Self/Nonself 1:4, 314-322; October/November/December 2010 © 2010 Landes Bioscience

Immunogenicity of protein therapeutics
The key causes, consequences and challenges

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Key words: immunogenicity, deimmunization, protein therapeutic, T cells, neutralizing antibody

The immunogenicity of protein therapeutics has so far proven to be difficult to predict in patients, with many biologics inducing undesirable immune responses directed towards the therapeutic resulting in reduced efficacy, anaphylaxis and occasionally life threatening autoimmunity. The most common effect of administrating an immunogenic protein therapeutic is the development of a high affinity anti-therapeutic antibody response. Furthermore, it is clear from clinical studies that protein therapeutics derived from endogenous human proteins are capable of stimulating undesirable immune responses in patients, and as a consequence, the prediction and reduction of immunogenicity has been the focus of intense research. This review will outline the principle causes of the immunogenicity in protein therapeutics, and describe the development of pre-clinical models that can be used to aid in the prediction of the immunogenic potential of novel protein therapeutics prior to administration in man.

Introduction

The expectation that 'self' derived protein therapeutics (comprised of human germline sequence), such as recombinant human cytokines and 'human' antibodies, will avoid immunogenicity due to central tolerance is clearly flawed. There are now many examples of recombinant proteins (e.g., IFNβ[1-3] IFNα[4,5] GM-CSF[6] and human anti-TNFα[7,8] antibodies) which stimulate host immune responses that are directed against the therapeutic. **Table 1** summarizes the frequency of anti-therapeutic antibodies (obtained from package inserts detailing clinical trial and post-approval data) observed against a number of FDA-approved biologics. Generation of anti-therapeutic antibodies involves stimulation of multiple components of the immune system, and therefore the immunogenicity of protein therapeutics cannot necessarily be attributed to a single factor. Indeed, stimulation of both adaptive (exemplified by the development of high affinity, highly specific antibodies and long lasting lymphocyte 'memory') and non-adaptive (often mediated by innate receptors which does not confer long-lasting protective immunity to the host) immune responses are normally involved in the development of a highly specific humoral response such as those directed against protein therapeutics. Such responses are normally polyclonal, and can have both a neutralizing and non-neutralizing effect on protein therapeutics. Anti-therapeutic antibodies that are detected in the serum of patients can comprise multiple isotypes (IgM, IgG and IgE) and sub-classes (IgG1-4) of heavy chain constant regions. In many instances such antibodies possess variable regions that bind with high affinity to the protein therapeutic, and will therefore have undergone somatic hypermutation of variable region genes. The ability to neutralize the protein therapeutic is a product of the B cell epitope(s) against which the humoral response is directed. For example, in the case of antibody therapeutics, human anti-mouse (HAMA) or human anti-human (HAHA) responses directed against the idiotype are typically neutralizing, and such responses have been observed for both humanized and fully-human antibodies.[9,10] For protein therapeutics that are derived from endogenous proteins that serve a non-redundant function (e.g., recombinant human erythropoietin), a neutralizing antibody response can cross-react with the endogenous protein resulting in morbidity and mortality.[11]

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Submitted: 08/10/10; Revised: 09/20/10; Accepted: 10/07/10
Previously published online:
www.landesbioscience.com/journals/selfnonself/article/13904
DOI: 10.4161/self.1.4.13904

Development of Immune Responses Directed Against Protein Therapeutics

Initial events that trigger the development of immune responses against protein therapeutics may occur independently of $CD4^+$ T-cell help. Such events can involve innate receptor activation (e.g., pattern recognition receptors, PRR) resulting in the stimulation of antigen presenting cells (APC), such as dendritic cells (DC) as well as B-cell subsets (reviewed in ref. 12–14). The involvement of innate receptors expressed on APC will greatly facilitate the development of a potent adaptive immune response.[15-19] It is possible that the biophysical properties of the protein therapeutic, such as glycosylation, as well as excipients that may be present in the drug and/or formulation could provide the initial stimulation via PRR on DC, resulting in effective maturation and expression of lymphocyte co-stimulatory receptors.[20,21] DC stimulated via PRR have an increased capacity to stimulate T cells and consequently support the generation of T-dependent (antigens that stimulate these responses are known as T-cell or thymus-dependent antigens) high affinity anti-therapeutic antibody response. Indeed, the biological activity of the

