ELSEVIER

Immunogenicity of biotherapeutics in the context of developing biosimilars and biobetters

Maria D.F.S. Barbosa[1]

CA Consultants, PO Box 2390, Iowa City, IA 52244, USA

Issues concerning the approval of biosimilars are currently being addressed by the US Food and Drug Administration and the European Medicines Agency. There appears to be a consensus that immunogenicity impacts comparability studies and the interchangeability of biosimilars. In addition, preclinical immunogenicity assessment and mitigation, if validated in clinical studies, might impact patient safety and development costs, and also facilitate the development of 'biobetters' and other protein therapeutics. This review addresses recent advances in the field of biosimilars and focuses on predictive immunology, with an emphasis on preclinical immunogenicity assessments of protein therapeutics other than vaccines and their corresponding clinical outcomes.

Introduction

'Man-made miracles' have been largely associated with either small-molecule drugs or protein therapeutics. The latter represent a class of drugs that could be used to treat a wide spectrum of diseases, from microbial infections to cancer and arthritis. The high costs of these effective protein drugs have been the subject of debate [1–3] and are generally attributed to the preclinical development costs of innovative products; the difficulties associated with their production and the maintenance of standards during development and marketing; and the absence of other effective treatment options. Immunogenicity could complicate either preclinical or clinical development [4–6]. Hence, effective ways of assessing and mitigating immunogenicity from early preclinical development stages onwards could impact patient safety and development costs.

The immunogenicity of protein therapeutics can cause hypersensitivity responses, anaphylaxis, anaphylactoid reactions, infusion reactions, decreased efficacy of the drug, among other consequences [4]. Activated B cells produce antibodies of five major classes, namely immunoglobulin (Ig) E, IgM, IgG, IgD and IgA. IgE has a role in immediate hypersensitivity reactions, whereas IgM accounts for a smaller percentage of the serum Ig and is the predominant 'early antibody', as it usually represents a major proportion of the primary antibody response; and IgG is the major Ig in serum, representing most of the secondary response. This review focuses on the adaptive immune responses resulting in antibody formation against protein therapeutics other than vaccines. Such anti-drug antibody (ADA) responses could affect the efficacy and/or safety of protein therapeutics and/or complicate interpretation of the toxicity, pharmacokinetic and pharmacodynamic data [5]. Safety concerns have been often associated with neutralizing ADA (nADA) [4].

Some antigens can activate B cells without major histocompatibility complex (MHC) class-II restricted T cell help. However, sustained antibody responses to most antigens depends upon T and B cells recognizing the antigen in a linked fashion [7,8]. For T-cell-dependent antibody responses, following uptake and processing by antigen processing cells (APC) such as dendritic cells, antigenic peptides are displayed at the APC surface, bound to MHC class II molecules. Peptides bound to the MHC proteins form complexes with T cell receptors, leading to the activation of T cells and, ultimately, B cell differentiation and antibody production [7,8]. The term 'epitope' is commonly used to designate peptides that bind to MHC proteins and/or elicit a T cell response. T cell activation also requires interactions between costimulatory molecules, such as CD28 and CD80/CD86 [7]. Some of the steps leading to T cell activation have been used for the development of assays to predict immunogenicity. In addition to genetic component, factors such as impurities, glycosylation and

E-mail address: mbarbosa@cacobio.com.

[1] Present address: Bristol-Myers Squibb, Princeton, NJ, USA.

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2011.01.011

the aggregation state of the protein might affect the antibody response [7,9].

Immunogenicity assessment and mitigation is a complex subject encompassing various methodologies. For clarity, those approaches are divided here into three major classes: (i) direct methods are defined as the clinical measurement of immune responses, including antibody responses against the protein drug; (ii) indirect methods are defined as technologies that measure and/or mitigate factors that might be implicated in immunogenicity, such as aggregation, impurities and subvisible particles; and (iii) predictive methods are those that attempt to mimic human immune responses to protein therapeutics, either *in silico*, *in vivo* or *in vitro*. The currently available *in vivo* predictive tools include transgenic animal models (for example expressing human leukocyte antigen (HLA) proteins or a given human therapeutic protein). The *in vitro* predictive tools include binding assays to identify epitopes and T cell activation assays. *In silico* epitope prediction methods are also available.

The expiration of patents for several therapeutic proteins has prompted much interest in logical paths for the development of biosimilars (in the USA, the term 'follow-on biologics' has also been used). 'Biosimilar' can be defined as a biotherapeutic similar to another one already marketed. One interesting aspect of the process is that biosimilars could offer the unique possibility of testing preclinical immunogenicity assessments in clinical settings. In addition, advances in assessing and mitigating immunogenicity in the context of biosimilars could be applied for the development of 'biobetters' (newer versions of marketed biotherapeutics, engineered for improved properties), of other protein therapeutics and also for comparability testing following manufacturing changes. This review provides highlights of the approval process for biosimilars in the USA and Europe, the associated immunogenicity concerns and the *status quo* of predictive immunology.

Approval of biosimilars in Europe

The European Medicines Agency (EMA) has released guidelines regarding '...a new biologic medicinal product claimed to be 'similar' to a reference medicinal product...' (Box 1). In addition, it has also provided concept papers and specific guidelines for the development of similar medicine products that contain recombinant proteins, such as erythropoietins, insulin, somatropin, interferon (IFN)-α, low-molecular-weight-heparins, granulocyte-colony stimulating factor (G-CSF) and follicle stimulating hormone and IFN-β (Box 1). Although much progress has been made and biosimilar products have been approved by EMA following reliable and rigorous evaluation, their bioequivalence and interchangeability is still a matter of debate [10–12].

