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Can we prevent immunogenicity of human protein drugs?

D W Scott, A S De Groot

ABSTRACT
Monoclonal antibodies have proved to be extremely valuable additions to conventional treatment for rheumatic diseases. However, despite the general trend towards “humanisation”, these drugs remain immunogenic in clinical settings, baffling drug developers. In principle, humanised and fully human monoclonal antibodies are “self” immunoglobulins and should be tolerated. In this overview, the factors that may influence this process, the nature of immunogenicity and methods to analyse and modify potential immunogenicity are discussed. Finally, novel approaches to “re-induce” immunological tolerance to these proteins, including gene therapy and the recognition of unique regulatory epitopes, are outlined.

IMMUNOGENICITY: A PERSPECTIVE
In the context of the immune response described above, consider the factors that may contribute to the immunogenicity of human protein therapeutics. Table 1 lists the factors intrinsic and extrinsic to the protein which may be involved in eliciting an immune response.

B-cell epitopes are a required component of anti-drug immune response. In addition, the presence and total number of T-cell epitopes (and “Tregitopes”) are critically important, as will be discussed in detail later. Suffice it to say that numbers matter. Clearly, however, human protein therapeutics are mostly “self.” However, there may be important differences in the protein sequences of the drug product (due to allotypes or idiotypes), which differ substantially from T-cell epitopes, presented in the course of thymic maturation and, therefore, T cells responding to the neo-epitopes may not have been deleted in the thymus. Moreover, there may be modifications of an antibody that are introduced during the manufacturing process and packaging, leading to aggregation, for instance. Aggregates tend to be highly immunogenic because they can activate APCs and are more easily phagocytised. In addition, post-translational modifications such as glycosylation differences may contribute to immunogenicity. Degradation products, which could contribute to immunogenicity of some protein drugs, can result during scale-up or in storage.

Generally speaking, humanised antibodies targeting soluble proteins tend to be less immunogenic than those targeting membrane antigens. Through their direct action on cell membrane targets, the latter antibodies may lead to cell death and necrosis, which can create a “danger signal”, thus promoting an immune response. There are many extrinsic factors—not related to the structure of the protein itself—that contribute to immunogenicity. Some therapeutics are delivered as an intravenous bolus, whereas others are given intramuscularly. The former route favours tolerance, whereas the latter more often leads to immunity since after intradermal or subcutaneous administration, there would be drainage to a local lymph node and, potentially, some inflammatory signals from the injection site. Moreover, APCs (dendritic cells) are abundant in skin tissue, effectively transporting antigens to the follicles of lymph nodes, where B cells reside. Again, these are generalisations.

If the patient has an underlying infection (“danger” personified), in the same location where the therapeutic drug is delivered (since all immune reactions are local reactions) this could influence...
the immune response to the drug. For example, injection of indwelling catheters may be associated with immune response to therapeutic FVIII. Moreover, patients with autoimmune disease may have an underlying immune system defect that led to autoimmunity in the first place. This may then favour immunogenic responses to proteins that might not normally trigger one. In contrast, if the patient is receiving immunosuppressive therapy, this can diminish immunogenicity.

The HLA type of the patient is another extrinsic factor; HLA determines whether T-cell epitopes derived from the protein therapeutic are presented to T cells, a key step in the generation of an immune response. Thus, HLA class II molecules have a groove into which processed peptides fit, like a hot dog in a bun. To be more specific, the peptide is linear, and the amino acid side chains (R groups) bind in pockets lining the bottom and sides of the HLA “bun”. The type of pocket available for R-group binding depends on the HLA. Thus, only certain peptides, that have the right R groups, will fit into the pockets of each HLA. The R groups defining binding to a specific HLA are called motifs. Some peptides have R groups that will fit into a number of different HLA pockets; such epitopes are called promiscuous.

When looking at a given protein (antibody) sequence, each antibody therapeutic may have certain unique sequences and amino acids which can fit into HLA binding pockets. Thus, by using algorithms that evaluate protein sequences for the presence of these motifs, one can define how many epitopes exist in a given therapeutic for each human HLA class II (or class I) and whether some of these are promiscuous, meaning that they could be immunogenic in the general population. Based on the number of such HLA-binding epitopes per unit (total number of epitopes divided by the total number of amino acids) one might successfully predict that certain monoclonal antibodies or protein therapeutics are likely to be more immunogenic than others. This led to the development of an "immunogenicity scale". However, as will be noted below, this may be truer for proteins that are foreign, but for proteins that are autologous, this simple summation of epitopes does not take into account the potential role of suppressor epitopes, or T regulatory epitopes, to be discussed below.

