Therapeutic proteins: room for improvement?

A novel, highly directed approach has been developed to overcome the problems of immunogenicity associated with therapeutic proteins.

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Protein-based drugs represent the cornerstone of the biotechnology industry. In 1997 peptide, recombinant and natural therapeutic proteins achieved worldwide sales of approximately US$17 billion (1). Proteins are attractive candidates for therapeutic use because they are the key molecules in all biological processes – exhibiting powerful, defined actions achieved through natural selection. Additionally, the advent of recombinant DNA technology has ensured the potential for unlimited production of almost any protein. However, there are disadvantages associated with therapeutic proteins which must be overcome if these drugs are to completely fulfil their potential (1). Proteins are large molecules whose structural complexity must be maintained to avoid compromising their function. This imposes limitations on the form and delivery mode of a drug. Their susceptibility to enzymatic digestion can preclude oral delivery; thus, to maintain effective circulating levels, frequent injection or continuous intravenous infusion is often necessary. However, large systemic doses of proteins may cause adverse reactions including immune responses.

Immunogenicity of existing therapeutic proteins

The administration of therapeutic proteins – even human ones – can cause immunogenicity. In the case of a protein deficiency disorder, the reason can be straightforward; the replacement therapy introduces a protein foreign to the host immune system and, not unexpectedly, an immune response ensues. However, an alternative explanation is required when proteins which already exist *in vivo* are used therapeutically. Normally, the human immune system is tolerant to physiological levels of human proteins, many of which have specific locations within the body. Therapeutic proteins are usually administered in high systemic doses. It is possible that, when human proteins are introduced *in vivo* at higher than physiological levels and in areas where they do not normally occur, this immunological tolerance may be broken leading to immunogenicity. Any neutralising antibodies produced can reduce or eliminate the effectiveness of the protein. Immunogenicity can preclude repeated administration of a drug, as the secondary immune response can be much more severe and may lead to anaphylaxis.

A number of therapeutic proteins have shown a clear indication of immunogenicity (Table 1) and it is notable that the production of neutralising antibodies can compromise the patient's response to treatment (Table 1, Refs 2 and 3).

Haemophilia A is an X-linked inheritable disease caused by reduced levels or the absence of coagulation factor VIII. Administration of exogenous factor VIII causes the production of anti-factor VIII antibodies in 30-40% of haemophilia A patients. To manage this clinically, tolerance to the protein

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Table 1. Therapeutic proteins which have shown an indication of immunogenicity

Protein	Indication	Immunogenicity	Reference
Insulin	Insulin-dependent diabetes	1. 78% (n = 9) of non-diabetics not previously exposed to insulin produced antibodies to semisynthetic insulin. 2. 100% (n = 7) non-diabetics not previously exposed to insulin produced antibodies to human insulin.	7 8
Interferon β1-a (IFN β1-a)	Relapsing/remitting multiple sclerosis	24% (n = 560) patients receiving 22μg IFNβ1-a produced neutralising antibodies.	9
IFN α	Hairy cell leukaemia	51% (n = 31) patients produced antibodies which neutralised recombinant IFN α *in vitro*. 37% of these patients showed clinical resistance to treatment.	2
IFN α	Chronic myeloid leukaemia	22% (n = 67) patients produced neutralising antibodies, of whom 73% were unresponsive to treatment. Of the 78% not producing antibodies, only 21% were unresponsive to treatment.	3
Granulocyte-macrophage colony stimulating factor (GM-CSF)	Metastatic colorectal carcinoma	95% (n = 20) patients produced GM-CSF binding antibodies. 47% of these individuals produced antibodies which neutralised biological activity of GM-CSF	10

is artificially induced by daily infusions of large doses of factor VIII over months to years (immune tolerance induction or ITI). Once tolerance is achieved, it is usually lifelong – but this is obviously an expensive way of reducing the incidence of immunogenicity. The cost of employing factor VIII to induce tolerance in a five-year old haemophiliac is estimated at US$ 1 million (4). However, costs must not only be measured financially, but also emotionally, as prolonged treatment of this nature is potentially very distressing to a child.

Despite the success of therapeutic proteins over a number of years, immunogenicity is still a significant problem and there is room for improvement to ensure these drugs are safer and more clinically effective.

