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Taking immunogenicity assessment of therapeutic proteins to the next level

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ABSTRACT

Therapeutic proteins provide innovative and effective therapies for numerous diseases. However, some of these products are associated with unwanted immunogenicity that may lead to clinical consequences such as reduced or loss of efficacy, altered pharmacokinetics (PK), general immune and hypersensitivity reactions, and neutralisation of the natural counterpart (e.g. the physiological hormone). Regulatory guidance on immunogenicity assessment needs to take into consideration a great diversity of products, indications and patient populations as well as constantly advancing manufacturing technologies. Such guidance needs to be sufficiently specific while, at the same time, allowing interactive discussion and adjusted benefit-risk weighing of each product on a case-by-case basis, e.g. for a unique treatment of a life threatening disease acceptable treatment risks may differ considerably from the ones in case of less serious disease. This theme was the focus of the international conference "Taking immunogenicity assessment of therapeutic proteins to the next level", held at the Paul-Ehrlich-Institut in Langen,

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Germany, on the 10–11. June 2010. The objectives of the conference were to highlight how the field could move from that of a mere description of risk factors to a system of risk assessment and mitigation, as well as an understanding of the impact of unwanted immunogenicity on the overall benefit/risk consideration for a medicinal product. More than 150 experts from industry, academia and regulatory authorities worldwide discussed the phenomenon of undesired immunogenicity from different perspectives. The conference focussed on issues relevant to three areas: (1) new European guidelines that are currently the subject of discussion; (2) testing strategies for immunogenicity assessment; and (3) scientific progress on the product-related factors that may contribute to the development of pathogenesis of immunogenicity, in particular in the field of protein aggregation and post-translational modifications. This report provides an overview of issues, insights, and conclusions that were discussed and achieved during the meeting.

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1. Introduction

For nearly 30 years, therapeutic proteins have revolutionised the treatment of numerous diseases. Different classes of bio-therapeutics have become available, such as antibodies, hormones, enzymes, growth and blood factors allowing major progress in treatment. However, it became obvious that many of these products bear the risk of an unwanted immune response (which will be referred to as “immunogenicity” throughout this report). The immunogenic profile of therapeutic proteins and the ensuing risk to patients is determined by a multiplicity of product-, process- and patient-related factors that have to be systematically evaluated during the clinical development of the therapeutic agent to ensure a proper benefit/risk assessment. Clinical consequences of immunogenicity range from no effect, a reduction or loss of efficacy to severe complications due to neutralisation of the natural counterpart or general immune system reactions. Often these “common rare” events have an incidence of less than 1% of treated patients and become only obvious at the late stage of phase III trials or after approval.

Research has added considerable knowledge about the pathogenesis of immunogenicity, comprising ‘classical’ immune reactions to neo-antigens as well as the breakdown of immune tolerance to self-antigens. At the same time, manufacturing and analytical characterisation technologies have changed and are constantly evolving. Thus, regulatory agencies are facing a highly dynamic process.

Regulations and guidelines have been developed to support drug developers and regulators to ensure the safety of therapeutic proteins. Such guidance is expected to be sufficiently specific but needs to be flexible enough to take into account ongoing technical and scientific progress. To provide a forum for discussion and taking the next steps to handle unwanted immunogenicity from a regulatory perspective, the International Association for Biologicals (IABS), the Paul-Ehrlich-Institute (PEI) (Federal Institute for Vaccines and Biologicals, Germany), and the International Federation of Pharmaceutical Manufacturers & Associations (IFPMA) organised an international conference on ‘Taking immunogenicity assessment of therapeutic proteins to the next level’ that was held in Langen, Germany, on the 10.–11. June 2010. Experts in the field of unwanted immunogenicity from industry, academia and regulatory agencies worldwide discussed the phenomenon of undesired immunogenicity from different angles.

The conference aimed at taking the regulatory assessment of immunogenicity risk to the next level: first, to enhance the dialogue on the application of regulatory guidance on identifying and controlling risks associated with undesirable immunogenicity and second, to discuss how unwanted immunogenicity is practically handled to achieve an appropriate overall benefit-risk estimation. Available guidance documents and the progress on new guidelines

that are currently under development were presented as well as case studies with regard to immunogenicity assessment and the pathogenesis of an unwanted immune response.

After an opening keynote lecture by the PEI president Klaus Cichutek, the first two sessions, chaired by Christian K. Schneider and Isabel C. Büttel (PEI, Germany), Harald Kropshofer (Hoffmann-La Roche, Switzerland) and Paul Chamberlain (NDA Advisory Board, France), respectively, provided a wide overview of existing and upcoming guidelines. The third session, chaired by Huub Schellekens (Utrecht University, The Netherlands) and Falk Ehmann (European Medicines Agency [EMA], United Kingdom [UK]), asked what we learned from case studies and how new insights could be incorporated into advanced testing strategies. The following sessions, chaired by Anthony Mire-Sluis (Amgen, United States of America [USA]), Jean-Hugues Trouvin, (Agence française de sécurité sanitaire des produits de Santé [AFSSAPS], France), Martina Weise (Federal Institute for Drugs and Medical Devices [BfArM], Germany), and Philippe Stas (Algonomics [Lonza], Belgium) reflected on the pathogenesis of immunogenicity. The panel discussion, chaired by Johannes Löwer (BfArM, Germany), focussed on immunogenicity data that are required during product development and in the post-marketing phase. Christian K. Schneider then summarised the conclusions and recommendations of the meeting.

