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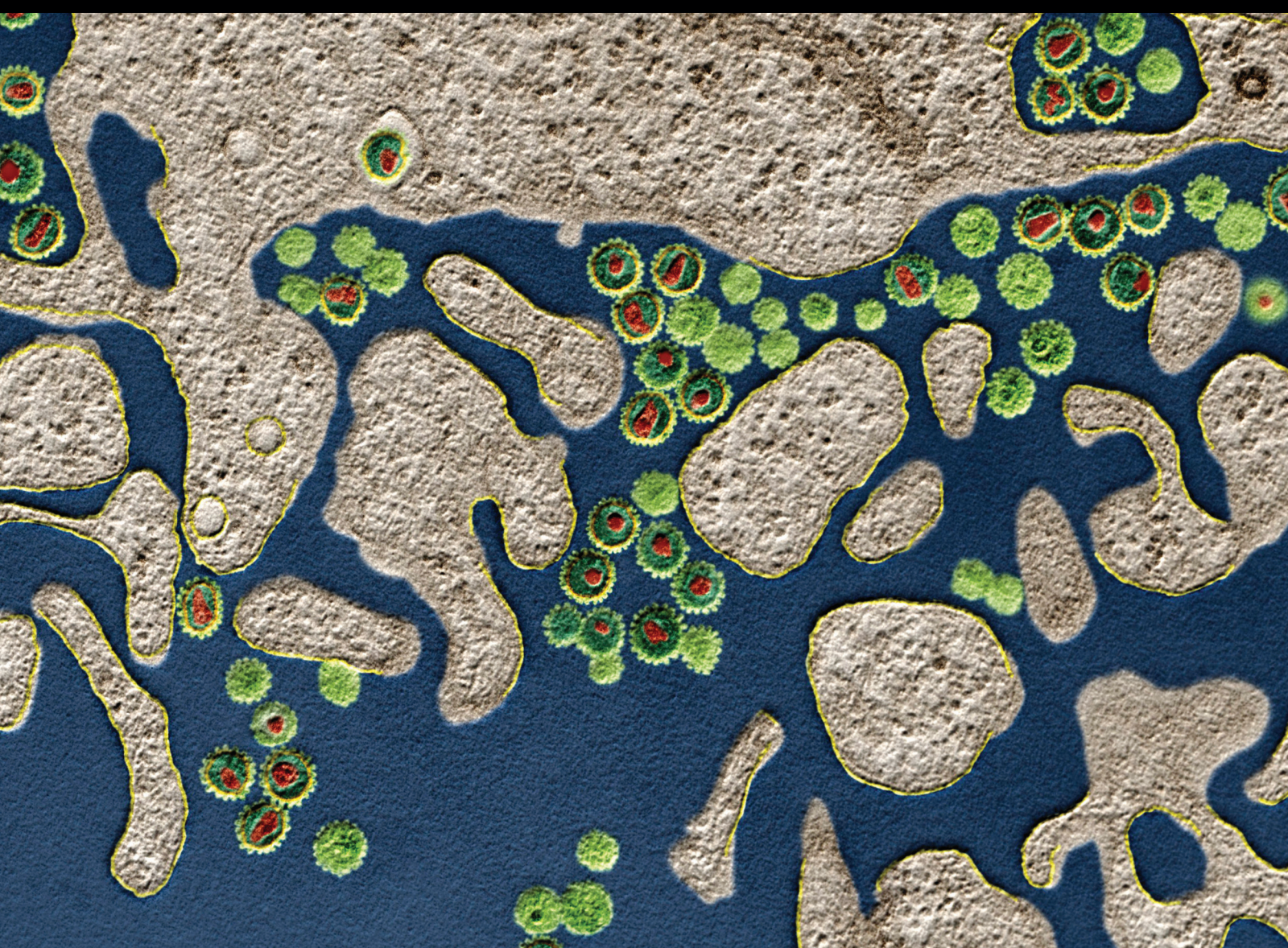
Recent Advances in Assessing Immunogenicity of Therapeutic Proteins: Impact on Biotherapeutic Development

Guest Editors: Yanmei Lu, Leslie A. Khawli, Shobha Purushothama, Frank-Peter Theil, and Michael A. Partridge



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Editorial Board

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Editorial

Recent Advances in Assessing Immunogenicity of Therapeutic Proteins: Impact on Biotherapeutic Development

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Biologics such as monoclonal antibodies, recombinant proteins, and novel protein scaffolds can elicit an unwanted immune response in patients. This response may produce neutralizing and/or nonneutralizing antidrug antibodies (ADA) that can impact drug pharmacokinetics, clinical efficacy, and patient safety. Therefore, it is critical to detect an immunogenic response and characterize ADA in both preclinical and clinical phases of development.

The special issue provides a snapshot of some of the ongoing efforts in the area of immunogenicity such as the prediction, detection, and characterization of the ADA responses as well as the emerging area of understanding how to deal with preexisting ADA. Although the topics adopted by the different papers are diverse, the common theme that unifies these papers is the need to solve the fundamental problems of immunogenicity by using various strategies. Some of the problems addressed by the papers in this thematic issue have a long history, such as the need to better understand the impact of immunogenicity on study outcomes, the corelation of ADA incidence to clinical relevance, and the need to improve our knowledge of manufacturing processes that impact immunogenicity.

M. A. Partridge et al. review the use of technologies such as SQI SquidLite, Genalyte Maverick System, and immunocapture-LC/MS for simultaneous detection and isotyping of ADA response. The pros and cons of using immune PCR for improved drug tolerance and sensitivity as well as the Gyrolab for decreased reagent use and automated workflow are also discussed. Selection of the appropriate technology platform (ELISA, Meso Scale Discovery, Gyrolab, and AlphaLISA) for improved assay sensitivity and drug/soluble target tolerance to reduce false positive rate has been discussed by J. Collet-Brose et al. The improvement in assay sensitivity and drug/soluble target tolerance allows the identification of a much greater ADA positive rate. S. Song et al. present their perspective on correlating unexpected high ADA incidence with clinical relevance to provide physicians with clinically meaningful immunogenicity results. Moreover, different approaches to the use of a generic ADA assay in preclinical testing are presented by M. Carrasco-Triguero et al. and M. Boysen et al. The merits of generic ADA assays include minimal assay development time, in part because these methods do not require reagents specific to the therapeutic molecule. These advantages may significantly advance the drug discovery timeline. A timely paper by J. Ruppel et al. discusses how to deal with the challenge of preexisting ADA to the hinge region of F(ab′)2 therapeutic molecule. The preexisting ADA levels vary considerably between animals, necessitating the use of an individual cut-point for each animal in order to detect treatment induced ADA. Lastly, R. J. Kubiak et al. present evidence that conjugated reagents formulated and stored in a histidine-sucrose buffer had superior assay performance compared with reagents in PBS.

Fully human therapeutic monoclonal antibodies have been developed in part to lower immunogenicity. Although making the antibody sequence more "self" reduces ADA

incidence, clinical data has shown that even "fully" human monoclonal antibodies can induce an antibody response. Knowledge accumulated over the years indicates that many other factors can affect the immunogenicity in addition to the antibody sequence. Therefore, it is important to review the underlying mechanism and identify critical contributing factors of antibody response. In this special issue, A. Kuriakose et al. discuss different causes of antibody responses that are related to inherent properties of the therapeutic molecule, processes in manufacturing the product, patient characteristics, and route of administration. This and another review article in the special issue (R. Jefferis et al.) elaborate on the association of posttranslational modification and immunogenicity.

In addition, a review paper by A. Smith et al. describes the enhanced understanding of the impact of immunogenicity on study outcomes. Specifically, the paper touched upon different preclinical in silico, in vitro, and in vivo tools for immunogenicity risk assessment and the effect of immunogenicity on PK/PD, efficacy, and safety of large molecule therapeutics.

Biotherapeutics have evolved from antibodies to novel modalities such as multidomain proteins, multispecific antibodies, nanobodies, and the like. The collection of papers in this thematic issue covers a range of topics in the field of immunogenicity, including understanding the causes of ADA development, predictive immunogenicity before administration in humans, mitigating interference in ADA assays, and examination of new technologies in ADA detection.

Acknowledgments

We would like to extend our thanks to all the authors, who have high quality submissions and provide great insight into the current state of the field, which has put us ahead in the quest for understanding the immunogenicity of therapeutic proteins. We would particularly like to thank the special issue reviewers, whose efforts substantially contributed to the improvement of the overall quality of this thematic issue.

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Review Article

Unraveling the Effect of Immunogenicity on the PK/PD, Efficacy, and Safety of Therapeutic Proteins

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Biologics have emerged as a powerful and diverse class of molecular and cell-based therapies that are capable of replacing enzymes, editing genomes, targeting tumors, and more. As this complex array of tools arises a distinct set of challenges is rarely encountered in the development of small molecule therapies. Biotherapeutics tend to be big, bulky, polar molecules comprised of protein and/or nucleic acids. Compared to their small molecule counterparts, they are fragile, labile, and heterogeneous. Their biodistribution is often limited by hydrophobic barriers which often restrict their administration to either intravenous or subcutaneous entry routes. Additionally, their potential for immunogenicity has proven to be a challenge to developing safe and reliably efficacious drugs. Our discussion will emphasize immunogenicity in the context of therapeutic proteins, a well-known class of biologics. We set out to describe what is known and unknown about the mechanisms underlying the interplay between antigenicity and immune response and their effect on the safety, efficacy, pharmacokinetics, and pharmacodynamics of these therapeutic agents.

1. Introduction

Biologics are some of the most promising innovations in modern drug therapy and represent a diverse array of molecular tools ranging in complexity and size. These biologically derived therapeutic agents are engineered by exploiting molecular and cellular machinery already found in nature. They are capable of performing complex and precise functions; their medicinal applications are seemingly endless. They greatly differ from the more traditional concept of a *drug*: the small molecule. Small molecules are typically orally administered, lipophilic, organic compounds that are low in molecular weight (<900 Da) and a nanometer in size [1]. These characteristics allow them to diffuse throughout the body, binding promiscuously to both target and off-target sites. On the other hand, biologics encompass a wide range of biomolecular products ranging in size from short peptides to organs for transplantation [2–5]. As these products increase in size and complexity, so do their molecular and functional profiles.

Biotherapeutics are a subclass of biologics that are synthesized using recombinant DNA and/or hybridoma technologies. They include monoclonal antibodies, cytokines, growth factors, hormones, and other regulatory peptides [6]. They differ from small molecule drugs in that they are often hydrophilic and interact with a far more discrete set of targets making them an attractive alternative to traditional small molecule therapies associated with severe toxicities such as chemotherapeutics and immunosuppressants. For instance, antitumor antibodies can be conjugated to small molecule payloads, markedly decreasing the effective dose and toxic effects of the moiety. Once considered science fiction, the advancement of biologic technology has made treatments like enzyme replacement therapies, gene therapy, and tissue engineering a reality.

Biologics have some clear advantages over small molecule drugs, but they are not a panacea. Due to their high antigenic potential, biologics frequently stimulate an immunogenic response [7, 8]. Some clinical investigations of a biotherapeutic have reported antidrug antibody (ADA) formation

incidence rates in >90% of patients [9–12]. These immunogenic responses sometimes result in altered efficacy and immunotoxicity profiles that can vary from patient to patient. Immunogenicity-related adverse events (AEs) may be as benign as a mild itchy rash or as serious as infusion reactions, anaphylaxis, and cytokine release syndrome (CRS) [13, 14]. As an example, tumor necrosis factor (TNF) inhibitors can activate autoantibodies leading to Drug-Induced Autoimmunity (DIA) [15]. Luckily, the majority of immunogenic responses are not toxic and do not lead to sustained autoimmune pathologies. Rather, encounters with ADAs affect efficacy by either accelerating or impeding clearance [16, 17]. It is in these instances that immunogenicity complicates the interplay of pharmacokinetics (PK) and pharmacodynamics (PD), which further obfuscates the development of a reliable preclinical model correlating with clinical events. Without these data, research and development (R&D) are severely impaired, further potentiating the already abysmal attrition rates (85.2%) of biologics once they reach phase 1 clinical trials [18]. As the library of biologics continues to grow, so does our need to understand how immunogenicity affects efficacy and safety. Here we set out to describe the biology of immunogenic response and its impact on the PK/PD of biologics. We do this in an effort to shed light on the challenges and pitfalls facing the successful development of safe and efficacious biologics.

2. Immunogenic Risk Factors and the Need for Predictive Tools

Attributes of both the biologic *and* the patient contribute to the risk of immunogenic response. The antigenicity of a therapy along with patient immune status can be used to predict the occurrence and severity of a potential immunogenic response. Patients with hyperreactive immune disease, autoimmunity, or history of biotherapeutic administration are all at an increased risk of reactions against biologics. Conversely, immunosuppressed patients are less likely to mount a response [19, 20]. Hypervigilant immune systems can be especially problematic when treating autoimmune diseases with biotherapeutics. For instance, an observational study monitored patients being treated for Crohn's disease with infliximab (IFX), a chimeric anti-TNF-alpha therapy [21]. It was found that patients with high anti-IFX antibodies in serum and low trough IFX levels correlated with lowered efficacy or poor IFX persistence. These clinical observations and immunoassays generated a PK profile of IFX that allowed clinicians to monitor Crohn's patients for bioanalytical markers that predicted treatment outcomes in the context of ADA. Nonetheless, this kind of data is often not applicable to biosimilars or other biotherapeutics and is difficult to validate across institutions administering identical therapies due to variances in sample collection, *in vitro* immunoassays, data analysis, and records. In order to standardize the clinical assessment of immunogenic response, procedures and standards must be applied and interpreted by predetermined criteria. The US Food and Drug Administration (FDA) and the European Medicine Agency (EMA) provide regulatory documents detailing such guidelines [22–24].

Genetically derived markers predicting immunogenicity could potentially be applied across all treatment groups and even across biotherapies. For instance, the Human Leukocyte Antigen (HLA) genes that encode for Major Histocompatibility Complex (MHC) class II haplotypes have been shown to play a role in the immunogenic response [25, 26]. A clinical study monitored patients diagnosed with Multiple Sclerosis (MS) treated with Interferon-Beta (INF-β). An association between a common MHC class II allele (DRB1*0701), present in 23% of the patients studied, and an increased likelihood of producing an ADA response against INF-β was discovered. Screening for this allele could guide future treatment plan decisions of MS patients. Establishing reliable and clinically relevant PK profiles of biotherapeutics and identifying genetic markers predictive of immunogenicity will allow clinicians to more effectively monitor and guide biotherapeutic administration.

2.1. Predicting Antigenicity. From discovery to clinic, immunogenicity must constantly be monitored, yet our current preclinical models have poor predictive power for clinically relevant immunogenicity [27]. For example, humanized therapies are designed to evade the immune system of patients, yet they are inherently antigenic in immunocompetent animal models and often exaggerate the antigenicity of the therapeutic agent. Nevertheless, the effort to combine preclinical data from *in silico*, *in vitro*, and *in vivo* methods remains worthwhile and may one day yield valid criteria for immunogenicity risk assessment.

In silico computational tools have broad applications in the identification and assessment of antigenicity. Some screen for leads such as cancer neoantigens and peptides for vaccine targets [28–30]. Others troubleshoot antigenicity by optimizing the design of peptide nanoparticles or by determining deimmunization methods for antileukemic L-asparaginase [31, 32]. As this technology matures and provides more reliable predictions, we will improve our ability to navigate the effects of immunogenicity.

In vitro modelling of antigenicity utilizes T cell assays, B cell assays, and mimotopes to identify potentially immunogenic epitopes [33, 34], whereas *in vitro* characterization of immunogenic response from serum samples includes qualitative and quasiquantitative immunoassays such as enzyme linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), and immunoradiometric assays (IRMAs) [35]. Cell-based assays remain a popular choice for modeling antigenicity via the immunophenotyping, proliferation tracking, and cytokine secretion profiling of immune cells [36, 37]. However, these techniques remain costly since they are labor and time intensive. *In vitro* modeling and *in vitro* characterization work together to describe mechanisms of immune activation as well as immunogenic markers/phenotypes belonging to both disease and nondisease states. Ideally, this information can be used to predict the magnitude and frequency of immunogenic response and to discern which patients are most at risk of developing this response. The limitations of the clinical utility of *in vitro* assays for immunogenic marker discovery were discussed in a previous section. However, predictive preclinical *in vitro* assays would help protect both

patients and companies from the expense and risk associated with failed clinical trials. The development of such assays can be informed by selecting clinically tested therapeutic proteins and then validated if the clinically observed antibody response can be reconstructed in the preclinical setting [38]. Expanding the repertoire of valid yet simplified assays will push forward our preclinical evaluation of biologic design hopefully improving biologic design at its earliest stages.

Preclinical *in vivo* methods monitoring the antigenicity of a therapy have failed to correlate with clinical events and are considered to have low predictive value for immunogenic response in humans according to the International Conference on Harmonisation (ICH) *S6* guidance. However, they still hold value in the preclinical investigation of immunogenicity [39]. Nonhuman primates have proved successful in the modeling of toxicology, but an analysis of their use in nonclinical trials revealed that they are not particularly good for evaluating efficacy or immunogenicity since they both under-/overpredicted immunogenic response [40]. Consequently, researchers are still searching for ways to model human immunogenic response *in vivo*. One strategy is to engineer transgenic mice to express human antigens that tolerize them to humanized biotherapeutics. In these mice, researchers were able to model immune complex aggregation and extended-dose related ADA formation which closely mirror the phenomena observed in humans [41]. Vaccines are often evaluated in transgenic mice with a humanized haplotype, which has proved to be an effective tool for predicting CD4+ T cell activation [42, 43]. Savvy strategies like these are gradually improving our preclinical *in vivo* investigations, howbeit animal modeling remains an unreliable predictor of clinically relevant immunogenicity.

Over the past few decades, investigators have developed sophisticated assessment strategies and significantly expanded our understanding of immunogenicity. While regulatory agencies have agreed upon and established international guidelines for certain preclinical standards, the nonclinical evaluation of allergy and pseudoallergy remains unclear [44].

3. Immunotoxicity

Immunotoxicity is not unique to biologics. Penicillin is a classic example of a potentially lethal hypersensitivity reaction to a drug therapy, and DIA can be induced via small molecule therapies as well. As early as the 1950s, drug-induced lupus was documented in response to hydralazine [45]. Since biologics have greater immunogenic potential, they are more likely to elicit immunotoxic effects. Here we will briefly discuss immunogenic response, emphasizing ADA formation, and how these can affect the toxicity profile of the therapy.

Interestingly, many biotherapeutic agents are immunomodulators capable of stimulating or suppressing immune function. This immunogenicity is engineered to accomplish a therapeutic goal, whereas unwanted immunogenic response may impair therapeutic outcome by affecting safety and/or efficacy. Immunotoxicology is associated with four derangements of immune function: immunosuppression, immunostimulation, hypersensitivity, and autoimmunity [46, 47]. These derangements can result in an unintentional blockade of endogenous function, CRS, infusion reactions, and anaphylaxis. While these AEs can have serious consequences, the majority of immunogenic response elicits mild AEs or remains clinically silent. Prior to the start of a clinical trial, it is necessary that researchers evaluate the therapy in order to establish appropriate criteria for monitoring immunogenicity and to define unacceptable immunotoxicity that would halt/end the study.

Immunologically based AEs are often ADA-mediated. The formation of ADA is triggered when the classical pathway recognizes a "foreign" antigen via antigen presentation cells or by B/T cell mediated breaking of tolerance to humanized recombinant therapies [17, 48]. The latter functions via two pathways: the T cell dependent (TD) and the T cell independent (TI) pathway. TD accounts for 90% of ADA formation and relies on antigen stimulation of CD4+ T cell, which mounts a full adaptive immune response. TD activating therapeutic agents have an epitope that engages CD4+ T cells thus generating high affinity IgGs and memory B cells leading to a more persistent and robust response. In contrast, TI-ADA are thought to arise from the direct stimulation of B cell receptors (BCRs) by molecular therapies containing patterned structural motifs, that is, polymeric repeats or carbohydrate molecules, capable of crosslinking BCRs. TI activation generates pentavalent IgM or low-affinity IgG antibodies that bypass T cell mediated affinity maturation and isotype switching. This immature antibody response fails to induce memory phenotypes. T cell epitope screening should be considered along with the construct design of the therapy in order to mitigate ADA response by either selecting for immunosuppressive phenotypes like Tregs or avoiding stable epitopes all together.

Each biologic must be evaluated case by case for potential immunotoxicity. Careful attention is required when considering bispecific antibodies, antibody drug conjugates, and other products lacking an endogenous analog since they are more likely to elicit ADA formation [49]. For instance, Hemophilia A is a life-threatening genetic disease in which patients lack an essential clotting factor and is treated with Factor VIII replacement therapy [50]. Unfortunately this therapy triggers ADA response in up to a third of patients and often worsens the condition in patients with high ADA titers. Even though supplementary recombinant proteins are often constructed from the native protein sequences already expressed in patients they can elicit ADA which also neutralized the native proteins resulting in thrombocytopenia and red-cell aplasia [51, 52]. Additionally, tissue deposition of immune complexes can precipitate disease such as nephrotic syndrome and drug-induced vasculitis [53, 54]. Preclinical models and clinical precedence can provide clues pointing to possible immunogenic outcomes of a biologic in the clinic; however, immunotoxicity remains an unpredictable threat to patient safety.

4. The Impact of Immunogenicity on PK/PD and Efficacy

In order for a therapy to be successful once it reaches market, clinicians must have confidence in its therapeutic potential

and be able to prescribe/administer the drug with a reliably effective dosing schedule. What the body does to the drug (PK) and what the drug does to the body (PD) need to be fully characterized and understood. This requires the accrual of data pertaining to treatment outcomes and clinical events that can be generalized to the biologic's target population. This has proven to be a challenging task for biotherapeutics, in part, because immunogenicity unpredictably perturbs PK/PD and efficacy. Regardless, the success of biologics would be improved by defining the impact of unwanted immunogenicity on PK profiles, especially since the primary reason for drug failure in phase II clinical trials is lack of clinical efficacy [55].

Small molecules and therapeutic proteins are comprised of entirely distinct molecular classes which are not only distinguished by their chemical profiles but their immunogenicity and PK profiles as well [17, 56]. Small molecule PK profiles can be interpreted from direct measurements of the active drug and its metabolites in serum; their ADME is well characterized and employs simplified compartment models. Their PD can be interpolated from relationships such as dose-exposure-response curves and does not require a nuanced understanding of the drug's mechanism of action. The PK/PD of biologics are driven by target-mediated drug disposition (TMDD) meaning that the high affinity of the therapy for its target (PD) strongly influences its PK. Theoretically, TMDD should yield a large volume of distribution with a high accumulation of therapy in its target tissue. However, tissue uptake can be slowed by a variety of mechanisms thus confining the therapy to its administration site. Currently, we do not understand ADME well enough to fully describe and characterize this interplay.

Serum measurements of biologics must consider the unbound, partially bound, and bound forms [57–59]. Distinguishing between the free and bound forms of the biologic and its metabolites is necessary in order to identify the effective fraction of bioactive drug in circulation. The terminal half-lives of these forms tend to vary, with the free form often displaying the shortest half-life [60]. Many of the well-established small molecule models employed by R&D fail to describe the PK/PD and ADME of biotherapeutics. Additionally, unwanted and unanticipated immunogenic response perturbs PK/PD thereby diminishing the predictive power of preclinical data. Our inability to describe and anticipate these phenomena hinders our ability to develop biologics that will prove efficacious and reliable once they reach clinical trial. Here we discuss what is known about immunogenicity's role in affecting PK/PD and how we hope to apply this knowledge in order to improve the predictive power of biologic models.

4.1. The Influence and Modes of Action of Immunogenicity on PK/PD Assessment. The immunogenic potential of biologics can be affected at any stage of their journey, starting with their route of administration or even after the biologic is cleared, by leaving a lasting impression on the immunomemory of the patient. Most biotherapeutics are large hydrophobic molecules that cannot survive the harsh conditions of the GI tract; therefore they have poor bioavailability by oral administration. Traditionally therapeutic proteins have been given intravenously (IV) maximizing bioavailability and minimizing immunogenic risk. However, this method is both inconvenient and expensive. Newer formulations allow for subcutaneous (SC), intraperitoneal, or inhalation routes [61–63]. SC administration is more cost effective and convenient than IV, yet it is often slow and incomplete. In particular for large molecules (>20 kDa) that tend to migrate more slowly through the extracellular matrix (ECM), SC poses an increased risk of ADA formation by dendritic cells processing which drain directly to the lymphatics. Depot formulations can aid absorption by including ECM degrading enzymes like hyaluronidase and by stabilizing proteins preventing degradation and aggregation [64]. Currently we are in need of formulations that can deliver an efficacious dose in affordable and convenient ways.

As was previously described, myriad patient and product-related factors can influence immunogenicity's effect on PK/PD and efficacy. ADAs are described by their mode of action, classifying them as either neutralizing ADAs (nADAs) or nonneutralizing ADAs (non-nADAs) [65]. The nADAs interact directly with biologics and/or bind to pharmacologically relevant sites, obscuring interactions between the therapy and its target, thereby decreasing the overall efficacy. Another classification categorizes ADA as sustaining or clearing, which indicates their influence on protein drug clearance [66]. Both nADA and non-nADA can either prolong or shorten the half-life of biologics. Most of the time immune complex formation, comprised of drug-ADA aggregates, will increase the clearance of the biotherapeutic. This phenomenon is most notable in patients with high affinity, high titer ADA. In some cases, isotype interactions with Fc receptors (FcR) also influence serum drug concentration. For IgGs, binding to the neonatal FcR (FcRn) increases bioavailability, possibly due to FcRn-mediated protection from catabolism at the injection site and/or draining lymphatics [67, 68]. Coupling immunoprotective technologies, such as Fc fusion proteins, with improved delivery formulations could stabilize circulating concentrations of biotherapeutics and their overall bioavailability. This effect will vary from patient to patient depending on factors like their MHC haplotypes and whether TD-ADA versus TI-ADA are triggered. Since we do not fully understand the patient and product-related factors governing this response, it is difficult to establish general guidelines defining an efficacious and reliable dosing regimen.

In order to characterize the effect of ADA on the PK profile of a biologic, an immunogenicity profile must be established. ADAs are a temporally evolving heterogeneous population circulating in serum alongside a multitude of endogenous proteins and antibodies [69, 70]. In most animals, ADAs become detectable within 2–4 weeks after the first dose administration and around 10 days in mice. They are usually polyclonal antibodies against multiple epitopes comprised of more than one isotype circulating at varying concentrations amongst a multitude of endogenous proteins. To overcome this daunting task of identifying and classifying ADA response, a standard bioanalytical schematic is employed: (1) sensitive screen, (2) confirmatory assay, and (3) functional characterization. The detection of these ADA is limited to capture antibody concentrations >20–1000 ng/mL

with Kd of K-9 to K-8, respectively [71–73]. Occasionally the therapeutic is administered at such a low dose that it is rapidly consumed by the target site. In this absence of detectable circulating drug, PD measurements of target binding can correlate drug exposure. This method of analysis can also be useful in instances where a therapeutic protein has bioactive metabolites not measured by PK assays [74].

Studies looking at therapeutic antibodies, such as natalizumab and IFX, observed an increase in clearance and a reduction in efficacy when ADAs were persistent rather than transient throughout the first 12 weeks of therapy. They also found that clearing ADA formation was detectable 8–16 weeks after treatment initiation [8, 75, 76]. These labile kinetics suggest that *when* a measurement is taken significantly, it impacts the interpretation of the bioanalytics; therefore, dosing and sampling schedules should be well-defined and include peak and trough measurements as well as time points sampled long after the circulating drug has been cleared. Higher concentrations of ADAs are more likely to impact PK profiles and interfere with bioanalytical measurements making simultaneous quantification of both the biologic and ADA difficult. This hampers the ability to track the time course of ADA response. As a result, the impact of immune response on biologics cannot be fully investigated and our understanding remains incomplete.

Ultimately, the development of a physiologically based pharmacokinetic (PBPK) model akin to the small molecule compartment model that also accounts for immunogenicity would be ideal. Both "top down" and "bottom up" approaches are being used to derive mathematical models of ADME. For example, biodistribution coefficients (BC) can estimate tissue specific distribution of protein fragments based on molecular weight and plasma concentrations [77]. The BC50 values for most tissues were found to be ~35 kDa. Clinical derivation of these models could be achieved with a meta-analysis of clinical PK bioanalytical data, but inconsistent reporting of bioanalytical parameters regarding ADA response makes studies incomparable between institutions [78]. Fortunately, by collecting sparse or dense samples from many clinical studies, Population PK (PopPK) allows for the inclusion of data from a variety of un-/balanced designs. Most advantageously, PopPK looks at the target population of interest receiving clinically relevant doses rather than the healthy subjects in traditional PK studies. These studies enable the identification and measurement of variances and can point to potential explanations if identifying factors (demographic, environment, and pathophysiology) are found to correlate with altered PK. Currently, groups are working to validate parameters and refine models relating PK and immunogenicity; they have yet to design a reliable model generalizable to a diverse array of biotherapeutics.

ADAs affect both the clearance and efficacy of biologics. Building off of this basic tenant we have been able to partially classify the interplay between immunogenicity and PK/PD, yet we are unable to fully describe these phenomena. Unlike the PK/PD of small molecules, which began with bioanalytical measurements that later yielded theories to describe those measurements, the PK/PD of biologics is mostly understood through its apparent differences from small molecule ADME. We cannot rely on our current bioanalytical techniques to predict or describe PK/PD or clinical efficacy in the context immunogenic response.

5. Strategies for Overcoming the Pitfalls of Immunogenicity

As we have discussed, the PK/PD and the ADME of biologics can be dramatically affected by immunogenic response. So how then can we mitigate the consequences of those effects? We can approach this challenge from both R&D and clinical perspectives.

5.1. Biotherapeutic Production and Design. Design and manufacturing processes can mitigate or exacerbate the inherent antigenicity of biotherapeutics. Good manufacturing processes and quality assurance recognize the inherent heterogeneity of biologic batches. Antigenic impurities such as endotoxin contamination and detergents, aggregate formation, and variation in biologic activity must be kept to a minimum in order to ensure a safe and reliably efficacious product [79]. Careful handling of the protein product throughout the entire manufacturing process is critical in preventing aggregate formation. Not only are aggregated and misfolded proteins less efficacious, but also they precipitate immune complex formation and immunogenic response. Data suggest that proteins exposed to stress, freeze-thawing, and pH shifts with surface active agents or hydrophobic surfaces are more likely to form aggregates that can crosslink BCRs thereby inducing TI-ADA formation [80, 81]. Additionally, some biotherapeutics do not tolerate high concentration formulations well and may require more frequent dosing [82]. By improving production processes and conservatively administering these therapies, we can prevent the acquisition of these unnecessary antigenic properties.

