggregates may be induced at every step of the production process as well as during storage, shipment, and product administration (cf. Chap. 5). Aggregation is believed to be one of the main causes of immunogenicity of e.g., human growth hormone and rhIFNβ-1b (Moore and Leppert 1980; Barnard et al. 2013). However, aggregation is not the only risk factor and products containing small amounts of aggregates might also be very immunogenic.

Chemical modification of human proteins e.g., oxidation, might lead to formation of neo-epitopes which might be recognized by a patient's immune system and trigger ADA. Moreover, as demonstrated in numerous animal studies, antibodies induced by

chemically modified proteins can be cross-reactive with unmodified protein (Jiskoot et al. 2016).

Besides aggregates and chemically modified proteins, substances derived from the production process, such as host cell components, resins from chromatographic columns, enzymes used to activate the product and monoclonal antibodies used for affinity purification, may end up in the final product. Impurities may also be introduced by components of the formulation, may leak from the container and sealing of the product or may be introduced during the fill and finish steps. These impurities may be immunogenic by themselves and thereby elicit an immune response different from that to the therapeutic protein. Moreover, lipopolysaccharides from bacterial host cells and G-C rich bacterial host cell DNA are examples of impurities that can act as adjuvants. Antibodies induced by impurities may lead to general immune reactions such as skin reactions, allergies, anaphylaxis and serum sickness.

Formulation

Recombinant human therapeutic proteins are often highly biologically active and the doses may be at the μg level, making it a technological challenge to formulate the product and keep it stable with a reasonable shelf-life (cf. Chap. 5). The importance of designing a proper formulation in avoiding immunogenicity is highlighted in two historical cases. In the case of rhIFNα-2a, a large difference in immunogenicity was noted among different formulations (Fig. 7.2). A freeze-dried formulation, containing human serum albumin as a stabilizer that according to its instructions could be kept at room temperature, was more immunogenic in a clinical study than other formulations. It appeared that at room temperature rhIFNα-2a became partly oxidized. This led to the formation of aggregates, which most likely were responsible for the immune response (Ryff 1997). Interestingly, studies in animal models have confirmed that aggregates induced by oxidation are particularly immunogenic degradants (Jiskoot et al. 2016).

In the second case, an ADA-mediated severe form of anemia (pure red cell aplasia; PRCA) occurred after the formulation of an epoetin-α product was changed (Casadevall et al. 2002). Human serum albumin was replaced by glycine and polysorbate 80. How this formulation change led to a higher incidence of immunogenicity is still not certain, but it has been postulated that the new formulation is slightly less stable, resulting in aggregate formation when the product is not appropriately handled (Schellekens and Jiskoot 2006).

Route of Administration

Historically the subcutaneous route was considered to be the most immunogenic and intravenous the least immunogenic among administration routes used for therapeutic proteins (Schellekens 2002b). However, head-to-head comparison of administration routes is challenging, as the treatment regimen and/or the formulation have to be adjusted to compensate for altered pharmacokinetics and usually decreased bioavailability upon subcutaneous injection (Richter et al. 2012), all of which may influence the risk of an immune response. Subcutaneous formulations usually differ from intravenous ones because subcutaneous administration generally requires lower injection volumes. Overall, the influence of the administration route on immunogenicity seems to be strongly product dependent. Whereas for some products the subcutaneous route indeed seems to be associated with a higher immunogenicity risk as compared to the intravenous route, for others the influence of administration route seems to be negligible (Hamuro et al. 2017). Nevertheless, immunogenicity may occur after administration of a therapeutic protein via any route of application.