Improving the clinical characteristics of therapeutic proteins

Protein-based therapy has been well established, first with the naturally-occurring products, and more recently with their recombinant counterparts. Developments in DNA/protein technology now mean that proteins can be re-engineered to enhance desirable clinical characteristics without detriment to protein structure or function. For example, proteins may be engineered to improve stability, circulatory half-life, potency, receptor-binding affinity and efficacy. The potential clinical benefits may include lower and less frequent dosage as well as more "patient-friendly" modes of delivery.

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Examples of the differing approaches to re-engineering proteins include those of the biotechnology companies Maxygen and Applied Molecular Evolution (AME). Maxygen employs DNA shuffling of gene fragments to produce novel recombined genes, whilst AME uses its patented gene synthesis to alter an initial gene to create a library of diverse genes. Proteins are expressed and the variants screened for improvements in desirable characteristics whilst avoiding structural perturbation.

Enhancement of the characteristics of a therapeutic protein may prove to be clinically beneficial. However, the production of an altered version of an existing protein may result in the creation of new T (and B) cell epitopes, so that these re-engineered molecules constitute a new class of potentially immunogenic proteins. It is therefore surprising that few technologies exist which can eliminate immunogenicity in order to generate a safer, more effective therapeutic protein.

Eliminating immunogenicity

PEGylation is a method which has attempted to eliminate immunogenicity in therapeutic proteins. Polyethylene glycol (PEG) is a neutral, water-soluble non-toxic polymer which has a functional hydroxyl group at either end of the molecule to which proteins can be covalently attached. In order that the final therapeutic molecule does not become cross-linked, one of the –OH groups is removed to form monomethoxy PEG (mPEG). In aqueous solution,

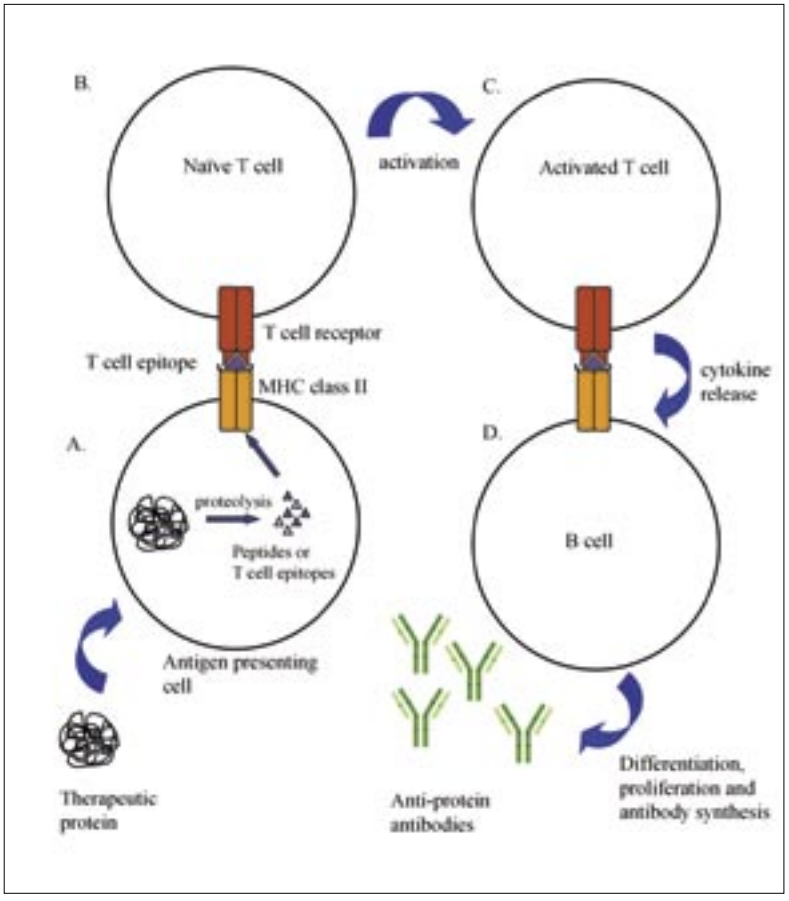

Figure 1. The importance of T cell epitopes in the generation of a mature antibody response. The administered therapeutic protein is taken up by an antigen-presenting cell (APC) and is proteolytically digested (A). Some of the peptides (T cell epitopes) are expressed on the surface of the APC in conjunction with the MHC class II molecule and can be recognised by the T cell receptor on a naïve T cell (B). This activates the T cell (C), which subsequently releases cytokines to drive B cell differentiation into a plasma cell (D). Antibodies directed against the therapeutic protein are secreted by the B cell.