Table 1. FDA approved (a) antibody therapeutics (adapted from http://www.fda.gov/BiologicsBloodVaccines/ucm133705.htm) showing the level of reported immunogenicity observed in patients from prescribing information available at http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm

Antibody name	Company	Type	Target	Indication(s)	Reported immunogenicity
Muromanab (OKT3)	Ortho Biotech	Murine	CD3	Allograft rejection	25% (24)
Abciximab (Reopro)	Centocor (Johnson & Johnson)	Chimeric Fab	GPIIb/IIIa	PTCA adjunct	6%–44% (36)
Rituximab (Rituxan)	Genentech (Roche)/Biogen Idec	Chimeric	CD20	Non-Hodgkin lymphoma	11% (2578)
Daclizumab (Zenapax)	Hoffman LaRoche	Humanized	IL2R	Transplant rejection	14–34%
Trastuzumab (Herceptin)	Genentech (Roche)	Humanized	Her2/neu	Breast cancer	<1%
Palivizumab (Synagis)	MedImmune (Astra Zeneca)	Humanized	RSV F	RSV prophylaxis	0.7%–2% (1002–639)
Basiliximab (Simulect)	Novartis	Chimeric	IL2R	Transplant rejection	1–2% (138–339)
Infliximab (Remicade)	Centocor (Johnson & Johnson)	Chimeric	TNFα	RA/Crohn	10–15%
Arcitumomab (CEA-scan)	Immunomedics	Murine	CEA	Colorectal cancer	<1% (3/400)
Canakinumab (Ilaris)	Novartis	Human	IL-1β	Cryopirin-associated periodic syndrome	0% (64)
Fanolesomab (Neutrospec)	Palatin Tech.	Murine	CD15	Imaging for appendicitis	0–16.6% (30–54)
Imciromab (Myoscint)	Centocor (Johnson & Johnson)	Murine	Myosin	Cardiac imaging for MI	<1% (914)
Capromab (Prostascint)	Cytogen	Murine	PSMA	Prostate cancer diagnostic	8%–19% (27–239)
Nofetumomab (Verluma)	Boehringer Ingelheim	Murine	40 KDa glycoprotein	Detection of SCLC	6% (53)
Gemtuzumab (Mylotarg)	Wyeth Pharma (Pfizer)	Humanized	CD33	Acute myeloid leukemia	0% (277)
Alemtuzumab (Campath)	Ilex Pharma (Genzyme)	Humanized	CD52	B cell chronic lymphocytic leukemia	1.9–8.3% (133–211)
Ibritumomab (Zevalin)	Idec Pharma (Biogen Idec)	Murine	CD20	Non-Hodgkin lymphoma	1.3% (446)
Adalimumab (Humira)	Abbott	Human	TNFα	RA/Crohn/PsA/JIA/Ankylosing spondylitis/plaque psoriasis	2.6%–26%
Omalizumab (Xolair)	Genentech (Roche)	Humanized	IgE	Asthma	<0.1% (1723)
Efalizumab (Raptiva)	Genentech (Roche)	Humanized	CD11a	Psoriasis	6.3% (1063)
Tositumomab (Bexxar)	GSK	Murine	CD20	Non-Hodgkins lymphoma	11% (230)
Cetuximab (Erbitux)	Imclone (Eli Lilly)	Chimeric	EGFR	Colorectal cancer	5% (1001)
Bevacizumab (Avastin)	Genentech (Roche)	Humanized	VEGF	Colorectal, breast, renal and NSCL cancer	0% (~500)
Panitumumab (Vectibix)	Amgen	Human	EGFR	Colorectal cancer	4.6% (613)
Ranibizumab (Lucentis)	Genentech (Roche)	Humanized	VEGF	Macular degeneration	1–6%
Eculizumab (Soliris)	Alexion Pharma	Humanized	C5	Paroxysmal nocturnal hemoglobinuria	2% (196)
Natalizumab (Tysabri)	Biogen Idec	Humanized	α-4 integrin	MS & Crohn	9% (627)
Golimumab (Simponi)	Centocor (Johnson & Johnson)	Human	TNFα	RA/PsA/Ankylosing spondylitis	4% (1425)
Cetolizumab pegol (Cimzia)	UCB	Humanized	TNFα	RA/Crohn	8% (1509)
Ofatumumab (Arzerra)	GSK	Human	CD20	CLL	0% (79)
Ustekinumab (Stelara)	Centocor (Johnson & Johnson)	Human	IL-12/IL-23	Plaque psoriasis	3–5% (743–1198)
Tocilizumab (Actemra)	Genentech (Roche)	Humanized	IL-6R	Rheumatoid arthritis	2% (2876)
Denosumab (Prolia)	Amgen	Human	RANKL	Osteoporosis	<1% (8113)