2. Update on regulatory approaches

2.1. General guidelines

Therapeutic proteins are highly complex molecules because of the diversity of characteristics that are inherent to both the drug substance or product itself and its manufacturing process. In addition to these product-related factors, several patient- and regimen-related factors may contribute to immunogenicity. It became obvious that the heterogeneity of testing strategies, analytical methods, and sampling schedules impede the systematic assessment of immunogenicity between the different classes of products and between products of the same class although they are used in comparable conditions. Therefore, a comprehensive guideline on the ‘Immunogenicity Assessment of Biotechnology-derived Therapeutic Proteins’ was developed by the Committee for Medicinal Products for Human Use (CHMP) that came into effect in April 2008. The guideline advocates a risk- and science-based multidisciplinary approach to immunogenicity and serves as a roadmap for developers of therapeutic proteins and regulators, providing general recommendations from a marketing authorisation perspective [1].

Christian K. Schneider, who is the rapporteur of this guideline, introduced the key principles of immunogenicity assessment. The prediction of both incidence and clinical significance of

immunogenicity is still problematic. Therefore, the recommended approach is to apply suitably sensitive bioanalytical methods to detect host responses to the drug product and to relate these to clinical correlates of pharmacokinetics (PK), efficacy and safety. The current, commonly used, and in most cases recommended bioanalytical approach, is a three-stage process consisting of screening, confirmation, and characterisation: If blood samples are found to be positive for anti-drug antibodies (ADA) during screening, these samples are then subjected to a confirmatory assay (e.g. competitive inhibition ELISA) ensuring that ADA are binding specifically. Having established that positive findings do not result from non-specific interactions such as with materials in the assay milieu (e.g. plastic, other proteins), ADA need to be characterised. Typically, this characterisation includes assessment of the neutralising capacity of ADA. Furthermore, assays for relevant biomarkers and/or pharmacokinetic measurements should complement ADA characterisation and analysis of their *in vivo* impact.

Clinical consequences of immunogenicity may be comprised of acute consequences, such as anaphylaxis or infusion reactions, non-acute consequences (e.g. loss of efficacy), cross-reactivity with and neutralisation of natural endogenous counterparts, and delayed hypersensitivity. Therefore, the clinical outcome of unwanted immune responses differ widely depending on the affinity, class, amount and persistence of ADA generated, the epitope recognised by the biotherapeutic protein, and the ability of ADA to activate complement. This diversity of causes and consequences underlines the importance of the systematic evaluation of immunogenicity during clinical trials. Christian K. Schneider reported that, consequently, the extent of clinical, non-clinical and assay data required for approval was the focus of the discussion during consultation on this guideline. Considering the complexity of biotherapeutic products, the question of whether the guideline might be too general was also addressed. In response, it was emphasised that the guideline, being a first step, provides only generally applicable principles. In fact, European regulators were of the opinion that maintaining flexibility is important to allow for development of new technologies evolving in the field of immunogenicity assessment [2]. This statement was supported by several commentators in the audience who favoured an approach that more closely linked the bioanalytical considerations with assessment of the actual significance of the clinical impact of undesirable immunogenicity. In a second step, guidelines that are more product class-specific are now drafted, for example on immunogenicity assessment of monoclonal antibodies (mAb).

Due to their clinical mode of action, which clearly distinguishes mAb from other classes of therapeutic proteins and due to the risk of assay interference, assessing immunogenicity of mAb is on an assay level technically challenging. Robin Thorpe from the National Institute for Biological Standards and Control (NIBSC, UK) reported on the upcoming EMA guideline on 'Immunogenicity Assessment of Monoclonal Antibodies Intended for *in vivo* Clinical Use' [3]. This guideline will cover particular issues regarding the screening and confirmatory assays used for mAb. Guidance on strategies to assess neutralising capacity of ADA against mAb will be given in the guideline. Furthermore, principles of a risk-based approach that incorporates both the probability and the severity of potential clinical consequences are discussed. Risk-stratification is an important tool, even if it is accompanied by a certain degree of uncertainty. Generally, incidence and consequences of immunogenicity require a case-by-case approach and appropriate clinical trials. The draft of this guideline on immunogenicity assessment of mAb was finalised in March 2010 and was sent out for public consultation in November 2010 after an internal consultation process.

2.2. Comparability exercise and biosimilars

Manufacturers of therapeutic proteins frequently change the manufacturing processes, e.g. to improve products characteristics, to increase scale, or to change formulation (e.g. free from plasma-derived components like albumin). Changes in the manufacturing process require demonstration of comparability of the pre- and post-change product to ensure that quality, safety, and efficacy of the post-change product are not adversely affected. However, even small modifications of the biotherapeutic molecule might alter an established immunogenic profile, and process-related impurities or degradation products, with immunogenic potential, might occur after production- and supply chain-related changes. Therefore, the risk of increased immunogenicity poses a particular challenge to the demonstration of comparability.

Jean-Hugues Trouvin presented two relevant guidelines that cover the quality, non-clinical and clinical issues of comparability assessment after a product change (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use [ICH] [4], CHMP [5]). He emphasised that the potential for altered immunogenicity needs to be considered even if comparative physicochemical and biological data on product quality do not indicate any difference. Because the predictability of non-clinical studies for the evaluation of immunogenicity is low, routine monitoring of patient samples might be required during clinical trials. In this respect, the extent of immunogenicity studies (clinical evaluation prior to or after authorisation of the change) might be based on a risk analysis that pays regard to both the nature of any observed differences and their potential clinical impact. Emerging technologies might provide additional data for the further evaluation of potential immunogenicity induced by the change introduced in the process.

The "biggest change" to a therapeutic protein, however, would be the independent development of a manufacturing process by a new manufacturer, which is the situation for biosimilars (i.e., a "copy" version of an already licensed originator product). While process changes within the innovator product can already entail significant technical and regulatory challenges, development and approval of biosimilars might be even more challenging because biological molecules, in contrast to generic chemical drugs, are more complex and thus inherently less well characterised than chemicals. The introduction of new production processes, new formulations, and new containers and closures might affect molecule characteristics and the impurity content of the biosimilar version of therapeutic proteins (in a positive or negative way). New production processes might impact post-translational modifications and the higher order structure of proteins. Impurities such as degradation products, host cell proteins, and leachates might be immunogenic themselves or might act as an adjuvant enhancing immunogenicity. In contrast to these product-specific factors, patient-specific factors potentially affecting immunogenicity such as dose and frequency of therapy, route of administration and host immune competence are usually known from the use of the original product.