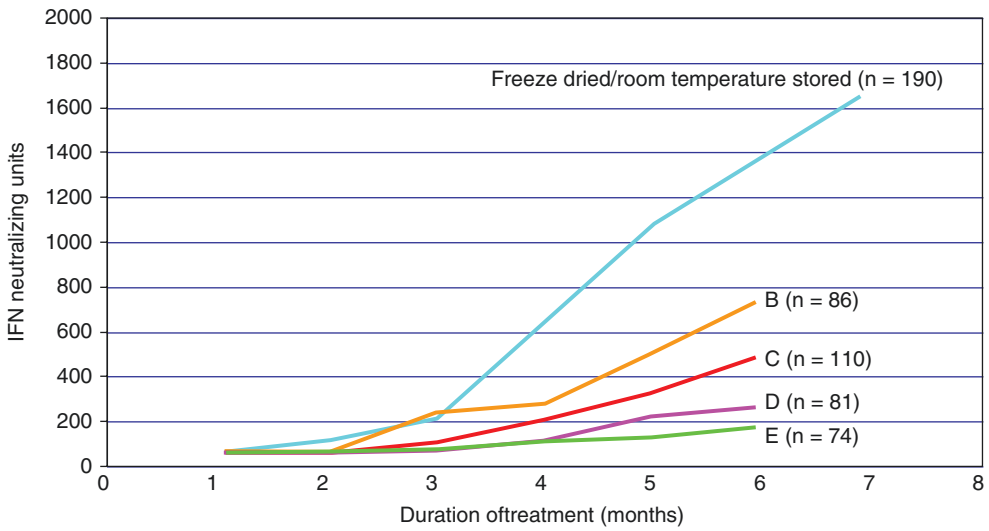


Figure 7.2 Immunogenicity differences between rhIFNα-2a formulations in patients (figure adapted from Ryff 1997)

Dose

The effect of the dose is not quite clear. There are studies with the lowest incidence of ADA formation in the highest dose group (Maini et al. 1999; Food and Drug Administration 2002; Ross et al. 2000). However, such data should be interpreted with caution. In the highest dose group the higher therapeutic protein levels in the circulation may interfere with the assay, or the measured ADA level may be lower by increased immune complex formation.

Patient Features

Several patient related factors may influence the incidence of ADA formation. For example, many of the patients receiving monoclonal antibodies are immune compromised by diseases such as cancer or by immune suppressive treatment. These patients are less likely to produce ADA than patients with a normal immune status. An opposite effect can happen in patients suffering from chronic infections (like hepatitis): these patients may be more prone to development of ADA, as observed for rhIFNα-2 (Antonelli and Dianzani 1999).

There are several indications that circumventing B-cell tolerance is HLA-type dependent. This may explain, at least in part, why some individuals or populations do and others do not produce ADA when treated with the same product. For instance, associations between HLA-type and ADA formation were reported for infliximab (Billiet et al. 2015).

In many patients treated with therapeutic proteins, ADA are found before the start of the treatment (Gorovits et al. 2016). These so-called pre-existing ADA might be formed against structures, e.g., common glycans that closely resemble epitopes present in the therapeutic protein. In many of these patients the pre-existing ADA do not seem to have any impact on therapy safety or efficacy. However, since some patients who are tested positive for pre-existing ADA might be more susceptible to acute side effects or development of high titers of ADA, pre-existing ADA are considered as an immunogenicity risk factor.

Assays for Antibodies

Assays are probably an important factor influencing the reported incidence of ADA induction by therapeutic proteins. In the published studies with rhIFNα-2 in patients with viral infections the incidence of ADA induction varied from 0% to more than 60% positive patients. A similar variation has been seen with rhIFNβ (van Beers et al. 2010). This variation must be assay related. Evaluations of the performance of different test laboratories with blind panel testing showed a more than 50-fold difference in titers found in the same sera. Thus, any reliable comparison between different groups of patients when looking for a clinical effect of ADA or studying factors influencing immunogenicity can only be made if the antibody quantification is done with a well-validated assay in the same laboratory.

This situation persisted for evaluation of antibody formation against TNF inhibitors (infliximab, adalimumab), resulting in incidence rates varying from 0 to 87% in case of adalimumab (Vincent et al. 2013). Several factors account for these large discrepancies between studies, but one factor in particular was found to be of prime importance: drug interference. In particular, for therapeutic monoclonal antibodies high doses are used resulting in high concentrations of the therapeutic antibody in the blood. Then serum samples will contain considerable amounts of therapeutic protein that may interfere with the detection of ADA. Accordingly, many efforts have been made to design assays that overcome this interference, resulting in assays that are 'drug-tolerant' to variable degrees (Bloem et al. 2015).

