

Chapter 19

Assessing the Risk of Undesirable Immunogenicity/ Allergenicity of Plant-Derived Therapeutic Proteins

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Summary

Therapeutic proteins have an intrinsic potential to induce undesirable immune and allergic responses. The nature of the expression system and the control of the manufacturing process represent extrinsic factors that could modify this potential. Accordingly, regulatory agencies require sponsors to assess the risk of clinical immune and allergic responses that could be associated with the production of therapeutic proteins in transgenic plants. Since factors related to the clinical use of the product – including the immune status and genetic background of subjects – are also relevant, the risk assessment needs to balance the probability of a response induced by a plant-specific factor relative to the likely consequences for the specific product and therapeutic indication.

Key words: Immunogenicity, Allergenicity, Risk assessment, Plant-derived biopharmaceuticals, Plant-derived therapeutic proteins, Host anti-product antibodies.

19.1. Introduction

The subject of this chapter is the production of recombinant protein-based medicinal products that are associated with a potential risk of inducing an *undesirable* host anti-product immune response. This excludes vaccines from the scope, although plant-specific glycosylation might well modify the immunogenic capacity of a vaccine – with implications for the benefit to risk profile.

The assessment of the risk of such undesirable immunogenicity is a major aspect of the regulatory assessment of therapeutic proteins, commencing in the pre-clinical phase, continuing

throughout clinical development, and then further qualified in the post-marketing setting. The most obvious clinical risk is the induction of allergic reactions mediated by IgE that is specific for plant glycans or plant-derived product impurities. But other risks might be associated with indirect effects on molecular structure and antigen presentation caused by differential (non-mammalian) processing.

It is recognised that modifications to the manufacturing process, including a change in the cell line used to express the product, may impact on the potential immunogenicity of a therapeutic protein (1). This reflects the potential of these complex molecules to undergo variable degrees of post-translational structural modification that may alter their capacity to interact with elements of the immune system.

Given the qualitative and quantitative differences that exist between plant and mammalian protein expression systems, it is considered that plant-derived therapeutic proteins could present altered glycoprotein structural motifs to induce an undesirable immune response (2). In addition, since there is less experience in the control of the manufacturing process for plant-derived proteins, particular attention will have to be given to the characterisation of product- and process-related contaminants, and their potential impact on the immunogenicity of the product.

Accordingly, the evaluation of the probability of inducing a humoral *and/or* cell-mediated immune response will represent an essential aspect of the overall risk assessment of a novel plant-derived therapeutic protein. The probability must then be balanced relative to the potential clinical consequences of an anti-product immune or allergic response, as well as the intended conditions of use (patient population, duration of exposure, route of administration, etc.) of the product and its advantages over alternative therapies.

As a cautionary note, it is important to be aware that most biopharmaceutical products have the potential to be immunogenic in at least a small population of human subjects (3, 4).

19.2. Legal Basis for Medicinal Product Registration

The legal bases in the European Union for the registration of medicinal products derived from transgenic higher plants are Regulation 726/2004 and Directive 2001/83/EC as amended. This implies a pan-European review of a marketing authorisation application via the Centralised procedure, although clinical trials remain within the jurisdiction of individual Member States

within the scope of Directive 2001/20/EC. The containment provisions of Directive 2001/18/EC also apply to commercial plant-derived medicinal products.

In the USA, medicinal products derived from bioengineered plants are regulated by Food and Drug Administration (FDA), according to the legal basis of *either* the Public Health Service Act *or* the Federal Food, Drug, and Cosmetic Act.

It is anticipated that many of the plant-derived candidate products will be new versions of already marketed biopharmaceutical products that are manufactured using mammalian cell substrates. In such cases, the principles of establishing comparability with the originator product, within the legal provisions for “biosimilar” or “follow-on protein products”, could apply (5).