In her talk, Martina Weise summarised the main EU requirements concerning biosimilars. The current understanding is that the biosimilar and the innovator product should be identical on the amino acid level and any difference needs to be justified, e.g. in post-translational modifications. For assessment of immunogenicity, human data are always required. Animal data, even if potentially useful to detect major differences in immune response, are considered not to be predictive. An optimised antibody testing strategy with detailed sampling protocols, a sensitive validated screening assay and further characterisation of ADA, if detected, is requested. Due to the general lack of standardised assays comparative

immunogenicity data (on both the biosimilar and the reference product) are usually required for interpretation of the results. The pre-licensing immunogenicity database is expected to exclude excessive immunogenicity of the biosimilar relative to the reference product [6] but further data may be requested post-marketing, especially when rare and serious ADA-related adverse reactions have been encountered with the reference product or the substance class. Martina Weise emphasised that, because of these regulations, biosimilars in the EU show close resemblance to their reference product with respect to quality, efficacy and safety. In this respect, global consistency is needed because 'copy versions' of innovator biologicals are licensed in various other countries without a clear regulatory pathway and based on different data requirements. Moreover, such non-innovator products are often called "biosimilars" despite the lack of a (thorough) comparison with the original product and even in the presence of clear differences. Therefore, WHO has developed the 'Guidelines on evaluation of similar biotherapeutic products' which in principle is in line with the EU requirements [7].

Current guidelines for the development of biosimilars may already cover some general aspects applicable to biosimilar mAbs [6,8]. However, the complex nature of antibodies demands more specific regulatory guidance, including physicochemical and biological characterisation, as well as non-clinical and clinical development. Christian K. Schneider reported on the EMA Workshop on biosimilar mAb held in 2009 [9]: The main conclusion had been that current guidelines, since their recent update, might be sufficient for quality assessment of mAbs, but that more specific guidance is required to solve the following questions: i) How should a reference mAb and a biosimilar mAb be compared in a non-clinical model when the relevant species is a non-human primate? ii) Is it possible to establish similar clinical efficacy in a feasible study design? iii) Is it possible to extrapolate efficacy (or activity) from one indication to another? iv) If so, can safety also be extrapolated? v) Finally, how should unwanted immunogenicity be handled?

Christian K. Schneider noted that immunogenicity, especially that of biosimilar mAb may require a re-consideration of regulatory principles, since therapeutic or diagnostic mAbs do not have endogenous counterparts as do the currently licensed biosimilars (e.g. erythropoietins). Nevertheless, systematic evaluation of immunogenicity is important since it has the potential for significant clinical implications such as impaired efficacy, immune complex disease, injection site reactions, and infusion reactions. Moreover, ADA to biosimilar mAb may also cross-react with the reference medicinal product or *vice versa* and hamper further treatment cycles.

Obviously, different regulatory requirements need to be met for changes in the manufacturing process and for biosimilars. However, as emphasised by Joerg Windisch (Sandoz, Austria), these different requirements share common scientific principles. He demonstrated that in both cases the magnitude of the difference is not always decisive. For example, the fundamental bioprocess parameters of the manufacturing process of a licensed erythropoietin-derivative were changed considerably. Although certain quality attributes were altered, the change was finally accepted based on the totality of data [10]. In contrast, relatively small changes of the manufacturing process may sometimes cause severe clinical effects. In the case of an erythropoietin, organic compounds leaching from uncoated rubber stoppers and a new formulation were associated with increased immunogenicity that resulted in Pure Red Cell Aplasia (PRCA) through neutralisation of drug and endogenous protein [11]. Joerg Windisch therefore proposed a differential risk-based approach for immunogenicity assessment both of innovator products after a manufacturing change, and of

biosimilars (N.B. the proposal for such approach was included in the systematic strategy proposed in this article).

The regulatory requirements that are recommended, either for the approval of a biosimilar product or after a manufacturing change were thoroughly discussed during the panel discussion. The discussion centred on the question of how immunogenicity should be addressed post-marketing. The term 'pharmacovigilance', which describes the detection, assessment, understanding and prevention of adverse effects after the launch of a product, is perfectly apropos for such adverse effects as immunogenicity. Because neither onset nor incidence of immunogenicity is predictable, and the size of the population followed during the clinical studies performed for the development of biosimilars will likely be too small to detect rare events, post-marketing surveillance monitoring of potential immunogenicity was considered essential. The immunogenicity guideline, as well as the biosimilar guidelines, state that immunogenicity should always be addressed in the risk management plan which has to be submitted with the Marketing Authorisation Application (MAA). Thus for biosimilars, the same approach is applied as when a change is made to the manufacturing process of an authorised recombinant protein; comparability needs to be demonstrated by physicochemical and biological comparison, and if changes in the immunogenic profile of the therapeutic protein cannot be ruled out, sometimes supported by clinical and/or post-marketing safety surveillance.

3. Update on testing strategies

3.1. The risk-based approach

Risk is defined as the product of probability and consequences. Therefore, risk-based strategies for immunogenicity assessment involve various factors influencing the probability of an unwanted immune response and the severity of any clinical consequences due to unwanted immunogenicity. More specifically, (i) the identification of the risk factors; (ii) an impact assessment that balances probability vs. severity vs. detectability; and (iii) the risk management strategy. The probability depends mainly on patient- and product-related factors. Typically, the likelihood of immunogenicity is considered lower if the therapeutic protein consists solely of human sequences, the drug is highly purified, and administered intravenously in a single dose. In contrast, therapeutic proteins of non-human origin, product impurities, and subcutaneous administration (in comparison to intravenous administration) would usually pose a higher risk. The severity of an unwanted immune response might be determined by questions such as: Is there an endogenous version of the protein? Is that counterpart encoded by a single human gene sequence? Does it possess a unique function? Does it represent the sole therapy available? Finally, early ADA detection provides data that helps to evaluate whether an unwanted immune response can be correlated with clinical effects. Where needed, the ability to detect ADA accurately and as soon as possible may help contribute to reducing the overall risk to patients.