The relationship between therapeutic antibody concentrations and ADA is important, not only for the detection of ADA, but also for the impact of ADA on efficacy. Often the main consequence of ADA formation is the reduction of 'active' protein concentrations. Thus, small amounts of ADA without a noticeable effect on the protein concentrations may be detected with a modern drug-tolerant assay but these ADA have no impact on efficacy (van Schouwenburg et al. 2013a).

Recommendations for Antibody Assays

There is a lack of standardization of assay methodology, and only a few reference and/or standard antibody preparations are available. Nevertheless, a number of papers appeared with recommendations for setting up and validating immunoassays for immunogenicity testing (Mire-Sluis et al. 2004; Shankar et al. 2008, 2014). A brief discussion of these recommendations follows below.

A single assay may not be sufficient to evaluate the immunogenicity of a new therapeutic protein. A number of assays may have to be used in conjunction. Most antibody assay strategies are based on a two-tier approach: a screening + confirmation assay to identify the ADA positive sera, followed by further characterization, e.g. determination of the ADA titer, affinity and isotype. Especially quantification may be useful, given that ADA levels can vary widely across individuals and the potential clinical impact of an ADA response usually relates to the extent of antibody formation.

In general, the screening assay is a binding assay, often a bridging type of assay (either ELISA –enzyme-linked immunosorbent assay (cf. Chap. 3) or electrochemoluminescence) with the radio-immune-precipitation methodology as an alternative. Screening assays are

designed for optimal sensitivity to avoid false negatives, and often, the cut-point (i.e., threshold positive/negative) for the assay is set at a 5% false positive level by using a panel of normal human sera and/or untreated patient sera representative of the groups to be treated. The results would have to be evaluated in conjunction with a confirmatory assay that evaluates those samples found positive in the screening assay. The confirmatory assay may be the same screening assay, in which excess unlabeled therapeutic protein is added to evaluate if the signal is reduced. Moreover, a more strict confirmatory cut-point is defined to make sure that only 'true' positive samples are identified as such.

One issue that complicates cut-point determination is the possible existence of pre-existing antibodies. In rare cases (e.g., cetuximab, see above) these might have clinical consequences. More often, pre-existing antibodies towards a therapeutic protein may be detected in a (small) fraction of individuals without a clear clinical impact (Xue and Rup 2013). This needs to be dealt with on a case by case basis (Gorovits et al. 2016). One common type of pre-existing antibodies that is found in most rheumatoid arthritis patients but also in a few percent of the general population is the so-called 'rheumatoid factor', low-affinity antibodies binding to the Fc portion of human IgG. There is no evidence for these antibodies being relevant in immunogenicity assessment and measures should be taken to avoid their measurement (van Schie et al. 2015b). Antibodies to PEG groups attached to proteins are another commonly observed phenomenon, although their accurate measurement is still in its infancy, hampering the evaluation of their potential risk (Krishna et al. 2015; Schellekens et al. 2013).

The assay for neutralizing antibodies is in general a modification of the potency assay for the therapeutic protein product. The potency assay is in most cases an *in vitro* cell based assay. A predefined amount of product is added to the serum and a reduction of activity is then evaluated in the bioassay. An appropriate alternative is the competitive ligand binding assay, which evaluates reduction in target binding (Finco et al. 2011). The latter type of assays is much easier to set up and validate, and may be preferred unless there is a risk of antibodies formed to the therapeutic protein that neutralize its activity via a mechanism other than preventing target binding.

ISSUES SPECIFICALLY RELATED TO MONOCLONAL ANTIBODIES

The first generation of monoclonal antibodies was of murine origin. They induced an immune response in the majority of patients, as foreign proteins should trigger a classical vaccine-type immune response. This so-called HAMA response (human antibodies to murine antibodies) was a major restriction in the clinical success of these murine antibodies. Over the years, however, methods were introduced to (fully) humanize monoclonal antibodies (cf. Chap. 8). Recombinant DNA technology was used to exchange the murine constant parts of the immune globulin chains with their human counterparts resulting in chimeric monoclonal antibodies. The next step was to graft murine complementarity determining regions (CDRs), which determine the specificity, into a human immune globulin backbone creating humanized monoclonal antibodies. The final step was the development of transgenic animals, phage display technologies and other developments allowing the production of fully human monoclonal antibodies. The assumption that human monoclonal antibodies would have no immunogenicity proved wrong. Although humanization has reduced the immunogenicity, even completely human monoclonal antibodies may induce antibodies, as illustrated in Fig. 7.3.