The frequency of anti-therapeutic antibody responses (both neutralizing and non-neutralizing antibodies) observed in patients is shown as % and the size of the patient group evaluated in immunogenicity studies given in brackets.

Table 1. FDA approved (b) other biologics (adapted from http://www.fda.gov/BiologicsBloodVaccines/ucm133705.htm) showing the level of reported immunogenicity observed in patients from prescribing information available at http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm (continued)

Biologic name	Company	Type	Target	Indication(s)	Reported immunogenicity
Prolastin	Talecris biotherapeutics	PD	α1-proteinase inhibitor	α1-antitrypsin deficiency	None reported
Aralast	Baxter Healthcare	PD	α1-proteinase inhibitor	α1-antitrypsin deficiency	None reported
Zemaira	Aventis Behring (CSL Behring)	PD	α1-proteinase inhibitor	α1-antitrypsin deficiency	None reported
Kogenate FS	Bayer (Bayer Schering Pharma)	rHu	Factor VIII	Hemophilia A	15%
ReFacto	Genetics Institute (Wyeth)	rHu	Factor VIII	Hemophilia A	30%
Zyntha	Wyeth (Pfizer)	rHu	Factor VIII	Hemophilia A	2.2% (89)
NovoSeven	NovoNordisk	rHu	Factor FVII	Hemophilia	<1%
Benefix	Wyeth (Pfizer)	rHu	Factor IX	Hemophilia B	3%
ATryn	GTC Biotherapeutics	rHu	Anti-thrombin	Thromboembolism	None reported
BabyBIG	California Department of Health Services	PD	Botulism Immune Globulin Intravenous Human	Infant botulism	
Berinert	CSL Behring	rHu	C1 Esterase Inhibitor	Angioedema	
Cinryze	Lev Pharmaceuticals	rHu	C1 Esterase Inhibitor	Angioedema	
Rhophylac	CSL Behring	PD	Rho(D) Immune Globulin	ITP	0% (447)
Evithrom	OMRIX Biopharmaceuticals	rHu	Thrombin, Topical	Coagulation	3.3%
Recothrom	ZymoGenetics	rHu	Thrombin, Topical	Coagulation	1.2–1.5%
Wilate	Octapharma	PD	von Willebrand Factor	Coagulation	1.5–3%
Cerezyme	Genzyme	rHu	ß-glucocerebrosidase	Gacher Disease	15%
Exenatide or Byetta	Amylin Pharmaceuticals/Eli Lilly	R	Glucagon Like Peptide-1	Type II diabetes	6%
IntronA	Schering Corp (Bayer Schering Pharma)	rHu	IFNα	Leukemia, Kaposi sarcoma, hepatitis B/C	<3–13%
Betaseron	Bayer Schering Pharma	rHu	IFNβ	Multiple sclerosis	16.5–25.2%
NovoLog	NovoNordisk	rHu	Insulin analog	Type II diabetes	Transient antibodies
Leukine	Genzyme	rHu	GM-CSF	Preventing infection in cancer	2.3%
NEUPOGEN (Filgrastim)	Amgen	rHu	G-CSF.	Preventing infection in cancer	3%
Retavase	PDL Biopharma	rHu	TPA	Myocardial infarction, pulmonary embolism	0% (2400)
Humatrope	Eli Lilly	rHu	Growth hormone	Dwarfism	1.6%
Adagen	Enzon Pharmaceuticals	Bovine	ADA Adenosine deaminase	Inherited immunodeficiency	Not reported (SCID)
Pulmozyme	Genentech (Roche)	rHu	DNase I	Cystic fibrosis	2–4%
Procrit	Amgen	rHu	EPO	Anemia in chronic renal disease	
Proleukin	Novartis	rHu	IL-2	Oncology	<1%