These legal instruments are supported by quality, safety and efficacy guidelines that define the general scope of the data to be submitted in support of an application to market a product. Because product- *and/or* process-specific considerations often apply, actual data requirements should be discussed – typically before commencing clinical trials and then again before submission of an application for marketing authorisation – with concerned regulatory agencies.

19.3. Regulatory Guidance on Immunogenicity/Allergenicity Testing

19.3.1. Plant-Derived Products

Draft FDA guidance pertaining to plant-derived therapeutic proteins indicate that the potential risk of immunogenic and allergic responses *must* form part of the development programme for these products.

FDA Draft Guidance for Industry: *Drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals*, September 2002

This guidance explicitly directs sponsors to assess potential immunogenicity and allergenicity of bioengineered plants, including evaluation of the final product for allergenic determinants such as N-glycans.

19.3.2. Biopharmaceutical Products

The ICH S6 guideline on “*Preclinical testing of biotechnology-derived pharmaceuticals*” (6) contains helpful information on the scope of testing required. It is acknowledged that such testing is unlikely to be predictive of a clinical response, due to the inter-species differences in immune recognition. Nevertheless, it is necessary to measure the host anti-product immune

response to enable the impact on product exposure to be estimated. This should be performed as an integral part of the repeat-dose toxicology studies, using appropriately qualified analytical methods. Unfortunately, routine animal toxicology studies are not very sensitive for predicting allergic responses that occur in humans.

Guidance was recently finalised by the EU regulatory agencies to provide sponsors with a description of the risk factors to be considered as well as the methods to be applied to measure humoral and cell-mediated immune responses (7). This guidance is consistent with the multi-factorial risk approach developed by FDA (8, 9, 10). Product-specific guidelines, for example, those for monoclonal antibodies (11), should also be consulted.

An important aspect of the regulatory guidance in relation to a product comparability exercise – as would be required in the case of “biosimilar” or “follow-on protein products” – is that a *direct* comparison of the immunogenicity of the test *versus* reference product in an animal model might be required; according to the EU guideline (7), “*a clear difference in immunogenicity in the animal model would indicate non-comparability*”.

Industry “best practice” for bioanalytical methodology to measure host anti-product antibody responses is also available (12, 13). Since regulatory agencies place particular emphasis on the suitability of bioanalytical methods used to measure immunogenicity, sponsors should ensure that assay qualification and validation standards are strictly adhered to.

19.4. Strategy

An iterative immunogenicity risk assessment is an integral part of the development programme of most, if not all, biopharmaceutical products. This assessment begins in the pre-clinical phase and then continues into the post-marketing phase. The experience accumulated with the particular product, and with other products in the same class, is then used to refine the ongoing risk management plan.

During the development of a plant-derived, recombinant protein-based medicinal product, the sponsor would need to implement a non-clinical and clinical testing strategy that could effectively address a series of critical questions:

- Does the use of a plant expression system alter the intrinsic immunogenicity of the therapeutic protein relative to that expressed in a mammalian cell substrate?

- Are the manufacturing conditions adequately controlled to maintain product- and process-specific impurities within acceptable limits?
- Are the bioanalytical methods suitable to detect immune responses should these occur?
- Is the incidence and magnitude of host anti-product immune response in non-clinical models *comparable* for the plant-derived *versus* mammalian cell substrate-derived product?
- If differences in non-clinical immunogenicity are detected, could such differences impact on pharmacokinetic *and/or* pharmacodynamic parameters, and how might this affect the interpretation of the toxicology studies?
- What precautions should be incorporated into the design of clinical studies to minimise the risk of immunogenic *and/or* allergic reactions?
- Can the pivotal (Phase III) clinical studies be adequately powered to detect clinically significant immune or allergic responses, or will the risk qualification have to be deferred into the post-marketing phase?

These points are considered in the sections that follow.

19.5. Intrinsic Immunogenicity

The intrinsic immunogenicity of a biopharmaceutical product will depend on the presence of “self” and “foreign” structural motifs that are recognised by the host immune system (1, 3, 14, 15, 16).