Construct design is at the heart of biologic engineering. New and exciting therapies such as bispecific antibodies and antibody drug conjugates are expanding the utility and function of biotherapeutics, even so they may introduce antigenicity with their inadvertently novel structures. In fact, it was the drive to decrease antigenicity that evolved antibody therapies. Initial antibody therapies were murine-derived and found to be highly immunogenic. Eventually chimeric antibody technologies evolved until we achieved fully humanized antibodies. In theory, a humanized antibody, free of antigenic properties, should not be immunogenic. In reality, the immense variance of CD4+ T cell epitopes and MHC haplotypes allows for fully humanized antibodies to remain immunogenic in some patients [83, 84]. To mitigate this, deimmunization strategies can be used when designing protein therapeutics. This method utilizes *in silico* predictions of antigenic peptide sequences to identify potentially antigenic epitopes in a therapeutic protein. These peptides are then synthesized and their antigenicity is evaluated with *in vitro* binding assays to TCRs and MHC II receptors. Immunogenic epitopes are then mutated to a more tolerated sequence and subsequent constructs are iteratively screened. Tolerization is an ingenious twist on deimmunization that selects for and engineers biologics with Treg activating

epitopes that can induce tolerance of the biologic [85]. Combining both deimmunization with tolerization has proved an effective method for reducing immunogenic response. Posttranslational modifications of biologics also appear to influence immunogenicity. For instance, glycosylation can shield antigens from ADA binding and retard processing. In line with this finding, a synthetic kind of glycosylation known as PEGylation can prevent protein aggregation and mask antigenic epitopes thereby reducing immune response [86]. However, sometimes this process introduces an immunogenic linker. Additionally, some forms of xeno-glycation may have immunostimulatory effects. The risk is especially high when eukaryotic organisms like yeast are used to express proteins, since they may introduce their own microorganism specific glycosylation patterns. For instance, yeast posttranslational processing adds mannose glycan which can bind to mannose receptors on human immune cells [87]. Though this is not an exhaustive list of all the possible considerations that should be taken into account when developing biologics, these examples illustrate the usefulness of optimizing a biologic's design to minimize immunogenicity.

5.2. The Clinical Approach. Clinicians already manage immunogenicity associated with a variety of pathologies and treatments such as rheumatoid arthritis and hematopoietic stem cell transplantation. Clinical strategies to mitigate immunogenic response include dose manipulation and immunosuppressive agents such as methrotrexate and prednisone. Some patients diagnosed with Infantile Pompe disease formed ADAs when treated with the enzyme replacement therapy, recombinant human acid α-glucosidase (rhGAA) [88, 89]. A subgroup of patients form nADAs, but additional work showed that this subgroup can be tolerized against rhGAA by complementing the replacement therapy with combinatorial immunosuppressive therapy comprised of rituximab and methotrexate. Fortunately, this tolerance persisted even after termination of B cell recovery and immunosuppression. Dosing-through is another method that has also proven to be a successful strategy in overcoming the deleterious effects of ADA on efficacy [90]. For MS patients receiving long term IFN-s therapy, nADAs have also proven to be problematic. Interestingly, recent studies have shown that high doses of IFN-sv32 result in lower nADA titers due to the saturation of these nADAs and the restoration of IFN-s binding to its receptor. High doses of IFN-s can also induce long term high dose tolerance of the immune system.

6. Summary and Conclusions

The advancement of biologics has revolutionized targeted therapy. By utilizing biologically derived tools we can administer drugs with complex and precise functions, which often mimic the body's natural processes. However, it is this key characteristic of molecular mimicry that potentiates the antigenicity of biologics and leads to undesirable ADA-mediated outcomes such as immunotoxicity and decreased efficacy. ADAs against biologics can alter ADME thereby greatly confounding the interpretation of PK/PD assessments. Thus, it is vital that we develop bioanalytical tools that can reliably predict and model the complex interplay between immunogenicity and PK/PD. Outside of patient immune status and exposure history, we do not currently possess adequate tools or knowledge of immunogenic markers to identify which patient-product interactions carry an increased risk of immunogenic response. Though we described various preclinical *in silico*, *in vitro*, and *in vivo* tools used for immunogenic risk assessment, these methods are not recognized as predictive. We must rely on clinical investigations. As novel biotherapeutic proteins continue to shape the course of modern medicine, we must work to fill these knowledge gaps in order to ensure that patients receive safe and effective treatments.

Competing Interests

The authors declared no conflict of interests.

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References

[1] M. J. Macielag, "Chemical properties of antibacterials and their uniqueness," in *Antibiotic Discovery and Development*, T. J. Dougherty and M. J. Pucci, Eds., pp. 801–802, 2012.

[2] J. Maca, V. Dragalin, and P. Gallo, "Adaptive clinical trials: overview of phase iii designs and challenges," *Therapeutic Innovation and Regulatory Science*, vol. 48, no. 1, pp. 31–40, 2014.

[3] H. Kempf, B. Andree, and R. Zweigerdt, "Large-scale production of human pluripotent stem cell derived cardiomyocytes," *Advanced Drug Delivery Reviews*, vol. 96, pp. 18–30, 2016.

[4] N. Kubicki, C. Laird, L. Burdorf, R. N. Pierson III, and A. M. Azimzadeh, "Current status of pig lung xenotransplantation," *International Journal of Surgery*, vol. 23, pp. 247–254, 2015.

[5] A. M. Bailey, J. Arcidiacono, K. A. Benton, Z. Taraporewala, and S. Winitsky, "United states food and drug administration regulation of gene and cell therapies," *Advances in Experimental Medicine and Biology*, vol. 871, pp. 1–29, 2015.

[6] M. Wadhwa, I. Knezevic, H.-N. Kang, and R. Thorpe, "Immunogenicity assessment of biotherapeutic products: an overview of assays and their utility," *Biologicals*, vol. 43, no. 5, pp. 298–306, 2015.

[7] C. A. Weber, P. J. Mehta, M. Ardito, L. Moise, B. Martin, and A. S. De Groot, "T cell epitope: friend or foe? Immunogenicity of biologics in context," *Advanced Drug Delivery Reviews*, vol. 61, no. 11, pp. 965–976, 2009.

[8] R. Ponce, L. Abad, L. Amaravadi et al., "Immunogenicity of biologically-derived therapeutics: assessment and interpretation of nonclinical safety studies," *Regulatory Toxicology and Pharmacology*, vol. 54, no. 2, pp. 164–182, 2009.

[9] K. Kuus-Reichel, L. S. Grauer, L. M. Karavodin, C. Knott, M. Krusemeier, and N. E. Kay, "Will immunogenicity limit the use, efficacy, and future development of therapeutic monoclonal

antibodies?" *Clinical and Diagnostic Laboratory Immunology*, vol. 1, no. 4, pp. 365–372, 1994.

[10] E. Koren, L. A. Zuckerman, and A. R. Mire-Sluis, "Immune responses to therapeutic proteins in humans—clinical significance, assessment and prediction," *Current Pharmaceutical Biotechnology*, vol. 3, no. 4, pp. 349–360, 2002.

[11] H. Schellekens and N. Casadevall, "Immunogenicity of recombinant human proteins: causes and consequences," *Journal of Neurology*, vol. 251, supplement 2, pp. II4–II9, 2004.

[12] H. Schellekens, "Immunogenicity of therapeutic proteins: clinical implications and future prospects," *Clinical Therapeutics*, vol. 24, no. 11, pp. 1720–1740, 2002.

[13] T. Corona, C. Leon, and L. Ostrosky-Zeichner, "Severe anaphylaxis with recombinant interferon beta," *Neurology*, vol. 52, no. 2, p. 425, 1999.

[14] R. Stebbings, L. Findlay, C. Edwards et al., "'Cytokine storm' in the Phase I trial of monoclonal antibody TGN1412: better understanding the causes to improve preclinical testing of immunotherapeutics," *Journal of Immunology*, vol. 179, no. 5, pp. 3325–3331, 2007.

[15] M. De Bandt, J. Sibilia, X. Le Loët et al., "Systemic lupus erythematosus induced by anti-tumour necrosis factor alpha therapy: a French national survey," *Arthritis Research & Therapy*, vol. 7, no. 3, pp. R545–R551, 2005.

[16] V. Jawa, L. P. Cousens, M. Awwad, E. Wakshull, H. Kropshofer, and A. S. De Groot, "T-cell dependent immunogenicity of protein therapeutics: preclinical assessment and mitigation," *Clinical Immunology*, vol. 149, no. 3, pp. 534–555, 2013.

[17] H. Kropshofer and W. F. Richter, "Immunogenicity: its impact on ADME of therapeutic biologics," *Pharmaceutical Sciences Encyclopedia*, vol. 11, pp. 1–2, 2015.

[18] M. Hay, D. W. Thomas, J. L. Craighead, C. Economides, and J. Rosenthal, "Clinical development success rates for investigational drugs," *Nature Biotechnology*, vol. 32, no. 1, pp. 40–51, 2014.

[19] Y. Vugmeyster, J. Harrold, and X. Xu, "Absorption, Distribution, Metabolism, and Excretion (ADME) studies of biotherapeutics for autoimmune and inflammatory conditions," *AAPS Journal*, vol. 14, no. 4, pp. 714–727, 2012.

[20] P. Ragnhammar, H. J. Friesen, J. E. Frodin et al., "Induction of antirecombinant human granulocyte-macrophage colony-stimulating factor (Escherichia coli-derived) antibodies and clinical effects in nonimmunocompromised patients," *Blood*, vol. 84, no. 12, pp. 4078–4087, 1994.

[21] S. Ben-Horin, M. Yavzori, L. Katz et al., "The immunogenic part of infliximab is the F(ab′)2, but measuring antibodies to the intact infliximab molecule is more clinically useful," *Gut*, vol. 60, no. 1, pp. 41–48, 2011.

[22] US FDA Guidance, *Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products; Revised Draft Guidance for Industry; Availability*, FDA, Silver Spring, Md, USA, 2016.

[23] EMA, "Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins," Tech. Rep. EMA/CHMP/BMWP/14327/2006, EMA, London, UK, 2007.

[24] EMA, "Guideline on immunogenicity assessment of monoclonal antibodies intended for in vivo clinical use," Tech. Rep. EMA/CHMP/BMWP/86289/2010, EMA, London, UK, 2012.

[25] M. D. F. S. Barbosa, J. Vielmetter, S. Chu, D. D. Smith, and J. Jacinto, "Clinical link between MHC class II haplotype and interferon-beta (IFN-β) immunogenicity," *Clinical Immunology*, vol. 118, no. 1, pp. 42–50, 2006.

[26] V. Zota, A. Nemirovsky, R. Baron et al., "HLA-DR alleles in amyloid β-peptide autoimmunity: a highly immunogenic role for the DRB1*1501 allele," *Journal of Immunology*, vol. 183, no. 5, pp. 3522–3530, 2009.

[27] V. Brinks, D. Weinbuch, M. Baker et al., "Preclinical models used for immunogenicity prediction of therapeutic proteins," *Pharmaceutical Research*, vol. 30, no. 7, pp. 1719–1728, 2013.

[28] A. Desrichard, A. Snyder, and T. A. Chan, "Cancer neoantigens and applications for immunotherapy," *Clinical Cancer Research*, vol. 22, no. 4, pp. 807–812, 2016.

[29] R. Zaheer, C. L. Klima, and T. A. McAllister, "Expeditious screening of candidate proteins for microbial vaccines," *Journal of Microbiological Methods*, vol. 116, pp. 53–59, 2015.

[30] P. Carlos, V. Roupie, S. Holbert et al., "In silico epitope analysis of unique and membrane associated proteins from *Mycobacterium avium* subsp. paratuberculosis for immunogenicity and vaccine evaluation," *Journal of Theoretical Biology*, vol. 384, pp. 1–9, 2015.

[31] T. A. P. F. Doll, R. Dey, and P. Burkhard, "Design and optimization of peptide nanoparticles," *Journal of Nanobiotechnology*, vol. 13, no. 1, article 73, 2015.

[32] L. N. Ramya and K. K. Pulicherla, "Studies on deimmunization of antileukaemic L-asparaginase to have reduced clinical immunogenicity—an in silico Approach," *Pathology and Oncology Research*, vol. 21, no. 4, pp. 909–920, 2015.

[33] E. M. Moussa, J. Kotarek, J. S. Blum, E. Marszal, and E. M. Topp, "Physical characterization and innate immunogenicity of aggregated intravenous immunoglobulin (IGIV) in an in vitro cell-based model," *Pharmaceutical Research*, vol. 33, no. 7, pp. 1736–1751, 2016.

[34] V. Dhir, M. Fort, A. Mahmood et al., "A predictive biomimetic model of cytokine release induced by TGN1412 and other therapeutic monoclonal antibodies," *Journal of Immunotoxicology*, vol. 9, no. 1, pp. 34–42, 2012.

[35] L. Yin, X. Chen, P. Vicini, B. Rup, and T. P. Hickling, "Therapeutic outcomes, assessments, risk factors and mitigation efforts of immunogenicity of therapeutic protein products," *Cellular Immunology*, vol. 295, no. 2, pp. 118–126, 2015.

[36] V. Rombach-Riegraf, A. C. Karle, B. Wolf et al., "Aggregation of human recombinant monoclonal antibodies influences the capacity of dendritic cells to stimulate adaptive T-cell responses in vitro," *PLoS ONE*, vol. 9, no. 1, Article ID e86322, 2014.

[37] H. Kropshofer and T. Singer, "Overview of cell-based tools for pre-clinical assessment of immunogenicity of biotherapeutics," *Journal of Immunotoxicology*, vol. 3, no. 3, pp. 131–136, 2006.

[38] P. Gaitonde and S. V. Balu-Iyer, "*In vitro* immunogenicity risk assessment of therapeutic proteins in preclinical setting," *Methods in Molecular Biology*, vol. 716, pp. 267–280, 2011.

[39] V. Brinks, W. Jiskoot, and H. Schellekens, "Immunogenicity of therapeutic proteins: the use of animal models," *Pharmaceutical Research*, vol. 28, no. 10, pp. 2379–2385, 2011.

[40] P. J. K. van Meer, M. Kooijman, V. Brinks et al., "Immunogenicity of mAbs in non-human primates during nonclinical safety assessment," *mAbs*, vol. 5, no. 5, pp. 810–816, 2013.

[41] M. Haji Abdolvahab, A. Fazeli, A. Halim, A. S. Sediq, M. R. Fazeli, and H. Schellekens, "Immunogenicity of recombinant human interferon beta-1b in immune-tolerant transgenic mice corresponds with the biophysical characteristics of aggregates," *Journal of Interferon & Cytokine Research*, vol. 36, no. 4, pp. 247–257, 2016.

[42] P. Riese, S. Trittel, K. Schulze, and C. A. Guzmán, "Rodents as pre-clinical models for predicting vaccine performance in humans," *Expert Review of Vaccines*, vol. 14, no. 9, pp. 1213–1225, 2015.

[43] Y. Zeng, T. Gao, G. Zhao et al., "Generation of human MHC (HLA-A11/DR1) transgenic mice for vaccine evaluation," *Human Vaccines & Immunotherapeutics*, vol. 12, no. 3, pp. 829–836, 2015.

[44] J.-Y. Han, Y. Yi, A.-H. Liang et al., "Review on requirements of drug allergy or pseudoallergic reactions in pre-clinical evaluation," *Zhongguo Zhong Yao Za Zhi*, vol. 40, no. 14, pp. 2685–2689, 2015.

[45] A. T. Borchers, C. L. Keen, and M. E. Gershwin, "Drug-induced lupus," *Annals of the New York Academy of Sciences*, vol. 1108, pp. 166–182, 2007.

[46] J. Descotes and A. Gouraud, "Clinical immunotoxicity of therapeutic proteins," *Expert Opinion on Drug Metabolism and Toxicology*, vol. 4, no. 12, pp. 1537–1549, 2008.

[47] F. Dedeoglu, "Drug-induced autoimmunity," *Current Opinion in Rheumatology*, vol. 21, no. 5, pp. 547–551, Sep 2009.

[48] A. S. De Groot and D. W. Scott, "Immunogenicity of protein therapeutics," *Trends in Immunology*, vol. 28, no. 11, pp. 482–490, 2007.

[49] M. B. Hock, K. E. Thudium, M. Carrasco-Triguero, and N. F. Schwabe, "Immunogenicity of antibody drug conjugates: bioanalytical methods and monitoring strategy for a novel therapeutic modality," *The AAPS Journal*, vol. 17, no. 1, pp. 35–43, 2015.

[50] M. Ing, N. Gupta, M. Teyssandier et al., "Immunogenicity of long-lasting recombinant factor VIII products," *Cellular Immunology*, vol. 301, pp. 40–48, 2016.

[51] C. Pollock, D. W. Johnson, W. H. Hörl et al., "Pure red cell aplasia induced by erythropoiesis-stimulating agents," *Clinical Journal of the American Society of Nephrology*, vol. 3, no. 1, pp. 193–199, 2008.

[52] M. de Serres, B. Ellis, J. E. Dillberger et al., "Immunogenicity of thrombopoietin mimetic peptide GW395058 in BALB/c mice and New Zealand white rabbits: evaluation of the potential for thrombopoietin neutralizing antibody production in man," *STEM CELLS*, vol. 17, no. 4, pp. 203–209, 1999.

[53] T. E. Hunley, D. Corzo, M. Dudek et al., "Nephrotic syndrome complicating α-glucosidase replacement therapy for pompe disease," *Pediatrics*, vol. 114, no. 4, pp. e532–e535, 2004.

[54] R. G. Grau, "Drug-induced vasculitis: new insights and a changing lineup of suspects," *Current Rheumatology Reports*, vol. 17, no. 12, article 71, 2015.

[55] P. van der Graaf, "Influencing early portfolio decision making using preclinical M&S: how early is early and when is it too late?" in *Proceedings of the AAPS National Biotechnology Conference*, San Francisco, Calif, USA, 2010.

[56] S. Shi, "Biologics: an update and challenge of their pharmacokinetics," *Current Drug Metabolism*, vol. 15, no. 3, pp. 271–290, 2014.

[57] H. Salimi-Moosavi, J. Lee, B. Desilva, and G. Doellgast, "Novel approaches using alkaline or acid/guanidine treatment to eliminate therapeutic antibody interference in the measurement of total target ligand," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 51, no. 5, pp. 1128–1133, 2010.

[58] A. C. Bautista, D. Wullner, M. Moxness, S. J. Swanson, N. Chirmule, and V. Jawa, "Impact of matrix-associated soluble factors on the specificity of the immunogenicity assessment," *Bioanalysis*, vol. 2, no. 4, pp. 721–731, 2010.

[59] J. W. Lee, M. Kelley, L. E. King et al., "Bioanalytical approaches to quantify 'total' and 'free' therapeutic antibodies and their targets: technical challenges and PK/PD applications over the course of drug development," *AAPS Journal*, vol. 13, no. 1, pp. 99–110, 2011.

[60] M. P. Hall, C. Gegg, K. Walker et al., "Ligand-binding mass spectrometry to study biotransformation of fusion protein drugs and guide immunoassay development: strategic approach and application to peptibodies targeting the thrombopoietin receptor," *AAPS Journal*, vol. 12, no. 4, pp. 576–585, 2010.

[61] W. F. Richter and B. Jacobsen, "Subcutaneous absorption of biotherapeutics: knowns and unknowns," *Drug Metabolism and Disposition*, vol. 42, no. 11, pp. 1881–1889, 2014.

[62] S. Tamilvanan, N. L. Raja, B. Sa, and S. K. Basu, "Clinical concerns of immunogenicity produced at cellular levels by biopharmaceuticals following their parenteral administration into human body," *Journal of Drug Targeting*, vol. 18, no. 7, pp. 489–498, 2010.

[63] R. U. Agu, M. I. Ugwoke, M. Armand, R. Kinget, and N. Verbeke, "The lung as a route for systemic delivery of therapeutic proteins and peptides," *Respiratory Research*, vol. 2, no. 4, pp. 198–209, 2001.

[64] S. Rosengren, S. S. Dychter, M. A. Printz et al., "Clinical immunogenicity of rHuPH20, a hyaluronidase enabling subcutaneous drug administration," *The AAPS Journal*, vol. 17, no. 5, pp. 1144–1156, 2015.

[65] M. Krishna and S. G. Nadler, "Immunogenicity to biotherapeutics—the role of anti-drug immune complexes," *Frontiers in Immunology*, vol. 7, article 21, 2016.

[66] N. Chirmule, V. Jawa, and B. Meibohm, "Immunogenicity to therapeutic proteins: impact on PK/PD and efficacy," *AAPS Journal*, vol. 14, no. 2, pp. 296–302, 2012.

[67] S. A. Mousavi, M. Sporstøl, C. Fladeby, R. Kjeken, N. Barois, and T. Berg, "Receptor-mediated endocytosis of immune complexes in rat liver sinusoidal endothelial cells is mediated by FcγRIIb2," *Hepatology*, vol. 46, no. 3, pp. 871–884, 2007.

[68] E. S. Ward, S. C. Devanaboyina, and R. J. Ober, "Targeting FcRn for the modulation of antibody dynamics," *Molecular Immunology*, vol. 67, no. 2, pp. 131–141, 2015.

[69] G. Shankar, S. Arkin, L. Cocea et al., "Assessment and reporting of the clinical immunogenicity of therapeutic proteins and peptides-harmonized terminology and tactical recommendations," *AAPS Journal*, vol. 16, no. 4, pp. 658–673, 2014.

[70] J. Wang, J. Lozier, G. Johnson et al., "Neutralizing antibodies to therapeutic enzymes: considerations for testing, prevention and treatment," *Nature Biotechnology*, vol. 26, no. 8, pp. 901–908, 2008.

[71] A. R. Mire-Sluis, Y. C. Barrett, V. Devanarayan et al., "Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products," *Journal of Immunological Methods*, vol. 289, no. 1-2, pp. 1–16, 2004.

[72] G. Shankar, V. Devanarayan, L. Amaravadi et al., "Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 48, no. 5, pp. 1267–1281, 2008.

[73] M. Liang, S. L. Klakamp, C. Funelas et al., "Detection of high- and low-affinity antibodies against a human monoclonal antibody using various technology platforms," *Assay and Drug Development Technologies*, vol. 5, no. 5, pp. 655–662, 2007.

[74] J. A. Lofgren, S. Dhandapani, J. J. Pennucci et al., "Comparing ELISA and surface plasmon resonance for assessing clinical immunogenicity of panitumumab," *Journal of Immunology*, vol. 178, no. 11, pp. 7467–7472, 2007.

[75] M. K. de Vries, G. J. Wolbink, S. O. Stapel et al., "Inefficacy of infliximab in ankylosing spondylitis is correlated with antibody formation," *Annals of the Rheumatic Diseases*, vol. 66, no. 1, pp. 133–134, 2007.

[76] M. G. Tovey and C. Lallemand, "Immunogenicity and other problems associated with the use of biopharmaceuticals," *Therapeutic Advances in Drug Safety*, vol. 2, no. 3, pp. 113–128, 2011.

[77] Z. Li, B. Krippendorff, S. Sharma, A. C. Walz, T. Lavé, and D. K. Shah, "Influence of molecular size on tissue distribution of antibody fragments," *mAbs*, vol. 8, no. 1, pp. 113–119, 2016.

[78] L. Aarons, "Population pharmacokinetics: theory and practice," *British Journal of Clinical Pharmacology*, vol. 32, no. 6, pp. 669–670, 1991.

[79] S. K. Singh, "Impact of product-related factors on immunogenicity of biotherapeutics," *Journal of Pharmaceutical Sciences*, vol. 100, no. 2, pp. 354–387, 2011.

[80] K. D. Ratanji, J. P. Derrick, R. J. Dearman, and I. Kimber, "Immunogenicity of therapeutic proteins: influence of aggregation," *Journal of Immunotoxicology*, vol. 11, no. 2, pp. 99–109, 2014.

[81] A. S. Rosenberg, "Effects of protein aggregates: an immunologic perspective," *AAPS Journal*, vol. 8, no. 3, pp. E501–E507, 2006.

[82] M. J. Treuheit, A. A. Kosky, and D. N. Brems, "Inverse relationship of protein concentration and aggregation," *Pharmaceutical Research*, vol. 19, no. 4, pp. 511–516, 2002.

[83] D. R. Jandrlić, G. M. Lazić, N. S. Mitić, and M. D. Pavlović, "Software tools for simultaneous data visualization and T cell epitopes and disorder prediction in proteins," *Journal of Biomedical Informatics*, vol. 60, pp. 120–131, 2016.

[84] R. R. Mettu, T. Charles, and S. J. Landry, "CD4+ T-cell epitope prediction using antigen processing constraints," *Journal of Immunological Methods*, vol. 432, pp. 72–81, 2016.

[85] A. S. De Groot, F. Terry, L. Cousens, and W. Martin, "Beyond humanization and de-immunization: tolerization as a method for reducing the immunogenicity of biologics," *Expert Review of Clinical Pharmacology*, vol. 6, no. 6, pp. 651–662, 2013.

[86] R. Jefferis, "Glycosylation as a strategy to improve antibody-based therapeutics," *Nature Reviews Drug Discovery*, vol. 8, no. 3, pp. 226–234, 2009.

[87] A. Von Delwig, D. M. Altmann, J. D. Isaacs et al., "The impact of glycosylation on HLA-DR1-restricted T cell recognition of type II collagen in a mouse model," *Arthritis and Rheumatism*, vol. 54, no. 2, pp. 482–491, 2006.

[88] Y. H. Messinger, N. J. Mendelsohn, W. Rhead et al., "Successful immune tolerance induction to enzyme replacement therapy in CRIM-negative infantile Pompe disease," *Genetics in Medicine*, vol. 14, no. 1, pp. 135–142, 2012.

[89] K. P. Pratt, "Engineering less immunogenic and antigenic FVIII proteins," *Cellular Immunology*, vol. 301, pp. 12–17, 2016.

[90] F. Deisenhammer, "Interferon-beta: neutralizing antibodies, binding antibodies, pharmacokinetics and pharmacodynamics, and clinical outcomes," *Journal of Interferon & Cytokine Research*, vol. 34, no. 12, pp. 938–945, 2014.

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Research Article

SEC Based Method for Size Determination of Immune Complexes of Therapeutic Antibodies in Animal Matrix

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Therapeutic monoclonal antibodies (mAbs) represent a milestone in pharmacological development. Their superiority is based on the combination of high specificity, low toxicity, and long half-life that characterizes biologics. If biologics have Achilles' heel, it is their potential immunogenicity. To better understand the impact of the size of immune complexes of mAbs on anti-drug antibody (ADA) dependent adverse reactions in *Macaca fascicularis*, we developed an efficient high-throughput size exclusion chromatography- (SEC-) based methodology that enables analysis of the size, size distribution, and ratio of free and ADA-complexed mAb in serum allowing for assessment of formation and clearance of circulating ADA-mAb immune complexes (CIC).

1. Introduction

Therapeutic mAbs represent a mature medicinal technology with proven therapeutic benefit in many clinical disease indications. Their advantages over conventional small molecule drugs include their high specificity, long half-life, and low toxicity; however biologics are more likely than small molecule therapeutics to induce immune reactions. Fully humanized therapeutic mAbs, engineering advances resulting in fewer immunogenic CDR regions, improved formulations, and quality control and other developments have made therapeutic mAbs safer and highly beneficial for many patients around the world. Although many therapeutic mAbs still elicit an immune response in a certain subpopulation of patients (e.g., [1, 2]) and it is sometimes associated with hypersensitivity reactions (e.g., [3–7]), the clinical application of mAbs in general is well tolerated. However mAb-neutralizing immune response is not uncommon [8–10]. Therapeutic mAbs are tested for their safety (among other species) in nonhuman primates such as *Macaca fascicularis* (cynomolgus monkey) and have been observed to cause adverse reactions mediated by circulating immune complexes (CIC) formed by anti-drug antibody complexation with mAbs [11]. The immunogenicity and the associated risks of therapeutic antibodies in preclinical species are poorly predictive of their immunogenicity and risks in humans. One reason is the differences between the immune systems of humans and nonhuman primates [12]. Further, preclinical species may recognize the humanized backbone of therapeutic antibodies as foreign [13, 14]. Nevertheless, further characterization of CIC-dependent adverse reactions in preclinical species is of high interest to the pharmaceutical industry [15]. A more profound understanding of the underlying mechanisms would help to minimize the risks in animal models and the likelihood of a negative study outcome. One sequel of CIC-dependent postdose reaction (PDR) is activation of the complement system followed by inflammation, deposition of immune complexes, and subsequent tissue damage. CIC-mediated reactions are difficult to predict as many individual factors which differ among species and individuals (e.g., the ability to process, metabolize, and distribute CIC) contribute to the outcome. One factor, which might serve as a marker for CIC-dependent reactions, is the size of immune complexes [8, 16]. It has been shown that patients who develop an immune response against therapeutic antibodies without any adverse symptoms have small immune complexes, while patients with autoimmune diseases like lupus erythematosus have large immune complexes associated with aggressive disease progression [8, 17]. Various factors contribute to the size of immune complexes, including mAb concentration,

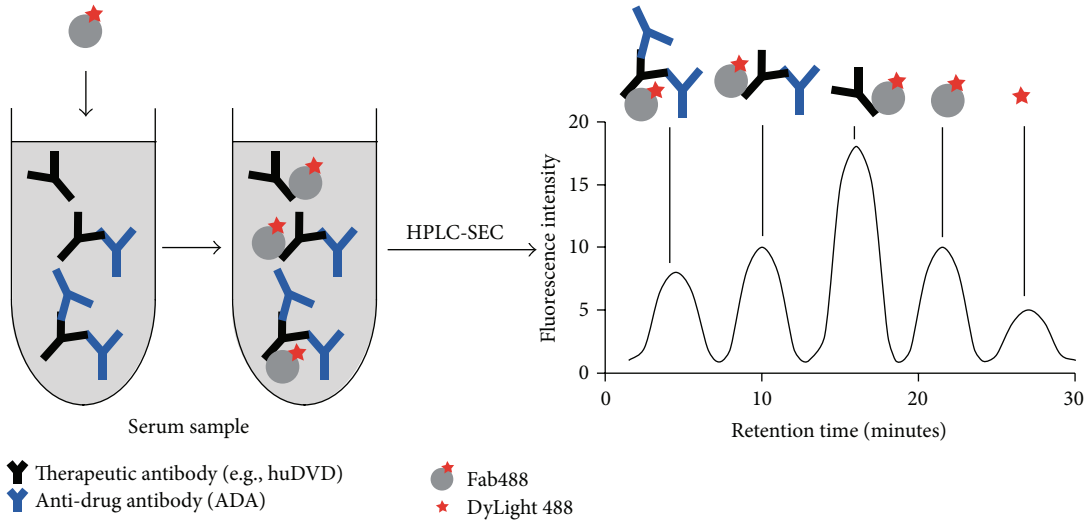


Figure 1: Principle of SEC assay. Fab488 is added to a serum sample. After incubation, Fab488 binds to the therapeutic mAb in the serum samples and to ADA complexes containing the therapeutic mAb (left). Formed complexes are separated by size exclusion chromatography (SEC) and the peaks quantified (right).

ADA concentration, antigen concentration, the affinities of the complex components, oligomer status, number of binding sites of complex components, and clearance mechanisms [18].

We developed an assay which characterizes the size, size distribution, and abundance of CIC in animal serum. The therapeutic mAb construct evaluated in these experiments was a humanized dual-variable domain immunoglobulin directed against soluble epitopes (huDVD) [19]. The assay was developed to assess CIC produced by cynomolgus monkeys following repeated parenteral administration of huDVD, some of which culminated in acute postdose hypersensitivity reactions. This assay enables quantification of free and complexed huDVD in one experiment. We used a DyLight 488-labeled Fab fragment of a monoclonal anti-human IgG_1 antibody (Fab488) to detect huDVD in serum. To quantify and characterize free and complexed huDVD we used size exclusion chromatography equipped with a laser-induced fluorescence (LIF) detector (Figure 1). The method achieved a sensitivity in the low microgram per milliliter range and was suitable for monitoring enrichment and clearance of free huDVD and huDVD-containing complexes of various sizes. We also monitored total amount of huDVD by western blot and quantified free concentration using a ligand biding assay.

2. Materials and Methods

2.1. Generation of Fab Fragments. Purified anti-human IgG antibody (8 mL, 3.5 mg/mL, AbbVie proprietary monoclonal mouse antibody) in digestion buffer (Thermo Fisher Scientific) was incubated with immobilized papain (2 mL, Thermo Fisher Scientific, 20341) for 24 hours at 37°C. The papain beads were removed by centrifugation. The Fc fragment was removed using a 5 mL Protein A column (HiTrap rProtein A FF, 5 mL, 17-5079-01, GE Healthcare) in PBS as binding buffer.