Approval of biosimilars in the USA

In March 23, 2010, President Obama signed into law the Patient Protection and Affordable Care Act (PPAC). The US Food and Drug Administration (FDA) has a record of expertise regarding the comparability of protein therapeutics [6,13] and the quotes in the following two paragraphs provide some information regarding the approval of biosimilars in the USA. They were sourced from http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/ucm215089.htm (accessed January 27, 2011). The PPAC Act:

BOX 1

EMA guidelines on similar biological medicinal products

(2005) Guidelines on similar biological medicinal products (http://www.ema.europa.eu/pdfs/human/biosimilar/043704en.pdf).(2006) Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substances: quality issues (http://www.ema.europa.eu/pdfs/human/biosimilar/4934805en.pdf).(2006) Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: nonclinical and clinical (http://www.emea.europa.eu/pdfs/human/biosimilar/4283205en.pdf).(2007) Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003947.pdf).(2010) Concept paper on the development of a guideline on similar biological medicinal products containing monoclonal antibodies (http://www.ema.europa.eu/pdfs/human/biosimilar/63261309en.pdf).(2010) Guideline on similar biological medicinal products containing monoclonal antibodies (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/11/WC500099361.pdf).(2010) Guideline on immunogenicity assessment of monoclonal antibodies intended for *in vivo* clinical use (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/11/WC500099362.pdf).

EMA concept papers and guidelines for the development of similar medicine products that contain recombinant proteins

(2010) Guideline on nonclinical and clinical development of similar biological medicinal products containing recombinant erythropoietins (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/04/WC500089474.pdf).(2006) Annex to guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: nonclinical and clinical issues. Guidance on similar medicinal products containing somatropin (http://www.ema.europa.eu/pdfs/human/biosimilar/9452805en.pdf).(2006) Annex to guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: nonclinical and clinical issues. Guidance on similar medicinal products containing recombinant human soluble insulin (http://www.ema.europa.eu/pdfs/human/biosimilar/3277505en.pdf).(2006) Annex to guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: nonclinical and clinical issues. Guidance on similar medicinal products containing recombinant granulocyte-colony stimulating factor (G-CSF). (http://www.ema.europa.eu/pdfs/human/biosimilar/313905en.pdf).(2008) Guideline on similar medicinal products containing recombinant interferon alpha (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003931.pdf).(2010) Concept paper on similar biological product containing recombinant interferon beta (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/04/WC500089210.pdf).(2010) Concept paper on similar biological medicinal products containing recombinant follicle stimulation hormone (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/04/WC500089208.pdf).(2008) Guideline on similar biological medicinal products containing low-molecular-weight heparins (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003928.pdf).

. . .'amends the Public Health Service Act (PHS Act) to create an abbreviated approval pathway for biological products that are demonstrated to be 'highly similar' (biosimilar) to or 'interchangeable' with an FDA-approved biological product. These new statutory provisions also may be referred to as the Biologics Price Competition and Innovation Act (BPCI Act) of 2009.

Immediately after the new statute was enacted, FDA formed a working group to plan the agency's approach to implementing the statute in order to ensure that the process of evaluation, review and approval of products within this newly-defined product category, will be achieved in a consistent, efficient and scientifically sound manner.

Paths for the approval of biosimilars and biobetters: immunogenicity concerns

Immunogenicity could hinder the development of new protein drugs and complicate the assessment of the comparability of biotherapeutics [6,14]. Risk-based strategies have been proposed for addressing immunogenicity issues often associated with the development of protein therapeutics [15]. According to the risk-based approach, recombinant erythropoietin (EPO) should be considered high risk, as it is has a nonredundant endogenous counterpart that mediates a unique function. Incidences of anti-EPO nADA have been implicated in the development of pure red cell aplasia (PRCA) [4,6]. Challenges regarding comparability issues with EPO were first addressed by the FDA in connection with Eprex® (erythropoietin-α) [6]. More recently, five biosimilars to Eprex® were approved by EMA [16].

The EMA regulations for the approval of biosimilars include the requirement for the demonstration of comparable clinical efficacy and safety ['The Marketing Authorisation (MA) application dossier of a biological medicinal product claimed to be similar to a reference medicinal product already authorized shall provide a full quality dossier. Comparable clinical efficacy and safety has to be demonstrated'; Box 1]. The comparability decisions for Retacrit® (epoetin zeta), a biosimilar to Eprex®/Erypo®, encompassed nonclinical and clinical testing and is used here as an example of the EMA decision-making process regarding immunogenicity issues with biosimilars [16].[a] The applicant presented a clinical immunogenicity report with 12-month data on 227 patients with renal anemia and a later update on 585 patients, and the size of that database was considered sufficient [16].[a] Serum samples for ADA determination were obtained before dosing and during the safety studies. Anti-EPO antibodies were tested with a validated radioimmunoprecipitation assay. A low incidence of ADA was observed in patients treated with either Retacrit® or the reference product. Moreover, in both cases, the patients were already ADA-positive before administration of the epoetin formulations. None of the ADA-positive patients showed indication of PRCA. Although testing for nADA was not required, a qualified and validated nADA was available for postmarketing testing.[a]

Regarding preclinical immunogenicity testing of therapeutic proteins, although a recent EMA document does not specifically determine the value of predictive immunology, its use is suggested ('Evolving *in vitro* and *in vivo* technologies, e.g. transgenic mouse models may be useful for evaluating the potential immunogenicity of a given protein product'; Box 1). However, without clinical data to validate preclinical assessments, it is generally difficult to know whether those predictions are relevant. The scarce available clinical data that compare preclinical predictions with clinical outcomes, which are reviewed below, stress the need for more studies that might validate the predictive tools.

Determination of interchangeability or substitutability for biosimilars

Immunogenicity could be a key factor affecting the determination of interchangeability and/or substitutability for biologics. The following is a statement by Janet Woodcock, Deputy Commissioner, Chief Medical Officer, FDA before the Committee on Oversight and Government Reform, US House of Representatives, on March 26 2007 [6]:

A finding by the agency that a follow-on protein product may be approved as safe and effective is distinct from a determination that the follow-on protein product would be substitutable for the referenced protein product. To establish that two protein products would be substitutable, the sponsor of a follow-on product would need to demonstrate through additional clinical data that repeated switches from the follow-on product to the reference product (and vice versa) would have no negative effect on the safety and/or effectiveness of the products as a result of immunogenicity. For many follow-on protein products – and in particular, the more complex proteins – there is a significant potential for repeated switches between products to have a negative impact on the safety and/or effectiveness. Therefore, the ability to make determinations of substitutability for follow-on proteins products may be limited.'