In the case of plant-derived therapeutic proteins, non-mammalian glycosylation and the presence of contaminants that contain xenodeterminants introduces additional *theoretical* risk factors: (1) provocation of immune/allergic responses in individuals sensitised to the foreign motifs; (2) an adjuvant effect that could increase the probability that immune tolerance to a co-presenting self-antigen might be broken, resulting in an autoimmune response. It is important to emphasise that these potential risks remain unproven with respect to the parenteral administration of plant-made biopharmaceutical products. Experience with marketed biopharmaceutical products, manufactured using mammalian cell substrates that contain non-human glycosylation profiles, has revealed immune responses directed against carbohydrate motifs that have been associated with clinically-significant adverse reactions including anaphylaxis (17). In addition, there is clear evidence that contaminating proteins can increase the immunogenicity of products such as human growth hormone (18).

The impact of plant-specific glycosylation needs to be understood in terms of the interdependence of the glycan and the peptide components of a glycoprotein; intra-molecular interactions between the respective components can affect the conformation of both glycan and peptide (19). Thus, the nature of the glycan may modify the conformation of a peptide that is presented by MHC Class II molecules to T-helper cells; possibly, carbohydrate residues themselves may be directly recognised by T-cell receptors.

In the context of potential modulation of T-cell recognition of glycopeptidic epitopes according to the nature of the glycan moiety, it could be instructive to compare the potential of a glycoprotein, produced in a plant expression system and in a mammalian system, to stimulate antigen-specific human T-cells *in vitro* (20); such assays can be performed in the presence of antigen presenting cells to mimic the effect of antigen processing. A comparable stimulation index for the plant- *versus* mammalian-derived glycoprotein might then provide reassurance that intrinsic immunogenicity had not been significantly altered, although this would not preclude the need for *in vivo* testing to qualify the relative risk. Even in the case that the plant-derived glycoprotein generated a higher stimulation index compared with the mammalian-derived protein, central and peripheral tolerance mechanisms may render the clinical risk as negligible.

Should an increased risk of immunogenicity be confirmed by *in vivo* studies, and the nature of the glycosylation suspected as being the causal factor, consideration could be given to the use of an alternate plant expression system. For example, it was found that alfalfa plants can generate a monoclonal antibody bearing N-glycans that are more “human compatible” than those produced in tobacco (21); although it is recognised that the actual glycosylation pattern may be product-specific.

19.6. Control of Manufacturing Process

Even if intrinsic molecular features of a particular plant-derived therapeutic protein were not to appear to present a higher risk of immunogenicity relative to the same protein expressed in a mammalian cell substrate, a number of extrinsic factors associated with the conditions of the manufacturing process could have an important influence. These include the presence of:

- Aggregates that could cross-link/stimulate B-cell receptors via presentation of repeating motifs
- Degradation products, for example, truncated variants, or other structural modifications that present neo-epitopes

- Incorrectly folded protein that contains modified conformational epitopes
- Process-related contaminants that might act as adjuvants
- Changes in the extent of post-translational modification that modify the tendency for protein aggregation.

Accordingly, regulatory agencies will expect thorough characterisation of the product, supported by validation of the manufacturing process, to demonstrate adequate control of these extrinsic factors.

Particular emphasis will be placed on the identification and minimisation of plant-derived protein contaminants, since these could be associated with a higher risk of inducing immune or allergic responses than the therapeutic protein itself (22). There might be an expectation for sponsors to develop bioanalytical methods to detect antibody responses to potential contaminating plant-derived proteins, as well as to the therapeutic protein, for application during non-clinical and clinical testing.

19.7. Bioanalytical Methods

During the pre-clinical development phase, the sponsor will need to develop bioanalytical methods of adequate sensitivity to:

- Measure host anti-product antibody responses in the relevant non-clinical and clinical sample matrices; *and* to
- Estimate the capacity of any detected antibodies to alter the pharmacokinetic behaviour *and/or* functional activity of the biopharmaceutical product.