2.2. Labeling of Fab Fragments with DyLight 488 for the Generation of Fab488. The purified Fab fragment (3 mg/mL) was labeled with a 10-fold molar access of DyLight 488 (Thermo Fisher Scientific, 20341) for 2 hours at room temperature in the dark. Excess label was removed on S200 16/600 column (28-9893-35, GE Healthcare) in PBS using ÄKTA-Explorer FPLC (GE Healthcare).

2.3. Purification of Polyclonal Antibodies Directed against the CDR of huDVD. Rabbits were immunized with huDVD (Eurogentec, 4 week immunization, "Speedy") and the collected serum depleted of antibodies recognizing common human IgG epitopes in a two-step procedure. 100 mg of human IgG (Sigma Aldrich, I4506) was added to the serum (200 mL) together with a 5% w/v solution of polyethylene glycol 6.000 (Fluka, 81253) and incubated at 4°C for 12 hours. The serum supernatant was harvested by centrifugation (1500 ×g/4°C, 20 minutes) and filtration (0.45 μm bottle-top filter, Merck Millipore) and incubated with 100 mg human IgG coupled to 4 mg CnBr Sepharose (GE Healthcare, 17-0430-01, coupling was performed according to the manufacturer's instructions (71-7086-00 AF)) for 2 hours at room temperature. The beads were removed using an Econo-Pac column (Bio-Rad Laboratories). The serum flow-through was incubated at 4°C for 12 hours with 100 mg of the drug antibody immobilized on 4 mg CnBr Sepharose. The beads were washed with 5 bed volumes of PBS and the bound polyclonal response was eluted with IgG elution buffer (Thermo Scientific, 21009). The elution was neutralized with 10% 2 M Tris pH 7.5. The purified polyclonal response was tested for drug specificity using an ELISA test.

2.4. Sample Preparation and Liquid Chromatography. An Alliance Waters 2795 system equipped with Bio-SEC-5 column (500 Å, 5190-2533, Agilent Technologies, Inc.) was used for liquid chromatography. All runs were performed at 0.2

or 0.3 mL/min in PBS. Serum samples were diluted 1 : 5 in a volume of 100–150 μL. 585 or 678 nM Fab488 was used (in final volume).

2.5. Fitting Operations. The binding equilibrium data points were fitted to a one site specific binding equation $Y = F + (F_{\max} - F) * ((((a + x + K_D)/2) - \text{sqrt}(((a + x + K_D)/2)^2 - a * x))/a)$ using GraphPad Prism. $F_{\max}$ is the maximum value of Y, F is the minimum value of Y, x is the total concentration of huDVD, a is the total concentration of Fab488, and K_D is the equilibrium dissociation constant. The equation is the solution of the quadratic equation for $[AB]$ derived from $K_D = ([A] * [B])/[AB]$, where $[A]$ is the free concentration of Fab488, $[B]$ is the free concentration of huDVD, and $[AB]$ is the concentration of the complex of Fab488 bound to huDVD. The % bound huDVD was calculated using the specific binding equation and the best-fit value for K_D.

2.6. Western Blot. Serum samples (0.5 μL per well) were separated on Criterion TGX Stain-Free Precast gels (Bio-Rad Laboratories) at 250 V for 30 minutes. The proteins were transferred via Trans-Blot Turbo Transfer System (Bio-Rad Laboratories) onto a nitrocellulose membrane (Trans-Blot Turbo Transfer Pack, Bio-Rad Laboratories). The nitrocellulose membrane was blocked for 5 hours at room temperature in TTBS, 2% BSA, and incubated o/n at 4°C in TTBS, 2% BSA, 3% milk powder, and 50 ng/mL of the AbbVie proprietary monoclonal mouse anti-human IgG antibody. The membrane was washed 5 times for 5 minutes and 3 times for 10 minutes in TTBS and 0.1% BSA. The membrane was incubated for 1 hour at room temperature in the standard ultrasensitive ABC staining solution (Thermo Fisher). The nitrocellulose membrane was washed 5 times for 5 minutes, once for 10 minutes, and twice for 5 minutes in TBS buffer. The membrane was incubated for 3 minutes in the dark with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher). The images were taken using VersaDoc Imaging System (Bio-Rad Laboratories). The analysis was done using Image Lab 5.1 software (Bio-Rad Laboratories).

2.7. SPR. All experiments were performed using a Biacore 3000 instrument (GE Healthcare) and buffer HBS-EP (GE Healthcare, BR100188). 1000 response units of anti-human IgG Fc antibody (Thermo Fisher Scientific, 31125) were immobilized on both flow cells of a CM5 chip (GE Healthcare, BR-1003-99) according to the manufacturer's instructions (GE Healthcare). After each injection cycle of huDVD over one flow cell (15 nM, 10 μL) and Fab488 over both flow cells (0.49–2000 nM, 50 μL/min, association 5 minutes, and dissociation 10 minutes) the chip was regenerated with 15 mM Glycine/HCl for 30 sec at 100 μL/min.

2.8. Supernatant Pellet Experiment. 678 nM Fab488, 830 nM huDVD, and 0, 1500, or 3000 nM ADA were incubated at 4°C for 5 h and 50 minutes to simulate the conditions of the SEC experiment for the sample, which was injected last. 30 μL of each sample was centrifuged at 15000 rpm in a table top centrifuge for 10 minutes and 24 μL were carefully removed. The samples were mixed with nonreducing sample buffer and subjected to SDS-PAGE. After Coomassie staining the bands were quantified using the Gel Doc Imager and the Image Lab 5.1 software (Bio-Rad Laboratories).

3. Results and Discussion

3.1. Generation of Fab488: A Fluorescence Labeled Anti-Human IgG Fab Fragment. To be able to detect huDVD in the presence of animal serum containing high concentrations of endogenous immunoglobulins, we chose a mouse monoclonal in-house generated antibody that recognizes human IgG_1 but not *Macaca fascicularis* IgG (data not shown). The mouse monoclonal antibody was digested with papain to generate monovalent Fab fragments that would not cross-link drug complexes and hence not create artificial complexes. The Fab fragment was purified and labeled with a tenfold molar excess of DyLight 488 and purified again to get rid of excess label to generate Fab488. The addition of Fab488 to untreated *Macaca fascicularis* serum resulted in the detection of 2 peaks: the main peak at 18 minutes (see Figure 2(a), peak 2), containing Fab488, and a smaller peak at 19.5 minutes, containing free DyLight 488 (see Figure 2(a), peak 1). The elution pattern of Fab488 corresponded well to its size (47150 kDa). Free DyLight 488 eluted later than expected for the calculated mass (1011 Dalton).

3.2. Characterization of the Interaction of Fab488 with huDVD Using SEC and SPR in 20% Naive Macaca fascicularis Serum. To determine the lower detection limit of huDVD by Fab488 we titrated huDVD from 0.23 to 500 μg/mL (1–2500 nM), added 678 nM Fab488 and 20% untreated *Macaca fascicularis* serum pool to each sample, and analyzed complex formation using size exclusion chromatography (SEC) with fluorescence detection. At 678 nM Fab488 huDVD should be bound to almost 100% to Fab488 also at lower concentrations of huDVD, giving highest possible intensity of the complex peak. As expected, the peak of free Fab488 declined with higher huDVD concentrations (Figure 2(a), peak 2, 18 minutes), while the peak of the huDVD-Fab488 complex (Figure 2(a), peak 3, 15 minutes) increased with increasing concentration of huDVD, reaching a maximum between 830 and 2500 nM of huDVD when no free Fab488 was detected. Peak 1, the free dye, remained unaffected. The limit of detection was 2 μg/mL of huDVD (10 nM) in the assay, which corresponded to 10 μg/mL in 100% serum. At this concentration the S/N ratio of the complex peak was greater than 1.5 (Figure 2(b)).

To establish the stoichiometry of the interaction of Fab488 and huDVD, we immobilized about 80 response units of huDVD on an anti-human IgG Fc coated CM5 SPR chip, bound saturating levels of Fab488 to huDVD, and compared the relative amount of response units. For 1 : 2 (huDVD : Fab488) stoichiometry we expected 36 response units of Fab488 binding to 80 response units of huDVD. We measured 37 response units, which confirm the 1 : 2 binding model (Figure 2(c)).

To establish the equilibrium dissociation constant of Fab488 to huDVD in serum, we repeated the experiment

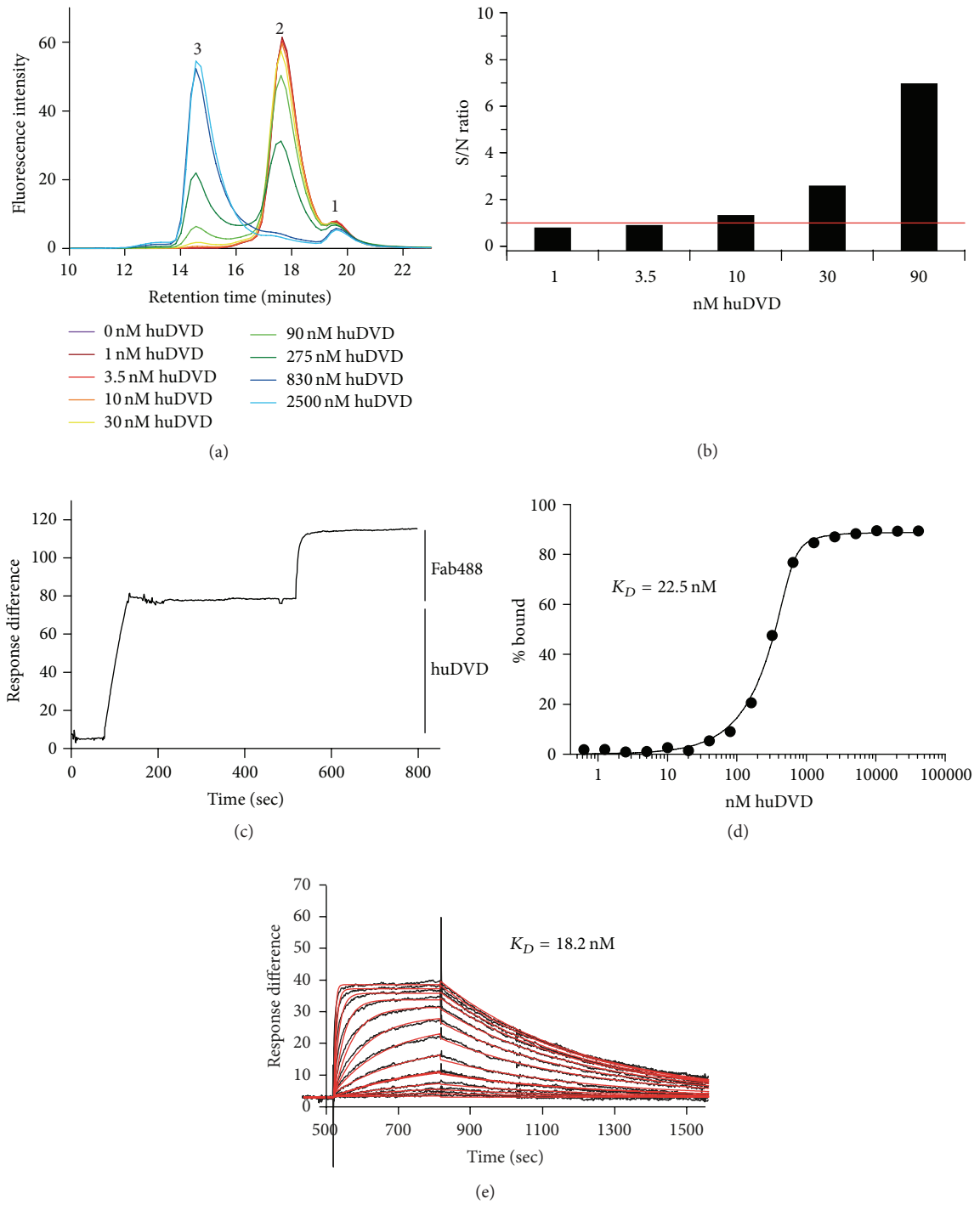


Figure 2: (a) Titration of huDVD on Fab488. 678 nM Fab488 and 1–2500 nM huDVD were incubated in 20% cynoserum as described in Section 2 and separated by size exclusion chromatography with fluorescence detection. Peak 1 is the free dye (DyLight 488), peak 2 is Fab488, and peak 3 is the complex of Fab488 and huDVD. (b) Determination of sensitivity. The signal to noise ratio (S/N ratio) of peak 3 was determined by dividing the percentage of the peak area of peak 3 in runs containing huDVD by the corresponding percentage of peak area at the same retention time of a control run containing no huDVD. (c) Stoichiometry. SPR signal showing the binding of huDVD to anti-human IgG Fc coated CM5 chip (0–200 sec) and the binding of Fab488 at saturation (500–800 sec). The average binding of huDVD was 78 response units and that of Fab488 37 response units (200000 and 47150 Da). (d) K_D of the binding of Fab488 to huDVD in 20% cynoserum determined by SEC. The % bound was calculated from the area of peak 3 as compared to total fluorescence as shown in experiment (a), plotted versus the concentration of huDVD, and fitted to the specific binding equation as described in Section 2 to determine the equilibrium dissociation constant (K_D). (e) K_D of the binding of Fab488 to huDVD determined by SPR. Association (500–800 sec) and dissociation (800–1500 sec) of Fab488 (0.49–2000 nM) to and from huDVD bound to an anti-human IgG coated chip. The data points (black curve) were fitted to a 1 : 1 Langmuir binding model (red curve).

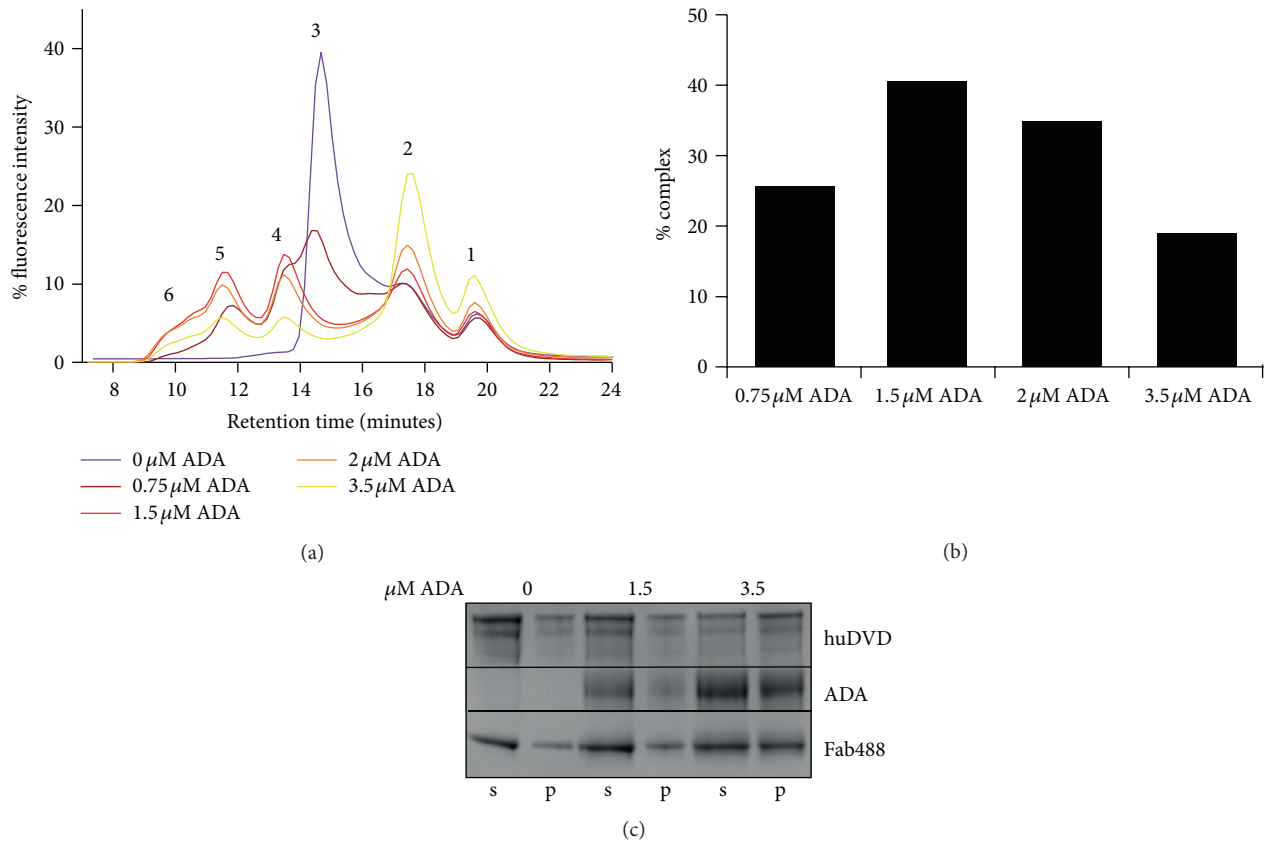


FIGURE 3: (a) Fab488-huDVD-ADA complexes prepared with ADAs purified from rabbit serum. 0.83 μM huDVD, 678 nM Fab488, and 0.75, 1.5, 2, and 3.5 μM ADA were incubated in 20% cynoserum as described in Section 2 and separated by size exclusion chromatography with fluorescence detection. Peak 1 is the free dye (DyLight 488), peak 2 is Fab488, peak 3 is the complex of Fab488 and huDVD, peak 4 is the complex of Fab488 and huDVD and one ADA, peak 5 is the complex of Fab488, one ADA, and two huDVD, and peaks under number 6 represent bigger complexes containing Fab488, huDVD, and ADA. (b) Quantification of fluorescence signal recovery in ADA complexes. Summed peak areas from (a) containing ADA (peaks 4–6) were set in relation to the fluorescence intensity of the whole elution profile and are shown as % complex. (c) Supernatant pellet experiment with ADA complexes. Coomassie stained SDS-PAGE showing the pellet (p) and the supernatant fraction (s) of samples containing 0, 1.5, or 3.5 μM ADA, Fab488, and huDVD prepared as in (a). The samples were treated as described in Section 2. The pellet fraction contained 20% of the reaction volume, whereas the supernatant fraction contained 80% of the reaction volume. Aggregation occurs when more than 20% of total protein amount are in the pellet fraction.

form Figure 2(a) using 585 nM Fab488 and 0.32–20560.8 nM huDVD with 22 data points for better fitting results. Because of the sensitivity the concentration of Fab488 used cannot be lower than the estimated equilibrium dissociation constant (K_D) (low nM range) and the lowest concentration of huDVD. However K_D can be fitted under these conditions using a one site specific quadratic binding equation derived from a 1 : 1 binding equilibrium, where the concentration of free huDVD is calculated as the total concentration minus the concentration of the complex. The concentration of huDVD was doubled for the fitting operation as compared to the concentration used in the experiment to account for the 1 : 2 stoichiometry based on the assumption that the binding of Fab488 to the 2 binding sites on one huDVD molecule is independent. Figure 2(d) shows the percentage of the complex plotted versus the concentration series of huDVD and the fitted curve giving K_D of 22.5 nM.

To confirm the result we determined K_D with a kinetic experiment using SPR. 80 response units of huDVD were bound to an anti-human IgG Fc coated CM5 SPR chip and the association and dissociation of a titration series of Fab488 was monitored (Figure 2(e)). K_D was fitted using a global 1 : 1 Langmuir model and gave a value of 18.2 nM confirming the result from the SEC experiment.

3.3. SEC Analysis of Complex Sizes and Size Distribution of Purified Rabbit ADA Directed against huDVD and huDVD in the Presence of 20% Naive Macaca fascicularis Serum. To determine the resolution of free huDVD and different complexes of huDVD and ADA, we preincubated a constant concentration of huDVD (830 nM) with increasing concentrations of rabbit ADA. The rabbit ADA material contained only the fraction that recognizes the CDR regions of huDVD. The samples were subsequently incubated with Fab488 and separated by size exclusion chromatography as described in the previous experiment. When no ADA was added (Figure 3(a), dark purple trace) the only peaks detected were for free DyLight 488 (Figure 3(a), peak 1), free Fab488 (Figure 3(a), peak 2), and Fab488-huDVD complex (Figure 3(a), peak 3). Two additional peaks occurred at a

substoichiometric ADA concentration (Figure 3(a), peaks 4 and 5, dark red trace). We interpreted these peaks as one huDVD bound to one ADA and two huDVDs bound to one ADA. With higher ADA concentrations, additional peaks at smaller retention times were detected (Figure 3(a), peak 6). These peaks could not be resolved any further with the chromatography setup employed. They increased with higher ADA concentrations (Figure 3(a), red, orange, and yellow trace).

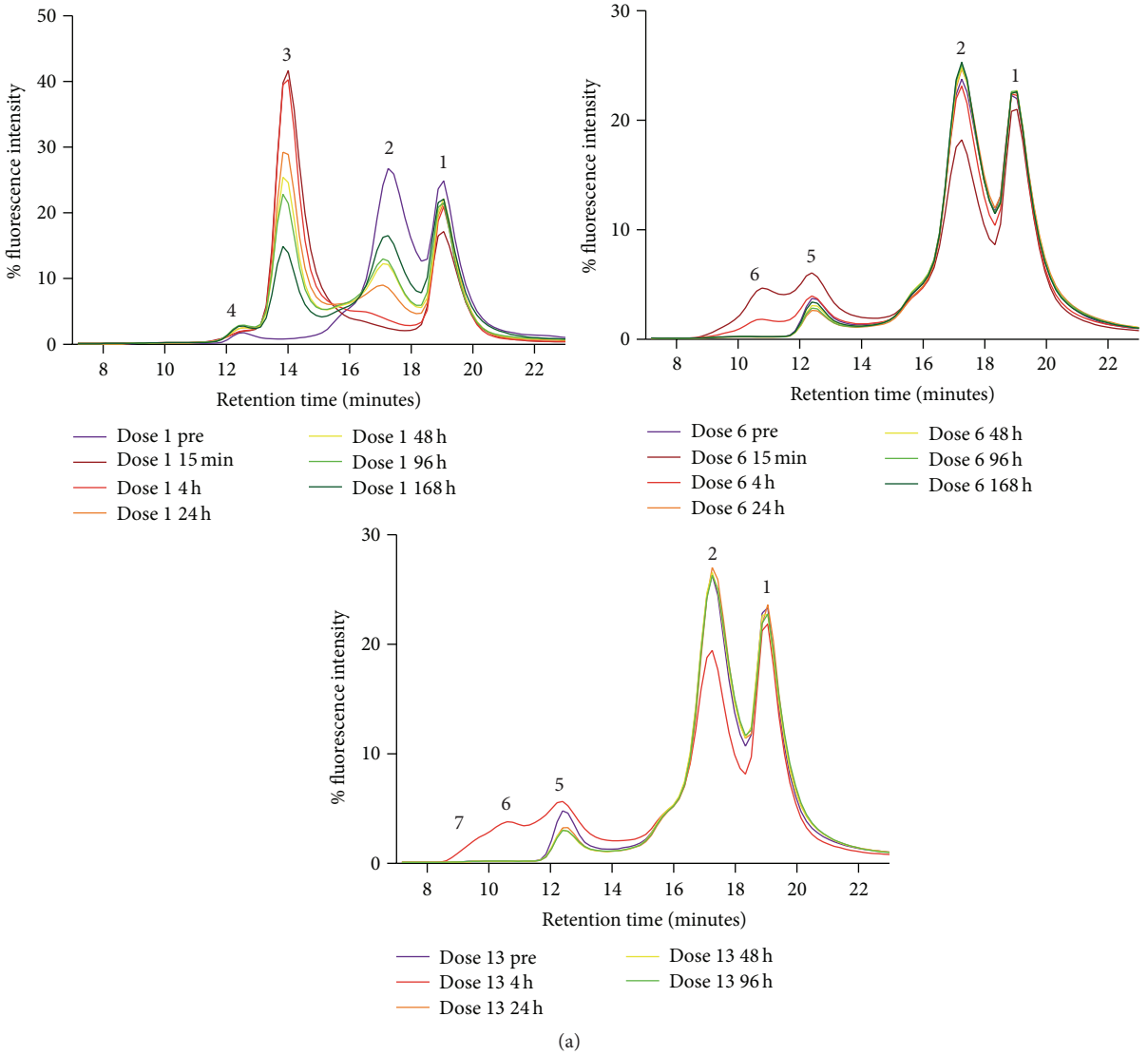
At 3.5 μM ADA we observed a relative increase in large complexes (Figure 3(a), yellow trace, peak 6); however we also observed an increase in free Fab488 (Figure 3(a), yellow trace, peak 2). Figure 3(b) shows the relative amount of detected ADA-huDVD complexes in Figure 3(a). The amount of detected complexes of huDVD and ADA reached a maximum at 1.5 μM ADA and was found to decline by up to 50% with higher ADA concentrations. To test whether the complexes precipitate with increasing ADA concentrations we repeated the experiment from Figure 3(a), fractionated the samples into a supernatant and pellet fraction, and analyzed the amount of proteins on SDS-PAGE (Figure 3(c)). Figure 3(c) shows that the amount of huDVD, ADA, and Fab488 increases in the pellet fraction (p) and decreases in the supernatant fraction (s). At the highest ADA concentration the quantification of the relative amounts of the proteins in the supernatant and pellet fractions showed that, at 3.5 μM ADA about 40% of huDVD, about 20% of ADA and about 20% of Fab488 go to the pellet. This is close to the decrease in complex detection observed in the SEC experiment, showing that complex precipitation might be the main limitation in the analysis of immune complexes by this method.

3.4. Application of the SEC Method to Macaca fascicularis Samples from a Toxicology Study with huDVD. Having demonstrated that the method described above enables specific detection of free huDVD and ADA-huDVD complexes in spiked animal serum, we then applied the method to toxicology study samples. The animals received 20 mg/kg of huDVD weekly for a total of 13 doses, subcutaneously, as an intravenous slow infusion or as a bolus intravenous infusion, and then entered an eight-week dose free recovery period. All study samples were analyzed for free drug and free ADA levels using conventional ligand binding assays. At the 1st, 6th, and 13th dose, a predose sample and samples at 15 minutes, 4 hours, 24 hours, 48 hours, 96 hours, and 168 hours after dose were taken. Figure 4(a) shows an example of an SEC profile. The animal in Figure 4(a) developed high ADA titers after dose 4. The first graph shows the chromatogram traces of the predose and postdose samples at dose 1. In the predose sample only the free dye peak (peak 1), the Fab488 peak (peak 2), and a small peak (peak 4) were detected. Peak 4 was present in all study animals and was likely a cross-reactivity of a serum component with Fab488. A Fab488-drug complex peak occurred in the following postdose samples (peak 3) which reached its highest intensity in the 15 minutes after dose sample (dark red chromatogram trace). The peak's intensity declined at later postdose time points. Figure 4(a), second graph, shows the chromatogram traces of the monkey predose and postdose samples at dose 6. No peak of the Fab488-drug complex was detected here (peak 3 in the first graph), but additional broad peaks were detected in 15 minutes and 4 hours after dose samples (peaks 5 and 6), which disappeared again at later postdose time points. These peaks corresponded to peaks 4 and 5 from Figure 3(a); therefore the complexes likely contain 1 ADA and 2 ADA molecules. Figure 4(a), third graph, shows the chromatogram traces of the monkey predose and postdose samples at dose 13. Similarly to dose 6, a specific huDVD-containing peak was only detected 4 hours after dosing. The peak pattern and abundance were very similar to the corresponding time point after dose 6, but there was also a significant broadening of the peak toward shorter retention times, indicating the existence of even larger immune complexes after dose 13. We had found that the binding of Fab488 to huDVD was in equilibrium after 1 hour and remained stable for at least 12 hours. Therefore we analyzed the predose and postdose samples of one animal from doses 1, 6, and 13 in one automated series, which took 10 hours and 50 minutes. We did not explore whether Fab488-huDVD and the fluorescence signal itself were stable for more than 12 hours.

3.5. Determination of Total Amount of huDVD with Western Blot and Free Concentration of huDVD with a Ligand Binding Assay and Comparison to the Results of the SEC Method. Next, we asked whether the decrease of the fluorescence signal of huDVD complex peaks beyond 4 hours after dose after doses 6 and 13 correlates with total huDVD clearance. The western blot in Figure 4(c) shows total amount of huDVD in the same study samples as analyzed in Figure 4(a). The relative clearance of total huDVD is very similar to the clearance of huDVD-Fab488 and huDVD-Fab488-ADA complexes from the SEC experiment in Figure 4(a). Figure 4(b) shows huDVD concentrations measured by ligand binding assay. The ligand binding assay was a bridging assay using one antigen to capture and the second antigen for detection. The lower limit of quantitation of the assay was 136 ng/mL in 100% serum; however the majority of measured concentrations were in the range of micrograms per milliliter, because the level dropped fast below the lower limit of quantitation. In all the samples that had measurable concentrations of huDVD in the ligand binding assay, we were also able to measure huDVD with the SEC assay. This was consistent with the SEC assay sensitivity of 10 μg/mL.

4. Conclusions

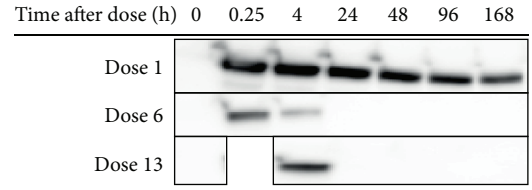
This paper demonstrates a method for fast and automated SEC based measurement of the concentrations of therapeutic mAbs and of therapeutic mAb complexes in the serum of animals. We present an engineered monovalent detection reagent that specifically detects humanized therapeutic mAbs with IgG_1 backbone in animal serum and complexes thereof. We showed that complexes formed with purified rabbit ADA material elicit a peak pattern very similar to the huDVD complexes in real samples of a toxicology study. We resolved three distinct complex sizes most likely representing complexes containing one ADA, two ADAs, and more ADAs. Finally, we showed that the sensitivity of our method is sufficient to be



(a)

Time after dose (h)	0	0.25	4	24	48	96	168
Dose 1 (μg/mL)	BQL	948	669	535	313	255	173
Dose 6 (μg/mL)	BQL	146	34.8	BQL	BQL	BQL	BQL
Dose 13 (μg/mL)	BQL	557	261	BQL	BQL	BQL	BQL

(b)



(c)

Figure 4: (a) SEC profile of toxicology study animal. 678 nM Fab488 was incubated with 20% serum samples from a cynotoxicology study with huDVD as described in Section 2 and separated by size exclusion chromatography with fluorescence detection. Peak 1 is the free dye (DyLight 488), peak 2 is Fab488, peak 3 is the complex of Fab488 and huDVD, peak 4 is unspecific binding, peak 5 is the complex of Fab488 and huDVD and one ADA, peak 6 is the complex of Fab488, huDVD, and two ADAs, and peaks under number 7 represent bigger complexes containing Fab488, huDVD, and ADA. Graph 1 shows the chromatographic traces of the predose sample (dose 1 pre) of one animal before the first dose and 15 minutes, 4 hours, 24 hours, 48 hours, 96 hours, and 168 hours after the first dose (from dark purple to dark green, dose 1 15 min–dose 1 168 h). Graph 2 shows chromatogram traces of the predose sample (dose 6 pre) of the same animal before dose 6 and 15 minutes, 4 hours, 24 hours, 48 hours, 96 hours, and 168 hours after the 6th dose (from dark purple to dark green, dose 6 15 min–dose 6 168 h). Graph 3 shows the chromatographic traces of the predose sample (dose 13 pre) of the same animal before dose 13 and 4 hours, 24 hours, 48 hours, and 96 hours after the 13th dose (from dark purple to green, dose 13 15 min–dose 13 96 h). The 15 minutes and 168 hours postdose samples were missing here. (b) PK profile of the same study animal measured with ligand binding assay. The concentration in μg/mL of huDVD was measured with an antigen-based ligand binding assay using Mesoscale Discovery ECL technology. The figure depicts the concentration in 100% serum in the predose and postdose samples of doses 1, 6, and 13 for the same animal as in (a). BQL indicates that the values were below the lower limit of quantitation. (c) Relative quantitation of total amounts of huDVD with western blot. The same study samples as in (a) and (b) were separated by nonreducing SDS-PAGE and analyzed with western blot as described in Section 2. The huDVD signal is shown.

easily applied to toxicology studies in *Macaca fascicularis* to quantify therapeutic mAbs and therapeutic mAb complexes and their size distribution over time. We demonstrated that the immune complexes of a therapeutic mAb at late dosing time points in *Macaca fascicularis* are very large compared with the complexes documented for patients [8].