Anthony Mire-Sluis explained how these parameters – probability, severity, detectability – define a preliminary risk score that allows selection of an appropriate testing strategy. In the case of a low-risk score approach, it might be sufficient to test retention samples at the end of the pivotal study, increase ADA assay sensitivity later in product development, and apply a less conservative approach to setting cut points. A high-risk score, however, would require real-time testing of samples, more sensitive assays early in development, and a more conservative approach to setting cut points. Anthony Mire-Sluis recommended that screening assays should detect at least IgM and all IgG subclasses in order not to miss out positive samples. However, the clinical diagnostic value of this

was challenged by some commenters during the conference. Moreover, late development of high affinity antibodies requires consideration. In a risk-based approach, the cut-off may have to be lower than normal in order to reduce the number of false positives rather than false negatives in order to capture all positive samples. Once a sample is shown to be positive, a confirmatory assay is required. The extent of subsequent ADA characterisation with regards to both sensitivity of neutralisation assays and comprehensiveness of antibody characterisation (immunoglobulin-isotype, affinity, time of occurrence, epitope mapping) might also be based on risk. Detection of IgM, for example, could enable early detection of an induced immune response, since IgM occurs prior to IgG formation, but would be an additional requirement not necessarily needed in all scenarios.

Daniel Kramer (Merck Serono, Germany) took up the issue of risk-based strategies for immunogenicity assessment and presented a method to rank therapeutic proteins into different immunogenicity risk classes. He proposed to define these risk classes by the severity of consequences (primary risk factors) and the incidence of ADAs (secondary risk factors). For primary risk factors, he suggested a flow chart with YES/NO branch points depending on the potential consequences of immunogenicity. According to these consequences, proteins are then categorised into low-, medium-, and high-risk proteins. Secondary risk factors comprise patient- and product-related factors such as the immune status of patients, the choice of biotherapeutic expression system, the treatment regimen, the route of administration, and the presence of aggregates or impurities in the final product. Daniel Kramer explicitly elaborated how risk classification entails different screening, confirmation, and characterisation assays to evaluate immunogenicity during both pre-clinical development and clinical phases. He explained that the flow chart was proven to be a helpful tool to rank therapeutic proteins according to their risk classes within his company. However, he emphasised that any immunogenicity strategy will always have to be adapted individually for each project. Therefore, he considered it indispensable to discuss the proposed strategy with the authorities as early in development as possible.

With regards to the mode of administration, Harald Kropshofer (F. Hoffmann-La Roche Ltd., Switzerland) demonstrated that the assumption that subcutaneous administration always increases the immunogenic potential of therapeutic proteins might be too general. He presented two studies that support the aforementioned concept [12,13]. Furthermore, he discussed other studies where no significant difference between the subcutaneous, intravenous, or intramuscular route of administration was found [14]. The higher immunogenicity of a biotherapeutic after subcutaneous administration could be explained by its access to Langerhans cells and dermal dendritic cells, the professional antigen presenting cells which are anatomically located there in very high number – depending on the exact skin layer involved. Harald Kropshofer pointed out that this might hold true only if proteins are dermally – but not hypodermally – administered, since drug proteins injected into the hypodermis might easily access blood vessels before they are taken up by antigen presenting cells, which are rare at that location of the skin. The fact that the immunogenic potential of a biotherapeutic may vary with the actual depth of each cutaneous injection obviously hampers comparisons within and between experimental studies. It also affirms that risk-based strategies are a powerful starting point for the immunogenicity assessment of therapeutic proteins but may require closer, case-by-case analyses.

3.2. Antibody kinetics and sampling – a question of timing

Several talks pointed out how multiple variables in the measurement of ADA impede the assessment of immunogenicity.

Several studies have shown that anti-TNF α mAb, such as infliximab and adalimumab, were often associated with ADA formation resulting in decreased drug concentrations [15], impaired clinical response [16,17] and hypersensitivity reactions [18]. However, other studies did not concur with such observations. For example, the ACCENT I study did not observe any effect of anti-infliximab antibodies (ATI) on clinical outcome in patients with Crohn's disease [19]. In his talk, Yehuda Chowers (Rambam Health Care Campus, Israel) explained that the reasons for this discrepancy are multifactorial and include population heterogeneity, methodological differences of ATI determination, and, most importantly, differences in sampling time points. He reported that 77% of patients with low drug levels and undetectable ATI subsequently developed ATI 8 weeks later, while the percentage of patients with inconclusive ATI data decreased from 50% to 20% at week 16 post-infusion [20].

A case study on natalizumab, presented by Meena Subramanyam (Biogen Idec, USA), further stressed the importance of a detailed analysis of ADA development kinetics and persistence with the associated clinical impact [21]. Natalizumab, a humanised mAb to the α -4 subunit of the VLA-4 receptor, is approved for use in relapsing forms of multiple sclerosis (MS) and, in the US, for Crohn's disease. In a randomised, double-blind, placebo-controlled study on the safety and efficacy of natalizumab in patients with relapsing remitting MS, ADA were detected in 9% of natalizumab-treated patients. Of these, 3% were transiently positive while 6% were persistently positive for antibodies. Interestingly, in over 90% of persistently positive patients, ADA were detected as early as week 12 of treatment; no subject developed persistent antibodies beyond 36 weeks of treatment. Patients who developed ADAs transiently had detectable antibodies at week 12, but were subsequently antibody negative. Besides the time to development of ADA, the persistence of the ADA response was also shown to strongly correlate with a decrease in natalizumab serum levels, diminished VLA-4 receptor saturation and consequently, a substantial decrease in natalizumab efficacy with relapse rates similar to that observed in the placebo arm. In striking contrast, in transiently ADA-positive patients, the decrease observed in circulating drug levels and in VLA-4 receptor saturation was completely reversed when ADA became undetectable in the serum between months 6 and 12. Moreover, the efficacy of the therapeutic as measured by disability progression and relapse rates in the transiently ADA-positive patients was completely restored and mirrored that of patients who remained ADA negative at study end [22]. In this context, details of an analytically validated, simplified immunogenicity ELISA that has been made available to physicians for use in monitoring patients suspected of having developed an ADA response to natalizumab was also presented.