IDENTIFYING AND MODIFYING EPITOPIES

Identification of epitopes can be accomplished by a number of methods. Originally, overlapping peptides could be synthesised and either used to immunise mice or added in vitro to stimulate responses from T cells of mice primed with the original protein; this can be an expensive and time-consuming process. Alternatively, the linear structure of a protein allows one to predict where epitopes might occur based on computer-driven searches for MHC class I and class II anchoring residues. These predictions can then be validated by in vitro binding assays, in which putative epitopes are incubated with plate-bound HLA proteins to establish a measure of binding affinity. Once peptide binding is validated, functional immunogenicity can be evaluated in several ways. In mice, animals of different strains can be immunised with whole proteins and the responses to designated peptides evaluated by T-cell assays ex vivo, for example, or the peptides themselves can be used to immunise mice. The first method relies on the protein being processed and measures immunodominant peptide recognition, whereas the latter method reflects immunogenicity itself. Because mouse and human MHC class II antigens differ greatly, it is best to do these analyses in HLA-transgenic mice. Final definition of an epitope would require confirming responses from human T cells.

After a series of likely peptides are identified and validated by several criteria, how do we use this knowledge to modulate immunogenicity? The process described below, which has been used successfully with several human protein therapeutics, is called "deimmunisation".

"DEIMMUNISATION" OF PROTEINS

Once the major epitopes have been identified, the next step takes advantage of the knowledge of which residues in a peptide are critical to "anchor" it into the MHC groove. New peptides can then be synthesised and each of these modified peptide epitopes can be assayed as described above for reactivity and immunogenicity. These residues can be targeted by site-directed mutagenesis to neutral residues that would not bind HLA but which are also not likely to significantly alter the folding and function of the modified protein. In some cases, these practical considerations may limit the number of epitopes that can be changed. Thus, the approach is to target promiscuous epitopes as the top priority. Once these steps are taken, a new recombinant protein can be produced which would lack the critical residues deemed likely to immunise the largest number of patients. Figure 1 shows the basic protocol for this approach.

PRINCIPLES OF TOLERANCE INDUCTION

Modification of residues to deimmunise a protein does not make it tolerogenic: it simply allows it to be ignored by the immune system. There are other circumstances in which one may need to induce tolerance to the native protein. This may be because deimmunisation might require modification of critical amino acid, such as those that are involved in the targeting function of the protein, or that contribute to its three-dimensional structure. Alternatively, it may be necessary to pre-treat an individual receiving an immunogenic product. Finally, tolerance protocols need to be developed to reverse ongoing and deleterious immune responses, such as in specific autoimmune diseases or after an immune response to a therapeutic product (eg, FVIII in haemophilia A).

During the past several decades, a variety of approaches have been developed for the induction of tolerance in experimental animals. Some of these approaches have progressed to clinical trials. Our goal herein is not to cover this area of research, which has been extensively reviewed elsewhere. Rather, the fundamental principles favouring tolerance will be outlined, after which we will focus on approaches developed in our laboratory and those of our collaborators. As noted in the introductory section, the route of immunisation with a protein significantly influences its immunogenicity and tolerogenicity. An intravenous injection favours tolerance, whereas subcutaneous/intramuscular injection leads to direct uptake by dendritic cells. These cells travel to the lymphoid follicles, where antigens are processed, and immune responsiveness

| Table 1 Factors affecting immunogenicity of human protein therapeutics |
|--------------------------|--------------------------|
| **Intrinsic** | **Extrinsic** |
| Self or non-self (polymorphisms) | HLA of patient |
| Presence of T-cell epitopes (absence of regulatory epitopes) | Route of administration |
| Formulation/packaging/aggregation | Underlying infection or pathology |
| Post-translational modifications | Immunosuppression or medication |
| Soluble or membrane target | |

Supplement
follows. In contrast, intravenous antigen is taken up by APCs in the spleen, primarily B cells. Oral introduction of antigens also can lead to a form of unresponsiveness due to processing of antigens in the gut immune system. Rather than reflecting a lack of responsiveness, this route may deviate the response toward mucosal immune responses like IgA formation. Alternatively, if co-stimulation is blocked (e.g., by CTLA-4-Ig or antibodies to CD80/CD86), anergy may ensue. The principle involved is to provide “signal 1” (T-cell epitopes in MHC) to the T cell in the absence of “signal 2” (co-stimulation). This approach has been widely employed in transplant models but has not been successful for other applications. Additional methods are based on treatments to block or subvert T-cell signalling using either antibodies to the CD3 co-receptor or drugs like rapamycin that inhibit downstream signalling pathways. Clinical trials of drugs employing these approaches are also in progress.