The advantages of the SEC based method with drug specific fluorescence detection are manifold: sample preparation is simple, involving only a sample centrifugation step and the addition of Fab488. A much lower sample dilution is required than with classical ligand binding assays, with the result that binding equilibria are only minimally perturbed and closer to real *in vivo* conditions. Further free and complexed mAb can be detected and quantified simultaneously. In contrast to the conventional ADA ligand binding assays routinely applied in industry, this method only quantifies ADA-mAb complexes and not free ADA, which often persists in the circulation after the therapeutic mAb has been cleared. Since the complexes are considered the potential pathogenic species, this assay allows not only monitoring the critical species but also keeping track of clearance of free therapeutic mAb.

The detection antibody used in this study, the Fab fragment of an anti-human IgG antibody with no cross-reactivity to *Macaca fascicularis* IgG, offers the opportunity of an generic assay setup as it binds to all humanized IgG_1 type therapeutic mAbs in any animal matrix. One cannot rule out the possibility that high ADA levels might impact the assay readout, because the accessibility of the binding site of the detection reagent on the mAb might be sterically hindered in large complexes. Additional use of Fab fragments against other constant epitopes and a reagent with very high affinity to partially outcompete bound ADA could improve complex detection. Nevertheless compromised detection of very large complexes can never be ruled out. Conventional ADA ligand binding assays also suffer from limitations, namely, limited drug tolerance, which can never be excluded completely although acid dissociation steps are employed. We found that larger ADA complexes formed using polyclonal rabbit ADAs tend to precipitate. The SEC method can only analyze complexes, which stay soluble. In addition we were not able to resolve large complexes containing more than three antibodies, although we could observe an increase in the large unresolved species formed *in vitro* with rabbit ADA or in *in vivo* in cynomolgus monkeys. Recent findings suggest that pathologic immune complexes might contain no more than 6 ADAs [18]. If our method could detect these species it is not clear however. Therefore the resolution of the method has still to be improved.

The sensitivity of our method was 10 μg/mL in 100% serum, which turned out to be very well suitable for the toxicology study analyzed here. We found that the complex peak detected at 10 μg/mL contains 98% of the total concentration of huDVD bound to Fab488. Hence, better sensitivity could only be achieved by enhancing the brightness of the Fab fragment and improving peak resolution and the signal to noise ratio. This could be achieved by lowering the concentration of Fab488, engineering a protein with more label conjugation sites, and using capillary columns.

In summary, we developed a new generic method for the detection of humanized therapeutic mAbs in animal matrix such as serum which allows the detection of free therapeutic mAbs and ADA-mAb complexes in one analytical run, thereby enabling the measurement of the clearance of both species. In addition this method has a high throughput with very low instrument and consumables costs.

Disclosure

Marta Boysen, Mario Richter, Ingeborg Dreher, Ralf Loebbert, and Laura Schlicksupp are AbbVie employees. AbbVie funding was involved in the design and performance of the experiments. AbbVie participated in the interpretation of data, review, and approval of the paper.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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References

[1] F. Baert, M. Noman, S. Vermeire et al., "Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease," *The New England Journal of Medicine*, vol. 348, no. 7, pp. 601–608, 2003.

[2] A. Jamnitski, G. M. Bartelds, M. T. Nurmohamed et al., "The presence or absence of antibodies to infliximab or adalimumab determines the outcome of switching to etanercept," *Annals of the Rheumatic Diseases*, vol. 70, no. 2, pp. 284–288, 2011.

[3] C. Steenholdt, M. Svenson, K. Bendtzen, O. A. Thomsen, J. Brynskov, and M. A. Ainsworth, "Severe infusion reactions to infliximab: aetiology, immunogenicity and risk factors in patients with inflammatory bowel disease," *Alimentary Pharmacology and Therapeutics*, vol. 34, no. 1, pp. 51–58, 2011.

[4] C. J. Van Der Laken, A. E. Voskuyl, J. C. Roos et al., "Imaging and serum analysis of immune complex formation of radiolabelled infliximab and anti-infliximab in responders and non-responders to therapy for rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 66, no. 2, pp. 253–256, 2007.

[5] F. Baert, M. De Vos, E. Louis, and S. Vermeire, "Immunogenicity of infliximab: how to handle the problem?" *Acta Gastro-Enterologica Belgica*, vol. 70, no. 2, pp. 163–170, 2007.

[6] M. Krishna and S. G. Nadler, "Immunogenicity to biotherapeutics—the role of anti-drug immune complexes," *Frontiers in Immunology*, vol. 7, article 21, 2016.

[7] T. Schaeverbeke, M. Truchetet, M. Kostine, T. Barnetche, B. Bannwarth, and C. Richez, "Immunogenicity of biologic agents in rheumatoid arthritis patients: lessons for clinical practice," *Rheumatology*, vol. 55, no. 2, pp. 210–220, 2016.

[8] P. A. van Schouwenburg, L. A. van de Stadt, R. N. de Jong et al., "Adalimumab elicits a restricted anti-idiotypic antibody response in autoimmune patients resulting in functional neutralisation," *Annals of the Rheumatic Diseases*, vol. 72, no. 1, pp. 104–109, 2013.

[9] N. Emi Aikawa, J. F. De Carvalho, C. Artur Almeida Silva, and E. Bonfá, "Immunogenicity of anti-TNF-α agents in autoimmune diseases," *Clinical Reviews in Allergy and Immunology*, vol. 38, no. 2-3, pp. 82–89, 2010.

[10] S. Garcês, J. Demengeot, and E. Benito-Garcia, "The immunogenicity of anti-TNF therapy in immune-mediated inflammatory diseases: a systematic review of the literature with a meta-analysis," *Annals of the Rheumatic Diseases*, vol. 72, no. 12, pp. 1947–1955, 2013.

[11] J. R. Heyen, J. Rojko, M. Evans et al., "Characterization, biomarkers, and reversibility of a monoclonal antibody-induced immune complex disease in cynomolgus monkeys (*Macaca fascicularis*)," *Toxicologic Pathology*, vol. 42, no. 4, pp. 765–773, 2016.

[12] M. Warncke, T. Calzascia, M. Coulot et al., "Different adaptations of IgG effector function in human and nonhuman primates and implications for therapeutic antibody treatment," *The Journal of Immunology*, vol. 188, no. 9, pp. 4405–4411, 2012.

[13] R. Ponce, L. Abad, L. Amaravadi et al., "Immunogenicity of biologically-derived therapeutics: assessment and interpretation of nonclinical safety studies," *Regulatory Toxicology and Pharmacology*, vol. 54, no. 2, pp. 164–182, 2009.

[14] J. R. Rojas, R. P. Taylor, M. R. Cunningham et al., "Formation, distribution, and elimination of infliximab and anti-infliximab immune complexes in cynomolgus monkeys," *Journal of Pharmacology and Experimental Therapeutics*, vol. 313, no. 2, pp. 578–585, 2005.

[15] J. L. Rojko, M. G. Evans, S. A. Price et al., "Formation, clearance, deposition, pathogenicity, and identification of biopharmaceutical-related immune complexes: review and case studies," *Toxicologic Pathology*, vol. 42, no. 4, pp. 725–764, 2014.

[16] P. A. van Schouwenburg, S. Kruithof, C. Votsmeier et al., "Functional analysis of the anti-adalimumab response using patient-derived monoclonal antibodies," *The Journal of Biological Chemistry*, vol. 289, no. 50, pp. 34482–34488, 2014.

[17] K. S. K. Tung, R. J. DeHoratius, and R. C. Williams Jr., "Study of ciculating immune complex size in systemic lupus erythematosus," *Clinical and Experimental Immunology*, vol. 43, no. 3, pp. 615–625, 1981.

[18] C. A. Diebolder, F. J. Beurskens, R. N. De Jong et al., "Complement is activated by IgG hexamers assembled at the cell surface," *Science*, vol. 343, no. 6176, pp. 1260–1263, 2014.

[19] C. Wu, H. Ying, C. Grinnell et al., "Simultaneous targeting of multiple disease mediators by a dual-variable-domain immunoglobulin," *Nature Biotechnology*, vol. 25, no. 11, pp. 1290–1297, 2007.

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Review Article

Emerging Technologies and Generic Assays for the Detection of Anti-Drug Antibodies

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Anti-drug antibodies induced by biologic therapeutics often impact drug pharmacokinetics, pharmacodynamics response, clinical efficacy, and patient safety. It is critical to assess the immunogenicity risk of potential biotherapeutics in producing neutralizing and nonneutralizing anti-drug antibodies, especially in clinical phases of drug development. Different assay methodologies have been used to detect all anti-drug antibodies, including ELISA, radioimmunoassay, surface plasmon resonance, and electrochemiluminescence-based technologies. The most commonly used method is a bridging assay, performed in an ELISA or on the Meso Scale Discovery platform. In this report, we aim to review the emerging new assay technologies that can complement or address challenges associated with the bridging assay format in screening and confirmation of ADAs. We also summarize generic anti-drug antibody assays that do not require drug-specific reagents for nonclinical studies. These generic assays significantly reduce assay development efforts and, therefore, shorten the assay readiness timeline.

1. Introduction

Biotherapeutics often elicit unwanted immune response that produces nonneutralizing and/or neutralizing anti-drug antibodies (ADAs). A widely adopted tiered immunogenicity testing approach includes first the screening and confirmation of both types of ADAs using immunoassays. Testing for neutralizing antibodies, preferably using methods that reflect the drug's mechanism of action *in vivo*, may be followed up for the ADA positive samples [1]. The industry standard for immunogenicity testing of ADA screening and confirmation is the bridging immunoassay. In these assays the drug is labeled separately with different haptens or tags and any anti-drug antibodies present in a sample will form a bridge between the two labeled molecules. The most important advantage with this method is that all isotypes of ADA (IgG, IgM, IgA, etc.) can be detected [2]. This format can also be used in all species, as any immunoglobulin is capable of forming an immune complex with the two labeled drugs.

However, there are several significant disadvantages with the bridging assay format. Bridging assays can also detect preexisting antibodies that recognize the drug. Examples of such antibodies include agents found in particular diseased populations, such as rheumatoid factor that binds to Fc domains of immunoglobulins [3–5]. In other cases preexisting antibodies reactive to specific regions, such as anti-allotype [6] or anti-hinge region [7, 8] antibodies, were detected in bridging assays. In one case, preexisting IgE antibodies specific to an oligosaccharide present on the therapeutic have also been shown to cause a hypersensitivity reaction [9]. However, preexisting reactivity has generally not been shown to be a risk factor for posttreatment immunogenicity, especially for monoclonal antibody drugs, and in most cases the agent responsible is not identified [4, 10]. Minimizing this type of interference is a challenge in bridging assays and can confound the detection and interpretation of treatment-induced ADA.

In addition to immunoglobulins, other serum components can generate signal in the assay. Soluble, multimeric

drug targets can also form a bridge between the two labeled molecules, generating a false positive response [11, 12]. This is a particularly challenging problem, as soluble target levels generally increase after dosing with the drug, in some cases by substantial amounts [13], and the target-mediated assay signal needs to be reduced to below the assay cut point.

Although the bridging assay format allows detection of all isotypes of ADA, it does not identify the specific isotypes represented in the response. In addition, the bridging assay does not detect IgG4 subclass when monovalent due to Fab arm exchange [14], and the ADA response to biotherapeutics can include a substantial proportion of IgG4 [15–17]. However, in these cases there was also a significant IgG1 response. Hence, the bridging assay may underestimate the level of IgG4 ADA but would likely not generate a false negative assay response.

Drug interference is a major challenge in immunogenicity assays because the presence of drug may result in false negatives, which in turn underestimates the ADA incidence. Given these difficulties with the bridging assay format and drug tolerance issue in general, alternative platforms or strategies may be required for immunogenicity testing. The focus of this paper is to review some of the new technologies that address the challenges of bridging ELISA/MSD or complement bridging assays for the screening and confirmation of ADAs. These technologies allow identification of the specific isotypes and subclasses of ADA and can substantially reduce interference from soluble drug target. In addition, they may provide improved drug tolerance, low sample or reagent consumption, and better matrix tolerance. It is important to recognize that the ADAs detected by immunoassays may not necessarily be clinically relevant. The correlations of ADAs with clinical impact on safety, pharmacokinetics, pharmacodynamics, and efficacy can only be drawn when sufficient data has been accumulated during late stage of clinical development or even postauthorization clinical practice [18]. Finally, generic ADA assay methodologies that provide significant time saving in assay development are also discussed. Neutralizing antibody assays have been reviewed by others [1] and are outside of the scope of this paper.

2. Emerging Technologies for ADA Assays

2.1. Immunogenicity Assays Using Gyrolab. The Gyrolab platform, which incorporates microfluidics and partial automation, has been widely used as a bioanalytical tool in ligand binding assays [19]. Assays have been validated on the Gyrolab platform for quantification of biologic drug candidates (including human IgGs) to determine their pharmacokinetic properties [20, 21] and to measure the affinity of protein-protein interactions [22].

Recent industry meetings have also showcased bridging ADA assays being developed using the Gyrolab. The platform offers an ADA mixing CD where the key steps of an ADA assay, such as acid treatment, neutralization, and bridging reaction, are automated and performed within each CD microstructure [http://www.gyros.com/products/gyrolab-cds/]. Alternatively, since the technology is based on streptavidin coated beads [23], the sample preparation steps mentioned above can also be performed outside of the system [24] (similar to the MSD platform), followed by analysis on a regular Gyros CD (1000 nL or 200 nL). The Gyrolab ADA software also provides a statistical tool that can assist in cut point determination.

Among the advantages with the Gyrolab platform is the minimal use of critical reagents and sample volume due to the microfluidics [22]. For ADA assays using the ADA mixing CD, the run time per CD can be less than one hour (enabled by automating the acid and neutralization steps within the ADA CD). However, the relatively short incubation time for neutralization and bridging reaction in the ADA CD may not be sufficient for low affinity antibodies present in the samples to form the bridged complex. This can be addressed during development by optimizing the incubation times for the acid and neutralization/bridging steps.

Some of the disadvantages of the ADA CD include fewer sample throughputs per CD, 48 microstructures per CD compared to 96 or 112 (1000 or 200 nL CD used for PK assays). Also, the ADA CD has a maximum sample volume of 200 nL, which is 5 times less than the regular 1000 nL CD. Additional limitations to the Gyros platform include the cost of reagents and consumables, in particular the CDs. However, overall Gyros can be considered as an alternative platform for the development of ADA assays to improve workflow through automation or save on volume of critical reagents consumed.

2.2. Immunogenicity Assays Using iPCR. One of the key challenges reported for accepted methodologies for ADA testing is the interference from circulating drug. Several methodologies, prominent amongst them acid dissociation and ADA enrichment, have been reported for improving drug tolerance [25]. In addition, collecting samples after a wash-out period is also common, although this approach may not be practical in multidose efficacy studies. One way to improve drug tolerance in ADA assays is by incorporating large dilutions to reduce the concentration of drug in the sample. Technologies like immune-PCR (iPCR) that offer high sensitivity may allow for detection of ADA in diluted samples. In two reports describing ADA assays using iPCR [26, 27], the bridging assay format was used. The divalent ADA molecule forms a bridge between drugs immobilized on the plate and biotin labeled drug. The biotin labeled complexes were detected using a proprietary Imperacer® reagent, anti-biotin antibody conjugated to DNA, which is quantified by real time PCR [26].

In the first study, using a model system of goat anti-mouse IgG as protein drug mimic and a polyclonal rabbit anti-goat IgG mimicking the ADA, the assay could tolerate drug levels 2000-fold greater than ADA levels (10 ng/mL of the ADA detected in the presence of 20 mg/mL of drug), probably due to larger initial dilution of the sample causing dissociation of ADA-drug complexes. The high sensitivity of the platform due to signal amplification allows for the detection of low ADA levels. This is higher than what has been reported using sample pretreatment and acidification [25, 28, 29]. This model system also reported sensitivity in the order of 5 pg/mL [26].

The second report using iPCR for ADA detection pertains to the development of an assay to support clinical trials of

a receptor IgG1 fusion protein. Drug tolerance levels were reported as 1000-fold greater than ADA levels for the iPCR, versus 40-fold greater for the MSD platform. In addition, sensitivity (using the same positive control) was 20 ng/mL and 40 pg/mL for the MSD and iPCR platforms, respectively [27]. In addition, in this case study, using the iPCR platforms allowed an investigation of whether the inverse relationship between ADA incidence and dose administered was due to the inability of the assay to detect ADA in the presence of circulating drug or the induction of immune tolerance.

One of the advantages to using iPCR (in addition to improved drug tolerance) is that the PCR amplification enables large sample dilution, thereby reducing matrix effects [26]. While the iPCR assay platform has reported improved drug tolerance and sensitivity, the platform is dependent on proprietary reagents obtained from the vendor and is therefore less amenable to in-house method development. The need for rigorous analyst training has also been mentioned as a concern for obtaining reproducible results [27].

2.3. Simultaneous Detection and Isotyping of an ADA Response

2.3.1. SQI SquidLite Technology. Additional characterization of the immune response may require the development of assays to isotype the ADA detected. SQI Diagnostics SquidLite technology platform offers the option of ADA detection and isotyping in the same well, potentially eliminating the need for multiple assays and reducing the amount of sample volume needed. SQI's technology prints microarray spots of the drug on an activated glass surface. This is followed sequentially by sample addition and the addition of fluorescent labeled detection antibodies [30]. Using this technology platform, users have reported improved sensitivity and drug tolerance compared to an ELISA or ECL platform [30]. The advantage of the SQI platform is the ability to multiplex when isotyping is needed and the automated system can potentially increase throughput. However, the platform uses proprietary buffers and has a substantial upfront cost in instrument setup. In addition, initial method development is performed by SQI and workup is needed by the vendor to optimize spotting of the drug on the glass surface.

2.3.2. Genalyte Maverick System. Another technology platform that enables simultaneous ADA detection and isotyping of the immune response is the Genalyte Maverick. In this technology platform, anti-isotype capture probes are printed on the silicon photonic biosensor surface. Anti-drug antibody in the sample is captured via the Fc portion and the complex is detected using biotinylated drug followed by streptavidin coated beads causing a change of refractive index. The Maverick system is capable of detecting all immunoglobulin isotypes including monovalent IgG4. However, this platform requires sample pretreatment steps of acid dissociation followed by affinity capture of ADA using drug coated on 96 plates. The ADAs are eluted off the plate in low pH and neutralized before flowing over the sensor chip. Both platforms (SQI and Maverick) offer the advantage of automation. Maverick is a label-free detection that allows for real time kinetic binding results, whereas SQI is end point reading. However, unlike the SQI there is no user published literature as yet on the use of the Maverick and the currently available information is primarily provided by the vendor (http://www.genalyte.com/resources/).

2.3.3. Immunocapture-LC/MS. Liquid chromatography mass spectrometry (LC/MS) is becoming an important technology for large molecule drug development. The applications of LC/MS in quantifying endogenous protein biomarkers and biotherapeutics in biological matrices are increasing. Generic LC-MS assays that measure human mAbs or Fc fusion proteins have been successfully used for PK bioanalysis [31, 32]. The use of LC-MS for immunogenicity testing has also emerged in the recent years [33, 34]. In this special issue, Chen et al. reported an immunocapture-LC/MS assay for simultaneous ADA detection and isotyping [35]. The authors identified proteolytic derived surrogate peptides that are unique to each human immunoglobulin isotype and subclass by LC/MS. Human sera containing preexisting ADA against a therapeutic protein as determined by ECL bridging assay were used to demonstrate the LC/MS ADA assay technology. To increase assay sensitivity, immunopurification was first performed on these human sera using two techniques. One method is to use biotinylated drug to enrich the ADA. The other is to spike excess drug into the sera to saturate the ADA binding sites followed by using a mouse monoclonal antibody against drug to capture the ADA-drug complex. The universal peptides specific to each isotype/subclass were semiquantitated using LC/MS and purified human Ig isotype standards. LC/MS ADA assay will become a valuable technology for immunogenicity testing given LC/MS's advantage in multiplexing capability and high specificity. However, additional improvements are needed to further reduce the endogenous Ig interference of the immunocapture-LC/MS assay, and the immunocapture step itself may lead to a higher level of interlaboratory variability. Furthermore, successful use of the platform requires strong LC/MS capabilities which may not be available in standard bioanalytical laboratories. Serial analysis by LC/MS is also generally lower throughput compared to ligand binding assays performed in a 96-well immunoassay plate. Finally, the application of LC/MS in clinical immunogenicity testing needs to be demonstrated in patient samples treated with biotherapeutics.

3. Generic ADA Assay Methodologies

ADA assays typically use drug-specific reagents that require substantial time and resources to prepare and characterize. Generic ADA assays that can be applied to all human monoclonal antibody therapeutics have been reported for nonclinical applications [36, 37]. These "off-the-shelf" assays require minimal assay optimization and can be used for early candidate selection studies right up to IND-enabling GLP studies.

3.1. Generic ADA ELISAs for Nonclinical Studies. Immune-complex assays have been used to measure immunogenicity in mouse and cynomolgus monkey studies [36, 37]. The assays used anti-human constant region antibodies to capture the drug and anti-mouse or anti-cynomolgus IgG

species-specific antibodies to detect the drug-ADA complex. By preincubating the sample with excess drug, the immune-complex assay measures total ADA levels. Since the first report of the assay in 2010, the monkey ADA assay has been modified and successfully used by Carrasco-Triguero et al., for not only monovalent human IgG therapeutic antibodies, but also antibody-drug conjugates (see this special issue) [38]. The modified version uses a commercially available anti-human IgG Fc specific monoclonal antibody as coat and an anti-monkey IgG polyclonal antibody for detection. In addition, the paper also described a simple methodology for determining in study cut points. This assay format has superior drug tolerance and adequate sensitivity that detected equal or greater number of ADA positive responses compared to therapeutic-specific bridging ELISA. One of the drawbacks of the generic total ADA assay is that this assay format does not allow for mapping ADA domain specificity, which was typically done by spiking in other drug-related constructs. Another difference is that bridging ELISAs detect many different isotypes whereas the ADA-drug complex assay is limited to IgG isotype. Furthermore, the complex assay method is not likely applicable to the detection of ADA in humans dosed with human monoclonal antibodies.

3.2. Universal ADA ECL Immunoassays for Nonclinical Studies. To simplify ADA assay development during nonclinical studies, Bautista et al. reported a universal indirect species-specific immunoassay (UNISA) for ADA detection in three animal species: mouse, rat, and cynomolgus monkey [39]. The format of these assays is to coat the therapeutic on carbon surface plates to capture ADA, which is detected with a species-specific antibody. Unlike the immune-complex assay, this assay format allows for mapping the ADA specificity to different regions of the biotherapeutic molecule.

In a follow-up paper, Bautista et al. validated the UNISA using a single assay condition across four representative therapeutic monoclonal antibodies [40]. The paper established a universal cut point, but the suitability of the cut point would need to be assessed for each new drug candidate. The paper also demonstrated similar assay sensitivity (2–10 ng/mL) and drug tolerance (272–403 ng/mL) with the positive control and defined acceptance criteria based on the four human monoclonal antibodies tested. As with the immune-complex assays, the UNISA uses generic reagents and assay conditions which is a considerable advantage in nonclinical studies. First, the coat antibody does not require labeling and is ready to use as it is. Second, using one assay condition across different therapeutic antibodies saves the assay development time. In addition, the streamlined immunogenicity assessment strategy speeds up the assay validation. As with the immune-complex generic ADA assay, the use of specific detection antibodies precludes detection of all isotypes of ADA. In addition, the method has limited application in the clinical setting.

3.3. Generic Human Anti-PEG Antibody Assays Using Acoustic Membrane Microparticle Technology. Polyethylene glycol (PEG) is a synthetic polymer that has wide applications, from the cosmetics industry to the biomedical field [41–43]. However, preexisting anti-PEG antibody and antibody against PEG moiety induced by PEGylated therapeutics have been observed in animal models and humans. Preexisting anti-PEG antibodies may result in an increased incidence of immunogenicity and/or in hypersensitivity reactions when patients are treated with PEGylated biotherapeutics [42].

Current immunoassays to detect anti-PEG antibody responses have suffered from a lack of well-characterized positive controls and poor specificity due to the high background seen in these assays [44]. Recently the development of a generic anti-PEG antibody assay using custom generated anti-PEG antibody and the Acoustic Membrane Microparticle Technology (AMMP or ViBE® workstation) platform has been reported [45]. In this assay, a magnetic bead conjugated with biotin PEG and bound anti-PEG antibody is detected on a Protein A functionalized sensor. The change in mass due to the bound complex changes the frequency of an oscillating piezoelectric membrane proportional to the amount of analyte bound [46]. The AMMP assay overcomes the issue of high background seen in other anti-PEG antibody assays as only beads that have the complex of PEG-anti-PEG antibody stick to the sensor. Other serum components that may bind to Protein A sensor surface do not contribute to a significant change in mass. The anti-PEG antibody assay sensitivity with this custom positive control was 800 ng/mL on the AMMP compared to the 50–500 μg/mL range seen with the MSD and AlphaLISA platforms with the same positive control [45]. The use of a Protein A surface increases the assay sensitivity owing to the multiple IgG binding domains on Protein A. However, direct binding methods also detect IgM which may be a substantial component of any anti-PEG response [42]. During method development on the AMMP platform, it is important to determine the extent of protein coverage on the bead surface, the need for additional wash steps (and their impact on the ability to detect low affinity ADA). A particularly important consideration for anti-PEG antibody assays is the removal of surfactants like Tween that have a framework structure similar to PEG.

4. Conclusion

The interpretation of immunogenicity data from bridging ADA assays has been widely accepted by industry and regulators for approval and marketing of biotherapeutics. Given the ubiquity of the bridging assay platform, many of the technologies discussed here have been targeted for particular uses (e.g., ADA isotyping, obtaining high sensitivity, or improving drug tolerance). However, immunogenicity data from marketed products that have been extensively studied indicate that ADA positive patients identified using assays with higher drug tolerance have a lower correlation with clinical efficacy [18]. This suggests that more sensitive and drug tolerant assays may not always help interpret the impact of ADA on clinical outcomes. Nevertheless, regulatory authorities continue to request additional details on the nature of immunogenic responses to ensure patient safety. The alternative platforms described in this review may help overcome some of the limitations of the bridging assay or

provide options for further characterization of antibodies against therapeutic proteins.

Disclosure

The views and opinions expressed in this paper are those of the authors and do not necessarily reflect the position of UCB Pharma, Regeneron Pharmaceuticals, or Genentech.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Shobha Purushothama and Michael A. Partridge contributed equally to this paper.

References

[1] G. Shankar, C. Pendley, and K. E. Stein, "A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs," *Nature Biotechnology*, vol. 25, no. 5, pp. 555–561, 2007.

[2] A. R. Mire-Sluis, Y. C. Barrett, V. Devanarayan et al., "Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products," *Journal of Immunological Methods*, vol. 289, no. 1-2, pp. 1–16, 2004.

[3] H. Myler, T. Felix, J. Zhu, M. Hruska, and S. P. Piccoli, "Measuring biotherapeutics with endogenous counterparts and pre-existing antibodies: an interferon case study," *Bioanalysis*, vol. 6, no. 8, pp. 1113–1122, 2014.

[4] K. A. Van Schie, G.-J. Wolbink, and T. Rispens, "Cross-reactive and pre-existing antibodies to therapeutic antibodies—effects on treatment and immunogenicity," *mAbs*, vol. 7, no. 4, pp. 662–671, 2015.

[5] B. Gorovits, J. McNally, C. Fiorotti, and S. Leung, "Protein-based matrix interferences in ligand-binding assays," *Bioanalysis*, vol. 6, no. 8, pp. 1131–1140, 2014.

[6] S. M. Tatarewicz, G. Juan, S. J. Swanson, and M. S. Moxness, "Epitope characterization of pre-existing and developing antibodies to an aglycosylated monoclonal antibody therapeutic of G1m17,1 allotype," *Journal of Immunological Methods*, vol. 382, no. 1-2, pp. 93–100, 2012.

[7] S. Ben-Horin, M. Yavzori, L. Katz et al., "The immunogenic part of infliximab is the F(ab')2, but measuring antibodies to the intact infliximab molecule is more clinically useful," *Gut*, vol. 60, no. 1, pp. 41–48, 2011.

[8] T. Rispens, H. De Vrieze, E. De Groot et al., "Antibodies to constant domains of therapeutic monoclonal antibodies: anti-hinge antibodies in immunogenicity testing," *Journal of Immunological Methods*, vol. 375, no. 1-2, pp. 93–99, 2012.

[9] C. H. Chung, B. Mirakhur, E. Chan et al., "Cetuximab-induced anaphylaxis and IgE specific for galactose-α-1,3- galactose," *The New England Journal of Medicine*, vol. 358, no. 11, pp. 1109–1117, 2008.

[10] L. Xue and B. Rup, "Evaluation of pre-existing antibody presence as a risk factor for posttreatment anti-drug antibody induction: analysis of human clinical study data for multiple biotherapeutics," *The AAPS Journal*, vol. 15, no. 3, pp. 893–896, 2013.

[11] M. Carrasco-Triguero, C. Mahood, M. Milojic-Blair et al., "Overcoming soluble target interference in an anti-therapeutic antibody screening assay for an antibody-drug conjugate therapeutic," *Bioanalysis*, vol. 4, no. 16, pp. 2013–2026, 2012.

[12] Z. D. Zhong, S. Dinnogen, M. Hokom et al., "Identification and inhibition of drug target interference in immunogenicity assays," *Journal of Immunological Methods*, vol. 355, no. 1-2, pp. 21–28, 2010.

[13] N. Nishimoto, K. Terao, T. Mima, H. Nakahara, N. Takagi, and T. Kakehi, "Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease," *Blood*, vol. 112, no. 10, pp. 3959–3964, 2008.

[14] M. H. Hart, H. De Vrieze, D. Wouters et al., "Differential effect of drug interference in immunogenicity assays," *Journal of Immunological Methods*, vol. 372, no. 1-2, pp. 196–203, 2011.

[15] T. E. Barger, D. Wrona, T. J. Goletz, and D. T. Mytych, "A detailed examination of the antibody prevalence and characteristics of anti-ESA antibodies," *Nephrology Dialysis Transplantation*, vol. 27, no. 10, pp. 3892–3899, 2012.

[16] P. A. Van Schouwenburg, T. Rispens, and G. J. Wolbink, "Immunogenicity of anti-TNF biologic therapies for rheumatoid arthritis," *Nature Reviews Rheumatology*, vol. 9, no. 3, pp. 164–172, 2013.

[17] P. A. Van Schouwenburg, C. L. Krieckaert, M. Nurmohamed et al., "IgG4 production against adalimumab during long term treatment of RA patients," *Journal of Clinical Immunology*, vol. 32, no. 5, pp. 1000–1006, 2012.

[18] P. A. Van Schouwenburg, C. L. Krieckaert, T. Rispens, L. Aarden, G. J. Wolbink, and D. Wouters, "Long-term measurement of anti-adalimumab using pH-shift-anti-idiotype antigen binding test shows predictive value and transient antibody formation," *Annals of the Rheumatic Diseases*, vol. 72, no. 10, pp. 1680–1686, 2013.