During the panel discussion, participants agreed that the frequency and duration of sampling need to enable a clear differentiation between transient and persistent ADA development, as well as the detection of delayed ADA responses after chronic treatment (1 year). More generally, the sampling schedule should always include baseline sampling and, because the onset of immune response cannot be predicted, samples should be obtained more frequently during the early phase of a clinical trial. It was further noted that the sampling schedule needs to be standardised for each product on a case-by-case basis.

Tom Platts-Mills (University of Virginia, USA) reported on a phenomenon that was observed in the Southeast of the United States where pre-existing antibodies cross-reacted with cetuximab thereby causing hypersensitivity reactions in 20% of patients who had received the first infusion of the mAb [23]. Analysis of pre-treatment sera revealed the presence of IgE antibodies specific for a foreign oligosaccharide present on the Fab portion of cetuximab [24]. Specific IgE antibodies were also found in approximately 20%

of non-cancer subjects in this area and are likely induced by tick bites sensitizing people to this specific oligosaccharide [25]. The presence of the oligosaccharide galactose alpha-1,3-galactose on the Fab part of cetuximab reflects the properties of the mouse cell line, SP2/0, used to produce cetuximab; the mouse cell line NS0 may also add galactose alpha-1,3-galactose residues. The widely used CHO cell line was considered not to add galactose alpha-1,3-galactose; however a recent publication has reported that this property can vary between CHO clones [26]. The occurrence of pre-existing antibodies exhibiting cross-reactivity raised the question, whether or not a specific pre-treatment screening would be required in high-risk regions.

In addition to ADA kinetics, the value of immunoglobulin class and subclass determination for the assessment of immunogenicity was discussed throughout the whole meeting. ADA towards therapeutic proteins are predominantly of the IgG1 subclass. However, treatment of chronic diseases requiring repeated doses over long time periods can result in a switch of the antibody response to IgG4 and, possibly, later to IgE. For example, neutralising antibodies against erythropoietin in patients who subsequently developed PRCA predominantly belong to the IgG4 subclass, and, as reported above, IgE antibodies specific for the galactose alpha-1,3-galactose epitope, present on the Fab portion of cetuximab, have been reported, with consequent hypersensitivity reactions. Roy Jefferis (University of Birmingham, UK) pointed out that an additional parameter that may potentiate immunogenicity is the heavy chain allotype of the antibody therapeutic when there is a miss-match with the heavy chain genotype of the patient [27]. Therefore, ADA characterisation should link allotype profiling of the subjects to the determination of subject ADA specificity for the idiotype and/or allotype of the therapeutic where this might be relevant for the risk assessment.

With regard to the capacity of ADAs to neutralise therapeutic antibodies, Peter Lloyd (Novartis Biologics, UK/Switzerland) explained how immunogenicity signals could be interpreted by integrating information on pharmacokinetics (PK) and pharmacodynamics (PD). The presence of immunogenicity is often apparent in the pharmacokinetic behaviour of a monoclonal antibody, for example rapid clearance of immune complexes. The format of the PK assay, free or total mAb, should also be taken into account when interpreting the overall picture, since ADAs may interfere with the ability to measure drug concentration. Perhaps more importantly, markers of target binding such as ligand capture (soluble ligand) or receptor occupancy (cell surface ligand) contain valuable information regarding the ability to neutralise the target. For example, a soluble ligand-mAb complex is often cleared more slowly than the natural ligand. This leads to an accumulation of inactive ligand-mAb complexes in the systemic circulation, which is a useful marker of ligand capture [28]. If ligand capture is impaired by the presence of ADAs, then the total ligand concentration in serum would be expected to decrease. This is a direct marker of activity in terms of target binding and the ability to maintain target suppression. For cellular targets, the mAb-ligand complex often takes on the elimination characteristics of the ligand. If the ligand is turning over relatively rapidly compared with the clearance of IgG then so called "Target Mediated Drug Disposition" is observed. At low doses the mAb is cleared rapidly, predominantly by binding to the target. At high doses, when saturation of the target is achieved, then clearance of the mAb is predominantly via the normal routes for IgG clearance. In this case, the presence of neutralising ADA may alter the degree of ligand saturation achieved with a given dose of the mAb. Taking into account the impact of immunogenicity on PK/PD, Peter Lloyd concluded that the need for specific neutralisation assays might be obviated in certain cases by the use of these alternative markers of functional activity and an integrated approach when interpreting immunogenicity.

3.3. Prediction of immunogenicity

To avoid the potentially severe consequences of an unwanted immune response, research is geared towards the prediction of immunogenicity before entering clinical trials. Appropriate animal models might in some cases help to enable for a comparative immunogenicity assessment during pre-clinical development. Earlier in drug development, *in vitro* strategies for immunogenicity assessment might be complemented by *in silico* screening procedures.

Philippe Stas (Algonomics [Lonza], Belgium) presented case studies of *in silico* predictions demonstrating the relevance of this method for discovery, selection, and optimisation of biotherapeutic lead compounds. Because T-cell epitopes are required for sustained immunogenicity, protein candidates might be prioritised and selected according to their relative T-cell epitope content. Philippe Stas explained how structure-based *in silico* methods further allow the mapping of linear epitopes from human leukocyte antigen molecules derived from a wide genetic background [29]. The *in silico* tools allow for a relative ranking of comparable drug candidates, but are not recommended for making comparisons between different drug classes. Such analyses may provide a data-driven rationale for selection of candidate molecules for drug development that minimises immunogenicity risk through molecular re-engineering approaches.