Recombinant human (rHu), plasma derived (PD) and recombinant (r) products are indicated. Companies that acquired a majority stake holding in any of the companies that performed the initial drug development of the biologics listed are shown in brackets

protein therapeutic can itself have an adjuvant effect resulting in high levels of immunogenicity in patients.[6] One possible explanation for immunogenicity associated with GM-CSF treatment (to help prevent infection during cancer therapy) is the fact that GM-CSF has an 'adjuvant effect' and efficiently activates DC, monocytes and lymphocytes. In contrast a G-CSF a similar size molecule also used in the treatment of cancer but with no immunomodulatory activity is non-immunogenic.

T-independent stimulation of B cells [antigens that stimulate these responses are known as T-cell or thymus-independent (T-independent) antigens] in which the protein therapeutic bypasses the need for T-cell help may occur if the protein forms a multimeric structure that can effectively cross-link the B-cell receptor (BCR) to an extent where co-stimulation from T cells is not required for an anti-therapeutic antibody response.[22] It is envisaged that aggregated proteins could act in a manner similar to that described for T-independent type 2 antigens,[23] via the polyclonal activation of splenic marginal zone B cells (MZ B cells). However, a number of factors regarding humoral responses against T-independent type 2 antigens do not support

this hypothesis, including the fact that T-independent type 2 antigens are highly ordered protein structures (e.g., viral coat proteins) which enables the presentation of B-cell epitopes in a regular (5–10 nm) and repeating manner. Such regular repeating epitopes are critical for the activation of MZ B cells.[24,25] Whether such organized protein structures are formed in aggregates that may be in formulations of protein therapeutics is at present unclear. The involvement of T cells also cannot be ruled out for T-independent type 2 antigens, since MZ B cells can efficiently present antigen to T cells.[25] Stimulation of splenic MZ B cells in humans is characterized by production of IgM, and is not sufficient alone to induce class switch recombination to multiple IgG subclasses which are commonly observed in human immune responses against protein therapeutics.[25]

There is, however, a link between aggregated or multimeric proteins and enhanced immunogenicity. Studies have shown that aggregated protein therapeutics can stimulate enhanced humoral immune responses in a variety of models. Braun et al.[26] showed that aggregated, but not monomeric, recombinant human IFNα was able to break tolerance and result in the generation of IFNα specific antibodies in human IFNα transgenic mice. A more detailed study of the immunogenicity of recombinant human IFNα aggregates with different physiochemical properties in human IFNα transgenic mice showed that maintenance of a 'native'-like confirmation was associated with more immunogenic aggregates.[27] The precise mechanism behind the influence of aggregates in the immunogenicity of protein therapeutics has not been defined, but earlier studies on antibody feedback regulation in the immune system provide an interesting view of how antibodies that form immune complexes with soluble or particulate antigen are able to influence the progression of an immune response. Administration of IgG to mice in combination with particulate antigen suppressed the humoral response,[28] whereas combined administration of IgM and IgG3 enhanced antibody responses.[29] Suppression by IgG is likely to be dependent on masking B-cell epitopes and suppression is dependent on antibody affinity,[30,31] engagement of FcγRIIb and/or enhanced phagocytic clearance of immune complexes.[32-35] By contrast enhancement of responses by IgM and IgG3 to antigens administered at sub-optimal doses in mice is dependent on fixing C1q of the classical complement cascade, since mice treated with cobra venom factor or lacking CR1/CR2 are impaired in their ability to mount an enhanced antibody response.[36-38] A single IgM molecule can activate complement, and mouse IgG3 has the capacity to spontaneously multimerize to allow binding of C1q resulting in the subsequent activation of C3.[39] Since it is possible to produce both IgM and IgG3 (in mice only) independently of T cells it is feasible that these isotypes are an important early trigger in initiating the first stages in the immune response cascade against preparations of protein therapeutics that may contain very low levels of aggregates. It should also be noted that certain antigens (e.g., derived from bacterial membranes) are effective in activating the alternative complement cascade in the absence of immune complexes and may also ultimately result in fixing C3d and effectively opsonising the protein for uptake by macrophages via C3d receptors (CR2).[40]