[19] C. Eriksson, C. Agaton, R. Kånge et al., "Microfluidic analysis of antibody specificity in a compact disk format," *Journal of Proteome Research*, vol. 5, no. 7, pp. 1568–1574, 2006.

[20] I. Magana, C. R. Macaraeg, L. Zhang, M. Ma, and T. M. Thway, "Validation of a microfluidic platform to measure total therapeutic antibodies and incurred sample reanalysis performance," *Bioanalysis*, vol. 6, no. 19, pp. 2623–2633, 2014.

[21] X. F. Liu, X. Wang, R. J. Weaver et al., "Validation of a gyrolab™ assay for quantification of rituximab in human serum," *Journal of Pharmacological and Toxicological Methods*, vol. 65, no. 3, pp. 107–114, 2012.

[22] H. Salimi-Moosavi, P. Rathanaswami, S. Rajendran, M. Toupikov, and J. Hill, "Rapid affinity measurement of protein-protein interactions in a microfluidic platform," *Analytical Biochemistry*, vol. 426, no. 2, pp. 134–141, 2012.

[23] A. M. Given, P. M. Whalen, P. J. O'Brien, and C. A. Ray, "Development and validation of an alpha fetoprotein immunoassay using Gyros technology," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 64-65, pp. 8–15, 2012.

[24] A. Mikulskis, D. Yeung, M. Subramanyam, and L. Amaravadi, "Solution ELISA as a platform of choice for development of robust, drug tolerant immunogenicity assays in support of drug development," *Journal of Immunological Methods*, vol. 365, no. 1-2, pp. 38–49, 2011.

[25] H. W. Smith, A. Butterfield, and D. Sun, "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," *Regulatory Toxicology and Pharmacology*, vol. 49, no. 3, pp. 230–237, 2007.

[26] M. Spengler, M. Adler, A. Jonas, and C. M. Niemeyer, "Immuno-PCR assays for immunogenicity testing," *Biochemical and Biophysical Research Communications*, vol. 387, no. 2, pp. 278–282, 2009.

[27] D. Jani, E. Savino, and J. Goyal, "Feasibility of immuno-PCR technology platforms as an ultrasensitive tool for the detection of anti-drug antibodies," *Bioanalysis*, vol. 7, no. 3, pp. 285–294, 2015.

[28] J. S. Bourdage, C. A. Cook, D. L. Farrington, J. S. Chain, and R. J. Konrad, "An Affinity Capture Elution (ACE) assay for detection of anti-drug antibody to monoclonal antibody therapeutics in the presence of high levels of drug," *Journal of Immunological Methods*, vol. 327, no. 1-2, pp. 10–17, 2007.

[29] D. Sickert, K. Kroeger, C. Zickler et al., "Improvement of drug tolerance in immunogenicity testing by acid treatment on Biacore," *Journal of Immunological Methods*, vol. 334, no. 1-2, pp. 29–36, 2008.

[30] J. Mora, A. Given Chunyk, M. Dysinger et al., "Next generation ligand binding assays—review of emerging technologies' capabilities to enhance throughput and multiplexing," *The AAPS Journal*, vol. 16, no. 6, pp. 1175–1184, 2014.

[31] M. T. Furlong, Z. Ouyang, S. Wu et al., "A universal surrogate peptide to enable LC-MS/MS bioanalysis of a diversity of human monoclonal antibody and human Fc-fusion protein drug candidates in pre-clinical animal studies," *Biomedical Chromatography*, vol. 26, no. 8, pp. 1024–1032, 2012.

[32] H. Li, R. Ortiz, L. Tran et al., "General LC-MS/MS method approach to quantify therapeutic monoclonal antibodies using a common whole antibody internal standard with application to preclinical studies," *Analytical Chemistry*, vol. 84, no. 3, pp. 1267–1273, 2012.

[33] H. Jiang, W. Xu, C. A. Titsch et al., "Innovative use of LC-MS/MS for simultaneous quantitation of neutralizing antibody, residual drug, and human immunoglobulin G in immunogenicity assay development," *Analytical Chemistry*, vol. 86, no. 5, pp. 2673–2680, 2014.

[34] H. Neubert, C. Grace, K. Rumpel, and I. James, "Assessing immunogenicity in the presence of excess protein therapeutic using immunoprecipitation and quantitative mass spectrometry," *Analytical Chemistry*, vol. 80, no. 18, pp. 6907–6914, 2008.

[35] L.-Z. Chen, D. Roos, and E. Philip, "Development of immunocapture-LC/MS assay for simultaneous ADA isotyping and semiquantitation," *Journal of Immunology Research*, vol. 2016, Article ID 7682472, 14 pages, 2016.

[36] K. Stubenrauch, K. MacKeben, R. Vogel, and J. Heinrich, "Generic anti-drug antibody assay with drug tolerance in serum samples from mice exposed to human antibodies," *Analytical Biochemistry*, vol. 430, no. 2, pp. 193–199, 2012.

[37] K. Stubenrauch, U. Wessels, U. Essig, R. Vogel, and J. Schleypen, "Evaluation of a generic immunoassay with drug tolerance to detect immune complexes in serum samples from cynomolgus monkeys after administration of human antibodies," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 52, no. 2, pp. 249–254, 2010.

[38] M. Carrasco-Triguero, H. Davis, Y. Zhu et al., "Application of a plug-and-play immunogenicity assay in cynomolgus monkey serum for ADCs at early stages of drug development," *Journal of Immunology Research*, vol. 2016, Article ID 2618575, 14 pages, 2016.

[39] A. C. Bautista, H. Salimi-Moosavi, and V. Jawa, "Universal immunoassay applied during early development of large molecules to understand impact of immunogenicity on biotherapeutic exposure," *The AAPS Journal*, vol. 14, no. 4, pp. 843–849, 2012.

[40] A. C. Bautista, L. Zhou, and V. Jawa, "Universal immunogenicity validation and assessment during early biotherapeutic development to support a green laboratory," *Bioanalysis*, vol. 5, no. 20, pp. 2495–2507, 2013.

[41] X. Hu, L. Miller, S. Richman et al., "A novel PEGylated interferon beta-1a for multiple sclerosis: safety, pharmacology, and biology," *Journal of Clinical Pharmacology*, vol. 52, no. 6, pp. 798–808, 2012.

[42] H. Myler, M. W. Hruska, S. Srinivasan et al., "Anti-PEG antibody bioanalysis: a clinical case study with PEG-IFN-λ-1a and PEG-IFN-α2a in naive patients," *Bioanalysis*, vol. 7, no. 9, pp. 1093–1106, 2015.

[43] J. T. White, M. Crossman, K. Richter, M. Berman, J. Goyal, and M. Subramanyam, "Immunogenicity evaluation strategy for a second-generation therapeutic, PEG-IFN-β-1a," *Bioanalysis*, vol. 7, no. 21, pp. 2801–2811, 2015.

[44] H. Schellekens, W. E. Hennink, and V. Brinks, "The immunogenicity of polyethylene glycol: facts and fiction," *Pharmaceutical Research*, vol. 30, no. 7, pp. 1729–1734, 2013.

[45] H. Dong, J. R. Mora, C. Brockus et al., "Development of a generic anti-PEG antibody assay using bioscale's acoustic membrane microparticle technology," *The AAPS Journal*, vol. 17, no. 6, pp. 1511–1516, 2015.

[46] S. D. Chilewski, W. M. Dickerson, J. R. Mora, A. Saab, and E. M. Alderman, "Evaluation of acoustic membrane microparticle (Ammp) technology for a sensitive ligand binding assay to support pharmacokinetic determinations of a biotherapeutic," *The AAPS Journal*, vol. 16, no. 6, pp. 1366–1371, 2014.

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Research Article

Storage Conditions of Conjugated Reagents Can Impact Results of Immunogenicity Assays

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Consistent performance of anti-drug antibody (ADA) assays through all stages of clinical development is critical for the assessment of immunogenicity and interpretation of PK, PD, safety, and efficacy. The electrochemiluminescent assays commonly employed for ADA measurement use drug conjugated with ruthenium and biotin to bind ADA in samples. Here we report an association between high nonspecific ADA responses in certain drug-naïve individuals and the storage buffer of the conjugated reagents used in a monoclonal antibody ADA assay. Ruthenylated reagents stored in phosphate-buffered saline (PBS) buffer had increased levels of aggregate and produced variable and high baseline responses in some subjects. Reagents stored in a histidine-sucrose buffer (HSB) had lower aggregate levels and produced low sample responses. In contrast to PBS, conjugated reagents formulated in HSB remained low in aggregate content and in sample response variability after 5 freeze/thaw cycles. A reagent monitoring control (RMC) serum was prepared for the real-time evaluation of conjugated reagent quality. Using appropriate buffers for storage of conjugated reagents together with RMCs capable of monitoring of reagent aggregation status can help ensure consistent, long-term performance of ADA methods.

1. Introduction

Protein-based therapeutic drugs have an inherent potential to elicit undesired immune response in human subjects. The impact of treatment-induced anti-drug antibody (ADA) responses may range from inconsequential to potentially life-threatening. Regulatory agencies mandate testing for the presence of ADA in all phases of clinical development and require assessments of potential impact on safety, drug exposure, and efficacy [1–5]. It is therefore crucial to ensure that ADA testing results are accurate and consistent throughout the drug development cycle by implementing long-term maintenance and monitoring of the functional integrity of critical reagents [6–8].

One of the common assay formats for ADA evaluation is the solution phase bridging electrochemiluminescent (ECL) assay, which typically provides high levels of sensitivity and drug tolerance combined with ability to detect most ADA isotypes. In this format, the ECL signal is generated by ADA simultaneously binding two different conjugated forms of the drug: one biotinylated and one conjugated with a ruthenium complex. Conjugation chemistry requires the protein being labeled to be in a buffer free of primary and secondary amines. To achieve this, proteins are typically buffer-exchanged into phosphate-buffered saline (PBS) prior to the chemical reaction. For convenience, conjugated proteins are frequently maintained in the PBS buffer after labeling since PBS is compatible with a large variety of analytical methods, including those used to determine protein concentration. The use of PBS for long-term storage of proteins is fairly common as evidenced by the many commercially available antibodies formulated in PBS and stored at −20°C or below.

While PBS is convenient and widely used, numerous literature reports demonstrate that this buffer is far from ideal for cryostorage of proteins. Freezing of sodium phosphate buffers leads to precipitation of dibasic sodium salts which in turn causes a significant drop of pH. For example, pH of a 50 mM sodium phosphate solution may drop from 7.00 when measured at 25°C down to 3.36 when measured at −30°C [9]. In addition, formation of the $Na_2HPO_4 \cdot 12H_2O$ crystals leads to a local increase of protein concentration due to sequestration of water from the solution [10]. Localized high protein concentration combined with low pH and the presence of the liquid-solid interface on the surface of the dibasic sodium phosphate crystals may stimulate protein unfolding and aggregation [11]. Problems related to precipitation of dibasic sodium phosphate crystals may be eliminated by the use of 50 mM potassium phosphate containing 6.5% sucrose (a cryoprotectant), which was proposed by Staack et al. as an adequate buffer for long-term cryostorage of most antibodies [6]. As an alternative to cryostorage, long-term refrigeration of proteins at 5°C can eliminate problems caused by aggregation; however it presents a separate set of challenges such as potential for microbial contamination, protein hydrolysis reactions, and possibility of assay interference caused by the use of protein stabilizers (e.g., bovine serum albumin).

It should be remembered that even well-developed formulation buffers may not be able to completely eliminate protein degradation and formation of protein aggregates which are driven by a complex interplay between the storage temperature, protein concentration, and formulation buffer components as well as by the rate of cooling/thawing and the storage container material and size [12, 13]. Selection of formulation buffers suitable for long-term storage of critical reagents used in ADA assays may be of crucial importance due to emerging evidence that aggregation of conjugated reagents can play a critical role in generation of reliable immunogenicity data [14].

An ECL solution phase bridging assay on Meso Scale Diagnostics (MSD) platform for detection, confirmation, and titration of anti-drug antibodies against a therapeutic human monoclonal antibody was developed and validated by MedImmune. The method was subsequently transferred to a second laboratory site to support clinical studies for two disease indications (diseases A and B). The initial assay transfer was deemed successful based on the performance of the provided quality control samples: negative controls and low and high level ADA positive controls gave responses within the ranges established during the original method validation when tested at the second site. Unexpectedly, however, screening and confirmatory cut points obtained at the second laboratory site were significantly higher than those obtained during the original method validation in the same disease populations. For both disease A and disease B, the responses of serum samples from drug-naïve subjects were more variable and many were much higher than those observed during original validation. High responses were detected only in certain individuals but not in the negative control pool which suggested that the high sample-to-sample variability could be caused by aggregation of conjugated reagents similar to that reported by Tatarewicz et al. [14]. Tatarewicz and colleagues demonstrated that the presence of very small amounts (0.55%) of high molecular aggregates in the preparations of ruthenylated monoclonal antibody used in a bridging electrochemiluminescent immunogenicity assay caused a significant increase of baseline ADA responses in subjects with rheumatoid arthritis. While both biotinylated and ruthenylated reagents showed a similar level of aggregation, it was the ruthenylated conjugate that was found to be chiefly responsible for elevated signal. Based on this report and our own accumulated experience with the ECL immunogenicity assays, we evaluated reagent aggregation and its impact on ADA assays focusing on the ruthenylated conjugate as being more critical for assay performance than its biotinylated counterpart.

We performed an investigation into the effects of different formulation buffers on aggregation of ruthenylated antibody and the resulting ADA responses of drug-naïve subjects. Two storage buffers were compared: phosphate-buffered saline (PBS: 3 mM dibasic sodium phosphate, 1 mM potassium phosphate, and 155 mM NaCl, pH 7.4) and histidine-sucrose buffer (HSB: 25 mM histidine, 250 mM sucrose, pH 6.0) as examples of formulations that may, respectively, promote and prevent protein aggregation. Our selection of the histidine-sucrose buffer was driven by the relative ease of its preparation and its general similarity to most formulation buffers used for long-term cryostorage of monoclonal antibodies. We found that relatively small amounts of aggregate present in the ruthenylated reagent stored in PBS caused a disproportionately high increase of nonspecific signal in some individual serum samples. Replacing PBS with histidine-sucrose buffer (HSB) effectively eliminated protein aggregation and significantly reduced the amount of sample-to-sample variability in the immunogenicity assay. We also developed a reagent monitoring control comprised of pooled individual sera that are sensitive to the aggregation level of conjugated reagents and implemented it for monitoring of reagent quality during long-term clinical studies.

2. Materials and Methods

2.1. Reagents. Positive ADA assay controls were generated by MedImmune and consisted of affinity-purified goat polyclonal antibody directed against the idiotype of the human monoclonal antibody drug. Individual human serum samples and pooled human serum were obtained from Bioreclamation (Hicksville, NY). Multiarray high binding streptavidin-coated plates (MA6000), Blocker A, Read Buffer 4XT, and ruthenium (II) sulfo-trisbipyridine N-hydroxysuccinimide ester (Sulfo-Tag) were obtained from Meso Scale Diagnostics (Gaithersburg, MD). EZ-Link biotin sulfo-N-hydroxysuccinimide ester and 40 kDa Zeba spin desalting columns were purchased from Life Technologies. G75 Sephadex was purchased from Sigma-Aldrich. All antibody and antibody conjugates concentrations were measured using Pierce BCA protein assay kit with bovine gamma globulin standards (Life Technologies). Histidine was purchased from JT Baker, and sucrose was purchased from EMD Millipore.

2.2. Conjugation of the Therapeutic Antibody. Prior to conjugation, the human therapeutic monoclonal antibody was buffer-exchanged into PBS (3 mM dibasic sodium phosphate, 1 mM potassium phosphate, and 155 mM NaCl, pH 7.4), using a 40 kDa Zeba spin desalting column. Conjugations were performed by treating a 2 mg/mL solution of the antibody for 1 hour at room temperature either with NHS-Sulfo-Tag at a 15 : 1 challenge ratio or with sulfo-NHS-biotin at a 10 : 1 challenge ratio. Ruthenylated antibody was split into two portions; the first portion was purified using a 70 mL bed volume of G75 Sephadex media equilibrated with PBS, pH 7.4. The PBS eluate was diluted to 1 mg/mL with PBS and stored as single use aliquots (250 μL/vial) at −80 ± 10°. The second portion was purified using a 70 mL bed volume of G75 Sephadex media equilibrated with 25 mM histidine·HCl, pH 6.0. The eluate in the 25 mM histidine buffer was diluted to 1 mg/mL using the same buffer and concentrated sucrose solution so that the final buffer formulation was 25 mM histidine and 250 mM sucrose, pH 6.0 (henceforth referred to as histidine-sucrose buffer or HSB). Biotinylated antibody was purified using a 70 mL bed volume of G75 Sephadex media equilibrated with 25 mM histidine·HCl, pH 6.0. The eluate was diluted to 1 mg/mL with HSB and stored as single use aliquots (250 μL/vial) at −80 ± 10°. For normal use, individual aliquots of both ruthenylated and biotinylated reagents were thawed and stored at 5 ± 3°C for up to one month before being discarded.

2.3. Stability Study Design. Ruthenylated antibody solutions prepared in PBS buffer and HSB were placed in 500 μL polypropylene cryovials equipped with O-ring screw caps at 250 μL/vial. The aliquots were subjected to up to 5 freeze/thaw cycles. Each cycle consisted of freezing the aliquots for approximately 16 hours (overnight) at −80 ± 10°C (nominal) and then thawing them at 5 ± 3°C (nominal) for approximately 8 hours. Control aliquots (never frozen) were stored at 5 ± 3°C. Control aliquots and aliquots subjected to 1, 3, and 5 freeze/thaw cycles for both buffer formulations were analyzed by High Performance Size-Exclusion Chromatography (HPSEC) and used in the immunogenicity assay.

2.4. High Performance Size-Exclusion Chromatography. Levels of aggregation were assessed by HPSEC on an Agilent HPLC-1200 system (Agilent Technologies, Santa Clara, CA) operated by Agilent OpenLab software. High resolution TSKgel Super SW mAB HR column (4 μm, 250 Å, 7.8 mm ID × 300 mm) from Tosoh Bioscience (Tosoh Bioscience, Montgomeryville, PA) was isocratically eluted by 0.1 M sodium sulfate and 0.1 M sodium phosphate buffer, pH 6.8, and protein elution was monitored by UV absorbance at 280 nm. Wyatt Technologies DAWN EOS MALS detector coupled with UV detection was used in Size Exclusion Chromatography Multiangle Light Scattering (SEC-MALS) analyses. Structure designations were made based on comparisons with stressed antibody reference standard analyzed by SEC-MALS. Column performance was verified by running a gel filtration standard protein mixture (Bio-Rad, Hercules, CA).

2.5. Immunogenicity Assay and Cut Point Determination. Serum samples were diluted 50-fold with assay buffer (phosphate-buffered saline/10% MSD Blocker A/0.1% Tween 20, pH 7.2) containing a mixture of the ruthenylated and biotinylated forms of the drug at 0.8 μg/mL and 0.4 μg/mL, respectively. Samples were incubated for 17–22 hours with gentle agitation at room temperature (RT) in the dark. High binding streptavidin-coated (HB-SA) 96-well MSD plates were blocked with MSD Blocker A (150 μL/well) for approximately 2 hours at RT and washed four times with wash buffer (PBS/0.1% Tween 20, pH 7.2). The reaction mixture was transferred to the blocked HB-SA plate (50 μL/well) and incubated for 1 hour at RT with gentle agitation in the dark. Plates were washed four times with the wash buffer prior to addition of 2x MSD Read Buffer T (150 μL/well) and read immediately on MSD Sector 6000 Imager. Screening and confirmatory evaluations were performed on the same assay plate, each in duplicate wells. Confirmatory wells were treated the same as the screening assay wells except for the addition of excess unconjugated drug in the overnight incubation step (final concentration 10 μg/mL). Screening and confirmatory data were obtained by testing 48 drug-naïve human serum samples each from disease A and disease B patient populations using conjugated reagents stored in PBS or in HSB each exposed to 1 F/T cycle. Different sets of A and B disease samples were used for each reagent storage buffer. Each sample was tested in duplicate wells in four separate experiments performed by two different operators on two different occasions, generating a total of 192 screening and confirmatory data points for each disease indication. Each assay plate contained a set of system suitability controls consisting of a negative control measured in four duplicate wells (NQC) and low and high ADA positive controls, each measured in two duplicate wells (LQC and HQC: 15 ng/mL and 1000 ng/mL, resp., of goat anti-idiotypic antibody in NQC). The NQC consisted of a commercial serum pool collected from multiple healthy human male and female donors.

The screening assay responses were expressed as S : B (signal to baseline) values by dividing the mean signal of an individual sample by the mean response of NQC measured on the same assay plate. Confirmatory assay percent inhibition values were calculated as % Inhibition = 100% [1 − (Response in the presence of drug)/(Response in the absence of drug)]. The screening and confirmatory cut points were obtained with the respective false positive rates set to 5% and 1% using statistical methods described in detail elsewhere [15, 16].

2.6. Preparation of the Reagent Monitoring Control. Individual serum units from patients diagnosed with disease B (160 in total) were analyzed in the screening assay using ruthenylated and biotinylated reagents stored in PBS and subjected to one freeze/thaw cycle. Sera with S : B signals at or above the original validation screening cut point (31 in total) were retested using both conjugated reagents stored in PBS and in HSB. Samples that changed classification from negative when tested using reagents in HSB to positive when

Table 1: Summary of ruthenylated antibody analysis by HPSEC. Ruthenylated antibody was stored at 1 mg/mL in PBS) or in HSB. Each preparation was subjected to multiple freeze-thaw (F/T) cycles by cycling between −80 ± 10°C (approximately 16 hours) and 5 ± 3°C (approximately 8 hours) prior to analysis by HPSEC.

Retention time [min]	Molecular weight [kDa]	Structure designation	% peak area							
			0 F/T cycles		1 F/T cycle		3 F/T cycles		5 F/T cycles	
			PBS	HSB	PBS	HSB	PBS	HSB	PBS	HSB
11.45	~1900	Unordered multimer	0.09	0.0	0.22	0.0	0.44	0.0	0.31	0.0
12.45	~360	Dimers, trimers	2.6	0.2	2.9	0.2	3.6	0.2	2.8	0.2
14.89	~150	Monomer	97.3	99.7	96.9	99.7	95.9	99.6	96.9	99.7

tested with reagents stored in PBS and also showed increase of the S : B signal by >30% (10 in total) were pooled together to create a reagent monitoring control (RMC). The RMC was aliquoted into single use vials and stored at −80 ± 10°C.

3. Results

3.1. Aggregation of Ruthenylated Antibody. Chromatograms and results of the HPSEC analysis of the ruthenylated antibody stored in HSB or in PBS and subjected to 0 (stored at 5 ± 3°C), 1, 3 and 5 freeze/thaw (F/T) cycles are shown in Figure 1 and Table 1.

Ruthenylated antibody stored in HSB was 99.6% monomeric and contained about 0.2% of dimers/trimers with no unordered multimers. It was very stable and showed virtually no changes of aggregate and monomer content upon repeated freeze/thaw cycles. On the other hand, the preparation stored in PBS contained unordered multimers (peak at 11.45 minutes with molecular weight of approximately 1900 kDa) and had >10 times more dimers/trimers (peak at 12.45 minutes with molecular weight of approximately 360 kDa) than the HSB preparation, even prior to freezing. The area of the dimers/trimers peak remained relatively unaffected by the number of freeze/thaw cycles and ranged between 2.6% and 3.6% of the total protein. The unordered multimer peak, however, increased with the number of freeze/thaw cycles starting with 0.09% prior to freezing and growing to 0.44% after three freeze/thaw cycles. The rise of unordered multimer content upon repeated freeze/thaw cycles correlated well with the increased responses in the immunogenicity assay (see Figure 2) suggesting that these larger aggregates may be directly responsible for the elevation of ECL signal intensity.

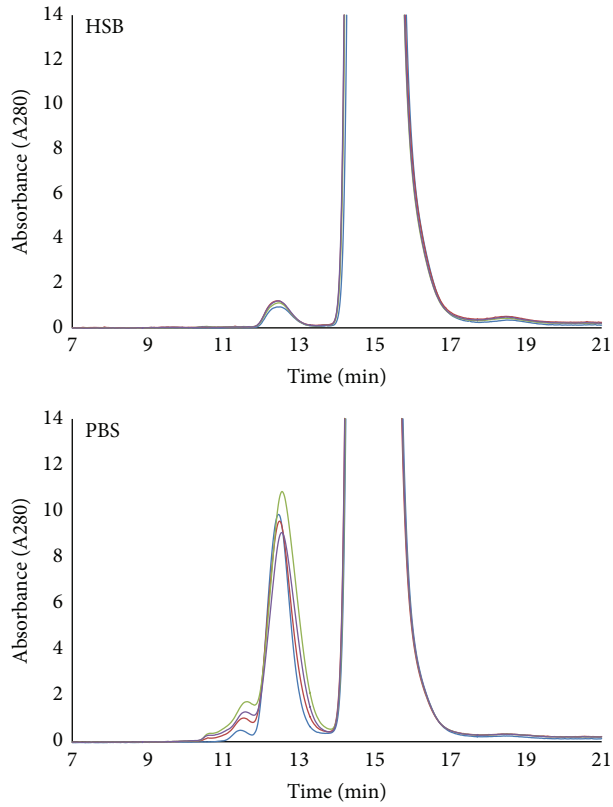

Figure 1: HPSEC traces of ruthenylated antibody conjugate stored at 1 mg/mL in PBS or in HSB. Each preparation was subjected to multiple freeze-thaw cycles by cycling between −80°C (approximately 16 hours) and 5°C (approximately 8 hours) prior to analysis by HPSEC: the light blue color: 0 F/T cycles; the red color: 1 F/T cycle; the green color: 3 F/T cycles; and the purple color: 5 F/T cycles. The retention times, structural designations, and peak areas are shown in Table 1.

3.2. Impact of Reagent Aggregation on the Screening Assay Results. Serum samples from drug-naïve individuals (8 from disease A and 8 from disease B) were tested in the screening assay alongside a set of system suitability controls: NQC, LQC, and HQC. All assays utilized biotinylated antibody reagent prepared in HSB and stored at −80 ± 10°C until time of use. The samples and controls were analyzed using ruthenylated antibody stored in HSB or in PBS that was subjected to 0, 1, 3 and 5 freeze/thaw cycles, and all four freeze/thaw cycles were analyzed on the same plate. The S : B value for each sample was calculated by dividing its mean response by the mean response of NQC analyzed using ruthenylated reagent subjected to the same number of freeze/thaw cycles (Figure 2).

The signal to baseline (S : B) responses of samples tested with ruthenylated antibody prepared in HSB were very consistent and were not affected by the number of freeze/thaw cycles applied to the ruthenylated reagent. For all samples in both disease groups the absolute percent difference of response across 5 F/T cycles of ruthenylated reagent was ≤14.7%. Of the eight disease A samples, one was consistently classified as positive and one measured slightly above

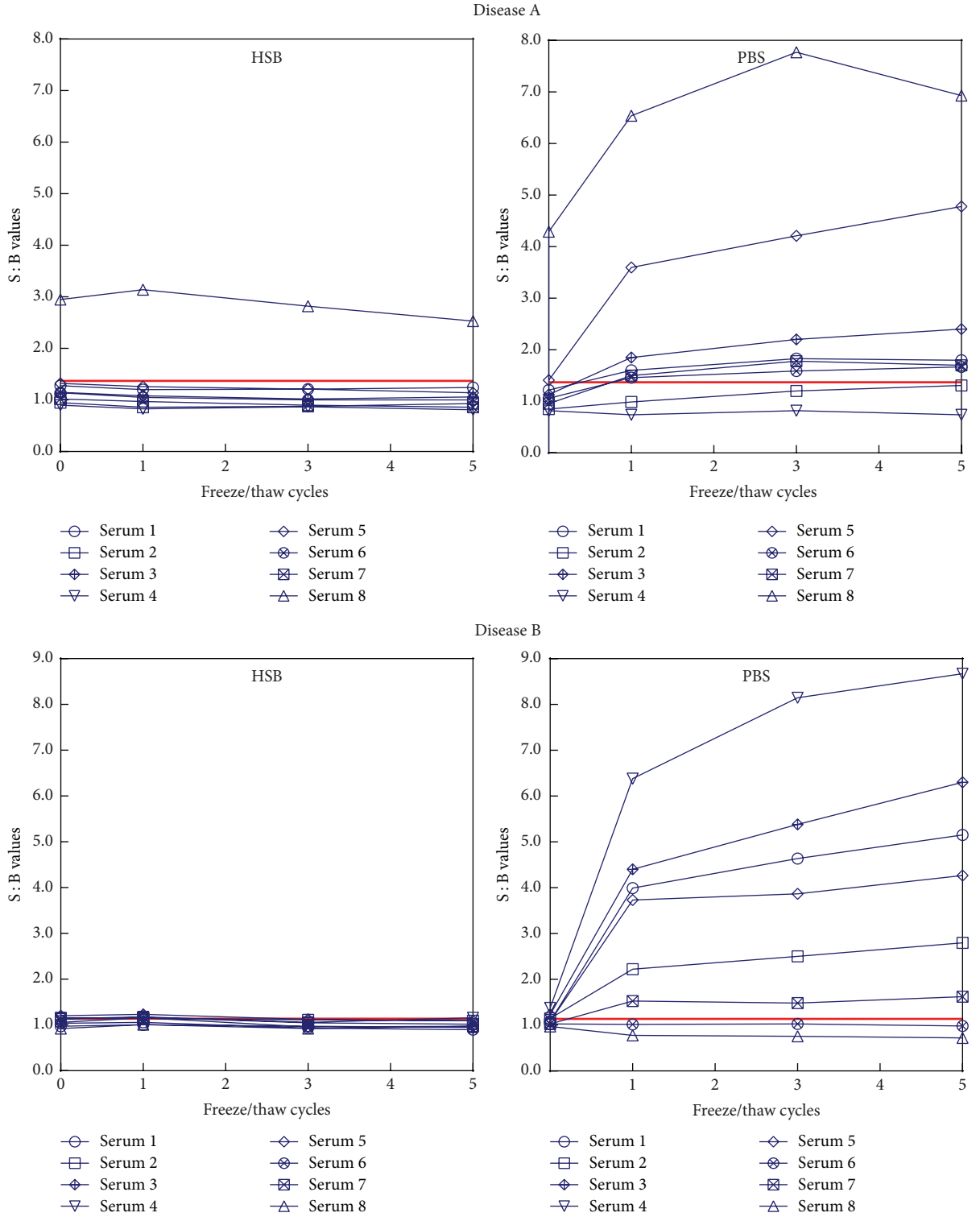

Figure 2: Screening responses of individual disease A and disease B serum samples tested using ruthenylated antibody reagents stored in PBS and in HSB. The solid red line on each graph indicates screening cut point values for disease A (1.37) and disease B (1.14) populations obtained during method validation.