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the PEG molecule becomes heavily hydrated and is a dynamic structure which sweeps out its exclusion volume, thus resisting the approach of other molecules. This apparently reduces immunogenicity by blocking the access of antibodies to B cell epitopes within the protein molecule. Additionally, PEGylated proteins exhibit an increase in half-life and reduction in immune clearance which contribute to improved efficacy.

Enzon Inc has shown that PEG-Intron A (PEGylated IFNα) can be administered weekly instead of the three-times-weekly doses of the non-PEGylated form normally used. The approach is not consistent, however, since PEGylated thrombopoietin (TPO) induces the production of neutralising antibodies in some patients, and this has led to the discontinuation of clinical trials involving PEGylated-TPO in the US (5). A more directed strategy is desirable to successfully and reliably eliminate immunogenicity in therapeutic proteins.

Biovation has developed a novel technology, based on immunological principles, to directly address the issue of immunogenicity caused by therapeutic proteins. To understand this, it is necessary to have an appreciation of how an immune response to a foreign protein is generated (Figure 1). A therapeutic protein can be taken up by an antigen-presenting cell (APC), such as a dendritic cell. Peptide fragments are generated by proteolytic degradation of the protein. Some of the peptides can bind to major histocompatibility complex (MHC) class II molecules, and are referred to as T cell epitopes. The MHC II-bound peptides are then presented on the surface of the APC where they can be recognised by naïve T cells. Activation of the T cells leads to the secretion of cytokines and the differentiation of B cells to antibody-producing plasma cells. Thus, T cell epitopes are essential to the stimulation of a mature antibody response, and removal of these epitopes from a therapeutic protein can eliminate immunogenicity. Biovation has developed a toolbox of technologies specifically to identify and remove T cell epitopes from a protein whilst maintaining its structure and function.

DeImmunisation: identification of T cell epitopes

A combination of three methods is used to identify T cell epitopes within a therapeutic protein.

Peptide Threading is an in *silico* approach which identifies peptides which bind to MHC class II molecules (also known as human leucocyte antigen or HLA). A number of the three-dimensional structures of the numerous HLA molecules have been solved by X-ray crystallography and, in conjunction with homology modelling, this has enabled the structures of different MHC alleles to be predicted. Peptides bind to MHC class II molecules via a specific binding cleft which has pockets radiating from it to accommodate the amino acid side chains (Figure 2). To identify all potential T cell epitopes within a given protein sequence, overlapping 13mers are assessed for binding within the MHC binding cleft and a binding score for each peptide is calculated.

Peptide-MHC Binding is an *in vitro* method which uses a collection of human cell lines carrying a repertoire of different MHC class II alleles. Typically, synthetic peptides from protein sequences are tested for displacement of control biotinylated peptides. Following cell lysis, MHC class II molecules are immunoprecipitated and tested for peptide

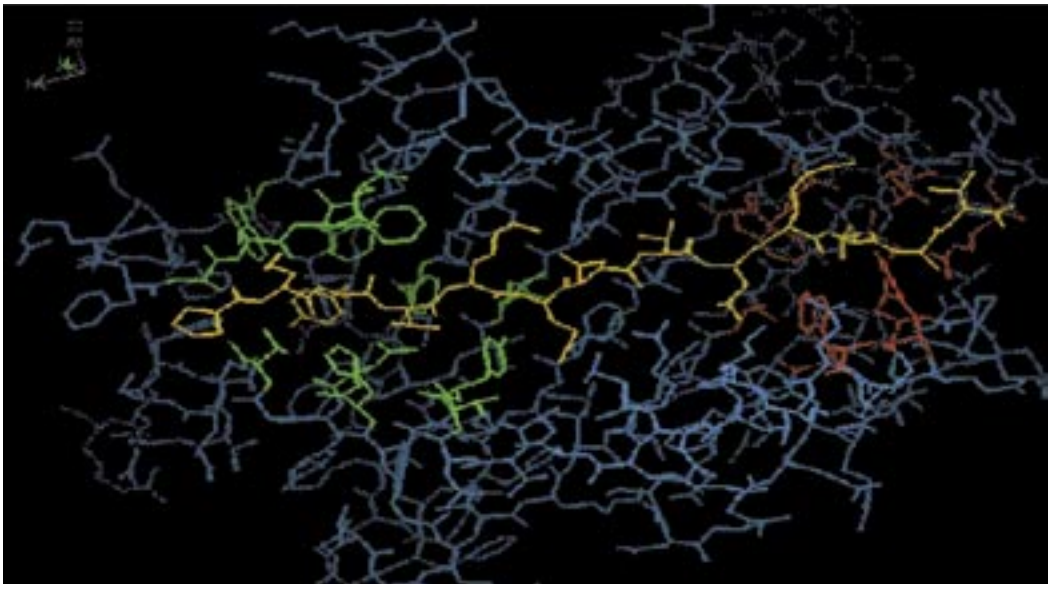

Figure 2. Computer-generated image of the MHC class II binding groove in complex with a peptide. The MHC class II molecule is depicted in blue and the peptide backbone in yellow. The residues of the five MHC class II binding pockets which interact with the side chains of the complexed peptide are shown in green and red.