The generation of immune complexes against both particulate and soluble antigens as well as immune complex independent activation of the alternative complement cascade will lead to enhanced uptake by APC via FcR and complement receptors (CR1 and CR2). Getahun et al. have shown[41] that transfer of D011.10 transgenic T cells into syngenic Balb/C mice that were immunized with IgG2a anti-TNP/TNP-Ova (without adjuvant) resulted in an enhanced T cell and Ova specific antibody response. These observations are consistent with an increase in presentation of T-cell epitopes to T-cells via enhanced uptake and processing through the APC via FcR-mediated uptake of immune complexes. Drug formulations are known to influence the conformation of protein therapeutics, such as multimerization and aggregation (reviewed in ref. 42). It is therefore possible that initial activation events involve the interaction of antibodies with the protein therapeutic to form immune complexes, and subsequent triggering of immune response suppression or enhancement is dependent on the conformation of the protein therapeutic and the binding of antibodies with specific isotypes. It should be noted that in naive patients, the amount of protein specific antibody is likely to be very low, although even small amounts of multimeric IgM binding to aggregated protein will be sufficient to activate complement. Pre-existing IgG antibodies in patients might be specific for a protein therapeutic that is derived from an endogenous counterpart, for example a recombinant human cytokine. Such antibodies may be produced as a result of incomplete central tolerance whereby low endogenous levels of protein expressed in specialized tissues will not facilitate deletion of auto-reactive B- and T-cell clones during lymphocyte development. Indeed transient and non-pathological autoimmune responses have been detected in healthy individuals and are probably suppressed by mechanisms involving peripheral tolerance such as regulatory T cells (Treg cells). For example, the development of anti-FVIII specific antibodies in healthy individuals has been frequently observed, and is thought to be produced as a result of tissue damage and activation of the clotting cascade.[43,44] These responses have no obvious detrimental effect on healthy individuals presumably due to their transient appearance. Peripheral tolerance may be broken by treating patients with high doses of protein, particularly for chronic diseases, which will enable the production of antibodies specific for the protein therapeutic. Indeed the specificity of the humoral response may change during treatment as a result of B-cell epitope spreading, and this will be dependent on T-cell help which can be enhanced by the initial non-neutralizing antibody response allowing more efficient uptake of the protein by APC, and consequently the presentation of T-cell eptiopes.

Antigen Presentation and T-Dependent Responses

The sequence of events leading to the generation of a T-dependent humoral immune response against a protein therapeutic probably involves rapid induction of a T-independent response leading to expansion (1,000-fold) of epitope specific B cells that can increase the probability of a cognate interaction with $CD4^+$ T cells.[45] In parallel to this process, antigen will be phagocytosed

by professional APC such as DC. If the therapeutic is injected sub-cutaneously, immature 'steady state' DC in the epidermis (e.g., Langerhans cells) will phagocytose and process the protein via the MHC class II processing pathway. For immature DC, activators or 'danger signals' that include proinflammatory factors, such as complement and chemokines as well as bacterial or viral products (LPS, CPG motifs and dsRNA), can enhance the activation of DC.[46-48]