As discussed, multiple factors may cause the immunogenicity of human therapeutic proteins including monoclonal antibodies. One of them is aggregation. In fact, in classical studies done more than 40 years ago aggregated immunoglobulin preparations were used to break B-cell tolerance (Weigle 1971). More recently, several preclinical studies have indicated that aggregates enhance the immunogenicity of monoclonal antibodies (reviewed by Jiskoot et al. 2016). However, also mAb products containing very low amounts of aggregates may be highly immunogenic. In contrast to other recombinant human proteins, monoclonal antibodies, even when fully human, may expose foreign epitopes in their complementarity-determining regions (CDR). Analysis of several monoclonal antibodies indeed confirmed that, in most cases, foreign $CD4^+$ T cells epitopes are found primarily within the CDR sequence (Harding et al. 2010).

Monoclonal antibodies have properties that may contribute to their immunogenicity. They can activate T-cells by themselves and may boost the immune response by their Fc functions such as macrophage activation and complement activation. Indeed, removal or modification of specific sugar units from N-linked glycosyl chains from the Fc part of the immunoglobulin may reduce Fc function and lead to a diminished immunogenicity (Liu 2015).

The molecule to which an antibody binds also influences its immunogenicity. Monoclonal antibodies targeting cell bound antigens generally induce a higher level of ADA than those targeting soluble targets (Harding et al. 2010). Monoclonal antibodies directed to antigens on immune cells with the purpose of inducing immune suppression also suppress an immunological response.

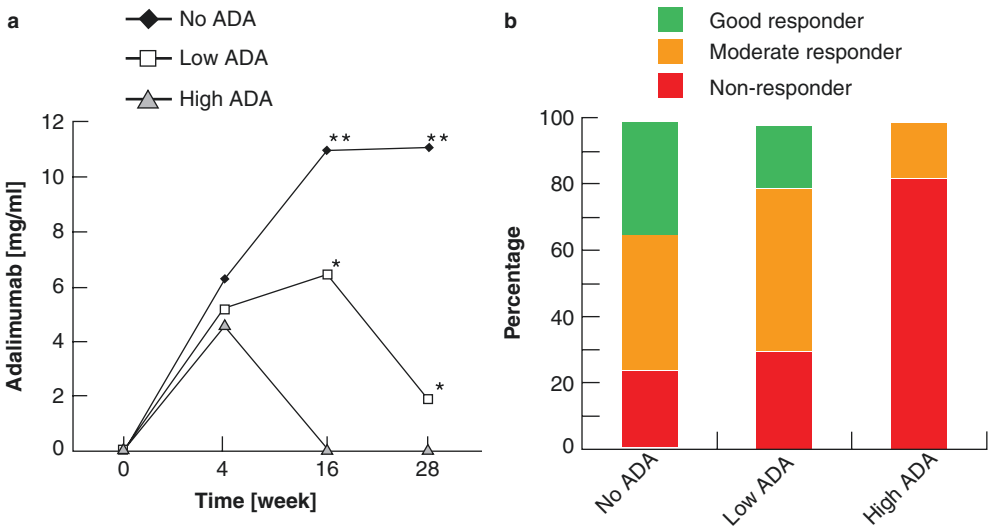


Figure 7.3 ■ Impact of immunogenicity on the serum level and treatment efficacy of fully human monoclonal antibody adalimumab. (**a**) The concentration of adalimumab in serum is altered by the presence of ADA. Low ADA titer corresponds to 12–100 AU/mL, high ADA titer corresponds to >100 AU/mL of ADA. *p < 0.05, **p < 0.01. (**b**) In patients with high ADA titers treatment is hampered to a higher extent than in patients with moderate or no ADA (figure adapted from Bartelds et al. 2011)