Direct methods for immunogenicity assessment: ADA responses and clinical outcomes

ADA responses to protein therapeutics might be of no consequence, or might alter pharmacokinetics and/or pharmacodynamics and/or cause adverse clinical effects. Possible deleterious consequences of immune responses to protein therapeutics have been reviewed, and might include hypersensitivity reactions, (IgE-mediated) anaphylaxis, anaphylactoid reactions, serum sickness or other reactions [4,17]. The ability to detect and characterize ADA to biotherapeutics has advanced in recent years, supported by the publication of immunogenicity 'white papers' [5,18–20] and regulatory guidelines(Box 1).[b] However, several challenges remain that need to be tackled as consensus strategies continue to evolve. The advantages and disadvantages of various ADA detection assays have been reviewed elsewhere [18]. Hence, this section only briefly

[a] EMA (2007) Retacrit: scientific discussion (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000872/WC500054374.pdf).

[b] FDA (2009) Guidance for industry – assay development for immunogenicity testing of therapeutic proteins (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf).

mentions some drawbacks of those methods and highlights advances in the field.

It has been acknowledged that ADA detection methods are semiquantitative in nature[b] [18,20] and, typically there are no standard sets of controls for the various assays used throughout the industry, which complicates the comparison of results. In addition, in many instances, it is more technically challenging to detect low-affinity ADA, and their associations with clinical sequelae might have been overlooked. In general, high-titer and high-affinity neutralizing ADA can be detected with assays that are easier to implement and, therefore, it might be more straightforward to correlate their presence with clinical effects. Progresses in detecting low-affinity antibodies should aid a better understanding of their significance and/or correlation with adverse events. Annex 2 of EMA 'Guidance on Immunogenicity Assessment of Biotechnology-derived Therapeutic Proteins' suggests two methodologies to detect ADA, namely a binding assay and a surface plasmon resonance (SPR) assay (Box 1). SPR assays have been effective in detecting clinically relevant antibodies against a fully humanized IgG1 monoclonal antibody used in clinical trials with patients with colon cancer [21]. The drug itself is often the main source of interference in ADA assays [18]. Despite its low drug tolerance, a SPR-based biosensor technology (Biacore®) was more effective for the detection of lower affinity antibodies against panitumumab, a fully human anti-epidermal growth factor receptor monoclonal antibody, than an ELISA assay that incorporated an acid dissociation step to reduce drug interference [22]. The ELISA was more sensitive for the detection of higher affinity antibodies and tolerated the presence of 100-fold molar excess of the drug, whereas the SPR-based method tolerated equal molar amounts of the drug [22]. An acid-dissociation step significantly increased the drug tolerance on a SPR assay, and the method was capable of detecting 92–100% of ADA against a chimeric monoclonal antibody (drug Y) in clinical samples spiked with 1 mg/ml of drug Y [23]. SPR technologies do not require detection labels [22,23]. In addition, higher throughput detection-label-free technologies that could be used to monitor ADA responses are now available [24,25]. The ForteBio Octet® system was compared with a step-wise bridging ELISA and a Meso-Scale Discovery (MSD) homogeneous bridging assay for the detection of an investigational therapeutic monoclonal antibody, CNTOX [24]. The Octet® system was capable of detecting lower affinity ADA and was more drug tolerant than were the ELISA and MSD methods [24]. Low drug tolerance of the assays can hamper the detection of ADA and that is a particular concern for biotherapeutics, such as antibodies that are dosed at high levels and have long half-lives [26]. Approaches to improve the drug tolerability of ADA assays have been described elsewhere [23,26–30]. The most common strategy is to use an acid-dissociation step to detect ADA in the presence of excess drug in the serum [23,26,29]. In addition to the assay format used and drug interference, other constraints to the comparison of ADA responses in different clinical studies could include the strategy used to determine cut-off point for the assay, and the extent of assay validation and maintenance of standards in various labs [20,31]. Considering that the ultimate goal of the comparability exercise is to assure patient safety and drug effectiveness, clinical outcomes and adverse events need to be taken into account. In some cases, the apparent absence of ADA responses against the drug might be a reflection of weaknesses of the detection assay [32]. This might be especially challenging during the development of biobetters that have structural modifications and/or have been 'de-immunized' and for which a true comparator does not exist.

Indirect methods for immunogenicity assessment

Similar to the comparability exercise for biosimilars, establishing comparability following manufacturing changes requires careful analysis and understanding of the risks involved (Box 1) [6,12,33,34]. ADA responses to Eprex® resulting in pure PRCA is a well-known case, in which immunogenicity seemed to be related to manufacturing changes, including a formulation change and the use of a different packaging system (a prefilled syringe) [6]. Analytical methods are available for the evaluation of parameters such as purity, aggregation, glycosylation and others that might be involved in immunogenicity [33,35–39]. Impurities might act as adjuvants (Box 1) and, in some cases, immunogenicity responses to the impurity itself might be problematic. ADA responses to Factor V contamination of thrombin purified from cows has been connected with coagulopathy [40,41], and a recombinant thrombin has been recently approved [42,43]. Particular glycosylation patterns might be immunogenic [37,44], and some protein aggregates might trigger immunogenicity [9,45,46].[c] Traditional animal models are not predictive of immunogenicity in humans, but transgenic mice overexpressing given human genes have been used to compare the immunogenicity of proteins based on their differential aggregation, although limited clinical data are available [45,47]. Whereas some authors have advocated that the mechanism involved is breakage of B cell tolerance [45,47,48], other work suggests T cell involvement in those ADA responses [49].[d] Therefore, these transgenic mouse models are discussed further in the section below covering 'predictive immunology'. As is the case when using direct methods for immunogenicity testing, to make assessments using indirect methods could be even more challenging for biobetters, owing to the unavailability of a comparator.

Predictive immunology: MHC class II associations with ADA responses

The foundation of immunogenicity prediction tools is the assumption that the uptake and processing of a biotherapeutic by APCs and the subsequent presentation of peptides bound by MHC class II proteins to T cells are steps required to elicit a sustained antibody response against that protein drug. As part of a validation process for those predictive assays, it is necessary to ascertain those presumed associations between HLA types and antibody responses to biotherapeutics other than vaccines. HLA genes encode MHC class II proteins. Cells from individuals of given HLA types express unique MHC class II proteins, which can bind and present different epitopes to T cells. Therefore, the immunogenicity of biotherapeutics might vary among individuals, depending on their HLA type. Several assays available for predicting the immunogenicity of protein therapeutics are based on steps of the T cell

[c] Rosenberg, A. (2010) What we don't know may be hurting us: a case for assessment of the fate of therapeutic proteins in vivo. Workshop on Protein Aggregation and Immunogenicity. July 22–20, Breckenridge, CO, USA.