One key function of immature DC is antigen capture and this can occur via a number of different receptors including members of the C-type lectin family (DEC-205, DCIR, Dectin-2 and CLEC-1), the immunoglobulin superfamily (DORA and ILT3), heat shock protein receptors, toll-like receptors (e.g., TLR-3 expressed on immature DC), and Fc receptors (FcγR and FcεR). As discussed above, one important factor in enhancing DC capture of therapeutic proteins may be the early formation of immune complexes with circulating IgM or IgG, which has been established to result in potent antibody responses in mice, and involves activation of complement and FcR binding. For example, in an experimental model the co-administration of IgG/KLH complexes results in a 1,000-fold enhancement (compared to KLH alone) in the IgG anti-KLH response.[49] There is at least some circumstantial evidence for this in humans whereby small circulating immune complexes have been detected in patients receiving technetium-labelled infliximab.[50] The presence of these immune complexes correlated with rapid uptake of infliximab in the liver and spleen, which is facilitated through interactions between antibody, FcR and complement-complement receptors.[51] Thus, for protein therapeutics, initiation of anti-therapeutic antibody response may be dependent on the formation of immune complexes that can bind to APC through complement receptors or via the FcR.

Evidence that T-cell help is a central component of the pathway that results in antibody responses against protein therapeutics comes from a number of observations including the prominence of high affinity responses comprising multiple IgG isotypes, such as those observed against infliximab, adalimumab and FVIII.[52,53] Indeed, T-cell subset polarization has been linked to the success of FVIII therapy in severe hemophiliacs where the predominance of Th2-driven IgG4 FVIII-specific antibodies correlates with the production of high inhibitor (neutralizing anti-FVIII antibodies) titres.[54] By contrast, patients with FVIII inhibitors receiving immunosuppressive therapy have a predominance of Th1 driven IgG1 and IgG2 FVIII antibodies which are associated with a more successful therapeutic outcome.[55] In some instances, T cells or T-cell help is targeted pharmacologically during treatment with protein therapeutics in order to treat the disease and/or reduce the immunogenicity of the protein therapeutic. The most frequent methods include the use of immunosuppressive therapies (such as methotrexate when administering infliximab for treating rheumatoid arthritis) or induction of neonatal tolerance, which is a primary strategy in reducing the incidence of inhibitors against FVIII (reviewed in ref. 56). It should be noted that other methods to reduce immunogenicity have also been adopted for therapeutic antibodies, such as infliximab, that induce peripheral tolerance where an inverse dose-immunogenicity relationship has been observed with high concentrations of antibody resulting in a reduction in the incidence of immunogenicity.[57] One interesting method for inducing peripheral tolerance that may eventually be applied in a therapeutic context is the administration of proteins via a mucosal surface. Preliminary studies in mice with human IFNα and IFNβ have shown that anti-IFNα and IFNβ IgG responses are inhibited by oromucosal administration of the proteins.[58] Induction of tolerance via this route is dependent on a unique class of 'tolerizing' DC and regulatory T cells (which suppress immune responses), which further highlights the importance of T cells in the development of anti-therapeutic antibodies.[59]

There has been considerable effort to establishing the role of MHC class II-restricted T-cell epitopes present in protein sequences and the development of humoral immune responses against protein therapeutics. Several studies have directly measured in vitro $CD4^+$ T-cell responses against protein therapeutics (or peptide derivatives). Such studies have shown that many therapeutic proteins (including recombinant human proteins) contain potent T-cell epitopes.[60-64] Stickler et al.[65] used human in vitro T-cell assays to show the importance of T-cell epitopes in the immunogenicity of human IFNβ1b where the antibody response to deimmunized IFNβ1b (in which T-cell epitopes were mapped and removed by amino acid mutation) was compared in Balb/C mice. The immunogenicity of IFNβ1b was ameliorated by removing T-cell epitopes from the protein sequence. Moreover, removal of a single immunodominant but not a sub-dominant T-cell epitope, was sufficient to avoid an immune response.[60] In a similar in vitro study the immunogenicity of human IFNβ (Rebif®) was attributed to the efficient uptake and processing of IFNβ1a by monocyte-derived DC in different clinical buffer formulations.[66] Thus for recombinant human IFNβ1a, the formulation may influence protein conformation, leading to the possible development of protein multimers that can be efficiently engulfed by DC which may result in enhanced presentation of T-cell epitopes to $CD4^+$ T cells. Analysis by in vitro T-cell assays revealed that the new Rebif® formulations were significantly less immunogenic than the existing formulation. These results were confirmed after a phase IIIb clinical trial with one new formulation that induced significantly fewer neutralizing anti-IFNβ antibodies in patients treated with the new formulation compared to standard Rebif®.[67,68]