Although for a number of therapeutic proteins more injections and higher doses are associated with a higher immune response, for therapeutic antibodies this may not apply. In fact, lower doses and episodic treatment are usually associated with more antibody formation (Han and Cohen 2004). Although this may in part be the result of ADA assays not detecting antibodies in the presence of high therapeutic protein concentrations (see previous section), transient antibody formation has been reported for both infliximab and adalimumab, using drug-tolerant assays to measure the ADA response (van de Casteele et al. 2013; van Schouwenburg et al. 2013b). A phenomenon called 'high-dose tolerance' might be operational, where soluble antigen overwhelms the immune system.

Another important aspect when studying the immunogenicity of monoclonal antibodies is timing of the blood sampling of patients. These products may have a relatively long half-life (up to several weeks) and the circulating product may interfere with the detection of induced antibodies and may lead to false negative results. Sampling sera up to 20 weeks after the patient has received the last injection may be necessary to avoid the interference of circulating therapeutic monoclonal antibodies. Also natural antibodies, soluble receptors and immune complexes may interfere with assays and lead to either false positive or false negative results.

CLINICAL EFFECTS OF INDUCED ANTIBODIES

The list of protein products with clinically relevant immunogenicity-related side effects is growing (Schellekens 2002a; Malucchi and Bertolotto 2008). The most common consequence is loss of efficacy. Sometimes this loss can be overcome by increasing the dose or changing to another product.

Clinical consequence	Examples of therapeutic proteins
Loss of efficacy	• Animal and recombinant human (rh) insulin • Factor VIII (both natural and rh) • Rh Interferon alpha 2 • Rh Interferon beta • Rh Interleukin 2 • Human chorionic gonadotropin • Monoclonal antibodies
Neutralization of endogenous protein	• Rh megakaryocyte-derived growth factor • Epoetin
No apparent biological consequence	• Rh growth hormone • Rh insulin
General immune effects 1. Allergy 2. Serum sickness 3. Anaphylaxis 4. Injection site reactions	• Various therapeutic proteins

Table 7.2 ■ Examples of clinical consequences of immunogenicity of therapeutic proteins (adopted from Schellekens 2002a; Malucchi and Bertolotto 2008)

The most dramatic and undisputed complication occurs when the antibodies to the product cross-neutralize an endogenous factor with an important biological function. This has been described for a megakaryocyte growth and differentiation factor which induced antibodies cross-reacting with endogenous thrombopoietin (see Table 7.2). Volunteers and patients in a clinical trial developed severe thrombocytopenia and needed platelet transfusions. Because of this complication, the product was withdrawn from further development.

Another example is the upsurge of PRCA (see above) associated with a formulation change of epoetin-α marketed outside the USA. The antibodies

induced by the product neutralized the residual endogenous erythropoietin in these patients, resulting in a severe anemia that could only be treated with blood transfusions.

ADA can also influence the side effects of therapeutic proteins. The consequences are dependent on the cause of the side effects. If the adverse effects are the results of the intrinsic activity of the protein, antibodies may reduce the side effects, as it is the case with rhIFNα-2. Sometimes the mitigation of the side effects is even the first clinical sign of the induction of ADA.

With some products the side effects are caused by the ADA formation. This is in general the case when the product is administered in relatively high doses, such as with some monoclonal antibodies. Symptoms caused by immune complexes, such as delayed type hypersensitivity and serum sickness, are related to the level of antibodies induced.

The general effects caused by an immune reaction to a therapeutic protein such as acute anaphylaxis, hypersensitivity, skin reaction, serum sickness etc. are relatively common when large amounts of non-human proteins are administered. These effects are relatively rare for recombinant human proteins administered in relatively low amounts, but they are still relatively common during treatment with high doses of monoclonal antibodies.