Although meeting participants generally questioned the significance of animal models for predicting the probability (as opposed to consequences) of immunogenicity in humans, animal models may in some cases be of value for the comparative immunogenicity assessment of new product candidates. For example, several chemically modified human factor VIII products for the treatment of haemophilia A have been developed with the aim of extending the half-life of Factor VIII. Because any chemical or molecular modification of a protein might create new immunogenic epitopes or generate structures that could stimulate the innate immune system, it is reasonable to compare their potential immunogenicity to the non-modified factor VIII molecule before entering clinical development.

Birgit Reipert (Baxter BioScience, Austria) reported on the development of two new mouse models of haemophilia specifically designed for comparative immunogenicity assessment during pre-clinical development of modified factor VIII proteins. One of these models expresses a *human factor VIII* cDNA as a transgene which causes the development of immunological tolerance to native human factor VIII. When immune-tolerant mice are treated with a modified human factor VIII that expresses new immunogenic epitopes, tolerance breaks down and antibodies against human factor VIII develop. Therefore, this model allows for the exclusion of high-risk candidates early during pre-clinical development. The other mouse model of haemophilia expresses a human MHC-class II protein that is associated with an increased risk for the development of antibodies against factor VIII in patients [30]. As all murine MHC-class II genes are completely knocked out in this model, factor VIII peptides that drive anti-factor VIII immune responses are presented by the human MHC-class II protein. Although such models have their limitations, e.g. the human MHC-class II complex is usually highly polymorphic not consisting of only one or two haplotypes, they might help to identify high-risk candidates before entering clinical development. The final immunogenicity assessment still requires clinical studies.

The importance of prospectively evaluating immunogenicity in clinical studies, at best in a harmonised way for a given product class, was further stressed by Rainer Seitz (PEI, Germany) who presented data on neutralising antibodies, so called inhibitors, complicating treatment with plasma-derived and recombinant factor VIII products. In the 1990s, inhibitors were observed in two clusters of

patients who had been treated with and were tolerant to other factor VIII products for many years. The increased incidence was associated with a change in the production process to introduce new virus inactivation steps [31,32]. It was also suggested that a switch to recombinant factor VIII products may increase the inhibitor risk [33]. Rainer Seitz reported on the ongoing revision of the guideline for the clinical investigation of recombinant and human plasma-derived factor VIII products [34], and stressed the importance of harmonisation of clinical criteria and antibody detection/quantitation assays to allow for an inter-product comparison.

4. In focus: aggregation & post-translational modifications

4.1. Aggregation

One of the major issues throughout the whole conference was the intrinsic tendency of therapeutic proteins to form aggregates. It was suggested that protein aggregates contribute to the immunogenicity of therapeutic protein preparations, either by revealing new epitopes or by forming highly repetitive motifs. Although there have been some reports [35,36] that suggest a causal link between levels of aggregates and the immunogenic potential of biopharmaceutical products, open questions remain as regards incomplete physicochemical definition of the aggregates and by the presence of other immunogenic stimuli in the product. It appears that protein aggregates may contribute to the immunogenicity of therapeutic protein preparations either by revealing new epitopes or by forming highly repetitive motifs. Although these particulates can be present in very high numbers, their mass is often rather

small. Furthermore, the wide size range and diversity of protein aggregates hampers their characterisation.

Jack Ragheb (FDA, USA) summarised the highlights of an IABS-FDA meeting on protein particulates and immunogenicity. According to his talk, there is growing consensus that characterisation of protein particulates might be required in comparability studies after manufacturing changes. However, improved methods and standardisation of definitions will be needed to characterise protein aggregates adequately. Jack Ragheb further pointed out that current methods to determine routinely the number and size of particles are limited for two reasons. First, particles < 10 µm in size are not addressed by current methods. Second, there is no single method available that covers the entire size range of aggregates that spans 3–4 logs. Therefore, orthogonal techniques are required to enumerate and characterise subvisible particles in order to correlate aggregate/particulates profiles with clinical consequences.

Jack Ragheb further explained which intrinsic factors contribute to the risk of immunogenicity induced by aggregates: Amount, size, solubility, and structure of aggregates as well as their strength of association.

A common perception is that aggregates contribute to immunogenicity by breaking B-cell tolerance, leading to activated B-cells that produce binding antibodies [37]. With a HuIFN-α2 transgenic mouse model, Huub Schellekens demonstrated that aggregation increases immunogenicity in a dose-dependent fashion. Preparations containing increasing proportions of soluble aggregates prepared by mixing native rHuIFN-α2b with oxidised rHuIFN-α2b increased immunogenicity in terms of both ADA titre and numbers of animals responding [38]. In contrast to the immune response