Models for Predicting Immunogenicity

Traditional animal models used in safety and toxicological studies for small molecule drugs are of limited use in the assessment of the potential for immunogenicity of protein therapeutics in humans, especially for human proteins where the animal will not be tolerant to these proteins. Differences in antigen processing and MHC class II binding between humans and in-bred strains of rodents will also influence the repertoire of T-cell epitopes presented to T cells and consequently affect the immunogenicity of the protein therapeutic. In order to address some of these limitations, novel transgenic mice and xenograft transplantation models have been generated.

One of the more difficult issues with regard to studying the mechanisms behind immunogenicity using animal models is the fact that proteins administered in the absence of adjuvant are inherently non-immunogenic, indeed this observation was termed 'immunologists' dirty little secret' by Charles Janeway.[69] Nevertheless, this has not hampered attempts to generate in vivo models that can be used to predict the immunogenicity of protein therapeutics. Mice expressing specific human HLA allotypes (and lacking endogenous mouse MHC class II) are frequently used for research to evaluate the involvement of human HLA alleles in diseases such as allergy and autoimmunity.[70,71] Such models may be particularly useful where immune tolerance against a specific recombinant therapeutic or class of therapeutics is induced by either transgenic expression of the protein of interest or induction of tolerance during neonatal development.[72,73] There are significant technical challenges associated with developing such multi-feature transgenic mice including: restricted diversity in HLA alleles (typically alleles would be selected to cover the main MHC class II supergroups),[74] inbred stains of mice express restricted TCR repertoire, knocking out the murine homolog of the protein therapeutic whereby the endogenous murine gene and the human transgene may not always serve the same function in mice.

Human immune system xenografts offer a promising alternative to transgenic mouse models. Such models are based on immunodeficient (including lack of endogenous NK cells) NOD *scid IL2Rγ*$^{-/-}$ or Rag2$^{-/-}$γc$^{-/-}$ mice in which human immune systems are transplanted by engraftment of human CD34^{+} stem cells.[75,76] CD34^{+} stem cells typically derived from human peripheral blood mononuclear cells (PBMC) or from human umbilical vein are engrafted into neonatal mice. Differentiation of the human CD34^{+} stem cells results in the population of various lineages in different immune compartments including lymphocyte, monocyte/myeloid, granulocyte and dendritic cell. The fact that these mice express human MHC class II and are tolerant to human immunoglobulins (or do not recognize human immunoglobulins as "foreign") may enable their use as a valuable in vivo model for the prediction of the immunogenicity of antibody therapeutics (e.g., humanized and/or fully human antibodies). There are however limitations in using these engraftment models including the fact that they are not tolerant against all human proteins, that there is no germline transfer of genes encoding human immune cells so that each mouse has to be engrafted on an individual basis, and that some studies have shown considerable variability in immune responses to antigens/factors that stimulate potent responses in humans.[77]

In addition to mouse models, non-human primates have commonly been used as a test system to evaluate the potential for immunogenicity of protein therapeutics. However, non-human primate MHC molecules are significantly different from the human equivalent making them less suited for use as models for immunogenicity. Indeed, studies using a marmoset model of experimental autoimmune encephalitis showed that, although the disease itself closely mimicked that found in humans, the peptide T-cell epitope driving the autoimmune response was different from that identified in humans.[78] Moreover, the marmoset MHC class II molecule involved was of a lineage not found in humans. The fact that human protein therapeutics may still be significantly different from the endogenous primate homolog, coupled with the ethical issues involved with using non-human primates also significantly limit the utility of non-human primates as a model for immunogenicity in the clinic.

The generation of an anti-therapeutic antibody response in patients appears to correlate with the ability to stimulate epitope-specific human T-cell responses.[79] Consequently tools to aide researchers in measuring CD4^{+} T-cell epitopes in both a quantitative and qualitative manner have been developed.