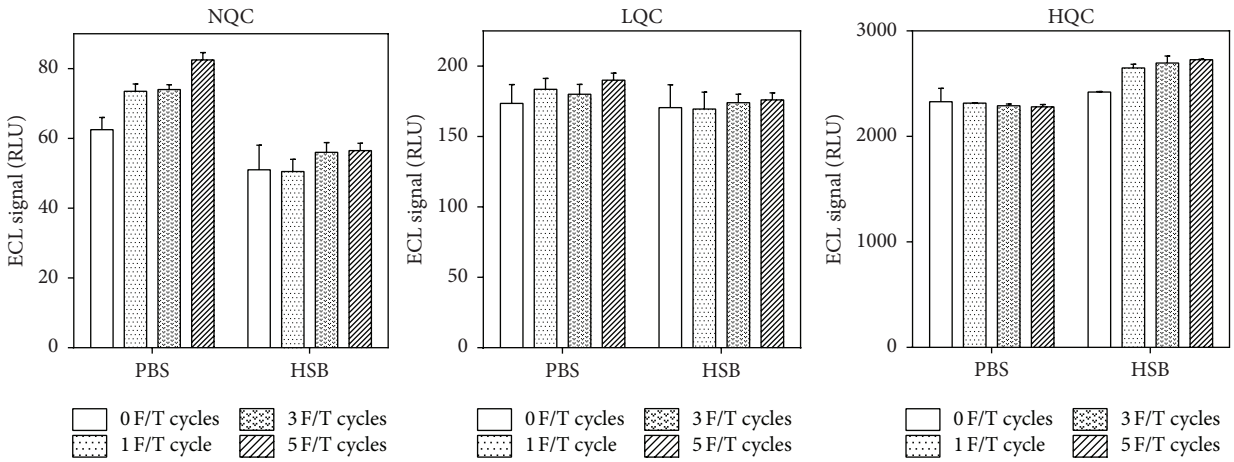

Figure 3: ECL signal in relative light units (RLU) of the system suitability controls: negative control (NQC: pooled serum) and low and high positive controls (LQC: 15 ng/mL, HQC: 1000 ng/mL of affinity-purified anti-idiotype goat polyclonal antibody in NQC) analyzed with reagents stored in PBS and HSB subjected to multiple freeze-thaw (F/T) cycles. Error bars show mean with standard deviation.

the screening cut point only with the 0 freeze/thaw cycles' reagent preparation. All eight disease B samples were consistently classified as negative. A dramatically different outcome was observed when the immunogenicity assay was performed with the ruthenylated drug stored in PBS. While only two of eight disease A samples were classified as screen positive for ADA using 0 F/T cycles' reagent, six measured positive after the first F/T cycle. For disease B samples, this number changed from one positive classification at 0 F/T cycles to six after the first F/T cycle. For the majority of samples, the change in classification status was accompanied by a severalfold increase of the S : B signals, most of which occurred upon the initial freezing of the ruthenylated antibody stored in PBS. The increased S : B ratios were due to increased sample responses and not to reduction of the NQC response (see Figure 3). In both disease A and disease B sets there were two samples whose responses were only slightly affected by the reagent storage in PBS. Overall, when the 0 F/T cycles' ruthenylated reagent was used to test the 16 samples, one measured ADA positive using the HSB preparation (6.3%) compared to 12 using the PBS preparation (75.0%). Storage of the ruthenylated reagent in PBS buffer or in HSB did not have an equally dramatic effect on the % inhibition values (data not shown). However, it should be noted that testing of samples with the reagent stored in PBS exposed to 0 and 5 freeze/thaw cycles resulted in changes of the positive/negative classifications of individual samples as well as the overall number of positive classifications. Three disease A samples (numbers 1, 5, and 8) and two disease B samples (numbers 4 and 6) were above the confirmatory cut point (29.9% and 21.2% for diseases A and B, resp.) when tested with the ruthenylated reagent stored in PBS at 0 F/T cycles. After exposure of the ruthenylated reagent to 5 F/T cycles, three disease A samples (numbers 3, 6, and 8) and no disease B samples were confirmed as positive. On the other hand, confirmatory responses for samples tested with the ruthenylated reagent stored in HSB remained constant after 5 freeze/thaw cycles with only two disease A samples (numbers 1 and 8) consistently classified as confirmed positive.

Unlike individual sera, control pool responses were not significantly impacted by the use of different storage buffers for the ruthenylated reagent. Application of multiple freeze/thaw cycles to the ruthenylated reagent did not produce a strong effect on responses of the assay quality controls (Figure 3). After 5 freeze/thaw cycles, the ECL signal of the low and high positive controls remained within ±12.7% of the values obtained with fresh (i.e., never frozen) reagents stored in either PBS buffer or in HSB. A more pronounced effect was observed for the negative control where 5 freeze/thaw cycles applied to the ruthenylated reagent stored in PBS led to a 30% increase of the signal generated with fresh reagents. This increase of the NQC signal is relatively small and cannot account for the observed severalfold upsurge of the S : B responses for some individual samples. It should be noted that, despite a positive trend in signal intensity of the assay controls related to the number of F/T cycles applied to the ruthenylated reagent, all responses remained within acceptable ranges established during the original method validation. Negative and positive control pools did not respond to changes in the conjugated reagent integrity and, therefore, are insufficient to monitor increases in sample responses associated with reagent aggregation.

3.3. Reagent Monitoring Control. As shown in Figure 3, a regular set of system suitability controls may be inadequate to detect degradation of conjugated reagents and the resulting increase of positive classifications. In order to prepare a control sensitive to aggregation status of the conjugated reagents, we focused on disease B population where the sample-to-sample differences caused by the presence of aggregates were more common and more striking (Figure 4).

We screened 160 individual serum units from individuals with disease B using conjugated reagents in PBS (1 F/T cycle) and selected 31 samples with responses above the screening

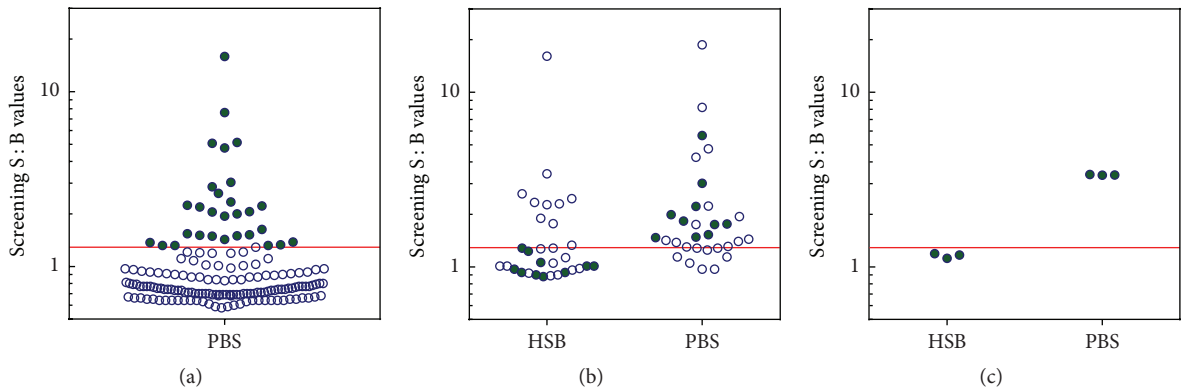

Figure 4: Preparation of the reagent monitoring control. (a) 160 commercial serum samples from drug-naïve subjects diagnosed with disease B were screened using conjugated reagents stored in PBS and subjected to one freeze/thaw cycle. Solid green circles indicate samples with S : B responses at or above the screening cut point. (b) Samples selected in step (a) were tested in the screening assay using conjugated reagents stored in PBS and in HSB. Solid green circles indicate samples that changed classification from negative when tested using reagents in HSB to positive using reagents in PBS. (c) Samples identified in step (b) were pooled together and measured three times using conjugated reagents stored in HSB and using reagents in PBS. Green circles in this panel represent the three separate measurements of the final RMC pool. Red line indicates assay cut point for disease B.

cut point. These samples were retested following the same procedure and on the same assay plate but using ruthenylated reagents stored in either PBS or HSB in order to identify sera that changed ADA classification status (negative to positive) and showed increase of the S : B signal by >30%. Ten samples met these criteria and were pooled to create a reagent monitoring control (RMC). When tested with aggregated reagents (in PBS), RMC responses increased approximately 3-fold in comparison to the result obtained with nonaggregated reagents (in HSB) thus demonstrating that RMC could be used to monitor reagent aggregation status during clinical studies.

3.4. Cut Point Determination in Drug-Naïve Disease A and Disease B Populations. The large differences in sample responses observed across different reagent storage buffers led us to suspect that even slight aggregation of the conjugated reagents may have a tremendous impact on the screening and confirmatory cut point values and, consequently, on the reported baseline reactivity in drug-naïve populations. Screening and confirmatory data were generated for disease A and disease B populations using conjugated reagents stored in PBS or HSB, each exposed to 1 F/T cycle. Screening and confirmatory data for the drug-naïve disease A and disease B populations and the resulting cut point values are shown in Figure 5.

As expected, screening cut points established using reagents stored in PBS (and containing aggregates) were much higher than those obtained with reagents stored in HSB (little or no aggregation) with the difference being approximately 4-fold for disease A population and almost 15-fold for disease B population. It should be noted that all cut point values were obtained after removal of statistical outliers following the customary procedures detailed in [15, 16]. The elevated screening cut point values obtained with the PBS reagent preparations were caused by broadening of signal distributions in both disease A and disease B populations. Confirmatory cut points obtained using reagents stored in PBS were also higher than those generated with reagents stored in HSB but the differences were less pronounced than those observed for the screening cut points. Increase of the confirmatory cut point for disease A samples was caused by a combination of broader distribution and increased median, while for disease B population increased median accounted for most of the change in the confirmatory cut point value.

4. Discussion

4.1. Aggregation of Conjugated Reagents. We conducted a stability evaluation of the ruthenylated monoclonal antibody and in lieu of storing the reagent for several years at <−70°C, which would be expected for its typical use during testing of clinical samples, we chose to subject the reagent to multiple freeze/thaw cycles which may simulate the stress of long-term cryostorage [13, 17]. We evaluated two storage buffers: phosphate-buffered saline (PBS: 3 mM dibasic sodium phosphate, 1 mM potassium phosphate, and 155 mM NaCl, pH 7.4) and histidine-sucrose buffer (HSB: 25 mM histidine, 250 mM sucrose, pH 6.0) in order to, respectively, enhance and reduce protein aggregation. Changes of protein purity caused by storage in these two buffers were monitored by High Performance Size-Exclusion Chromatography. Concentration of aggregates in the preparation of ruthenylated antibody stored in HSB remained constant at about 0.2% through five freeze/thaw cycles which provides assurance that conjugates stored in this buffer should be able to remain in cryostorage for years without any appreciable degradation. PBS proved less efficient in maintaining integrity of the conjugated antibody. An elevated amount of dimers and trimers (2.6%) appeared prior to freezing of the preparation in PBS which implies that certain degree of aggregation may be due to phosphate buffer alone and is not related

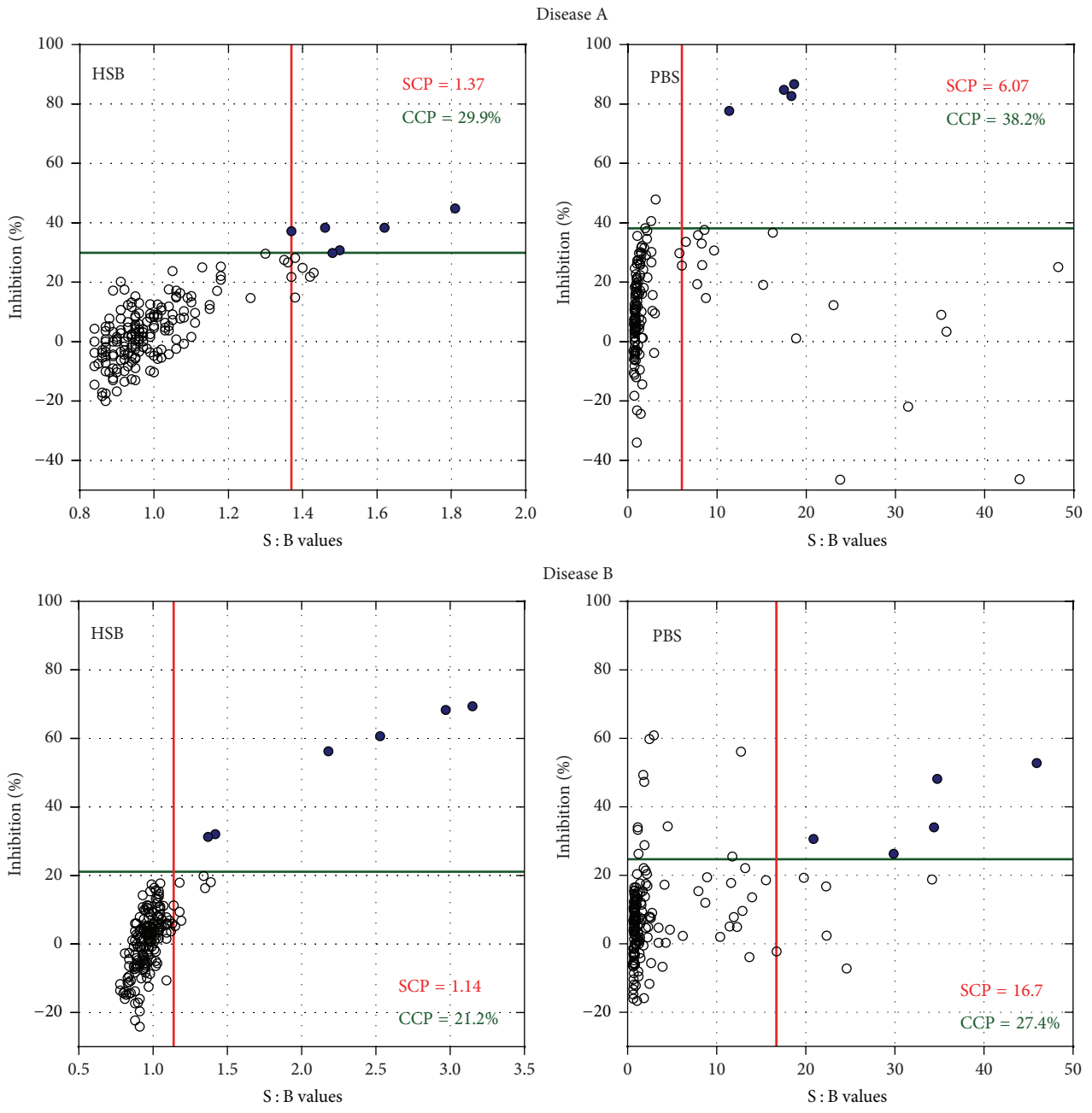

FIGURE 5: Screening and confirmatory responses of individual drug-naïve disease A and disease B samples tested using conjugated reagents stored in HSB or in PBS. The vertical red and horizontal green lines on each graph show screening (SCP) and confirmatory cut points (CCP), respectively. Open circles (○) indicate negative responses and closed circles (●) correspond to confirmed positives.

to protein degradation caused by freezing. This is underscored by the observation that the amount of dimers/trimers was not appreciably affected by the number of freeze/thaw cycles. Species that formed in response to the freeze/thaw induced stress were identified as unordered multimers with molecular weight of about 1900 kDa. Increased concentrations of unordered multimers in the ruthenylated antibody preparation were associated with the increased responses of selected individual samples in both disease A and disease B, suggesting that this species is directly responsible for high ECL responses. Despite formation of aggregates in PBS, it should be noted that the monomer content remained ≥95.9% throughout all five freeze/thaw cycles, which for most antibody applications would be considered acceptable and would not trigger a follow-up investigation. Our observation that the presence of ≤0.44% of high molecular aggregates can have a profound impact on the individual responses in the immunogenicity assay suggests that quality requirements for

conjugated reagents used in immunogenicity testing should be more stringent than those typically applied to monoclonal antibodies.

4.2. Nature of Matrix Interference. To the best of our knowledge, the publication by Tatarewicz et al. [14] is the only report linking the presence of aggregates in the conjugated reagents used for detection of ADA with high apparent baseline reactivity in a drug-naïve population. This report demonstrates that high signals in the ECL immunogenicity assay could be reduced by modifying the conjugated antibody formulation buffer with sucrose, a cryoprotectant, which was able to prevent formation of aggregates. Their study was performed using samples from patients with rheumatoid arthritis known to contain significant amounts of rheumatoid factor (RF) which may bind to aggregated immunoglobulins; however no correlation between the RF levels and the intensity of response in the ADA assay was detected. We also tested sixteen individual serum samples with RF ranging from 12.2 U/mL to 650.0 U/mL and we did not observe any correlation between the RF level and response in the screening assay using either of the two different formulations of the ruthenylated antibody (data not shown).

Using ruthenylated reagent stored in PBS, we observed high baseline reactivity in drug-naïve samples from patients diagnosed with disease A and disease B, two populations typically not associated with high levels of RF. Similar to the previous report, storage of conjugated reagents in PBS (containing no cryoprotectant) instead of HSB (containing 250 mM sucrose) caused formation of aggregates, which in turn resulted in a dramatic increase of the screening responses in both populations. Inexplicably, this increase was far from uniform: it reached as much as 6-fold for some individual disease A and disease B samples while other samples appeared to be unaffected. It is far from obvious how the aggregates present in the ruthenylated reagent can cause a significant signal increase for certain samples. One could hypothesize that high molecular weight aggregates (composed of 12-13 molecules based on the observed average molecular weight of 1900 kDa) are capable of amplifying ECL signal to a higher degree than a monomeric molecule, thus strongly enhancing otherwise insignificant sample-to-sample differences. Dramatic differences in behavior of individual serum samples in response to aggregated reagents can have a significant impact on screening and confirmatory cut point values as well as the number of positive classifications generated by the immunogenicity assay. Thus, integrity of conjugated reagents becomes of utmost importance for ensuring quality of immunogenicity data as discussed below.

4.3. Impact of Reagent Aggregation on ADA Responses in Drug-Naïve Populations. One can envision a scenario where, due to gradual degradation of conjugated reagents, screening and confirmatory responses of some individual samples show a marked increase similar to that illustrated in Figures 2 and 5. Such increases may be misinterpreted as developing immune responses, especially if ADA positive/negative classifications are made using cut points obtained with intact conjugated reagents. For example, screening and confirmatory cut point values generated in disease A and disease B populations using nonaggregated reagents (i.e., stored in HSB) are 1.37 and 29.9% and 1.14 and 21.2%, respectively. As shown in Figure 5, application of these cut points to data generated with aggregated reagents (i.e., stored in PBS) would result in a very high number of confirmed positive classifications. A common practice in clinical studies is to establish in-study cut points using either nontreated commercial samples or pretreatment patient samples prior to immunogenicity testing [18]. However, cut points obtained with aggregated reagents can be very high as shown in Figure 5. Cut point values for disease A and disease B populations obtained with reagents stored in PBS were 6.07 (screening) and 38.2% (confirmatory) and 16.7 (screening) and 27.4% (confirmatory), respectively. Such high screening cut point values would significantly reduce assay sensitivity and drug tolerance and may be invalid for sample testing.

It is expected that loss of reagent integrity should be detected by unacceptable performance of the system suitability controls resulting in assay failure that should preclude reporting of inaccurate immunogenicity results. As shown in Figure 3, however, aggregation of ruthenylated reagents had negligible effect on performance of both positive and negative assay controls, thus raising the likelihood of unwittingly reporting immunogenicity results that are heavily "contaminated" with false positives. In order to eliminate such a possibility, we generated a reagent monitoring control (RMC) by pooling together individual serum samples selected for their ability to strongly increase their ECL signal in response to the presence of aggregates in conjugated reagents. As shown in Figure 4, RMC is classified as negative when nonaggregated reagents (stored in HSB) are used and positive when tested with aggregated reagents (stored in PBS). The observed 3-fold increase of S : B response upon testing with reagents stored in PBS makes RMC more sensitive to reagent aggregation than the traditional set of positive and negative controls and can be used as a real-time functional test of reagent quality to be performed together with testing of unknown samples.

The RMC is included in the confirmatory assay tier (4 replicates tested in the absence of added drug) for the purpose of monitoring the performance of reagents during sample analysis. RMC is expected to be classified as negative and repeated positive classifications of RMC should trigger a halt to sample testing until completion of an investigation into quality of conjugated reagents.

Due to difficulties in its preparation, RMC cannot easily replace the negative control pool but it can be used in addition to regular system suitability controls. RMC or related controls could also be included in functional testing of stress-induced stability of critical reagents and selection of appropriate formulation buffers similar to the approach described by Staack et al. [6]. While we expect that use of optimal formulation buffer and proper storage of conjugated reagents should prevent their degradation and subsequent changes of assay performance, RMC can be used as an additional precaution ensuring high quality of the immunogenicity data.

5. Summary

Integrity of conjugated reagents used for measurement of ADA in clinical samples is critical for generation of reliable immunogenicity data. Small amounts of aggregates present in preparations of conjugated reagents may lead to a spurious increase of ADA positive classifications creating an appearance of immune response developing in certain individuals. System suitability controls prepared in normal serum or plasma pools may be unaffected by the presence of aggregates and perform within specification thus failing to alert the investigators about potential problems with the quality of immunogenicity results. Presence of aggregates in conjugated reagents is best detected using individual samples known to be sensitive to reagent aggregation analyzed either individually or being pooled together to create controls for monitoring of reagent quality. Phosphate-buffered saline may be especially ill suited for cryostorage of proteins and its use should be discouraged. Presence of cryoprotectants such as sucrose in formulation buffers may eliminate or at least reduce aggregation of conjugated reagents during long-term storage at $<-70^{\circ}$C.

Competing Interests

All authors are employees of MedImmune LLC and own stock in Astra Zeneca.

References

[1] European Medicines Agency (EMA), *Guideline on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins*, 2007, http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003946.pdf.

[2] European Medicines Agency (EMA), *Guideline on Immunogenicity Assessment of Monoclonal Antibodies Intended for In Vivo Clinical Use*, 2012, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/06/WC500128688.pdf.

[3] EMA, "Draft guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins," 2015, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2015/10/WC500194507.pdf.

[4] FDA, "Draft guidance for industry: Assay development for immunogenicity testing of therapeutic proteins," 2009, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf.

[5] FDA, 2014, http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm338856.pdf.

[6] R. F. Staack, J. O. Stracke, K. Stubenrauch, R. Vogel, J. Schleypen, and A. Papadimitriou, "Quality requirements for critical assay reagents used in bioanalysis of therapeutic proteins: what bioanalysts should know about their reagents," *Bioanalysis*, vol. 3, no. 5, pp. 523–534, 2011.

[7] D. M. O'Hara, V. Theobald, A. C. Egan et al., "Ligand binding assays in the 21st century laboratory: recommendations for characterization and supply of critical reagents," *AAPS Journal*, vol. 14, no. 2, pp. 316–328, 2012.

[8] B. J. Geist, A. C. Egan, T.-Y. Yang, Y. Dong, and G. Shankar, "Characterization of critical reagents in ligand-binding assays: enabling robust bioanalytical methods and lifecycle management," *Bioanalysis*, vol. 5, no. 2, pp. 227–244, 2013.

[9] P. Kolhe, E. Amend, and S. K. Singh, "Impact of freezing on pH of buffered solutions and consequences for monoclonal antibody aggregation," *Biotechnology Progress*, vol. 26, no. 3, pp. 727–733, 2010.

[10] G. Gómez, M. J. Pikal, and N. Rodríguez-Hornedo, "Effect of initial buffer composition on pH changes during far-from-equilibrium freezing of sodium phosphate buffer solutions," *Pharmaceutical Research*, vol. 18, no. 1, pp. 90–97, 2001.

[11] M. C. Manning, D. K. Chou, B. M. Murphy, R. W. Payne, and D. S. Katayama, "Stability of protein pharmaceuticals: an update," *Pharmaceutical Research*, vol. 27, no. 4, pp. 544–575, 2010.

[12] L. A. Kueltzo, W. Wang, T. W. Randolph, and J. F. Carpenter, "Effects of solution conditions, processing parameters, and container materials on aggregation of a monoclonal antibody during freeze-thawing," *Journal of Pharmaceutical Sciences*, vol. 97, no. 5, pp. 1801–1812, 2008.

[13] S. K. Singh, P. Kolhe, A. P. Mehta, S. C. Chico, A. L. Lary, and M. Huang, "Frozen state storage instability of a monoclonal antibody: aggregation as a consequence of trehalose crystallization and protein unfolding," *Pharmaceutical Research*, vol. 28, no. 4, pp. 873–885, 2011.

[14] S. Tatarewicz, J. M. Miller, S. J. Swanson, and M. S. Moxness, "Rheumatoid factor interference in immunogenicity assays for human monoclonal antibody therapeutics," *Journal of Immunological Methods*, vol. 357, no. 1-2, pp. 10–16, 2010.

[15] R. J. Kubiak, L. Zhang, J. Zhang et al., "Correlation of screening and confirmatory results in tiered immunogenicity testing by solution-phase bridging assays," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 74, pp. 235–245, 2013.

[16] L. Zhang, J. J. Zhang, R. J. Kubiak, and H. Yang, "Statistical methods and tool for cut point analysis in immunogenicity assays," *Journal of Immunological Methods*, vol. 389, no. 1-2, pp. 79–87, 2013.

[17] B. A. Kerwin, M. C. Heller, S. H. Levin, and T. W. Randolph, "Effects of tween 80 and sucrose on acute short-term stability and long-term storage at -20°C of a recombinant hemoglobin," *Journal of Pharmaceutical Sciences*, vol. 87, no. 9, pp. 1062–1068, 1998.

[18] B. Gorovits, "Antidrug antibody assay validation: industry survey results," *AAPS Journal*, vol. 11, no. 1, pp. 133–138, 2009.

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Review Article

Immunogenicity of Biotherapeutics: Causes and Association with Posttranslational Modifications

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Today, potential immunogenicity can be better evaluated during the drug development process, and we have rational approaches to manage the clinical consequences of immunogenicity. The focus of the scientific community should be on developing sensitive diagnostics that can predict immunogenicity-mediated adverse events in the small fraction of subjects that develop clinically relevant anti-drug antibodies. Here, we discuss the causes of immunogenicity which could be product-related (inherent property of the product or might be picked up during the manufacturing process), patient-related (genetic profile or eating habits), or linked to the route of administration. We describe various posttranslational modifications (PTMs) and how they may influence immunogenicity. Over the last three decades, we have significantly improved our understanding about the types of PTMs of biotherapeutic proteins and their association with immunogenicity. It is also now clear that all PTMs do not lead to clinical immunogenicity. We also discuss the mechanisms of immunogenicity (which include T cell-dependent and T cell-independent responses) and immunological tolerance. We further elaborate on the management of immunogenicity in preclinical and clinical setting and the unique challenges raised by biosimilars, which may have different immunogenic potential from their parent biotherapeutics.

1. Introduction

Posttranslational modifications (PTMs) refer to enzymatic modifications that occur after translation, and which result in mature protein products. PTMs increase the functional diversity of the proteome, by the covalent addition of functional groups, proteolytic cleavage of regulatory subunits, or selective degradation of entire proteins. These modifications include glycosylation, acetylation, acylation, ADP-ribosylation, amidation, γ-carboxylation, β-hydroxylation, disulfide bond formation, phosphorylation, proteolytic processing, and sulfation and influence almost all aspects of normal cell biology and pathogenesis. Therefore, all living cells are tuned to use PTMs to regulate cellular activity. In addition, these PTMs have a major impact on evolution; multisite PTMs lead to a combinatorial explosion in the number of potential molecular states. Such complexity may provide the foundation for sophisticated forms of cellular information processing that are essential for the emergence of complex organisms [1].

In comparison with small molecule drugs, protein pharmaceuticals are complex entities; and since they are usually expressed in cellular systems, they are exposed to factors which could influence PTMs. The PTM profile is dependent on several factors including the type and differentiation status of the host cell, upstream and downstream manufacturing process, formulation, and storage conditions and microheterogeneities formed during fermentation and downstream processing. Naturally occurring PTMs have been associated with unwanted immunogenicity and autoimmune diseases. Recent studies have identified anticitrullinated protein antibodies, along with other antibodies to specific posttranslational modified proteins, as biomarkers in rheumatoid arthritis, psoriatic arthritis, periodontitis, and osteoarthritis [2]. It is hypothesized that such PTMs induce neoepitopes that can generate novel antibody specificities probably triggering autoimmunity. Given the multiplicity of possible PTMs, any variation in a recombinant protein's PTM profile relative to the natural product might be of concern and should be

evaluated. Adverse immune reactions can lead to clinical consequences, such as anaphylaxis, reduced drug half-life, and neutralization of the therapeutic protein as well as its endogenous human homologue [3, 4]. However, it should be noted that, of the very large number of patients treated with biotherapeutic proteins over the years, only a few are affected by undesirable immune responses [5, 6]. For example, a ~2% incidence of adverse reactions (attributable to anti-drug antibodies, ADAs) for insulin and an even rarer but more clinically serious effect for erythropoietin has been reported [7, 8]. These studies clearly indicate that some patients are more susceptible to immune responses than others.

In this review, we will identify some of the causes of immunogenicity in therapeutic proteins and will discuss the association of immunogenicity with PTMs, with other critical quality attributes of protein therapeutics, and with patient characteristics.

2. Immunogenicity and Its Causes

Immunogenic response to therapeutic molecules can generate anti-drug antibodies (ADAs), which can be either neutralizing or nonneutralizing. Neutralizing antibodies (NAbs) bind to sites in therapeutic proteins in such a way that they directly impair or abrogate the biological functions of therapeutic proteins [6, 9]. NAb responses have the potential to cause negative clinical consequences by neutralizing the therapeutic product and therefore reducing efficacy, as has been seen with factor VIII or streptokinase [10, 11]. This reduced efficacy would, in some cases, require the need to dose patients more frequently to get the desired clinical effect. The situation can further be aggravated if the NAbs neutralize not only therapeutic proteins but also the endogenous counterpart of the therapeutic agent, resulting in severe adverse consequences. Examples of drugs inducing ADAs which also inactivate autologous proteins include recombinant human thrombopoietin [12, 13], erythropoietin [5, 14, 15], GM-CSF [16], and many interferons [17–19]. The problem is most often seen with non-mAb therapeutic proteins with significant similarity to host proteins (except for a few amino acid changes or glycosylation differences) [13]. However, some non-mAb proteins such as insulin, factor XIII, and α interferons (IFNs) primarily induce nonneutralizing ADAs and the effect is not physiologically debilitating. In these cases, clinicians often continue treatment in the presence of ADAs. This may indicate that it is not always possible to break self-tolerance even when the self-protein is exogenously chronically administered. Overall, only a small percentage of treated patients develop adverse immunogenic reactions attributed to the formation of NAbs [6].

Most patients who develop ADA response to therapeutic proteins generate nonneutralizing antibodies (NNAbs). These antibodies bind to antigenic sites in the therapeutic proteins in ways that do not affect the therapeutic effects of these drugs. Examples are NNAbs generated against tumor necrosis factor receptor and recombinant human growth hormone [20]. In some cases NNAbs can accelerate the clearance of therapeutic proteins resulting in reduced drug efficacy [13]. Product- and process-related factors can affect immunogenicity by minor alterations in the tertiary structure of the molecule such as altered protein folding. Additionally, patient characteristics, dose, and route of administration of the biotherapeutics can also lead to an increased risk of immunogenicity [21]. This will be described in more detail in later sections.

2.1. Product- and Process-Related Causes of Immunogenicity. The first therapeutic insulin products in the 1920s were of bovine or porcine origin and were therefore immunogenic in humans. In some cases, fatal anaphylactic reactions were reported [22]. The molecular structure of proteins purified from animal sources is different from that of their human counterparts. Thus it is expected that these proteins will be seen as "foreign" by the human immune system. Interestingly, removal of proinsulin, C-peptide, glucagon, and somatostatin from porcine insulin preparations led to a remarkable decrease in immunogenicity [22]. These results suggested that the anti-insulin antibodies generated may have been against noninsulin proteins or adjuvant-like contaminants [23]. This observation indicated that deviation from the structure of the human homologue is not the only determinant of immunogenicity.

Impurities have been held responsible for the immunogenicity of several therapeutic proteins. Human growth hormone (hGH) derived from the pituitary glands of cadavers and from patients undergoing hypophysectomy had been used in hypopituitary children to stimulate their growth [43]. Fifty percent of treated children developed immune reaction to the first clinical grade hGH; and this was attributed to the presence of 40% to 70% aggregated hGH in the product [44]. Improvement of the purification process decreased the aggregates to less than 5%, which resulted in slower onset of antibody production. Resulting antibodies had high affinities but were significantly less persistent [4].