[d] Fradkin, A.H. *et al.* (2010) Immunogenicity of growth hormone aggregates in a murine model. Workshop on Protein Aggregation and Immunogenicity, June 20–22, 2010, Breckenridge, CO, USA.

activation pathway, and assume that sustained ADA responses require epitopes bound to MHC class II molecules and T cell activation [7]. Although this has been shown for vaccines for some time, only recently have clinical data become available for protein therapeutics with endogenous counterparts (PTEC). Establishing correlations between MHC class II binding epitopes and/or HLA types and ADA responses seems to be a more complex task in the case of PTEC, owing to tolerance mechanisms that prevent destruction of the host tissues. It is known that regulatory T cells are involved in tolerance mechanisms and it has also been suggested that, in the case of self-proteins, high affinity-binding epitopes are implicated in tolerance rather than in immunogenicity [7,50–52]. In addition, no statistically valid clinical associations between HLA types and ADA have been published for non-PTEC protein therapeutics other than vaccines. One also needs to keep in mind that vaccines contain adjuvants, whereas protein therapeutic formulations are optimized to minimize immunogenicity, and other factors besides binding epitopes are often involved in ADA responses. In addition to its possible use for the development of preclinical immunogenicity assays, HLA class II associations with ADA responses could be used to select patients who are less likely to mount an immune response. This section chronologically reviews the data available for associations between HLA types and antibody responses against PTEC.

Factor VIII

Earlier attempts to establish direct associations between HLA types and ADA responses against factor VIII (FVIII) were controversial, which could be attributable to the lack of consistent standards for the different studies [53–59]. Table 1 summarizes some of the parameters for those FVIII studies, in which ADA are often referred to as 'inhibitors'. In some cases, only low-resolution HLA typing data were obtained and no information about the assay used to detect antibodies was provided (patients were tested in different centers before the study). In addition, in none of the FVIII studies there is mention of patient immune status and/or whether the patients were treated with immunosuppressive drugs while undergoing FVIII therapy, which could have precluded antibody responses. The type of FVIII gene mutation and other genetic factors also affects differently the incidence of anti-FVIII antibody responses [60,61]. Another source of variation was the time at which antibodies were tested after the patients started FVIII treatment, which might have confused data interpretation owing to transiently detected antibodies or epitope spreading. In the study by Lippert *et al.* 'hemophiliacs were categorized as inhibitor patients if, at any time, a titerable anti-factor VIII inhibitor was detected in their plasma.' [53]. The authors concluded that ADA formation seemed to be associated with MHC class II genes either directly or with 'unidentified closely linked genes' [53]. Hay *et al.* found a high proportion of HLA-DRB*1501, DQB1*0602 and DQA1*0102 in patients who had developed anti-FVIII antibodies, with the association between DQA1*0102 and ADA reaching statistical significance [58]. The authors described the ADA-positive patients as having 'reached a historical maximum titer > 10 Bethesda units/ml' and it is not clear for how long they were treated with FVIII [58]. At the same time, a study by Oldenberg *et al.* was inconclusive, failing to identify any strong correlation between any HLA-allele and anti-FVIII antibody

TABLE 1

Associations between HLA types and immune responses against protein drugs[a]

RA	ADA assay	Total number of patients	TT	Patient IS	T-cell assay	HLA typing	HLA association with ADA	*P* value	Corrected *P* value	Ref
rHuEpo										
SC	RI	22	~0.8 year	NA	No	LR and HR	DRB1*09 DQB1*0309	≤0.001	NA	[66]
IFN-β (Betaferon®, Avonex® and Rebif®)										
SC	IC	508	4.8 years	NA	No	HR	DRB1*0401	0.0004 0.005[b]	0.012 (0.03)[b]	[65]
IM			4.3 years				DRB1*0408	0.003	0.048	
IFN-β (Betaserum®)										
SC	DC	39	≥1 year	NI	Yes	HR	DRB1*0701//DQA1*0201	0.0009	0.0094	[64]
SC	IC	39	≥1 year	NI	Yes	HR	DRB1*0701//DQA1*0201	0.0247	NA	[64]
FVIII										
NA	BM	260	≥0.4 year	NA	No	HR	DRB1*1501//DQB1*0602	NA	0.0423	[62]
NA	NA	176	NA	NA	No	LR and HR	DQA1*0102 DQB1*0602	NA NA	NA NA	[58]
NA	BM	71	NA	NA	No	HR	ND	NA	NA	[59]
NA	BM	55	NA	NA	No	HR	DQB1*0602[e]	NA	NA	[57]
NA	BM	170	NA	NA	No	LR[c]	ND[d]	NA	NA	[53]
NA	NA	44	NA	NA	No	LR	ND	NA	NA	[56]
NA	NA	65	NA	NA	No	LR	DR5	0.02	NA	[55]
NA	NA	57	NA	NA	No	LR	ND	NA	NA	[54]

[a] *Abbreviations*: BM, Bethesda method for antibody detection; DC, direct capture; HR, high resolution; IC, indirect capture; IM, intramuscular; LR, low resolution; NA, not available; ND, not detected; NI, not immunocompromised; Patient IS, patient immune status; RA, route of administration; RI, radioimmunoprecipitation; SC, subcutaneous; TT, treatment time (i.e. the length of time that the patients had been treated with the human recombinant protein).

[b] Validation group.

[c] Restriction fragment length polymorphism (RFLP) analysis was used in addition to HLA typing.

[d] Genetic association with antibody response observed using RFLP analysis.

[e] Increased in frequency but failing to reach statistical significance.

positive or negative status [59]. Despite the discrepancies, the overall data seemed to implicate MHC class II binding epitopes in anti-FVIII ADA responses. Recently, a study provided evidence for an association between HLA types and ADA responses against FVIII [61,62]. Indirect evidence for HLA type–MCH class II epitope association with anti-FVIII ADA responses came from the observation that, in patients with an established humoral response to FVIII, HIV infection caused disappearance of the anti-factor VIII antibodies following the decline in their T cell counts [63].