IMMUNOGENICITY RISK ASSESSMENT AND REDUCTION

The occurrence of immunogenicity is seldom a result of a single risk factor (cf. Fig. 7.1). Rather, several factors working synergistically may trigger immunogenicity. For example, the higher immunogenicity of interferon beta 1b (Betaferon) than interferon beta 1a (Avonex) is most likely the combined result of differences in treatment regimen and product quality (Bertolotto et al. 2004). Moreover, the immune mechanisms leading to antibody induction by therapeutic proteins are still not completely understood. Consequently, based on our current knowledge it is impossible to fully predict the immunogenicity of a new product in a patient population, let alone in individual patients. However, immunogenicity risk assessment and potential mitigation strategies are required by authorities for all new products. The most commonly used tools for assessing the relative immunogenicity risk are summarized in Table 7.3.

■ Tools to Assess Immunogenicity

According to current knowledge, $CD4^+$ T-cells are key players in immunogenicity. Therefore, one of the most commonly used approaches to assess immunogenicity risk is an *in silico* analysis of $CD4^+$ T-cell epitopes, i.e., short peptides within the protein sequence capable of binding to MHC II molecules. Multiple algorithms have been developed for this purpose (reviewed by Jawa et al. 2013). Generally speaking, the more high-affinity $CD4^+$ T-cell epitopes a protein contains, the higher is the expected risk of immunogenicity. Recent developments in understanding of the immune system allowed improving *in silico* prediction algorithms. Instead of assessing the overall/total number of $CD4^+$ T-cell epitopes, nowadays epitopes for effector and regulatory $CD4^+$ T-cells can be discriminated (Cousens et al. 2014). Combined anal-

Assessment tool		Pros	Cons
In silico	• $CD4^+$ T-cell epitope prediction • Regulatory T-cell epitope prediction	1. Fast and cheap 2. Useful as a first step of immunogenicity assessment 3. Allows design or selection of less immunogenic protein variant	1. Tendency to be over-predictive 2. Results based exclusively on primary sequence 3. The quality of results depends on degree of understanding of studied process 4. Translational power limited
In vitro	• Dendritic cell uptake and activation • $CD4^+$ T-cell activation • MHC II binding	1. Relatively fast and cheap 2. Enable studying formulated products (assessing factors other than primary sequence) 3. Some assays may allow studying biological effect	1. Quality of results strongly dependent on assay format 2. Cells from a large set of representative donors required 3. Focus on isolated immune cells 4. Translational power limited
In vivo	• Non-transgenic rodents • Transgenic rodents • Non-human primates	1. (Usually) presence of complete, functional immune system 2. Immune processes similar to those in patients 3. Various factors influencing immunogenicity can be studied 4. In some models, studying biological effect possible	1. Time-consuming and expensive 2. Translational power depends on protein and animal model 3. Immune system of used model differs from that of patient to various extents (non-transgenic mice vs transgenic mice)

Table 7.3 ■ Tools used for assessing protein immunogenicity (adopted from Brinks et al. 2013; Kijanka et al. 2012)

ysis of effector and regulatory CD4+ T-cell analysis may be more reliable than analysis of total CD4+ T-cell epitopes. However, as these approaches rely exclusively on the primary sequence of the protein and do not take into consideration other factors such as protein folding, posttranslational modifications, presence of impurities and complexity of the interaction between different immune cells, their prediction power is limited especially for self(-like) proteins.

Another approach is represented by a number of *in vitro* tools, such as CD4+ T-cell stimulation and MHC II binding tests. Although, similarly to *in silico* tools, these assays do not fully represent the complexity of the immune system, they do allow assessing the impact of formulation related factors on immunogenicity risk. Thus, they can be used for validation of *in silico* results and to measure relative immunogenicity of different protein variants or different formulations of the same protein.

A third type of immunogenicity assessment tool is a set of *in vivo* models ranging from wild type and transgenic mice to non-human primates (Jiskoot et al. 2016; Brinks et al. 2013). The main benefit of *in vivo* models is that the complexity of an animal's immune system is similar to that of patients. Thus, they might be used not only to assess the immunogenicity, but also to study immune mechanisms and to provide insight into the possible clinical effects of immunogenicity.