Humanization of monoclonal antibodies has significantly decreased immunogenicity, especially the intense immunogenicity (allergic reactions culminating in anaphylactic shock) observed with early murine antibodies, which generated a human anti-mouse antibody (HAMA) response. However, some humanized and even fully human sequence-derived antibody molecules still carry immunological risk. Often the cause for immunogenicity with these fully human molecules is associated with unique (nonhuman) sequences in the cluster of differentiation regions (CDRs) of these antibodies [24] and modifying certain amino acids in these regions could reduce immunogenicity risk. For example, despite being humanized, alemtuzumab induced binding antibodies and NAbs in 30% to 70% of patients. It was shown that pretreatment with an altered version of alemtuzumab, which no longer binds to its target, induced immunogenic tolerance to alemtuzumab itself [28]. Other fully human antibodies such as canakinumab, ofatumumab, and pembrolizumab induced ADAs at very low incidence (<0.5%) [30, 35, 39, 45]. Details of immunogenicity and NAbs reported for mAbs are shown in Table 1.

Intrinsic factors influence the immunogenicity of antibodies; for example, antibodies directed at cell surface markers are deemed to have a higher risk of immunogenicity than

Table 1: Immunogenicity of FDA-approved biologics.

SI number	Biologic	Type/target	Indications	Immunogenicity (% patients)		Reference
				Binding antibodies	Neutralizing antibodies	
(1)	Adalimumab	Human IgG1 antibody specific for TNF alpha	Rheumatoid arthritis, psoriasis	5%–89%	5%–89%	[24–27]
(2)	Alemtuzumab	Humanized monoclonal antibody which binds to CD52 on leukocytes	B cell chronic lymphocytic leukaemia	30%–70%	30%–70%	[28]
(3)	Belimumab	Human monoclonal antibody that inhibits the biologic activity of the soluble form of the essential B cell survival factor B-lymphocyte stimulator	Systemic lupus erythematosus	4.8%	3/11	[29] European Medicines Agency Assessment Report (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/002015/WC500110152.pdf)
(4)	Canakinumab	Human monoclonal antibody anti-IL-1β	Inflammatory diseases related to cryopyrin-associated periodic syndromes (familial cold autoinflammatory syndrome and Muckle-Wells syndrome)	0%	0%	[24, 30]
(5)	Cetuximab	Human/mouse chimeric monoclonal antibody that binds specifically to the extracellular domain of the human epidermal growth factor receptor	Colorectal cancer, squamous cell carcinoma of the head and neck	5%	Data not available	Drug label (http://pi.lilly.com/us/erbitux-uspi.pdf)

Table 1: Continued.

SI number	Biologic	Type/target	Indications	Immunogenicity (% patients)		Reference
				Binding antibodies	Neutralizing antibodies	
(6)	Denosumab	Human monoclonal antibody RANK ligand inhibitor	Treatment of postmenopausal women with osteoporosis at high risk for fracture To increase bone mass in men at high risk for fracture receiving androgen deprivation therapy for nonmetastatic prostate cancer To increase bone mass in women at high risk for fracture receiving adjuvant aromatase inhibitor therapy for breast cancer	<1%	0%	Drug label (http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/125320s5s6lbl.pdf)
(7)	Golimumab	Human monoclonal antibody anti-TNF-alpha	Rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis	4%	Data not available	[31–33]
(8)	Infliximab	Chimeric monoclonal antibody TNF blocker	Crohn's disease, ulcerative colitis, rheumatoid arthritis, ankylosing spondylitis, and psoriasis	Crohn's Disease: 10%, psoriatic arthritis: 15% psoriasis: 36–51%	Data not available	Drug label (http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/103772s5359lbl.pdf)
(9)	Ipilimumab	Human monoclonal antibody that binds to cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)	Melanoma	1.1% of 1024 evaluable patients tested positive for binding antibodies and 6.9% of 58 evaluable patients, who were treated with 0.3 mg/kg (dose cohort with the lowest trough levels) dose, tested positive for binding antibodies.	0%	Drug label (http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/103772s5359lbl.pdf)

Table 1: Continued.

SI number	Biologic	Type/target	Indications	Immunogenicity (% patients)		Reference
				Binding antibodies	Neutralizing antibodies	
(10)	Natalizumab	Human monoclonal antibody, which works against the cell adhesion molecule α4-integrin	Multiple sclerosis and Crohn's disease	9%	9%	Drug label (http://www.accessdata.fda.gov/drugsatfda_docs/label/2008/125104s033lbl.pdf)
(11)	Nivolumab	Human monoclonal antibody IgG4 PD-1 immune checkpoint inhibitor	Squamous non-small cell lung cancer, unresectable or metastatic melanoma, and disease progression following ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor	8.5%	0.7%	[34] Drug label (http://www.accessdata.fda.gov/drugsatfda_docs/label/2014/125554lbl.pdf)
(12)	Ofatumumab	Human monoclonal antibody anti-CD20	Rheumatoid arthritis, chronic lymphocytic leukaemia	0%	0%	[24, 35]
(13)	Panitumumab	Human monoclonal antibody against the epidermal growth factor receptor	Colorectal carcinoma	1–4.6%	0.8–1.6%	[24, 36–38] Drug label (http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/125147s080lbl.pdf)
(14)	Pembrolizumab	Human monoclonal antibody PD-1 blocking	Unresectable or metastatic melanoma and disease progression following ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor, metastatic NSCLC whose tumors express PD-L1	0.3%	0.3%	[39] Drug label (http://www.accessdata.fda.gov/drugsatfda_docs/label/2015/125514s004s006lbl.pdf)

Table 1: Continued.

SI number	Biologic	Type/target	Indications	Immunogenicity (% patients)		Reference
				Binding antibodies	Neutralizing antibodies	
(15)	Ramucirumab	Human monoclonal antibody vascular endothelial growth factor receptor 2 antagonist	Gastric cancer	6%	1%	Drug label (http://www.accessdata.fda.gov/drugsatfda_docs/label/2014/125477s002lbl.pdf)
(16)	Rituximab	Chimeric monoclonal antibody that targets the CD20 molecule expressed on the surface of B cells	Non-Hodgkin's lymphoma, rheumatoid arthritis	11%	Data not available	[40]
(17)	Secukinumab	Human monoclonal antibody against IL-17A	Plaque psoriasis	0.4%	3/10	[41] Drug information (http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/DermatologicandOphthalmicDrugsAdvisoryCommittee/UCM419023.pdf)
(18)	Siltuximab	Chimeric monoclonal antibody anti-IL-6	Multicentric Castleman's disease (a rare lymphoproliferative disorder) being human immunodeficiency virus-negative and human herpes virus-8-negative	0.2%	0%	Drug label (http://www.accessdata.fda.gov/drugsatfda_docs/label/2014/125496s000lbl.pdf)
(19)	Ustekinumab	Human monoclonal antibody that binds to the p40 protein subunit used by both the IL-12 and IL-23 cytokines.	Psoriasis	6.6%	Data not available	[42]
(20)	Vedolizumab	Humanised IgG1 monoclonal antibody that binds to the human $\alpha 4\beta 7$ integrin	Ulcerative colitis and Crohn's disease	4%	33/56	Drug label (http://www.accessdata.fda.gov/drugsatfda_docs/label/2014/125476s000lbl.pdf)

IgG: immunoglobulin G; IL: interleukin; TNF: tumour necrosis factor; CD: cluster of differentiation; RANK: receptor activator of nuclear factor kappa-B; PD-1: programmed cell death protein-1; PDL-1: programmed death ligand-1; NSCLC: non-small cell lung cancer.

those against soluble factors [24]. The reasons for this are not completely understood but may include antigen internalization and subsequent processing and presentation by target cells [24, 46]. A similar intrinsic factor is the presence of carbohydrate side-chains attached to the antibody via glycosylation sites, conferred by the amino acid sequence of the light chain constant region, the heavy chain constant region, or the V region itself [47, 48]. The presence of a galactose-alpha-1,3-galactose sugar within a carbohydrate structure on the Fab fragment of cetuximab was found to be associated with severe anaphylactic reactions to the antibody. Most patients were found to have preexisting immunoglobulin E (IgE) antibodies specific for the galactose-alpha-1,3-galactose sugars [49]. Notably, when cetuximab was manufactured in a cell line that could not add galactose-alpha-1,3-galactose to the antibody carbohydrate (a Chinese hamster ovary- [CHO-] derived manufacturing cell line), the product was much less immunogenic [49].

Other PTMs, such as glycation, deamidation, and oxidation of amino acid side-chains, may confer immunogenicity as well [24, 50]. Introducing additional N-linked glycosylation sites to create an erythropoietin (EPO) product with improved efficacy and catabolic half-life [51] did not result in increased immunogenicity [52]. However, it was noted that the subcutaneous administration of EPO was associated with pure red cell aplasia (PRCA) in some cases, because of NAbs generated to endogenous EPO. Although the cause of this reaction is still uncertain [53], extractables and leachates from the container are thought to have been responsible [54]. An increased concentration of anti-ESA IgG4 antibody is associated with the development of antibody mediated PRCA (amPRCA) [55].

2.2. Patient-Related Causes of Immunogenicity and Influence of Route of Administration. Generally, patients with impaired immune systems (e.g., cancer patients receiving chemotherapy) may be less likely to develop antibodies to therapeutic proteins than immunocompetent individuals [56, 57].

Exposure of patients to replacement therapeutic proteins that the patients cannot synthesize frequently leads to the generation of NAbs [58]. For example, NAbs frequently develop in severe congenital factor (F) XI-, FIX-, and FVIII-deficient patients treated with the missing therapeutic proteins [10, 59–61]. The inherent polymorphism of human proteins may be another contributing factor toward immunogenicity. This is especially applicable in hemophilia, where patients are predominantly categorized into two major groups. One group has large deletions, nonsense mutations, and intron 22 inversions leading to nondetection of FVIII protein in the plasma. This group, who have never been exposed to FVIII, generate anti-FVIII in more than 30% of treated patients. The second group of patients have small deletions, insertions, and point mutations and therefore might be innately tolerant to therapeutic FVIII administration. In this latter group, anti-FVIII antibody prevalence is less than 10% [62]. When mutation types are further subdivided based on their risk of antibody formation, patients with large deletions have highest risk (~75%), followed by patients with nonsense mutations and those with inversions of intron 22 [63–65]. Since small deletion/insertion mutations cause a frameshift, resulting in subsequent stop codon and truncated protein, a risk similar to that of a major deletion might be expected. Surprisingly, these patients had a relatively low risk (7.5%) [60]. This was attributed to polymerase errors which lead to restoration of reading frames for small deletion/insertion mutations. The small amount of endogenous FVIII produced was apparently sufficient to generate tolerance [63, 66]. However, mutations in the FVIII gene do not completely explain immunogenicity to therapeutic FVIII. Only one-third of the patients with intron 22 inversion develop anti-FVIII antibodies. Speculations explaining this include presentation of maternal FVIII to the fetal immune system to induce immunogenic tolerance and polymorphism in the immune systems of patients, which either hinders or synergizes anti-FVIII antibody formation [64, 67].

A meta-analysis conducted by Scharrer et al. indicated influence of race on the risk of antibody formation [68]. The incidence of antibody formation in African-Americans was double that in Caucasians (51.9%, 14 of 27, versus 25.8%, 51 of 191). This again indicates that genetic polymorphism has a role in immunogenicity. The incidence of antibody formation in siblings (50%) is significantly higher than in extended hemophilia A relatives (9%), suggesting that sibling risk is correlated [69]. However, nongenetic factors could also influence immune response as variations in response in monozygotic twins have been described [70]. Since the genetic defects in FVIII are assumed to be similar in siblings and extended relatives, polymorphisms in immune response genes may influence the risk of anti-FVIII antibody formation. An analysis of MHC class I and II alleles identified A3, B7, C7, DQA0102, DQB0602, and DR15 as risk alleles (relative risk: 1.9–4.0). MHC class I/II alleles C2, DQA0103, DQB0603, and DR13 were identified as protective alleles (relative risk 0.1–0.2) since they occurred more often in patients who did not develop antibodies than in those who did. However, sample sizes were generally too small for statistical confirmation of differences.

Immunogenicity is associated with the route of administration of a therapeutic molecule. The skin and the mucosal membranes make up the primary surface barriers to pathogens, and just beneath these lies the primary machinery to protect the body when these barriers are breached, abundant professional antigen-presenting cells (APCs). Routes of administration that involve the skin or mucosa thus may carry the greatest potential for an immunological response. Interestingly, exposure via gut mucosa is generally tolerogenic; however the challenge is the effective oral delivery with bioavailability of the biotherapeutic [71]. The probability of immune response is highest after subcutaneous injection, followed by intramuscular, intranasal, and intravenous routes. Subcutaneous administration generally localizes and prolongs the exposure of the protein to a small area within close proximity of lymph nodes, where B and T cells are present [72]. Lymphatic uptake can enhance exposure to APCs. Dendritic cells may potentially be activated if an adjuvant-like factor (e.g., impurities, host cell proteins, or endotoxin) or a danger signal is present. Peng et al. showed that changing the route administration reduces the frequency of ADAs

[73, 74]. Extensive clinical experience with pulmonary delivery of biotherapeutic insulin showed that patients with type 1 and type 2 diabetes switching from subcutaneous dosing resulted in larger ADA responses [75, 76]. The antibodies were of the immunoglobulin G (IgG) class, were not neutralizing, and had no impact on clinical efficacy and safety. The importance of route of administration is not accepted by all; Schellekens argues that the immune reaction is not predicated by route of administration but rather is inherent in a therapeutic molecule itself [77].

Generally, short-term therapy is less likely to be immunogenic than long-term therapy, although intermittent treatment is more likely to elicit a response than continuous therapy [78, 79]. Also, lower doses are generally more immunogenic than higher doses, as typically seen with mAbs where the phenotype is more tolerogenic. This may be because of the evolution of the immune system to be generally less tolerant of low-abundance proteins. This was observed in a primate study of adalimumab where 16/16 monkeys developed PAHA response at low dose while only 2/16 animals developed PAHA response at high dose [72]. Therefore in clinical practice, high-dose regimens are used as a mode of therapy to induce tolerance (e.g., for factor VIII) [80].

3. Mechanisms of Immunogenicity of Biotherapeutics

The immune system can generate antibodies to therapeutic proteins by two general mechanisms: one relies on T cell costimulation of B cells while the other is independent of T cell [58].

3.1. T Cell-Dependent Immune Response. Analysis of antibodies from clinical studies suggests that serious side effects are mainly driven by high levels of IgG antibodies, suggesting a T cell-dependent pathway. In fact, IgG antibodies make up the majority of the ADA responses [81]. Naïve B cells require two signals for their proliferation and differentiation into antibody secreting plasma cells. The first signal is generated by the direct binding of the antigenic protein to B cell receptors on naïve B cell surfaces. This protein is then internalized, processed, and returned to the surface as peptides bound to the MHC class II molecules. The second signal is delivered by the armed T helper (Th) cells, which recognize the same antigen (or a peptide within the antigen, a concept known as linked recognition), via the binding of the T cell receptors (TCRs) to the peptide: MHC class II complex on the surface of naïve B cells. Another interaction is governed by the binding of B7 on B cells to CD28 on T cells. B-T cell contact leads to the overexpression of the B cell costimulatory molecule CD154 (CD40L) on the Th cell surface and secretion of B cell stimulatory cytokines (IL-4, IL-5, and IL-6) by the Th cells. This in turn activates the B cells and leads to their differentiation into antibody-secreting (short- and long-lived) plasma B cells. Some of these activated B cells also become memory cells, which maintain the pool of long-lived plasma cells and react rapidly to rechallenge by producing short-lived plasma cells. This T cell dependent immune response is thus usually long-lasting and of high titer, particularly for foreign or exogenous proteins [81].

3.2. T Cell-Independent Immune Response. In the T cell-independent antibody response, the ability to bypass Th cell costimulation leads to a more rapid antibody response. This type of response is typically evoked by particulate antigens and sequences of microbial and viral origin [6] (repetitive epitopes termed *pathogen associated molecular patterns*). Antigens that are expressed on the surface of pathogens in an organized, highly repetitive form can activate specific B cells by cross-linking of antigen receptors in a multivalent fashion [82]. This activation is dependent on the formation of a small number of antigen receptor clusters, each of which contains approximately 10 to 20 antigen-bound membrane Ig (mIg) molecules [82]. These clusters induce local membrane association of multiple activated Btk (Bruton's tyrosine kinase) molecules, which results in long-term mobilization of intracellular ionized calcium. Such persistent calcium fluxes efficiently recruit transcription factors, and thereby induce T-cell-independent B cell activation and proliferation. While this first signal of multivalent mIg cross-linking can induce B cell proliferation, a second signal in the form of engagement of members of the Toll-like receptor (TLR) family could selectively induce Ig secretion in B cells that were activated by multivalent, but not by bivalent, antigen receptor engagement. Due to the lack of affinity maturation, this pathway typically results in an IgM-type response, which is transient, of low titer, and of poor specificity [72]. Changes to the structure of a therapeutic protein may alter its miscibility in ways that enhance aggregation or cause it to resemble a pathogen, thereby greatly increasing antigenicity.

Typically, an immune reaction can be triggered by most therapeutic proteins inducing antibody responses. Based on the trigger, the immune reaction can vary from low-titer, low-affinity, transient IgM antibody responses to high-titer, high-affinity responses, followed by class switching and IgG responses. Consequences of this transition can range from minimal to severe and life-threatening [72].

4. Posttranslational Modifications and Their Correlation with Immunogenicity

Most therapeutic proteins are synthesized in the endoplasmic reticulum (ER) and are eventually secreted. While some modifications occur before the proteins are secreted, others happen afterwards, during *in vitro* processing, including purification, formulation, and storage, and during administration into patients [83].

Modifications of proteins that occur in the ER, golgi, and exocellular spaces have been reviewed in detail by Fineberg et al. [75]. These modifications are disulphide bond formation, gamma carboxylation of glutamate residues, and beta hydroxylation of aspartate and asparagine residues in the ER; tyrosine sulfation, propeptide processing, O-linked glycosylation, phosphorylation, and amidation in the golgi; and deamidation, glycation, N-terminal pyroglutamate formation, oxidation, and proteolytic processing in the exocellular

spaces. Here, we will discuss protein structure, glycosylation, and chemical modifications.

Posttranslational modifications can have direct or indirect effects on immunogenicity. The modified part of the biotherapeutic itself could induce an immune response, or its presence can affect the tertiary structure of the protein subtly causing the biotherapeutic to become immunogenic [4].

4.1. Protein Structure. Primary amino acid sequence can affect protein structure, and hence immunogenicity, as is observed with animal-derived insulins [22]. For similar reasons, immunogenicity was higher for the first murine therapeutic antibodies, as compared to later chimeric, humanized, or fully human antibodies [84]. It is very interesting to note that while there are only 20 standard amino acids (19 amino acids and 1 imino acid), there are about 200 different functional amino acids after hydrolysis. The role of PTMs is thus significant [83]. Over the years, a significant number of modifications have been identified and several of them characterized [3, 85]. New epitopes in protein structure may be created due to the chemical modification of the protein, whereby new covalent crosslinks between amino acid residues are formed. These new protein structures could lead to the formation of aggregates, which may contain danger signals that greatly enhance immunogenicity.

4.2. Glycosylation. Glycosylation is the covalent addition of carbohydrate molecules (glycans) to the protein surface. It is the most common, complex, and heterogeneous PTM that can occur in both endogenous and therapeutic proteins [3, 86]. Almost half of the therapeutic proteins that are approved or in clinical trials are glycosylated [87]. The considerable heterogeneity in glycosylation profile of products can arise from the differences in the glycan itself (type, structure) or from the attachment pattern (site, extent of occupancy of possible sites). These variabilities may depend on the production and purification process [88]. Since glycans can influence the physicochemical (e.g., solubility, electrical charge, mass, size, folding, and stability) as well as the biological (e.g., activity, half-life, and cell surface receptor function) properties of proteins [89], any change with respect to the production or purification process, even in cell line, can alter glycosylation, thereby potentially altering physiological effects [4]. Glycosylation can have a direct or indirect impact on the immunogenicity of therapeutic proteins as well. The glycan structure itself can induce an immune response, or its presence can affect protein structure in such a way that the protein becomes immunogenic. Recent advances in analytical abilities, including matrix-assisted laser desorption ionization (MALDI), electrospray ionisation mass spectrometry (ESI-MS), and novel fluorescent tags for high performance liquid chromatography (HPLC), can help in effectively characterizing and picking up potential changes in glycan profile of therapeutics [90].

Over the past decade, at least four nonhuman carbohydrate structures that are able to induce an immune response in humans have been identified. They are galactose-α1,3-galactose (α-Gal epitope), N-glycolylneuraminic acid (Neu5Gc epitope), β1,2-xylose (core-xylose epitope), and α1,3-fucose (core-α1,3-fucose epitope) [4], of which the first two are well studied and are described here. The first observations of immune reactions against α-Gal and Neu5Gc were described in the context of xenotransplantation of pig organs in humans [91] and the targeting of vaccines to APCs in cancer immunotherapy. Autologous tumor cell membranes from solid tumors are processed to express α-Gal epitopes by incubation with neuraminidase, recombinant alpha1,3GT, and uridine diphosphate galactose [92].

Recently, the presence of α-Gal and/or Neu5Gc was demonstrated in several therapeutic mAbs [4], including cetuximab, a chimeric mouse-human IgG1 monoclonal antibody approved for use in colorectal cancer and squamous-cell carcinoma of the head and neck [4]. About 3% of patients develop severe hypersensitivity reactions within minutes after the first exposure to cetuximab, and a higher prevalence (up to 33%) may be seen in certain geographical regions. Most patients with hypersensitivity possess IgE antibodies against cetuximab before the start of therapy. These antibodies were found to be specific for the α-Gal epitope and related to IgE antibodies involved in anaphylactic reactions to red meat [4, 93]. All humans have IgA, IgM, and IgG antibodies against α-Gal, representing approximately 1% of circulating immunoglobulin. To our knowledge, the presence of IgA, IgM, and IgG antibodies against α-Gal did not correlate with accelerated clearance of cetuximab. Life-threatening hypersensitivity reaction with cetuximab was associated with preexisting IgE anti-α-Gal antibodies [94]. Qian et al. [95] demonstrated that the α-Gal epitopes are located in the Fab regions of the cetuximab antibody. The intravenous injection method and the presence of α-Gal on both Fab regions, which enables efficient cross-linking of IgE on mast cells, may explain the prompt immune reaction to cetuximab in a certain patient subset. The murine cell line SP2/0 used to produce cetuximab expresses the gene encoding for α1,3-galactosyltransferase, the enzyme responsible for the synthesis of the α-Gal epitope. Prevention of incorporation of the terminal α-Gal motif in therapeutic mAbs during production could help combat the problem of immunogenicity to an extent. Measures could include knocking out the gene for α1,3-galactosyltransferase in murine cells or using another expression system such as the CHO cells which may not produce the α-Gal epitope glycoform [96]. Other biotherapeutics like infliximab also have α-Gal epitopes located on Fc linked glycans but these were not found to be recognized by IgE anti-α-Gal antibodies. The relative low abundance of α-Gal epitopes and their location within the Fc region might be possible reasons for this lack of recognition. So far, IgE anti-α-Gal antibodies seem to have a significant importance to patients treated with cetuximab [94].

Humans synthesize the sialic acid N-acetylneuraminic acid (Neu5Ac) but are not able to synthesize Neu5Gc [4]. Consumption of Neu5Gc-rich foods, for example, red meat and milk products, allows for the accumulation of Neu5Gc on the surface of epithelial and endothelial cells [93]. As a result, the human immune system recognizes Neu5Gc as foreign and shows high levels of IgA, IgM, and IgG antibodies against Neu5Gc (0.1%–0.2% of circulating immunoglobulin) [93]. Injecting products that contain Neu5Gc in individuals with

preexisting antibodies can cause the formation of immune complexes that potentially activate complement or affect half-life of the drug. Ghaderi et al. [97] showed that the clearance of cetuximab increases significantly in mice when anti-Neu5Gc antibodies are preinjected. Maeda et al. [98] detected the presence of the Neu5Gc epitope in three commercial mAb pharmaceuticals produced in murine cell lines (cetuximab, gemtuzumab, and infliximab), whereas it was absent in other mAbs produced in CHO cell lines (tocilizumab, bevacizumab, and adalimumab). CHO cells are reported to be negative for α-Gal and Neu5Gc epitopes. However, these cells are capable of taking up these glycoforms from the cell culture media and metabolically incorporating them into the expressed protein [4]. Therefore, in addition to using these cell lines, media and other components should be void of components such as Neu5Gc [97].

Analysis of biotherapeutic mAbs purified from serum of subjects demonstrates that the PTM profile of the protein changes *in vivo*. Examples include deamidation at Asn-33 and oxidation at Trp-105 in the light chain and heavy chains, respectively, of two therapeutic mAbs [99]. Furthermore, a recent study shows that different levels of mannosylation of mAbs can have significant impact on pharmacokinetic parameters, including clearance and area under the curve (AUC) [100]; however, the increase in mannose did not impact immunogenicity rates [101]. Mannose receptors, expressed at high levels on DCs, mediate the capture, processing, and presenting of antigens (mannose-expressing glycoproteins) for an immune response. This response, depending on several factors, could either be immunogenic or tolerogenic [100, 102].

Glycans may also indirectly impact the immunogenicity of biotherapeutics through changes in the folding, solubility, or stability of the proteins. For example, recombinant human IFN β produced in *E. coli* is not glycosylated and is prone to aggregation leading to increased immunogenicity, as compared to the recombinant IFN β from CHO cells, where glycosylation reduces immunogenicity [4, 103].

4.3. Chemical Composition. Compared to glycosylation, other PTMs are less well understood [104, 105]. A biopharmaceutical may be chemically modified through accidental degradation in one of the many bioprocessing steps: fermentation, virus inactivation, purification, polishing, formulation, filtration, filling, storage, transport, and administration. Chemical modifications during bioprocessing may include deamidation, oxidation, isomerization, hydrolysis, glycation, and C/N terminal heterogeneity of the protein [106]. The susceptibility of an individual amino acid residue to chemical modification is dependent on neighboring residues; tertiary structure of the protein; and solution conditions such as temperature, pH, and ionic strength. Chemical modification may give rise to a less favorable charge, thus leading to structural changes or even the formation of new covalent crosslinks [107]. Covalent crosslinking could enhance immunogenicity by causing aggregation [108–110]. Multiple studies have indicated a strong correlation between aggregates and immunogenicity [89, 111–113]. Deamidation, isomerization, and oxidation have also been associated with potential immunogenicity [4].

Deamidation of proteins accelerates at high temperature and high pH and can occur during bioprocessing and storage. Deamidation of Asn and Gln contributes to charge heterogeneity of therapeutic proteins, determines the irreversible thermal denaturation of proteins at acidic and neutral pH, regulates the rate of protein breakdown, and could shorten *in vivo* half-life. Deamidation followed by isomerization of asparagine to isoaspartate (isoAsp) has been shown to alter protein structure, thereby potentially making the protein immunogenic [114]. Deamidation can be accompanied by some degree of oxidation, conformational changes, and fragmentation and aggregation, again posing a serious risk of enhanced immunogenicity [4].

Oxidative chemical modification of amino acid residues alters secondary and tertiary protein structures. This favors interaction between protein surfaces and subsequently leads to noncovalent aggregation [115]. Studies using metal-catalyzed oxidation (MCO) have shown that therapeutic proteins can aggregate and can also be immunogenic [4, 115]. Chemical stresses during manufacturing and storage can be caused by exposure to light or elevated temperatures and by the presence of oxygen, metal ions, or peroxide impurities from excipients. Trace amounts of iron, chromium, and nickel were found to leach into the formulation buffer via contact with the stainless steel surfaces typically used during bioprocessing [116]. Tungsten oxide-mediated oxidation caused precipitation of monoclonal antibodies and was pH-dependent [117]. Similarly in EPO, aggregation due to tungsten leachates from the container was associated with immunogenicity [54].

Despite limited information on the association of actual chemical modifications during biopharmaceutical manufacturing and immunogenicity, it is always prudent to be prepared for an untoward possibility. Preventative measures should include careful evaluation of buffers, surface materials, and conditions during manufacturing, transport, and storage. Extensive characterization of molecules using techniques like size exclusion chromatography, supported by orthogonal techniques like analytical ultracentrifugation (identifying aggregation) [118], circular dichroism (CD), and intrinsic fluorescence spectroscopy, can indicate deviations from secondary and tertiary structures. These steps incorporated into the process development will help in mitigating risks of immunogenicity.

5. Managing Immunogenicity

5.1. Managing Immunogenicity in a Preclinical Setting. The 2011 ICH S6 Guideline (preclinical safety evaluation of biotechnology-derived pharmaceuticals) describes the need for detection and characterization of antibodies in repeat-dose studies using animal models. However, relevant species must be used for *in vivo* studies, that is, one in which the target epitope is expressed. Immune responses are species-specific; therefore, induction is not entirely predictive of antibody formation in humans [119, 120]. Animal models are constrained by lack of genetic diversity which is a primary factor for

diverse immune response frequently observed in human beings [121]. Rodent models for immunogenicity testing are, therefore, less useful than animals that show a higher degree of homology with humans and more genetic diversity than inbred mouse strains, such as nonhuman primates; however, these are not widely used due to ethical constraints. Conventional nontransgenic animal models can be useful for highly conserved proteins, but a lack of immune tolerance to human proteins limits their use for immunogenicity testing. These animal models can be useful for comparing the immunogenicity of two similar products, that is, the immunogenicity of an originator and biosimilar product; this may not reflect the human situation but may provide a warning against advancement of a biosimilar if the immunogenicity profile observed differs from that of the originator.

Despite the limitations associated with the use of animals to predict immunogenicity, several transgenic animal models have been generated for this purpose. Transgenic mice are often the preferred *in vivo* model to predict immunogenicity as they are tolerant to the administered human protein [122, 123] and can be used to study the immunogenicity of biotherapeutic aggregates. In a study by van Beers et al., the IFNb-1a aggregate percentage and extent of denaturation were shown to influence the ability of aggregates to break tolerance in transgenic mice. In these experiments, immune tolerant mice were immunized with IFNb-1a formulations and antibody responses measured. Only noncovalently bound aggregates that retained some native epitopes were able to break tolerance resulting in a transient immune response; removal of aggregates prevented this breakdown of tolerance [123]. Additionally, mice expressing human MHC molecules can be used to compare antibody and T cell responses to vaccines and protein therapeutics [124]. High ADA titers were observed after injection of a metal catalyzed, oxidized, and aggregated IgG1 sample in nontransgenic and transgenic mice [4]. Therapeutic interferons oxidized and aggregated via the same metal-catalysis method were able to overcome the immune tolerance of transgenic mice that were immune tolerant for the administered human proteins [125, 126]. The transgenic mice also developed antibodies against oxidized and aggregated rhIFNβ-1a treated with H_2O_2 [126], but not against oxidized rhIFNα-2b treated with H_2O_2 [89], probably due to the absence of aggregation. Use of animal models in immunogenicity testing is discussed more extensively in the review by Brinks et al. [121].