For high throughput screening of sequences in silico peptide-MHC class II algorithms have been developed that predict the ability of a peptide to bind to MHC class II. In addition to the advantage of rapidly screening large numbers of sequences these methods are considerably less expensive than traditional in vitro MHC class II binding assays which are time consuming and expensive. Current in silico tools are also reasonably accurate in terms of predicting MHC class II binding peptides against a large number of HLA alleles. The major limitation common to all methods of MHC class II binding analysis (in silico and in vitro) is the high level of false positive peptides that are identified as binding MHC class II but which fail to stimulate T-cell responses both in vitro and in vivo. Over-prediction is largely due to the inability of in silico tools to take into account other factors that influence the formation of epitopes, such as protein/peptide processing, recognition by the T-cell receptor (TCR), and T-cell tolerance to peptides.[80]

In vitro T cell culture systems offer the advantage of a direct measurement of antigen specific activation of T cells and are able to therefore overcome some of the limitations, in particular over-prediction, observed with in silico methods. PBMC are typically used as a source of CD4^{+} T cells for in vitro T-cell assays and these are stimulated with whole proteins or peptides derived from therapeutic proteins using a variety of in vitro culture formats. In vitro T-cell priming responses can be detected in healthy donors; furthermore there is evidence showing that the repertoire of T-cell epitopes recognized in non-primed healthy donors is represented in the primed patient population PBMC.[61,62,81,83-87] This has enabled T-cell epitope maps to be generated using healthy donors that are relevant to the patient group.[43,61-63] Data from in vitro T-cell assays, such as the number and potency (immunodominance) of T-cell epitopes, can be used to determine the relative risk of immunogenicity between multiple variant therapeutic proteins during pre-clinical development. In addition, strategies can be employed to reduce immunogenicity by removing T-cell epitopes through targeted amino acid substitutions (reviewed in ref. 88). Accurate detection of T-cell epitopes using in vitro T-cell assays requires selection and testing of peptides against large cohorts of individuals to ensure that a broad spectrum of MHC class II allotypes are present. The recent development of methods to store large well characterized banks of HLA-typed individuals has facilitated the judicious selection of cohorts of donors for studies which closely represent the frequency of the major MHC class II allotypes expressed in the world population. Qualitative and quantitative measurement of T-cell epitopes by in vitro

T-cell assays has enabled strategies to remove T-cell epitopes (such as deimmunization) to be developed. Such strategies optimally rely on the use of in vitro methods for epitope identification combined with in silico tools for the design and selection of mutant peptides that can be incorporated into optimized T-cell epitope-depleted protein sequences which have reduced capacity for MHC binding.

Reducing Immunogenicity by Design

Depleting T-cell epitopes from protein therapeutics (by deimmunization) has proven to be successful and has lead to a number of deimmunized proteins progressing into clinical trials. The majority of these deimmunized protein therapeutics are antibodies, and none of these proteins has elicited any significant level of undesirable immunogenicity in patients to date. One of the more clinically advanced deimmunized antibodies targets prostate specific membrane antigen, and several clinical trials have been performed using this antibody (conjugated to radiolabels or the toxin DM1) with no anti-therapeutic antibody responses observed in any patient.[89-92] Overall, this clinical data provides support for the importance of T-cell epitopes in the generation of anti-therapeutic antibody responses and indicate that deimmunized protein therapeutics may provide a safer less immunogenic class of new biologic.

The way forward for dealing with issues associated with immunogenicity in protein therapeutics is likely to involve a combined approach whereby, new molecules can be generated through rational sequence design (via methods such as deimmunization) and where the lead proteins are also tested in the appropriate animal model and/or in vitro assay during pre-clinical development from which the least immunogenic leads can be selected. Testing of other product-specific triggers of immune responses should be considered including: appropriate physicochemical analysis of the therapeutic; aggregation, post-translational modifications, and effects of excipients that may be present in the final product formulation. Such a combined approach will undoubtedly facilitate the generation of protein therapeutics with reduced potential for immunogenicity.

Acknowledgements

The authors would like to acknowledge the kind assistance of Drs. Robin Thorpe, Laura Perry, Frank Carr and Tim Jones in proof reading this manuscript.

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