Recombinant IFN-β

A study published in 2006 investigated the association between MHC class II haplotypes and the immunogenicity of IFN-β (Table 1) while eliminating many of the hurdles that had confused the interpretation of previous studies with FVIII [64]. For example, patients with multiple sclerosis (MS) enrolled in the IFN-β study were undergoing Betaserum® therapy for at least one year and had not been treated with steroids or immunosuppressive drugs during that period [64]. At the time of the study, three IFN-β formulations (Avonex®, Rebif® and Betaserum®) were being marketed in the USA. Avonex® and Rebif® are produced in Chinese hamster ovary cells, whereas Betaserum® is produced in *Escherichia coli* and has a cysteine to serine mutation at position 17. Although patients reportedly mounted immune responses against all three formulations, a higher percentage of patients (~40%) developed anti-Betaserum® antibodies [64]. Aggregation might be an important factor triggering immunogenicity of protein therapeutics [9] and the use of only one IFN-β formulation removed sources of variation, such as aggregation, and also variations owing to the production system [64]. In addition, T cells of both ADA-positive and ADA-negative subjects enrolled in the IFN-β study were tested with an ELISPOT assay that measured cytokine release, further confirming T cell functionality [64]. Regarding antibody detection, two different assays were used for the primary antibody screen: a direct capture and an indirect capture sandwich assay with a highly sensitive detection system (Table 2). Patient samples were collected more than one day after the final Betaserum® administration, when the IFN-β concentrations in the blood were expected to be lower than 1 nM [64]. This study identified a strong association between the haplotype DRB1*0701-DQA1*0201 and anti-IFN-β antibodies detected with a direct capture assay (Table 1) [64]. Only a weak association was observed when the statistical analysis was performed using the results from the indirect capture antibody assay and DRB1*0701-DQA1*0201 [64]. DRB1*0701 is a common allele among Caucasians and has been reported to occur at a frequency of approximately 15% or higher [64].

In 2008, another IFN-β study described strong associations between HLA-DRB1*0401 and HLA-DRB1*0408 anti-IFN-β antibodies, although IFN-β antibodies were only detected with one binding assay (indirect capture) and it is not clear what steps were taken to avoid drug interference in the assay [65]. The other assay used in the study was based on *MX1* (MxA) gene expression (neutralizing assay) and the results were not used to test the associations with HLA types. It is also not known from the report on the study whether the immune status of the patients and their T cell functionality was monitored. In addition, the patients were treated with three different IFN-β formulations for long periods of time (Table 1), which might have led to epitope spreading and included other sources of variability, such as differential aggregation and impurities [7]. Many patients might have had transient antibodies that were not detected after more than four years. Other differences between studies, including, but not restricted to, sensitivity of the antibody detection assay and dilution of the serum (Table 2), and the strategy used to determine the assay cut-off point might further explain the different results and emphasize the need for standardization and uniform reporting of data. Finally, it is interesting that none of the above-mentioned IFN-β studies found associations between DRB1*1501 and the development of anti-IFN-β antibodies, and this result is further discussed below.

Recombinant erythropoietin (EPO)

An association between HLA-DRB1*09-DQB1*0309 (alleles common in the Thai population) and the incidence of anti-EPO antibodies was recently described [66]. In addition to the negative controls (ADA-negative patients), the study included 22 patients who were positive for anti-EPO antibodies, 19 of whom had developed PRCA.

In vivo, in vitro and *in silico* tools available for preclinical immunogenicity predictions

MHC class II binding epitope determination tools typically overpredict the immunogenicity of protein therapeutics. Many of the epitopes that bind to MHC molecules might not activate T cells and some might be involved in tolerance mechanisms rather than in immunogenicity [7,52]. The *in silico* tools available for epitope determination can be structure based or derived from experimental data and pros and cons can be associated with either methodology [7]. Various binding assay formats for epitope determination are available, using either purified MHC proteins or cells expressing MHC molecules [7]. Epitopes can also be determined following uptake, processing and presentation by dendritic cells [67].

T cell activation assays can have several different readouts, the most common of which are the measurement of thymidine incorporation during T cell proliferation and cytokine production in ELISPOT assays [64,68]. The assays can be performed using either naïve T cells primed *in vitro* or T cells primed *in vivo*. Many different assay formats are also available, which use either peripheral blood mononuclear cells (PBMC) or cells (T cells and monocytes) purified

TABLE 2

Variable assay conditions used for the detection of anti-drug antibodies[a]

Drug	ADA assay	Sample binding time (hours)	Sample dilution	Detection system	Ref
IFN-β (Betaferon®, Avonex® and Rebif®)	Indirect capture ELISA	1.5	1:100	PCGA	[65]
IFN-β (Betaserum®)	Indirect capture	2.0	1:10	TRF	[64]
	Direct capture	1.0	1:10	TRF	[64]

[a] *Abbreviations*: PCGA, peroxidase-conjugated goat anti-serum to human IgG; TRF, time-resolved fluoroimmunoassay.

from PBMC [64,68]. *In vitro* differentiation of monocytes yields dendritic cells, which are pulsed with the antigen and subsequently incubated with T cells [64,68]. More recently, an assay to detect 'danger signals' has also been proposed, which measures dendritic cell responses to external stimulus [69]. However, no dendritic cell receptors for the 'danger signals' have yet been identified.

Different types of transgenic mouse model are available for preclinical immunogenicity testing. For example, one type expresses human HLA genes [70], whereas another comprises mice that overexpress the human protein to be tested, thus developing tolerance to it [45,47]. Immunogenic versions of the same protein (such as more aggregated versions) would be able to break tolerance in the immunotolerant mice, whereas a nonimmunogenic formulation should not induce an ADA response [45].

The identification and use of peptides that induce tolerance (Tregitopes) has also been proposed for immunogenicity mitigation [52]. Although this is an interesting concept, it awaits clinical validation.

Overview of immunogenicity predictions and their corresponding clinical outcomes

First case study: IFN-β and epitope predictions

A predictive nonclinical study published in 2004 tested IFN-β overlapping peptides in a T cell assay that evaluated proliferation by measuring thymidine incorporation. Samples from 87 community donors were used for the assay, and the IFN-β peptides were tested for their ability to prime naïve T cells *in vitro*. The authors identified a MHC class II epitope with high affinity binding to DR15 and DQ6 *in vitro* and concluded that those alleles were involved in IFN-β immunogenicity [68]. Subsequent clinical studies refuted the results from those *in vitro* predictions, as no association was found between DRB1*1501 (and/or DQB1*0602) and ADA responses against IFN-β [64,65].