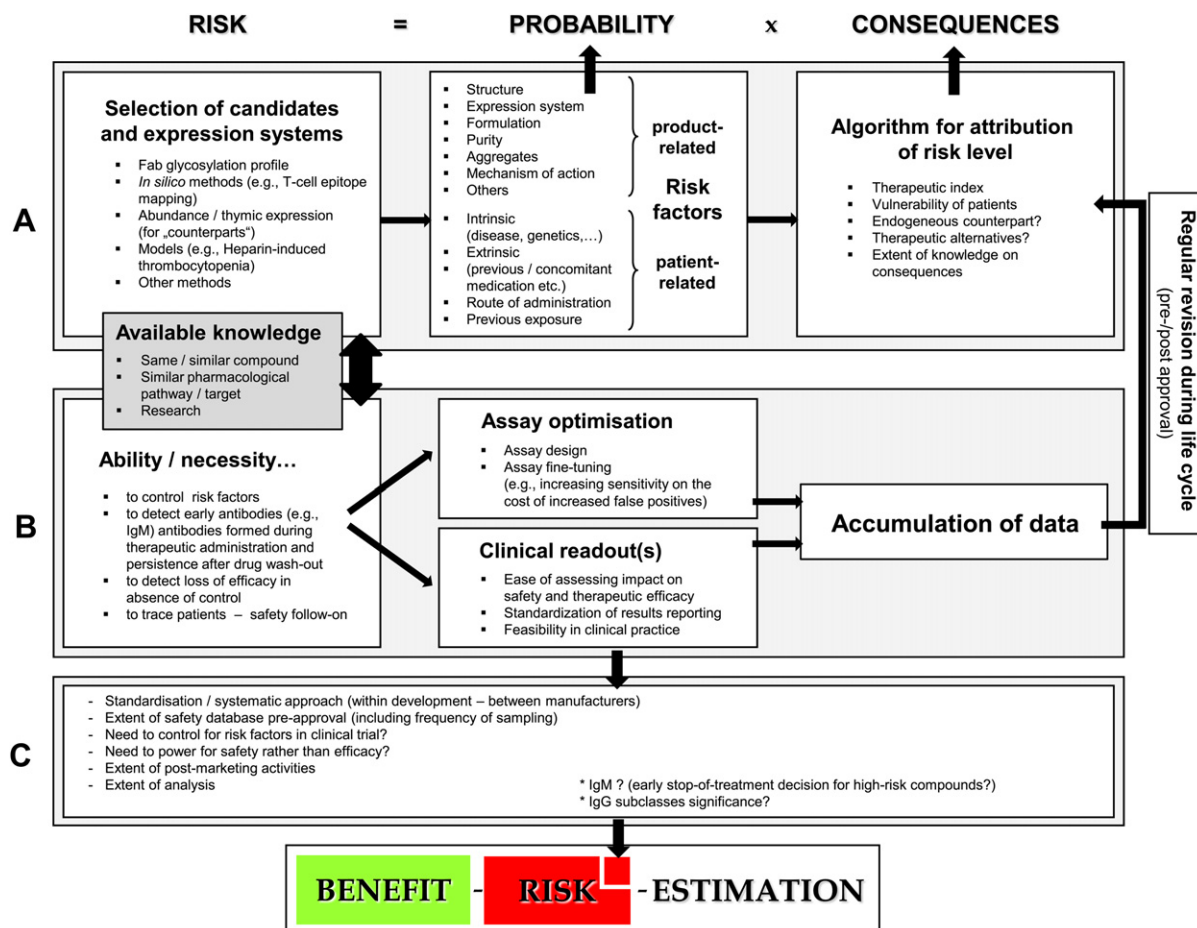


Fig. 1. Framework of risk estimation. This figure combines different approaches of risk identification (A), risk assessment (B), and risk mitigation (C).

against foreign antigens, breaking of self-tolerance results in a slow increase of ADA production that predominantly occurs after long treatment and disappears after the end of treatment. Additionally, Huub Schellekens postulated that aggregates may not induce an immune response by breaking tolerance but rather by by-passing it.

Andreas Greinacher (University of Greifswald, Germany) presented a detailed analysis of how even aggregates of endogenous proteins could induce an antibody response. During heparin-induced thrombocytopenia, the basic chemokine platelet factor 4 (PF4) forms multimolecular complexes with heparin, a polyanionic substance [39]. The resulting charge neutralisation allows PF4 to form clusters in which PF4 tetramers undergo close association. These complexes then induce binding of anti-PF4/heparin antibodies, thereby activating intravascular platelets. As a consequence, this mechanism paradoxically increases the risk for thrombosis in heparin treated patients [40]. Andreas Greinacher concluded that the detailed evaluation of the underlying mechanisms of how aggregates can trigger antibodies to endogenous antigens might provide important insights into the immune response to distinct biotherapeutics [41].

4.2. Post-translational modifications

Another major subject of the conference was the impact of post-translational modifications of therapeutic proteins on immunogenicity. Glycosylation, one of the most frequent post-translational modifications, is often discussed as having effects on the activity, stability, pharmacokinetics, and immunogenicity of therapeutic proteins. Because glycosylation is a species- and cell type-specific process, differences in glycosylation patterns are often found between endogenous and recombinant proteins and between recombinant proteins that are manufactured under different conditions [42].

Generally, 15–20% of normal polyclonal IgG molecules are N-glycosylated in the variable regions of the light and/or heavy chain [43]. For example, 21 distinct oligosaccharide structures were identified within the Fab and Fc region of cetuximab [44]. Roy Jefferis demonstrated how the complex oligosaccharide structure within the CH2 domains of the Fc region impacts Ig-effector functions and biologic efficacy [45]. He pointed out that whilst Fab glycosylation may be beneficial for solubility, the addition of non-human type N-linked oligosaccharides might affect activity and immunogenicity; therefore the glycoforms present should be characterised and controlled.

Amy Rosenberg (FDA, USA) included in her talk an overview of additional post-translational modifications. She provided some examples of how post-translational modifications such as aldehyde formation, oxidation, or deamidation can induce unwanted immune responses. In mice, introduction of aldehydes residues by glycolaldehyde rendered a model self-antigen immunogenic and converted a relatively non-immunogenic malaria antigen into an effective immunogen [46]. Deamidation products of murine peptides resulted in strong B- and T-cell autoimmune responses [47]. Oxidation of IFN- α products correlated with neutralising ADA in patients with chronic hepatitis C [48].

Post-translational or chemical modifications that occur during manufacturing or storage may be well controlled. However, as Amy Rosenberg pointed out, some of these modifications are more likely to occur *in vivo* where they remain undetected. Therefore, it could be important to track therapeutic proteins *in vivo*, particularly at their site of action.