Anti-product antibody responses should be described in terms of:

- Incidence
- Magnitude of binding response (corresponding to the titre at the assay cut point)
- Neutralisation capacity for product and endogenous counterparts
- Timing of onset and duration
- Identity of immunoglobulin class/sub-class.

A combination of methods (primary screen, confirmatory assay, cell-based assay for neutralisation, isotyping assay, etc.) will be required to generate the requisite data (7). Alternate ligand-binding formats, for example, enzyme-linked immunosorbent assay (ELISA), multiplex assays, radioimmunoprecipitation *and/or* BIAcore, may be used.

The suitability of these bioanalytical methods must be justified by presentation of data that demonstrate:

- Specificity for the authentic drug product, related proteins and endogenous counterparts
- Benchmarking of limit of quantitation relative to an appropriate positive control antibody
- Optimisation of sample dilution factor relative to sensitivity, matrix effects and interference by residual drug product
- Validity of assay cut point to exclude false negatives whilst minimising false positives.

The most important aspect is the need to demonstrate that the primary screening assay has adequate sensitivity to enable earliest possible detection of binding antibodies that might represent a risk for longer-term administration of the drug product.

Competitive assay formats should be used to confirm that binding responses are effectively inhibited by a modest excess of the solution-phase antigen. Such assay formats can provide a rank-order estimation of binding affinity to related antigens.

Care needs to be exercised to distinguish *antigenicity* from immunogenicity: the observation that antibodies can bind to N-glycans expressed on one protein does not imply that these N-glycans will be immunogenic when present on a therapeutic protein that is administered parenterally at modest dose levels.

A major challenge for understanding the antigenicity of plant-derived glycans is the practical challenge of isolating highly purified core glycans that can be used in such competitive assays; this can seriously limit the interpretation of the specificity of anti-glycoprotein antibodies, that is, whether the antibodies recognise a carbohydrate, peptide or glycopeptide epitope.

The potential cross-reactivity of IgE antibodies demands particular attention to avoid misinterpretation of potential for induction of clinically significant allergic responses (23), as does the analysis of pre-existing antibodies arising from environmental exposure. Since product-specific IgE is likely to be present at much lower levels – possibly, by a factor of several orders of magnitude – than IgG, depletion of the IgG fraction might be necessary to eliminate competition for binding to the antigen (24).

19.8. In Vivo Immunogenicity Evaluation in Non-Clinical Models

Although the evaluation of immunogenicity in non-clinical species is unlikely to be predictive of the response of the response in humans, there could well be merit in comparing the incidence and

magnitude of the host anti-product antibody response to a given therapeutic protein that has been manufactured using alternate manufacturing methods/expression systems. Essentially, the sensitivity of the immune system to detect structural modifications in the biopharmaceutical product enables the host anti-product antibody response to serve as a surrogate “product quality” parameter.

Another approach to understand the contribution of plant N-glycans to the immunogenicity of a glycoprotein is to test the immunogenicity of homologous proteins manufactured using different expression systems. In this way, it was demonstrated that a murine monoclonal antibody produced in transgenic plants was not immunogenic in mice following parenteral administration in the presence of an adjuvant (22). Of course, this does not imply a lack of immunogenicity in other species, but such does help to scale the probability of an undesirable immune response associated with particular plant N-glycans.

Transgenic mouse models (25) might enable comparison of the probability of different product forms, for example, a cytokine manufactured using alternate expression systems, inducing a breaking of tolerance to a self-protein, although species differences in the antigen processing/presentation machinery limit absolute predictive power for the clinical scenario.

Mice bearing functional elements of the human immune repertoire, created by engraftment of human CD34+ stem cells into the tissue of new-born immunodeficient mice, offer the potential of an immunogenicity screening model that reflects human-like immune stimulation and tolerance mechanisms (26).