Second case study: IFN-β and 'danger signals'

A T cell activation assay has also been used to evaluate the immunogenicity of IFN-β in a limited number of formulations and the results reportedly correlated with the clinical immunogenicity [71]. It is probable that different 'danger signals' in the different formulations were causing the differential T cell proliferation in the *in vitro* assay, although as discussed above, no receptors for such danger signals in dendritic cells have yet been identified [69,71,72].

Third case study: IFN-β and transgenic mice

Transgenic C57Bl/6 mice overexpressing human IFN-β and that are immune tolerant for IFN-β have also been used to test unformulated Avonex (IFN-β-1a in 100 mM sodium phosphate, 200 mM sodium chloride, pH 7.2), Avonex with human serum albumin and Betaseron® [47]. The authors state that 'to test the immunogenicity of a marketed formulation, wild-type and transgenic mice were injected daily i.p. with 5 μg Betaseron' (marketed as Betaferon® in Europe) [47]. In the study, IFN-β-1a was less immunogenic in the transgenic mouse model than was Betaseron® [47]. A more recent study used both the original C57Bl/6 mouse and a hybrid offspring from crossings of transgenic C57Bl/6 mice with wild-type FVB/N mice to study the immunogenicity of three commercial rh-IFN-β products, Rebif®, Avonex® and Betaferon® [45]. All three formulations were immunogenic in wild-type hybrid offspring, whereas only Betaferon® was capable of breaking tolerance in the transgenic hybrid mice, consistent with clinical results for the higher immunogenicity of Betaferon® [45,64]. The studies using IFN-β transgenic mice did not report the levels of IFN-β in blood from wild-type or transgenic animals [45,47], information that might be important for comparing results from studies with other protein therapeutics.

Fourth case study: EPO

The prediction of immunodominant T cell epitopes in EPO was the basis for engineering variants considered less immunogenic by *in vitro* testing [73], but no clinical data have yet been reported. Two regions within EPO (amino acids 91–120 and 126–155) were predicted to contain epitopes capable of binding to numerous HLA-DR types [73]. Available clinical data from other studies indicate an association between specific HLA types and ADA responses (Table 1). In addition, tolerance mechanisms need to be considered when evaluating possible determinants of endogenous protein immunogenicity [7,52].

Fifth case study: fusion protein, peptide and antibody

A fusion protein consisting of two 24 amino acid peptides attached to human Fc fragment (FPX) was administered either intravenously or subcutaneously to 76 human volunteers. Overall, 37% of those volunteers developed anti-FPX antibodies after one single injection [74]. However, only 15 of the subjects enrolled in the FPX study provided blood samples for *in vitro* T cell assays and were HLA typed, and they included four anti-FPX antibody-negative and 11 anti-FPX antibody-positive volunteers with various different alleles. Hence, it was not possible to draw conclusions substantiated by statistical analysis regarding the HLA type of those subjects and the clinical outcome [74]. Another study compared peptides of FPX1 (FPX) to an unspecified therapeutic antibody (FPX2) in a T cell activation assay using PBMC from 34 naïve healthy subjects [75]. FPX1 peptides elicited IFN-γ secretion in 16 of the 34 PBMC pools, whereas no IFN-γ secretion was observed in the presence of FPX2. The authors discussed that the T cell activation by FPX1 was consistent with its higher clinical immunogenicity. However, no associations between HLA types and T cell activation were observed. No information was provided regarding the formulation for either FPX1 or FPX2 or whether any impurities, if present, could have affected the clinical immunogenicity of these quite different protein therapeutics. In addition, the assays used to monitor antibody detection against FPX2 in clinical tests were not disclosed, neither were the concentrations of the therapeutic antibody FPX2 in the serum samples and/or whether drug interference could have affected clinical immunogenicity estimation. The immune status of the patients treated with FPX2 was also not disclosed, whereas FPX was administered to healthy human subjects [74]. More studies with nonendogenous proteins will be important to clarify possible HLA-type associations with antibody responses.

Efforts currently underway to derive recommendations regarding predictive immunology

Efforts are currently being made within the scientific community to better understand, assess and mitigate immunogenicity (http://

www.aaps.org/inside/focus_groups/TherPro/index.asp; accessed January 27, 2011). As data are made public and additional immunogenicity assessments are tested in clinical studies, understanding should increase of the potential benefits and limitations of predictive immunology.

Conclusions

Validation of predictive methodologies to assess immunogenicity could impact comparability studies and the development of biobetters and other protein therapeutics. Comparisons of preclinical immunogenicity assessments for protein therapeutics and the corresponding clinical outcomes are important for the evaluation of predictive tools. It is also essential to distinguish self versus nonself proteins during those predictive exercises, because of tolerance mechanisms that prevent the destruction by the host of its own tissues. Moreover, during preclinical immunogenicity assessments for nonself proteins, it is necessary to differentiate between vaccines (which contain adjuvants) and other therapeutic proteins with formulations that are optimized to minimize immunogenicity. A limited amount of data is currently available that suggests a value for transgenic mouse models for immunogenicity predictions. However, more correlations between preclinical studies and clinical data will be relevant for clarifying the value of predictive immunology.

Acknowledgements

The author thanks the anonymous reviewers for their constructive comments.