binding using avidin-enzyme conjugates. Peptide-MHC binding data shows an excellent correlation with peptide threading and provides concise data for a wide range of MHC allotypes.

The Human T Cell Proliferation Assay involves the generation of overlapping peptides from a whole protein sequence; these are then tested for their ability to stimulate the proliferation of T cells. Blood taken from naïve donors is fractionated into components containing antigen-presenting cells and T cells and test peptides are added. If the peptide binds to MHC on the APC and is recognised by the T cell receptor, T cell activation and proliferation occurs, which can be measured by tritiated thymidine uptake.

The combination of these techniques allows the accurate identification of peptide sequences within a protein which are capable of binding to MHC class II antigens and stimulating T cell proliferation *in vitro*.

DeImmunisation: elimination of T cell epitopes

Once a T cell epitope is identified ... it can usually be eliminated by a single amino acid substitution

Once a T cell epitope is identified by the techniques described, it can usually be eliminated by a single amino acid substitution. There is an inherent flexibility within the method which makes it possible to eliminate a potential T cell epitope by substituting any one of the amino acids which bind to the five main anchoring pockets of the MHC-peptide binding cleft. Epitopes encompassing part of an active site or functionally critical region of the protein can usually be removed by substitution of adjacent residues where they fall into one of five anchor positions for the peptide-MHC binding interaction. In some instances, conservative substitutions can be made with reference to a homologous protein or structural model in order to maximise the likelihood of maintaining functional activity.

DeImmunisation: proof of the concept

The scientific principles which underpin DeImmunisation make it an attractive theoretical concept – the ultimate form of humanisation. It was essential to validate the concept practically. Biovation chose to DeImmunise staphylokinase, a small protein with profibrinolytic activity, produced by certain strains of *Staphylococcus aureus*. It is used as a thrombolytic agent to treat acute myocardial infarction and ischaemic stroke. Therapeutic administration of staphylokinase causes production of antibodies, some of which may have neutralising activity and compromise the efficacy of the drug (6). Biovation has demonstrated that a DeImmunised form of staphylokinase does not induce human T cell proliferation *in vitro*, thus providing further support for the technology.

DeImmunisation has been further corroborated by *in vivo* murine studies. A human antibody was DeImmunised with respect to murine MHC II alleles. The resulting antibody which had DeImmunised variable regions combined with murine constant regions did not elicit an immune response when administered to mice.

The ultimate validation of the technology can only be provided by clinical administration of a

DeImmunised antibody in humans. J591 is the first DeImmunised antibody to enter clinical trials. This antibody is specific for prostate specific membrane antigen (PSMA) and is in Phase I/II clinical trials for the treatment of prostate cancer. So far, over 35 patients treated have shown no evidence of immunogenicity.

Conclusion

There is no doubt that proteins are important in disease therapy for a broad range of indications. Despite their success in different diseases, new methods are being applied to improve their clinical potential. However, some of the methods can result in the introduction of new, potentially immunogenic sequences in these proteins. Even though immunogenicity is known to be a major obstacle to clinical success, limited work has been done to address the issue. Biovation has developed a novel, highly directed approach aiming to solve this problem, and indeed proof of concept studies and the results of early clinical trials suggest a firm validation of the technology.

Tehmina Amin gained her PhD in 1996 from the University of Kent (Canterbury, UK) whilst working on the production of monoclonal antibodies with novel specificities for use as probes to investigate protein folding and assembly in ***Escherichia coli.*** *She spent a short period working on the functional analysis of genes in* ***Saccharomyces cerevisiae*** *before undertaking a postdoctoral position at the University of Aberdeen involving the construction of phage display antibody libraries to identify novel auto-antigens in recurrent miscarriage. Dr Amin made the transition into business development by taking up a position at Biovation Ltd in August 2001.*

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The ultimate validation of the technology can only be provided by clinical administration of a DeImmunised antibody in humans

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