Allergenic potential of therapeutic proteins is not straightforward to assess using animal models, but comparison of the IgE and IgG responses to a protein in wild-type mice might offer the potential to signal an increased allergenic risk for some products (27). The limitation of this approach is that the humoral response is studied in isolation of symptomatology.

19.9. Impact on Pharmacokinetics and/or Pharmacodynamics

Understanding the potential of a host anti-product antibody response to alter the pharmacokinetics of a biopharmaceutical product is an essential aspect of the interpretation of non-clinical toxicology studies (6), since such a response could substantially limit the systemic exposure to the product during chronic administration. If neutralising anti-product antibodies are generated, these could diminish the primary pharmacodynamic response to the product, thereby resulting in a potential underestimation of the dose-limiting toxicity.

In the case of relatively high sequence homology between a human therapeutic protein and its counterpart in the animal species, host anti-product antibodies could neutralise the activity of the endogenous protein (28). Since this would represent a high-risk factor, suggestive of a potential for inducing a clinical autoimmune response, measurement of the onset and reversibility of a reduction in relevant biomarkers of the physiologic response could be important to reduce concerns about long-lasting neutralisation of an endogenous counterpart of the drug product (7).

Moving into the clinical development stage, definition of the relationship of the timing of an antibody response to an impact on biomarkers represents an important safety consideration. The early detection of an antibody response could allow withdrawal of treatment to preclude adverse reactions; although the relationship between levels of antigen-specific IgE and clinical manifestations of Type I hypersensitivity is uncertain (29). For plant-derived therapeutic proteins, particular attention will need to be given to the specificity of an immune response, that is, to determine whether this is directed to human or plant-related immunogens. Even if there are detectable responses directed toward peptidoglycan or glycan responses, these might be of much lower affinity – and therefore, of negligible clinical relevance – compared with antibodies that recognise B- or T-cell epitopes related to the human protein sequence (30). In the case of therapeutic antibodies, the differential glycosylation could have much greater significance for the Fc functionality relative to potential for immunogenicity (31).

Ultimately, the immunogenicity risk will need to be addressed by clinical evaluation.

19.10. Minimising Risk for Clinical Study Subjects

A number of extrinsic factors have been identified that may influence the probability of induction of a host anti-product antibody response (7, 16). These include:

- Route of administration
- Concomitant medication/immune competence
- Dose level and frequency
- Patient factors, for example,
 - Human leukocyte antigen (HLA) type
 - Nature of underlying disease
 - Fc_γR polymorphism.

It is important to remember that these risk factors are not independent variables.

Because biopharmaceutical products may induce immune responses that have life-threatening consequences (32), albeit in rare circumstances, the sponsor will need to design clinical studies with reference to the following considerations:

- Appropriate timing and duration of monitoring of immune response, that is, before administration, and at suitable intervals post-doing to (1) minimise interference by residual circulating drug; *and* (2) detect antibodies formed after cessation of treatment; *and* (3) monitor decline of detected antibodies
- Incorporation of validated bioanalytical methods to enable correlation between pharmacokinetic parameters, pharmacodynamic markers and antibody levels
- Treatment strategy for potential immune-mediated adverse events (infusion reactions and hypersensitivity response)
- Precautions against potential allergenic responses, for example, skin-prick testing using relevant antigens before administration of plant-derived therapeutic proteins.

It may be worthwhile comparing the potential of the product to stimulate histamine release from passively sensitised basophils collected from patients with IgE to relevant plant proteins or carbohydrates as a test of allergenic potential (33).

A number of strategies can be effective in managing adverse events arising from host antibody responses to chronic administration of biopharmaceutical products (34) in non-life-threatening indications. These include:

- Exclusion of subjects due to HLA type
- Reduced rate of infusion (or stop completely)
- Shortened time interval between infusions
- Use of pre-medication (e.g. steroid or non-sedating antihistamine)
- Concurrent immunomodulation (e.g. azathioprine or methotrexate)
- Infusion of a combination of IV fluids, antihistamines and steroids in response to adverse reactions
- Switching to another agent [e.g. an alternate anti-TNF (tumour necrosis factor) monoclonal antibody]
- Monitoring of vital signs during administration to provide early detection of infusion reactions
- Use of a dose regimen with maintenance infusions scheduled to induce tolerance.