References

1 Engelberg, A.B. *et al.* (2009) Balancing innovation, access, and profits – market exclusivity for biologics. *N. Engl. J. Med.* 361, 1917–1919
2 Wheadon, D.E. (2010) Market exclusivity for biologics. *N. Engl. J. Med.* 362, 661–662 author reply
3 Pollock, A. and Zagari, M. (2010) Market exclusivity for biologics. *N. Engl. J. Med.* 362, 661–662 author reply
4 Rosenberg, A.S. (2003) Immunogenicity of biological therapeutics: a hierarchy of concerns. *Dev. Biol.* 112, 15–21
5 Ponce, R. *et al.* (2009) Immunogenicity of biologically-derived therapeutics: assessment and interpretation of nonclinical safety studies. *Regul. Toxicol. Pharmacol.* 54, 164–182
6 Woodcock, J. *et al.* (2007) The FDA's assessment of follow-on protein products: a historical perspective. *Nat. Rev. Drug Discov.* 6, 437–442
7 Barbosa, M.D. and Celis, E. (2007) Immunogenicity of protein therapeutics and the interplay between tolerance and antibody responses. *Drug Discov. Today* 12, 674–681
8 Sauerborn, M. *et al.* (2010) Immunological mechanism underlying the immune response to recombinant human protein therapeutics. *Trends Pharmacol. Sci.* 31, 53–59
9 Rosenberg, A.S. (2006) Effects of protein aggregates: an immunologic perspective. *AAPS J.* 8, E501–507
10 Schellekens, H. (2008) The first biosimilar epoetin: but how similar is it? *Clin. J. Am. Soc. Nephrol.* 3, 174–178
11 Schellekens, H. and Moors, E. (2010) Clinical comparability and European biosimilar regulations. *Nat. Biotechnol.* 28, 28–31
12 Hughes, D.A. (2010) Biosimilars: evidential standards for health technology assessment. *Clin. Pharmacol. Ther.* 87, 257–261
13 Antman, E.M. *et al.* (1990) Treatment of 150 cases of life-threatening digitalis intoxication with digoxin-specific fab antibody fragments. *Circulation* 81, 1744–1752
14 Korner, J. and Aronne, L.J. (2004) Pharmacological approaches to weight reduction: therapeutic targets. *J. Clin. Endocrinol. Metab.* 89, 2616–2621
15 Koren, E. *et al.* (2008) Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J. Immunol. Methods* 333, 1–9
16 Schellekens, H. (2009) Assessing the bioequivalence of biosimilars. The Retacrit case. *Drug Discov. Today* 14, 495–499
17 Hansel, T.T. *et al.* (2010) The safety and side effects of monoclonal antibodies. *Nat. Rev. Drug Discov.* 9, 325–338
18 Mire-Sluis, A.R. *et al.* (2004) Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J. Immunol. Methods* 289, 1–16
19 Gupta, S. *et al.* (2007) Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J. Immunol. Methods* 321, 1–18
20 Shankar, G. *et al.* (2008) Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J. Pharm. Biomed. Anal.* 48, 1267–1281
21 Ritter, G. *et al.* (2001) Serological analysis of human anti-human antibody responses in colon cancer patients treated with repeated doses of humanized monoclonal antibody A33. *Cancer Res.* 61, 6851–6859
22 Lofgren, J.A. *et al.* (2007) Comparing ELISA and surface plasmon resonance for assessing clinical immunogenicity of panitumumab. *J. Immunol.* 178, 7467–7472
23 Sickert, D. *et al.* (2008) Improvement of drug tolerance in immunogenicity testing by acid treatment on Biacore. *J. Immunol. Methods* 334, 29–36
24 Li, J. *et al.* (2011) Detection of low-affinity anti-drug antibodies and improved drug tolerance in immunogenicity testing by Octet((R)) biolayer interferometry. *J. Pharm. Biomed. Anal.* 54, 286–294
25 Rich, R.L. *et al.* (2010) Biosensor-based fragment screening using FastStep injections. *Anal. Biochem.* 407, 270–277
26 Patton, A. *et al.* (2005) An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen. *J. Immunol. Methods* 304, 189–195
27 Lofgren, J.A. *et al.* (2006) Detection of neutralizing anti-therapeutic protein antibodies in serum or plasma samples containing high levels of the therapeutic protein. *J. Immunol. Methods* 308, 101–108
28 Neubert, H. *et al.* (2008) Assessing immunogenicity in the presence of excess protein therapeutic using immunoprecipitation and quantitative mass spectrometry. *Anal. Chem.* 80, 6907–6914
29 Smith, H.W. *et al.* (2007) Detection of antibodies against therapeutic proteins in the presence of residual therapeutic protein using a solid-phase extraction with acid dissociation (SPEAD) sample treatment prior to ELISA. *Regul. Toxicol. Pharmacol.* 49, 230–237
30 Bourdage, J.S. *et al.* (2007) An Affinity Capture Elution (ACE) assay for detection of anti-drug antibody to monoclonal antibody therapeutics in the presence of high levels of drug. *J. Immunol. Methods* 327, 10–17
31 Savoie, N. *et al.* (2010) 2010 white paper on recent issues in regulated bioanalysis & global harmonization of bioanalytical guidance. *Bioanalysis* 2, 1945–1960
32 Clark, M. (2000) Antibody humanization: a case of the 'Emperor's new clothes'? *Immunol. Today* 21, 397–402
33 Chirino, A.J. and Mire-Sluis, A. (2004) Characterizing biological products and assessing comparability following manufacturing changes. *Nat. Biotechnol.* 22, 1383–1391
34 Chirino, A.J. and Mire-Sluis, A.R. (2005) State of the art analytical comparability: a review. *Dev. Biol.* 122, 3–26
35 Gabrielson, J.P. and Arthur, K.K. (2010) Measuring low levels of protein aggregation by sedimentation velocity. *Methods* 10.1016/j.ymeth.2010.12.030
36 Brinks, V. *et al.* (2010) Quality of original and biosimilar Epoetin products. *Pharm. Res.* 10.1007/s11095-010-0288-2
37 Beck, A. *et al.* (2008) Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and Fc–fusion proteins. *Curr. Pharm. Biotechnol.* 9, 482–501
38 Barnard, J.G. *et al.* (2010) Subvisible particle counting provides a sensitive method of detecting and quantifying aggregation of monoclonal antibody caused by freeze-thawing: insights into the roles of particles in the protein aggregation pathway. *J. Pharm. Sci.* 100, 492–503
39 Singh, S.K. *et al.* (2010) An industry perspective on the monitoring of subvisible particles as a quality attribute for protein therapeutics. *J. Pharm. Sci.* 99, 3302–3321
40 Israels, S.J. and Israels, E.D. (1994) Development of antibodies to bovine and human factor V in two children after exposure to topical bovine thrombin. *Am. J. Pediatr. Hematol. Oncol.* 16, 249–254
41 Neschis, D.G. *et al.* (2002) Coagulopathy as a result of factor V inhibitor after exposure to bovine topical thrombin. *J. Vasc. Surg.* 35, 400–402