Recently, the use of so-called “tolerogenic” APCs has gained popularity. The primary focus has been on tolerogenic immature dendritic cells (iDCs) that are pulsed with target antigens. Such iDCs express low amounts of CD80/CD86 and thus provide little co-stimulation. Maintaining this immature phenotype in vivo is an important caveat. Another approach has been to use B cells as tolerogenic APCs. Like iDCs, naïve B cells are low in co-stimulatory molecules. In contrast, even mature or activated B cells that express high levels of CD80/CD86 may still be tolerogenic under certain circumstances. We have taken advantage of these properties and combined gene therapy with the use of B cells as tolerogenic APCs. This approach has been successful in a variety of mouse and rat models for autoimmune diseases and haemophilia (see below and table 2). This approach is now moving forward to proof of concept in non-human primates.

NOVEL TOLERANCE METHODS: FROM GENE THERAPY TO TREGITOPES

Over a decade ago, our laboratory took advantage of the fact that B cells were tolerogenic APCs and combined the use B cells with immunoglobulin fusion proteins as tolerogens. Immunoglobulins, especially IgG subclasses, had long been known to be excellent tolerogenic carriers. This was based initially on their long half-life, ability to equilibrate throughout the body and to cross-link inhibitory Fc receptors. Therefore, we engineered antigens of interest in frame with an IgG heavy chain to create a platform for tolerance. The fusion protein itself was shown to be tolerogenic, a point we shall return to. The construct for this fusion immunoglobulin could then be retrovirally expressed in stem cells and B cells, which induced tolerance when given systemically to recipient animals. The hope was that the transduced B cells would be a factory for the fusion protein in vivo, thus providing a long-term source of the tolerogenic proteins. This was not entirely correct! We later found that very little protein was actually secreted. Rather, it was more critical for the B cells to express MHC class II molecules so that peptides from this fusion protein could be presented by these B cells. In addition, regulatory T cells were induced by this tolerogenic B-cell presentation of epitopes. Importantly, the presence of the IgG heavy-chain scaffold enhanced the ability of these fusion protein transduced B cells to be tolerogenic.

At this point, we began a collaboration with EpiVax based on their identification of a unique class of peptides in IgG molecules. They had discovered these peptides during their epitope mapping and deimmunisation programmes with human protein (especially IgG) therapeutics. They observed that all IgG subclasses contained epitopes in constant regions that were predicted to have strong binding to MHC class II, were promiscuous and conserved in multiple mammalian species. Yet, these epitopes did not appear to be immunogenic! They predicted that these epitopes might be regulatory—that is, they are recognised by Treg cells, and they coined the name “Tregitope”.

Further experimental data provided evidence that these Tregitopes would not only activate FoxP3-expressing T cells, they would also suppress effector T-cell reactions in vitro and in vivo. These studies have been performed with both human and mouse T-cell responses and go a long way towards explaining the efficacy of intravenous immunoglobulins as immunosuppressive agents (see Ephrem et al; Kaveri et al).

Thus, we now can envisage a new generation of protein therapeutics comprising a target epitope or protein domain fusion to these Tregitopes. This now reframes our picture of...
what makes a protein immunogenic. It is in part the sum of the epitopes, but those must now include the presence of Tregitopes (fig 2). The addition of Tregitopes may foster new tolerogenic therapeutics. The deletion of those Tregitopes may provide better vaccines for immunisation.

CONCLUDING REMARKS
A number of therapeutic protein developers have already incorporated in silico, ex vivo and in vivo preclinical immunogenicity screening protocols into their product development strategy. Mapping and modulating the epitopes contained in recombinant autologous and therapeutic proteins may reduce the chance that a biological agent will induce a T-cell mediated immune response. However, T-cell epitopes should not all be considered dangerous—in some cases, T-cell epitopes can be associated with a regulatory T-cell response. Regulatory T cells have the potential to help develop and maintain tolerance. Thus, means to identify T-cell epitope “friends” (Treg epitopes) and “foes” (effector epitopes) in the context of protein therapeutics and autoimmunity will enable us to harness these important mediators of immune response, enabling progress towards improved human health outcomes. Further new treatments to treat the responses to therapeutic development are in progress.

**REFERENCES**


**Table 2** Summary of applications using transduced B cells for tolerance

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</tr>
<tr>
<td></td>
<td></td>
<td>Block transfer of disease</td>
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