Certainly, atopic individuals should be excluded from clinical investigational studies of plant-derived biopharmaceutical prod-

ucts, and the decision to administer immunogenic therapeutic proteins to healthy volunteers made after very careful reflection.

19.11. Pre-Authorisation Studies *Versus* Post-Marketing Risk Management

Given the multi-factorial nature of the immunogenic response to biopharmaceutical products, and the relatively low incidence of immune-function-related adverse events for currently marketed products, the power of pre-authorisation clinical studies might not be adequate to identify or enumerate the risks for the broader target population. Even the incidence of immunogenicity may not be estimable on the basis of the population size included in the pre-authorisation programme; for example, anti-rhEPO (recombinant human erythropoietin) antibodies were detected in only 8 out of 5718 (0.14%) subjects that were asymptomatic for Pure Red Cell Aplasia (35).

Consequently, regulatory agencies will require, as part of a marketing authorisation application, the presentation of an effective risk management plan that provides appropriate surveillance of the probability and consequences of the immunogenicity of biopharmaceutical products. Most commonly, this would involve the establishment of a patient registry to identify “at risk” patients/populations. In a number of cases, regulatory agencies have imposed a post-authorisation commitment to undertake an interventional study to generate additional data on the incidence of antibody responses and adverse events in a suitably sized cohort (which may depend on the incidence detected in pre-authorisation studies) for an adequate duration (e.g. 12–18 months). As associations with patient genotype become more evident, it may be logical to consider HLA typing as part of such post-authorisation studies.

For plant-derived therapeutic proteins, the major regulatory concern is likely to be the incidence of allergic responses relative to the same protein manufactured using a mammalian expression system. In the case of a “biosimilar” or “follow-on protein product”, directly comparative clinical studies may be required to demonstrate therapeutic equivalence; an increased incidence of immunogenicity or allergic reactions could represent a “major” concern that might prejudice approvability. Nevertheless, the risk that such observations represent would always need to be assessed relative to the therapeutic benefits of the product; an increased incidence of anti-product immune responses would not necessarily affect the licensing of a plant-derived therapeutic protein if the overall benefit to risk profile was acceptable.

19.12. Conclusion: Balancing Prob- ability Versus Consequences

At the present time, the probability that plant-derived therapeutic proteins for parenteral administration, prepared using manufacturing process that are subject to the rigorous controls applied to marketed biopharmaceutical products, are associated with a real increased risk of provoking undesirable immune or allergic reactions is unknown; there simply is no data from controlled clinical studies available in the public domain that would enable an estimation of the risk. Until a number of different (non-vaccine) products have completed their clinical development and registration processes, the risk remains purely theoretical.

Nevertheless, because consequences of immune-mediated responses have been documented for various biopharmaceutical products manufactured using mammalian or bacterial expression systems (3, 17, 36, 37), it is necessary to apply the cautionary principle for plant-derived products.

The prevalence of food allergies attests to the capacity of plant-derived allergens to provoke serious hypersensitivity reactions, albeit in a restricted number of individuals. But, it is questionable whether highly purified, well-characterised, plant-derived protein therapeutic agents could be associated with a similar level of risk.

The methods developed for managing the risks associated with the expression of recombinant proteins in mammalian, fungal or bacterial expression systems will be equally suitable for plant-derived therapeutic proteins.

The most important consideration is that, having accumulated an immunogenicity/allergenicity database of adequate size and relevance to the proposed use of a product to enable an estimation of the “probability” vector, the risk for public health is then moderated by a stratification of the potential and demonstrated consequences (32). If there is no significant change in the overall benefit to risk profile, economic factors might then have the overriding influence on choice of expression system.

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