42 Ratner, M. (2008) Recombinant thrombin approved. *Nat. Biotechnol.* 26, 250
43 Ballard, J.L. *et al.* (2010) Safety and immunogenicity observations pooled from eight clinical trials of recombinant human thrombin. *J. Am. Coll. Surg.* 210, 199–204
44 Chung, C.H. *et al.* (2008) Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. *N. Engl. J. Med.* 358, 1109–1117
45 van Beers, M.M. *et al.* (2010) Hybrid transgenic immune tolerant mouse model for assessing the breaking of B cell tolerance by human interferon beta. *J. Immunol. Methods* 352, 32–37
46 Purohit, V.S. *et al.* (2006) Influence of aggregation on immunogenicity of recombinant human Factor VIII in hemophilia A mice. *J. Pharm. Sci.* 95, 358–371
47 Hermeling, S. *et al.* (2005) Development of a transgenic mouse model immune tolerant for human interferon Beta. *Pharm. Res.* 22, 847–851
48 van Beers, M.M. *et al.* (2010) Aggregated recombinant human interferon Beta induces antibodies but no memory in immune-tolerant transgenic mice. *Pharm. Res.* 27, 1812–1824
49 Fradkin, A.H. *et al.* (2009) Immunogenicity of aggregates of recombinant human growth hormone in mouse models. *J. Pharm. Sci.* 98, 3247–3264
50 Wing, K. *et al.* (2008) CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322, 271–275
51 Jain, N. *et al.* (2010) Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity. *Proc. Natl. Acad. Sci. U. S. A.* 107, 1524–1528
52 De Groot, A.S. *et al.* (2008) Activation of natural regulatory T cells by IgG Fc-derived peptide 'Tregitopes'. *Blood* 112, 3303–3311
53 Lippert, L.E. *et al.* (1990) Relationship of major histocompatibility complex class II genes to inhibitor antibody formation in hemophilia A. *Thromb. Haemost.* 64, 564–568
54 Frommel, D. *et al.* (1981) HLA antigens and factor VIII antibody in classic hemophilia European Study group of factor VIII antibody. *Thromb. Haemost.* 46, 687–689
55 Mayr, W.R. *et al.* (1984) HLA-DR and Factor VIII antibodies in hemophilia A. *Thromb. Haemost.* 51, 293
56 Aly, A.M. *et al.* (1990) Histocompatibility antigen patterns in haemophilic patients with factor VIII antibodies. *Br. J. Haematol.* 76, 238–241
57 Kapur, J.J. *et al.* (1994) Association between HLA-DQB1*0602 allele and antibodies to factor VIII in hemophilia A. *Hum. Immunol.* 40, 96
58 Hay, C.R. *et al.* (1997) HLA class II profile: a weak determinant of factor VIII inhibitor development in severe haemophilia A. UKHCDO Inhibitor Working Party. *Thromb. Haemost.* 77, 234–237
59 Oldenburg, J. *et al.* (1997) HLA genotype of patients with severe haemophilia A due to intron 22 inversion with and without inhibitors of factor VIII. *Thromb. Haemost.* 77, 238–242
60 Jacquemin, M. *et al.* (2003) CD4+ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A. *Blood* 101, 1351–1358
61 Astermark, J. *et al.* (2006) Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. *Blood* 107, 3167–3172
62 Pavlova, A. *et al.* (2009) Impact of polymorphisms of the major histocompatibility complex class II, interleukin-10, tumor necrosis factor-alpha and cytotoxic T-lymphocyte antigen-4 genes on inhibitor development in severe hemophilia A. *J. Thromb. Haemost.* 7, 2006–2015
63 Bray, G.L. *et al.* (1993) Loss of high-responder inhibitors in patients with severe hemophilia A and human immunodeficiency virus type 1 infection: a report from the Multi-Center Hemophilia Cohort Study. *Am. J. Hematol.* 42, 375–379
64 Barbosa, M.D. *et al.* (2006) Clinical link between MHC class II haplotype and interferon-beta (IFN-beta) immunogenicity. *Clin. Immunol.* 118, 42–50
65 Hoffmann, S. *et al.* (2008) HLA-DRB1*0401 and HLA-DRB1*0408 are strongly associated with the development of antibodies against interferon-beta therapy in multiple sclerosis. *Am. J. Hum. Genet.* 83, 219–227
66 Praditpornsilpa, K. *et al.* (2009) The association of anti-r-HuEpo-associated pure red cell aplasia with HLA-DRB1*09-DQB1*0309. *Nephrol. Dial. Transplant.* 24, 1545–1549
67 Rohn, T.A. *et al.* (2005) A novel strategy for the discovery of MHC class II-restricted tumor antigens: identification of a melanotransferrin helper T-cell epitope. *Cancer Res.* 65, 10068–10078
68 Stickler, M. *et al.* (2004) The HLA-DR2 haplotype is associated with an increased proliferative response to the immunodominant CD4(+) T-cell epitope in human interferon-beta. *Genes Immun.* 5, 1–7
69 Mueller, R. *et al.* (2009) Evaluation of the immuno-stimulatory potential of stopper extractables and leachables by using dendritic cells as readout. *J. Pharm. Sci.* 98, 3548–3561
70 De Groot, A.S. *et al.* (2008) Prediction of immunogenicity: in silico paradigms, ex vivo and in vivo correlates. *Curr. Opin. Pharmacol.* 8, 620–626
71 Jaber, A. and Baker, M. (2007) Assessment of the immunogenicity of different interferon beta-1a formulations using ex vivo T-cell assays. *J. Pharm. Biomed. Anal.* 43, 1256–1261
72 Jaber, A. *et al.* (2007) The Rebif new formulation story: it's not trials and error. *Drugs R. D.* 8, 335–348
73 Tangri, S. *et al.* (2005) Rationally engineered therapeutic proteins with reduced immunogenicity. *J. Immunol.* 174, 3187–3196
74 Koren, E. *et al.* (2007) Clinical validation of the 'in silico' prediction of immunogenicity of a human recombinant therapeutic protein. *Clin. Immunol.* 124, 26–32
75 Wullner, D. *et al.* (2010) Considerations for optimization and validation of an in vitro PBMC derived T cell assay for immunogenicity prediction of biotherapeutics. *Clin. Immunol.* 137, 5–14