5. Conclusions and recommendations

Assessment of the benefit-risk balance of therapeutic proteins is the key element of the scientific evaluation of a marketing

authorisation application, which encompasses also immunogenic potential in the context of clinical considerations. In his closing remarks, Christian K. Schneider summarised the different approaches for the assessment of unwanted immunogenicity that were proposed during the meeting. Furthermore, he integrated these approaches into a framework of risk estimation, risk assessment, and risk mitigation that would help taking practical measures. This was the aim of the conference, i.e. to not only describe risk factors, but to start considering how to manage and mitigate them.

This framework is a step-wise approach that comprises risk identification, risk assessment, and risk mitigation (Fig. 1A–C). Overall, it defines the risk of an unwanted immune response as the probability combined with the potential severity of consequences. For both dimensions – probability and consequences – there are various components that can often be defined before clinical testing is underway. Patient and product-related factors affecting probability have already been discussed above and in Fig. 1A. For consequences, factors like the presence of an endogenous counterpart, the vulnerability of the patient population, availability of alternative treatments and extent of knowledge on potential consequences are among the factors to be considered (see Fig. 1A).

Some considerations and measures that could already be taken, on a case-by-case basis, before candidate product selection (if possible) include: *in silico* methods for identification of immunogenic linear epitopes, choice of animal models, analysis of thymic antigen expression, and choice of the expression system (established expression system vs. expression system where more limited experience exists).

To determine the potential severity of consequences, any information gathered during the risk identification stage including incident statistics, previous data and available knowledge on the same or similar compounds as well as current research data should be reviewed and, moreover, regularly revised during the product life-cycle (Fig. 1A). It is conceivable that the perception of risk of a given biotherapeutic could change as data accumulates – from higher risk to lower risk, or *vice versa*.

After a risk determination has been performed, the possibility of risk detection and assessment should be undertaken, which means an evaluation of whether it is possible (or necessary) to control the factors identified (either to measure them, or better, to prevent them). It will also be necessary to consider if it is possible to detect in a given patient the clinical manifestations of an unwanted immune response. While infusion reactions may be detected relatively easily, it may be more difficult to evaluate loss of efficacy in a given patient if it is done without a concurrent control (i.e. in clinical practice). If no reliable endpoints or outcomes measures exist, the burden to establish in-depth the incidence and consequences of unwanted immune responses may fall more heavily on the clinical development programme prior to approval. Optimisation of corresponding assays and strategies as well as the establishment of clinical readouts (safety and efficacy) may help in re-defining the risk level classification during development.

After risk has been described and assessed, it is necessary to take the next step, i.e. to monitor, control and minimise the identified risks. There are multiple risk mitigation strategies that range from optimal standardisation of serological assays of the immune response to obtain more systematic and reliable approaches to considerations on how large both the pre-approval safety database and post-marketing activities have to be (Fig. 1C). Moreover, questions to be addressed may include:

- How can identified risk factors be avoided or controlled during clinical trials and after MAA?

- Should the clinical trial be designed to ensure statistical power on safety rather than efficacy? In some cases, efficacy may be established with fewer patients than the reliable description of immunogenicity prior to approval of a biotherapeutic whose risk is considered high and where a need for establishing the immunogenicity profile to this extent has been identified in the previous risk identification and assessment steps.

In this respect, discussants at the conference emphasised frequency of sampling and Ig-class- and IgG subclass analyses as additional details to be considered on a case-by-case basis. It needs to be emphasised that the immunogenicity of a therapeutic protein is, although important, only a part of the overall risk estimation that regulators have to take into account when deciding on the benefit/risk profile of a biotherapeutic prior to marketing approval [49].

Taken together, the field of unwanted immunogenicity has evolved considerably, leading to significant improvements in both knowledge and methodology. In conclusion, participants agreed that an overarching, flexible approach (including the ideas put forward in the currently available regulatory guidance), represents a good platform from which immunogenicity assessment of therapeutic proteins may start from. Supplementation of this basis with more specific recommendations with regard to distinct product classes or assay technologies and approaches seems appropriate but may be currently hampered by the present limitations of state-of-the-art technologies. The development of guidelines with regard to immunogenicity assessment of mAb and biosimilar mAb was appreciated by the audience.

Nevertheless, participants of the conference 'Taking immunogenicity assessment of therapeutic proteins to the next level' put forth the following recommendations:

- to explore the value of animal models and *in silico* prediction of epitopes during pre-clinical development;
- to define techniques for the enumeration and characterisation of aggregates and particulates (including subvisible particles <10 µm) in the final product;
- to further evaluate how route of administration impacts immunogenicity;
- to collect data on post-translational glycosylation and its impact on immunogenicity (and their correlation with hypersensitivity reactions);
- to consider pharmacokinetic and pharmacodynamic assays to complement immunogenicity assessment;
- to define immunogenicity risk classes and their impact on assay strategy;
- to consider whether it is instructive to routinely measure ADA's of the IgM, IgE and IgG isotypes, as well as IgG subclasses;
- to promote traceability to better monitor the safety profile of biotherapeutics
- to work on (clinical) readouts that physicians can use when treating patients in clinical practice that allow for identifying suspicious cases;
- to work on providing assays to treating physicians;
- to establish guidance concerning frequency of sampling;
- to globally harmonise scientific principles for the development, licensing and pharmacovigilance of biotherapeutics (terminology, definitions, data requirements).

The audience proposed that a follow-up conference of this format should be considered both to update and review specific scientific issues (such as aggregates, immune receptors, danger signals, infections, Ig-class switch) and to develop specific recommendations with regard to the immunogenicity assessment of therapeutic proteins.

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The views expressed in this article are the personal views of the authors and are not to be understood or quoted as being made on behalf of the EMA, one of its committees, or of the FDA, nor reflecting the position of the EMA, one of its committees, one of its Working Parties, or the FDA.

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