■ Reducing Immunogenicity

Several strategies are being applied to reduce immunogenicity. At an early stage of development, one may alter the amino acid sequence of the lead candidate molecule in order to remove potentially immunogenic or aggregate prone sequences (Griswold and Bailey-Kellogg 2016). Once the drug substance has been chosen, the immunogenicity can be reduced by redesigning the formulation and/or treatment regimen. As discussed previously, improvement in quality of the first therapeutic protein products resulted in considerable reduction of immunogenicity. Many protein therapeutics, especially mAbs, are used in combination with immunosuppressant medicines. Medicines such as methotrexate, rapamicin or aziothioprine can strongly inhibit formation of antibodies, but patients might be more prone to infections or malignancy (de Mattos et al. 2015). Another strategy is an induction of specific tolerance towards the protein. It has been observed that administration of high dose(s) of factor VIII may induce tolerance in hemophilia patients with antibodies to factor VIII (Aledort 1994). Another, recently proposed strategy for specific tolerance induction is co-administration of peptides recognized by regulatory T-cells (Tregitopes) with the protein of interest. These Tregitopes activate the suppressing (or regulatory) T-cells, which may result in tolerance towards the administered therapeutic protein (Jawa et al. 2013).

CONCLUSIONS

The most important points of this chapter are summarized in the following bullet points:

- Immunogenicity of therapeutic proteins is a commonly occurring phenomenon
- The clinical consequences can widely vary
- Validated detection methods are essential to study the immunogenicity of therapeutic proteins
- The prediction of immunogenicity in patients based on physico-chemical characterization and animal studies is not easy
- There is still a lot to be learned about why and how patients produce antibodies to therapeutic proteins

The growing awareness of the importance of immunogenicity of therapeutic proteins is illustrated by the adoption of a standard requirement in regulatory dossiers for new proteins and biosimilars to evaluate their immunogenicity in clinical trials (cf. Chap. 12).

SELF-ASSESSMENT QUESTIONS

■ Questions

1. Which factors contribute to unwanted immunogenicity of therapeutic proteins?
2. What are possible clinical consequences of antibody formation against biopharmaceuticals in patients?
3. Why do aggregates of recombinant human proteins induce antibodies that cross-react with the (non-aggregated) protein?
4. Explain the fundamental difference between (a) antibody formation in children with growth hormone deficiency treated against with recombinant human growth hormone and (b) antibody formation against epoetin in patients with chronic renal failure.
5. Give an example of a case that demonstrates that the formulation of a biopharmaceutical can affect the immune response.
6. Give at least 3 approaches that can be followed to reduce the immunogenicity of a biopharmaceutical.
7. Why is standardization of assays for detection of ADA important?
8. Why are ADA titers against a monoclonal antibody more difficult to determine accurately than antibodies against interferon?

■ Answers

1. See Fig. 7.1.
2. Reduction of therapeutic efficacy, (seldom) enhancement of efficacy, anaphylactic reactions, cross-reactivity with endogenous protein.
3. Aggregates can circumvent B-cell tolerance against native (like) epitopes; the more 'native like' the aggregate, the more likely cross-reactivity of the elicited antibodies with the monomer will occur.
4. (a) is the classical immune response versus (b) circumventing B-cell tolerance.
5. The examples given in the text are epoetin and interferon α.
6. Design another formulation, remove aggregates, change the glycosylation pattern of the protein or use amino acid mutants, use human(ized) versions of the proteins or select another route of administration. NB. Some of these approaches will lead to a new drug substance, which has implications for the way authorities will judge the procedure to be followed for obtaining marketing approval.
7. Different assay formats and blood sampling schedules give different answers and thus hamper direct comparison between studies. Therefore, it is difficult to compare the results obtained with different products that are tested for immunogenicity in different labs.
8. Monoclonal antibodies are often administered in high doses and have a long circulation time (days/weeks). This will likely cause interference with the ADA assay by the circulating therapeutic antibodies (resulting in false negatives or underestimation of antibody titers). Another possibility for interference is the occurrence of cross-reactivity of the reagents in the test for the ADA and the administered monoclonal antibody. With interferon a different situation is encountered: interferons are rapidly cleared and administered in low doses (microgram range); therefore, interferons will less likely interfere with the measurement of anti-IFN antibodies.

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