In vitro techniques can also be used to assess the immunogenic potential of therapeutic proteins. These could be used to predict the risk of immunogenicity in preclinical setting. The expression of APC-surface molecules differs following activation; for example, the expression of MHC (class I and II), costimulatory molecules, and cytokine receptors is enhanced. Flow cytometry is an *in vitro* technique that can be used to determine differences in cell surface molecule expression, indicative of APC maturation that may initiate T cell responses [127, 128]. T cell proliferation assays are also useful tools to study the activation and proliferation of T cells in the presence of antigen [129]. Additionally, the release of immunomodulatory cytokines can be characterized by enzyme-linked immunosorbent assay. This approach can be used to assess the quality of an induced immune response, as specific cytokines can be markers of Th1 (IL-12 and IFNγ) or Th2 immunity (IL-4 and IL-10). T cells that respond to a particular epitope *in vitro* can be labeled with MHC class II oligomers and sorted by flow cytometry; the phenotype of responsive T cells can then be determined using intracellular cytokine staining [124, 130]. Human peripheral blood mononucleated cells, when stimulated with aggregated monoclonal antibody, induce an adaptive T cell response characterized by CD4 T cell proliferation and release of cytokines like interleukin- (IL-) 1β, IL-6 and TNFα. These cytokines can be used as potential biomarkers for aggregate immunogenicity [129]. It should be noted that these *in vitro* techniques may indicate the probability of an immune response for a biotherapeutic but cannot predict its clinical consequences. Correlative studies with marketed biotherapeutics in these assays may refine these methods further, to enable prediction of relevant immunogenicity [4, 127].

In addition to the assays described above, *in silico* techniques have been developed for the prediction of antigenicity by identification of potential T cell epitopes [131]. *In silico* methods have been shown to successfully identify MHC class II-restricted epitopes within biotherapeutics [132]. Knowledge of aggregation-prone regions may also help in the design and selection of biotherapeutic candidates and reduce aggregation concerns [133]. For example, aggregation motifs that lack charge have been found in the light chain regions of mAbs, including Erbitux and Raptiva. This computational approach could, therefore, be useful to screen biotherapeutic candidates early in drug development [128].

Overall, preclinical methods have been focused on identifying potential immunogenicity associated with formation of aggregates often considered the "bête noire" for immunogenicity [134]. However, the challenge remains in identifying potential immunogenicity with low levels of aggregation induced naturally by PTMs (as described previously) especially in contexts of process change, shipping, and clinical use. The preclinical techniques to predict immunogenic potential described here are still exploratory. Developing more robust methods to predict possible immunogenicity attributable to PTMs should be the way forward to reduce clinical risk.

5.2. Managing Immunogenicity in the Clinic. Prior to treatment, patients should be screened for established biomarkers to check for potential immunogenicity. A retrospective analysis of cetuximab evaluated whether the presence of pretreatment IgE antibodies against cetuximab is associated with severe infusion reactions (SIRs) during the initial cetuximab infusion. This analysis used 545 banked serum or plasma samples from cancer patients participating in clinical trials. Patients with a positive test indicating the presence of pretreatment antibodies had a higher risk of experiencing an SIR. Although this test had low positive predictive value, it clearly indicated an association between the presences of preexisting IgE antibodies against cetuximab with SIRs, supporting prior association studies [135].

Infantile Pompe disease resulting from a deficiency of lysosomal acid α-glucosidase (GAA) requires enzyme replacement therapy (ERT) with recombinant human GAA

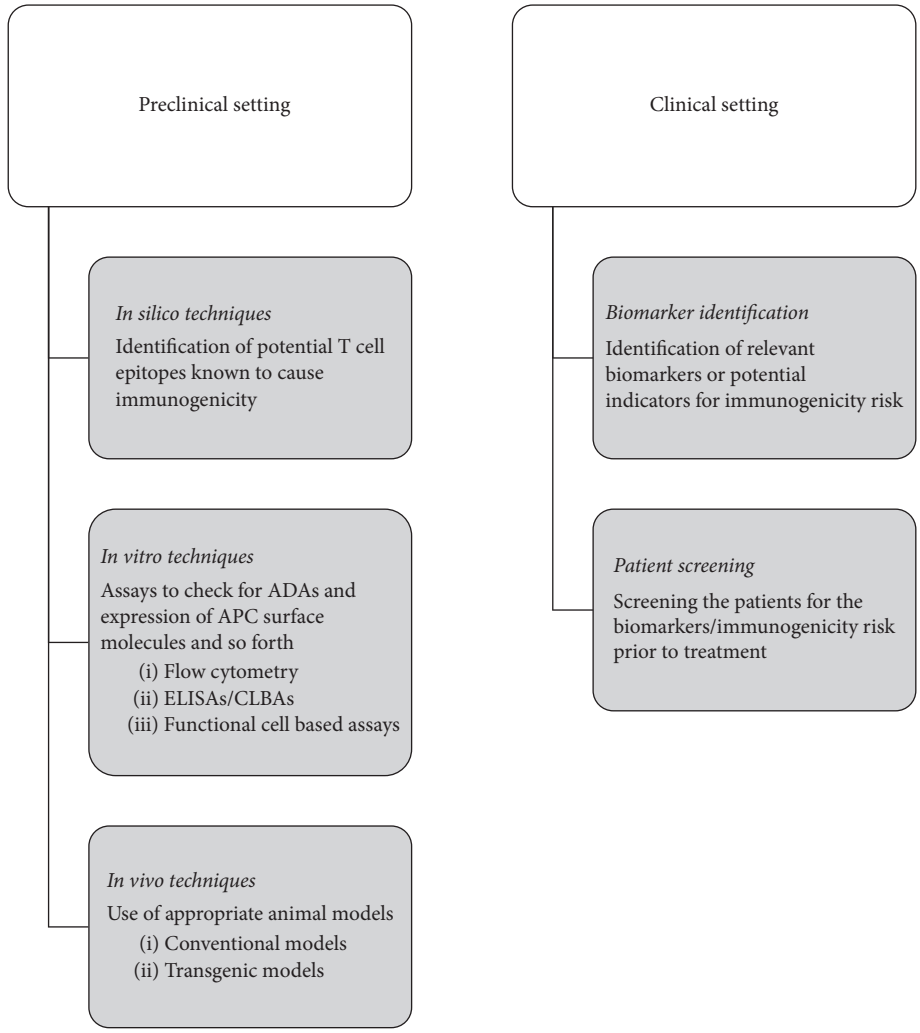

Figure 1: Management of immunogenicity in preclinical and clinical settings. ADAs: anti-drug antibodies; APC: antigen presenting cell; ELISA: enzyme-linked immunosorbent assay; CLBA: competitive ligand binding assay.

(rhGAA); immunogenicity can be managed with a combination of rituximab with methotrexate ± intravenous gamma globulins (IVIG). This is an option for tolerance induction of CRIM negative Pompe to ERT when instituted in the naïve setting or following antibody development [136].

With adalimumab, dosing over the NAb response is probably effective in recapturing symptomatic response. In patients with Crohn's disease, adalimumab dose escalation is effective for recapturing symptomatic response after secondary loss of response, but more than half of the patients eventually experience a tertiary loss of response [137]. An additional risk with dosing over the prescribed dose could involve adverse events such as serum sickness and hypersensitivity reactions [138]. Another strategy commonly adopted with anti-TNF therapeutics is to switch the biologic when a patient becomes refractive to a particular anti-TNF. In some cases, suppressing the immune response (formation of ADAs) with mild doses of methotrexate was seen to be beneficial [139]. Figure 1 gives a schematic representation of managing immunogenicity.

5.3. Managing Immunogenicity against Biosimilars. In recent years, follow-on biologics (or biosimilars) and generic protein therapeutics have become more prevalent as the patents associated with the original drugs expire. The first biosimilar reached the market almost a decade ago [140]; and biosimilar use has been steadily rising. Managing immunogenicity arising due to biosimilars is another challenge.

For small molecules approved in the EU, the generic paradigm applies; a product is pharmaceutically equivalent to a competitor molecule when it has the same qualitative and quantitative composition. If the products are shown through pharmacokinetic studies to have the same bioavailability, they are deemed bioequivalent. Generally, this is demonstrated in a limited number of studies in healthy volunteers [141]. Once products are deemed bioequivalent, they are assumed to be therapeutically equivalent and essentially similar in terms of benefits and risks *in vivo*.

However, such paradigm is not applicable for biopharmaceuticals. Biopharmaceuticals are large and intricate molecules and frequently subjected to extensive PTMs that

are sensitive to differences in manufacturing conditions [142]. Pharmaceutical equivalence for biopharmaceutical products cannot be directly demonstrated. Therefore, the biosimilar pathway was established. In this pathway, "biosimilarity" to an approved reference product must be demonstrated through an extensive comparability exercise. This exercise includes physicochemical studies, appropriate nonclinical studies, limited pharmacokinetic and pharmacodynamics studies, and comparative clinical studies to establish efficacy and safety (European Medicines agency, London 2006). The United States (US) Food and Drug Administration (FDA) has proposed a stepwise approach for providing totality of evidence of similarity between a proposed biosimilar product and a US-licensed (reference) product. This stepwise approach starts with the assessment of critical quality attributes that are relevant to clinical outcomes in structural and functional characterization in manufacturing process of the proposed biosimilar product. The FDA suggests that these critical quality attributes be identified first and then classified into three tiers depending upon their criticality: most (Tier 1), mild to moderate (Tier 2), and least (Tier 3) relevant to clinical outcomes [143]. However, even after demonstrating comparability, the products might not be similar in terms of risk of immunogenicity. Therefore, a detailed immunogenicity assessment is still warranted.

6. Conclusion

Recent years have seen an expansion in the development and manufacturing of protein therapeutic drugs, both in terms of number of molecules and in terms of global production capacity. In this review, we discussed the causes of immunogenicity which could be product-related (inherent property of the product or might be picked up during the manufacturing process), patient-related, or linked to the route of administration. We also discussed the impact of PTMs of therapeutic proteins on immunogenicity; and it is clear that some PTMs lead to increased immunogenicity. Managing immunogenicity in both preclinical and clinical settings is very important. With the advent of novel analytical technologies, there has been a dramatic enhancement of the capability to analyze and characterize therapeutics. Also, analysis of these proteins *in vivo* is critical to understand biological effects of PTMs. Relevant human immune system-specific animal models are now being established to study these biological effects. Future studies should focus on the development of sensitive diagnostics that can predict immunogenicity-mediated adverse events in small fraction of subjects that develop clinically relevant ADAs and hence mitigate the risk due to unwarranted immunogenicity.

Competing Interests

Anshu Kuriakose, Narendra Chirmule, and Pradip Nair are full time employees of Biocon Ltd.

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References

[1] O. N. Jensen, "Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry," *Current Opinion in Chemical Biology*, vol. 8, no. 1, pp. 33–41, 2004.

[2] A. N. Burska, L. Hunt, M. Boissinot et al., "Autoantibodies to posttranslational modifications in rheumatoid arthritis," *Mediators of Inflammation*, vol. 2014, Article ID 492873, 19 pages, 2014.

[3] N. Jenkins, "Modifications of therapeutic proteins: challenges and prospects," *Cytotechnology*, vol. 53, no. 1–3, pp. 121–125, 2007.

[4] M. M. C. van Beers and M. Bardor, "Minimizing immunogenicity of biopharmaceuticals by controlling critical quality attributes of proteins," *Biotechnology Journal*, vol. 7, no. 12, pp. 1473–1484, 2012.

[5] N. Casadevall, "Antibodies against rHuEPO: native and recombinant," *Nephrology Dialysis Transplantation*, vol. 17, supplement 5, pp. 42–47, 2002.

[6] I. Mukovozov, T. Sabljic, G. Hortelano, and F. A. Ofosu, "Factors that contribute to the immmunogenicity of therapeutic recombinant human proteins," *Thrombosis and Haemostasis*, vol. 99, no. 5, pp. 874–882, 2008.

[7] M. K. Ghazavi and G. A. Johnston, "Insulin allergy," *Clinics in Dermatology*, vol. 29, no. 3, pp. 300–305, 2011.

[8] I. C. MacDougall, S. D. Roger, A. De Francisco et al., "Antibody-mediated pure red cell aplasia in chronic kidney disease patients receiving erythropoiesis-stimulating agents: new insights," *Kidney International*, vol. 81, no. 8, pp. 727–732, 2012.

[9] P. Jolicoeur and R. L. Tacey, "Development and validation of cell-based assays for the detection of neutralizing antibodies to drug products: a practical approach," *Bioanalysis*, vol. 4, no. 24, pp. 2959–2970, 2012.

[10] M. G. Jacquemin and J.-M. R. Saint-Remy, "Factor VIII immunogenicity," *Haemophilia*, vol. 4, no. 4, pp. 552–557, 1998.

[11] U. Rosenschein, R. Lenz, J. Radnay, T. Ben Tovim, and L. A. Rozenszajn, "Streptokinase immunogenicity in thrombolytic therapy for acute myocardial infarction," *Israel Journal of Medical Sciences*, vol. 27, no. 10, pp. 541–545, 1991.

[12] J. Li, C. Yang, Y. Xia et al., "Thrombocytopenia caused by the development of antibodies to thrombopoietin," *Blood*, vol. 98, no. 12, pp. 3241–3248, 2001.

[13] G. Shankar, C. Pendley, and K. E. Stein, "A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs," *Nature Biotechnology*, vol. 25, no. 5, pp. 555–561, 2007.

[14] L.-C. Lim, "Acquired red cell aplasia in association with the use of recombinant erythropoietin in chronic renal failure," *Hematology*, vol. 10, no. 3, pp. 255–259, 2005.

[15] N. Casadevall, J. Nataf, B. Viron et al., "Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin," *The New England Journal of Medicine*, vol. 346, no. 7, pp. 469–475, 2002.

[16] P. Ragnhammar and M. Wadhwa, "Neutralising antibodies to granulocyte-macrophage colony stimulating factor (GM-CSF) in carcinoma patients following GM-CSF combination therapy," *Medical Oncology*, vol. 13, no. 3, pp. 161–166, 1996.

[17] Y. C. Q. Zang, D. Yang, J. Hong, M. V. Tejada-Simon, V. M. Rivera, and J. Z. Zhang, "Immunoregulation and blocking antibodies induced by interferon beta treatment in MS," *Neurology*, vol. 55, no. 3, pp. 397–404, 2000.

[18] V. R. Dharnidharka, C. Takemoto, B. M. Ewenstein, S. Rosen, and H. W. Harris, "Membranous glomerulonephritis and nephrosis post factor IX infusions in hemophilia B," *Pediatric Nephrology*, vol. 12, no. 8, pp. 654–657, 1998.

[19] H.-P. Hartung, F. Munschauer III, and H. Schellekens, "Significance of neutralizing antibodies to interferon beta during treatment of multiple sclerosis: expert opinions based on the Proceedings of an International Consensus Conference," *European Journal of Neurology*, vol. 12, no. 8, pp. 588–601, 2005.

[20] E. Koren, L. A. Zuckerman, and A. R. Mire-Sluis, "Immune responses to therapeutic proteins in humans—clinical significance, assessment and prediction," *Current Pharmaceutical Biotechnology*, vol. 3, no. 4, pp. 349–360, 2002.

[21] M. G. Tovey and C. Lallemand, "Immunogenicity and other problems associated with the use of biopharmaceuticals," *Therapeutic Advances in Drug Safety*, vol. 2, no. 3, pp. 113–128, 2011.

[22] G. Schernthaner, "Immunogenicity and allergenic potential of animal and human insulins," *Diabetes Care*, vol. 16, supplement 3, pp. 155–165, 1993.

[23] S. E. Fineberg, T. T. Kawabata, D. Finco-Kent, R. J. Fountaine, G. L. Finch, and A. S. Krasner, "Immunological responses to exogenous insulin," *Endocrine Reviews*, vol. 28, no. 6, pp. 625–652, 2007.

[24] F. A. Harding, M. M. Stickler, J. Razo, and R. B. DuBridge, "The immunogenicity of humanized and fully human antibodies: residual immunogenicity resides in the CDR regions," *mAbs*, vol. 2, no. 3, pp. 256–265, 2010.

[25] N. K. Bender, C. E. Heilig, B. Dröll, J. Wohlgemuth, F.-P. Armbruster, and B. Heilig, "Immunogenicity, efficacy and adverse events of adalimumab in RA patients," *Rheumatology International*, vol. 27, no. 3, pp. 269–274, 2007.

[26] T. R. D. J. Radstake, M. Svenson, A. M. Eijsbouts et al., "Formation of antibodies against infliximab and adalimumab strongly correlates with functional drug levels and clinical responses in rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 68, no. 11, pp. 1739–1745, 2009.

[27] R. L. West, Z. Zelinkova, G. J. Wolbink, E. J. Kuipers, P. C. F. Stokkers, and C. J. van der Woude, "Immunogenicity negatively influences the outcome of adalimumab treatment in Crohn's disease," *Alimentary Pharmacology and Therapeutics*, vol. 28, no. 9, pp. 1122–1126, 2008.

[28] A. J. Coles, "Alemtuzumab therapy for multiple sclerosis," *Neurotherapeutics*, vol. 10, no. 1, pp. 29–33, 2013.

[29] R. Furie, W. Stohl, E. M. Ginzler et al., "Biologic activity and safety of belimumab, a neutralizing anti-B-lymphocyte stimulator (BLyS) monoclonal antibody: a phase I trial in patients with systemic lupus erythematosus," *Arthritis Research and Therapy*, vol. 10, no. 5, article R109, 2008.

[30] E. Dhimolea, "Canakinumab," *mAbs*, vol. 2, no. 1, pp. 3–13, 2010.

[31] D.-Y. Chen, Y.-M. Chen, W.-T. Hung et al., "Immunogenicity, drug trough levels and therapeutic response in patients with rheumatoid arthritis or ankylosing spondylitis after 24-week golimumab treatment," *Annals of the Rheumatic Diseases*, vol. 74, no. 12, pp. 2261–2264, 2015.

[32] A. Chovel-Sella, R. Karplus, T. Sella, and H. Amital, "Clinical efficacy and adverse effects of golimumab in the treatment of rheumatoid arthritis," *Israel Medical Association Journal*, vol. 14, no. 6, pp. 390–394, 2012.

[33] I. Zidi, A. Bouaziz, W. Mnif, A. Bartegi, F. A. Al-Hizab, and N. B. Amor, "Golimumab therapy of rheumatoid arthritis: an overview," *Scandinavian Journal of Immunology*, vol. 72, no. 2, pp. 75–85, 2010.

[34] N. A. Rizvi, J. Mazières, D. Planchard et al., "Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): a phase 2, single-arm trial," *The Lancet Oncology*, vol. 16, no. 3, pp. 257–265, 2015.

[35] P. C. Taylor, E. Quattrocchi, S. Mallett, R. Kurrasch, J. Petersen, and D. J. Chang, "Ofatumumab, a fully human anti-CD20 monoclonal antibody, in biological-naive, rheumatoid arthritis patients with an inadequate response to methotrexate: a randomised, double-blind, placebo-controlled clinical trial," *Annals of the Rheumatic Diseases*, vol. 70, no. 12, pp. 2119–2125, 2011.

[36] A. Jakobovits, R. G. Amado, X. Yang, L. Roskos, and G. Schwab, "From XenoMouse technology to panitumumab, the first fully human antibody product from transgenic mice," *Nature Biotechnology*, vol. 25, no. 10, pp. 1134–1143, 2007.

[37] J. A. Lofgren, S. Dhandapani, J. J. Pennucci et al., "Comparing ELISA and surface plasmon resonance for assessing clinical immunogenicity of panitumumab," *The Journal of Immunology*, vol. 178, no. 11, pp. 7467–7472, 2007.

[38] D. Weeraratne, A. Chen, J. J. Pennucci et al., "Immunogenicity of panitumumab in combination chemotherapy clinical trials," *BMC Clinical Pharmacology*, vol. 11, article 17, 2011.

[39] L. Khoja, M. O. Butler, S. P. Kang, S. Ebbinghaus, and A. M. Joshua, "Pembrolizumab," *Journal for ImmunoTherapy of Cancer*, vol. 3, article 36, 2015.

[40] C. C. Mok, "Rituximab for the treatment of rheumatoid arthritis: an update," *Drug Design, Development and Therapy*, vol. 8, pp. 87–100, 2013.

[41] A. Karle, S. Spindeldreher, and F. Kolbinger, "Secukinumab, a novel anti–IL-17A antibody, shows low immunogenicity potential in human in vitro assays comparable to other marketed biotherapeutics with low clinical immunogenicity," *mAbs*, vol. 8, no. 3, pp. 536–550, 2016.

[42] H.-Y. Chiu, T. W. Chu, Y.-P. Cheng, and T.-F. Tsai, "The association between clinical response to ustekinumab and immunogenicity to ustekinumab and prior adalimumab," *PLoS ONE*, vol. 10, no. 11, Article ID e0142930, 2015.

[43] M. S. Raben, "Treatment of a pituitary dwarf with human growth hormone," *The Journal of Clinical Endocrinology and Metabolism*, vol. 18, no. 8, pp. 901–903, 1958.

[44] W. V. Moore and P. Leppert, "Role of aggregated human growth hormone (hGH) in development of antibodies to hGH," *Journal of Clinical Endocrinology and Metabolism*, vol. 51, no. 9, pp. 691–697, 1980.

[45] E. Van Cutsem, S. Siena, Y. Humblet et al., "An open-label, single-arm study assessing safety and efficacy of panitumumab in patients with metastatic colorectal cancer refractory to standard chemotherapy," *Annals of Oncology*, vol. 19, no. 1, pp. 92–98, 2008.

[46] P. Gogolák, B. Réthi, G. Hajas, and É. Rajnavölgyi, "Targeting dendritic cells for priming cellular immune responses," *Journal of Molecular Recognition*, vol. 16, no. 5, pp. 299–317, 2003.

[47] J. N. Arnold, M. R. Wormald, R. B. Sim, P. M. Rudd, and R. A. Dwek, "The impact of glycosylation on the biological function and structure of human immunoglobulins," *Annual Review of Immunology*, vol. 25, pp. 21–50, 2007.

[48] D. M. Sheeley, B. M. Merrill, and L. C. E. Taylor, "Characterization of monoclonal antibody glycosylation: comparison of expression systems and identification of terminal α-linked galactose," *Analytical Biochemistry*, vol. 247, no. 1, pp. 102–110, 1997.

[49] C. H. Chung, B. Mirakhur, E. Chan et al., "Cetuximab-induced anaphylaxis and IgE specific for galactose-α-1,3- galactose," *The New England Journal of Medicine*, vol. 358, no. 11, pp. 1109–1117, 2008.

[50] P. Eggleton, R. Haigh, and P. G. Winyard, "Consequence of neo-antigenicity of the 'altered self'," *Rheumatology*, vol. 47, no. 5, pp. 567–571, 2008.

[51] J. C. Egrie and J. K. Browne, "Development and characterization of novel erythropoiesis stimulating protein (NESP)," *British Journal of Cancer*, vol. 84, supplement 1, pp. 3–10, 2001.

[52] D. T. Mytych, T. E. Barger, C. King et al., "Development and characterization of a human antibody reference panel against erythropoietin suitable for the standardization of ESA immunogenicity testing," *Journal of Immunological Methods*, vol. 382, no. 1-2, pp. 129–141, 2012.

[53] J. Rossert, N. Casadevall, and K.-U. Eckardt, "Anti-erythropoietin antibodies and pure red cell aplasia," *Journal of the American Society of Nephrology*, vol. 15, no. 2, pp. 398–406, 2004.

[54] A. Seidl, O. Hainzl, M. Richter et al., "Tungsten-induced denaturation and aggregation of epoetin alfa during primary packaging as a cause of immunogenicity," *Pharmaceutical Research*, vol. 29, no. 6, pp. 1454–1467, 2012.

[55] D. K. Weeraratne, A. J. Kuck, N. Chirmule, and D. T. Mytych, "Measurement of anti-erythropoiesis-stimulating agent IgG4 antibody as an indicator of antibody-mediated pure red cell aplasia," *Clinical and Vaccine Immunology*, vol. 20, no. 1, pp. 46–51, 2013.

[56] G. Antonelli and F. Dianzani, "Development of antibodies to interferon beta in patients: technical and biological aspects," *European Cytokine Network*, vol. 10, no. 3, pp. 413–422, 1999.

[57] C. L. Wagner, A. Schantz, E. Barnathan et al., "Consequences of immunogenicity to the therapeutic monoclonal antibodies ReoPro and Remicade," *Developments in biologicals*, vol. 112, pp. 37–53, 2003.

[58] V. Jawa, L. P. Cousens, M. Awwad, E. Wakshull, H. Kropshofer, and A. S. De Groot, "T-cell dependent immunogenicity of protein therapeutics: preclinical assessment and mitigation," *Clinical Immunology*, vol. 149, pp. 534–555, 2013.

[59] M. G. Jacquemin, B. G. Desqueper, A. Benhida et al., "Mechanism and kinetics of factor VIII inactivation: study with an IgG4 monoclonal antibody derived from a hemophilia A patient with inhibitor," *Blood*, vol. 92, no. 2, pp. 496–506, 1998.

[60] J. Oldenburg, J. Schröder, C. Schmitt, H. H. Brackmann, and R. Schwaab, "Small deletion/insertion mutations within poly-A runs of the factor VIII gene mitigate the severe haemophilia A phenotype," *Thrombosis and Haemostasis*, vol. 79, no. 2, pp. 452–453, 1998.

[61] M. Franchini and P. M. Mannucci, "Inhibitors of propagation of coagulation (factors VIII, IX and XI): a review of current therapeutic practice," *British Journal of Clinical Pharmacology*, vol. 72, no. 4, pp. 553–562, 2011.

[62] R. Schwaab, H.-H. Brackmann, C. Meyer et al., "Haemophilia A: mutation type determines risk of inhibitor formation," *Thrombosis and Haemostasis*, vol. 74, no. 6, pp. 1402–1406, 1995.

[63] G. Kemball-Cook, E. G. D. Tuddenham, and A. I. Wacey, "The factor VIII structure and mutation resource site: HAMSTeRS version 4," *Nucleic Acids Research*, vol. 26, no. 1, pp. 216–219, 1998.

[64] J. Oldenburg, O. El-Maarri, and R. Schwaab, "Inhibitor development in correlation to factor VIII genotypes," *Haemophilia*, vol. 8, supplement 2, pp. 23–29, 2002.

[65] G. S. Pandey, C. Yanover, L. M. Miller-Jenkins et al., "Endogenous factor VIII synthesis from the intron 22-inverted F8 locus may modulate the immunogenicity of replacement therapy for hemophilia A," *Nature Medicine*, vol. 19, no. 10, pp. 1318–1324, 2013.

[66] M. Young, H. Inaba, L. W. Hoyer, M. Higuchi, H. H. Kazazian Jr., and S. E. Antonarakis, "Partial correction of a severe molecular defect in hemophilia A, because of errors during expression of the factor VIII gene," *American Journal of Human Genetics*, vol. 60, no. 3, pp. 565–573, 1997.

[67] J. Oldenburg, J. K. Picard, R. Schwaab, H. H. Brackmann, E. G. D. Tuddenham, and E. Simpson, "HLA genotype of patients with severe haemophilia a due to intron 22 inversion with and without inhibitors of factor VIII," *Thrombosis and Haemostasis*, vol. 77, no. 2, pp. 238–242, 1997.

[68] I. Scharrer, G. L. Bray, and O. Neutzling, "Incidence of inhibitors in haemophilia A patients—a review of recent studies of recombinant and plasma-derived factor VIII concentrates," *Haemophilia*, vol. 5, no. 3, pp. 145–154, 1999.

[69] J. Astermark, "Basic aspects of inhibitors to factors VIII and IX and the influence of non-genetic risk factors," *Haemophilia*, vol. 12, supplement 6, pp. 8–14, 2006.

[70] J. Astermark, E. Berntorp, G. C. White et al., "The Malmö International Brother Study (MIBS): further support for genetic predisposition to inhibitor development," *Haemophilia*, vol. 7, no. 3, pp. 267–272, 2001.

[71] R. Singh, S. Singh, and J. W. Lillard, "Past, present, and future technologies for oral delivery of therapeutic proteins," *Journal of Pharmaceutical Sciences*, vol. 97, no. 7, pp. 2497–2523, 2008.

[72] S. K. Singh, "Impact of product-related factors on immunogenicity of biotherapeutics," *Journal of Pharmaceutical Sciences*, vol. 100, no. 2, pp. 354–387, 2011.

[73] A. Peng, P. Gaitonde, M. P. Kosloski, R. D. Miclea, P. Varma, and S. V. Balu-Iyer, "Effect of route of administration of human recombinant factor VIII on its immunogenicity in hemophilia A mice," *Journal of Pharmaceutical Sciences*, vol. 98, no. 12, pp. 4480–4484, 2009.

[74] P. Perini, A. Facchinetti, P. Bulian et al., "Interferon-β (INF-β) antibodies in interferon-β1a- and interferon-β1b-treated multiple sclerosis patients. Prevalence, kinetics, cross-reactivity, and factors enhancing interferon-beta immunogenicity in vivo," *European Cytokine Network*, vol. 12, no. 1, pp. 56–61, 2001.

[75] S. E. Fineberg, T. Kawabata, D. Finco-Kent, C. Liu, and A. Krasner, "Antibody response to inhaled insulin in patients with type 1 or type 2 diabetes. An analysis of initial phase II and III inhaled insulin (Exubera) trials and a two-year extension trial," *Journal of Clinical Endocrinology and Metabolism*, vol. 90, no. 6, pp. 3287–3294, 2005.

[76] K. Hermansen, T. Rönnemaa, A. H. Petersen, S. Bellaire, and U. Adamson, "Intensive Therapy with Inhaled Insulin via the

AERx Insulin Diabetes Management System: a 12-week proof-of-concept trial in patients with type 2 diabetes," *Diabetes Care*, vol. 27, no. 1, pp. 162–167, 2004.

[77] H. Schellekens, "Factors influencing the immunogenicity of therapeutic proteins," *Nephrology Dialysis Transplantation*, vol. 20, supplement 6, pp. vi3–vi9, 2005.

[78] E.-M. Jahn and C. K. Schneider, "How to systematically evaluate immunogenicity of therapeutic proteins—regulatory considerations," *New Biotechnology*, vol. 25, no. 5, pp. 280–286, 2009.

[79] A. S. Rosenberg and A. S. Worobec, "A risk-based approach to immunogenicity concerns of therapeutic protein products, part 2: considering host-specific and product-specific factors impacting immunogenicity," *BioPharm International*, vol. 17, no. 12, pp. 34–42, 2004.

[80] I. Hwang and S. Park, "Computational design of protein therapeutics," *Drug Discovery Today: Technologies*, vol. 5, no. 2-3, pp. e43–e48, 2009.

[81] M. P. Baker and T. D. Jones, "Identification and removal of immunogenicity in therapeutic proteins," *Current Opinion in Drug Discovery and Development*, vol. 10, no. 2, pp. 219–227, 2007.

[82] Q. Vos, A. Lees, Z.-Q. Wu, C. M. Snapper, and J. J. Mond, "B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms," *Immunological Reviews*, vol. 176, pp. 154–170, 2000.

[83] X. Zhong and J. F. Wright, "Biological insights into therapeutic protein modifications throughout trafficking and their biopharmaceutical applications," *International Journal of Cell Biology*, vol. 2013, Article ID 273086, 19 pages, 2013.

[84] R. Jefferis and M.-P. Lefranc, "Human immunoglobulin allotypes: possible implications for immunogenicity," *mAbs*, vol. 1, no. 4, pp. 332–338, 2009.

[85] J. Seo and K.-J. Lee, "Post-translational modifications and their biological functions: proteomic analysis and systematic approaches," *Journal of Biochemistry and Molecular Biology*, vol. 37, no. 1, pp. 35–44, 2004.

[86] R. J. Solá and K. Griebenow, "Glycosylation of therapeutic proteins: an effective strategy to optimize efficacy," *BioDrugs*, vol. 24, no. 1, pp. 9–21, 2010.

[87] G. Walsh, "Biopharmaceutical benchmarks 2010," *Nature Biotechnology*, vol. 28, no. 9, pp. 917–924, 2010.

[88] Y. Vugmeyster, X. Xu, F. P. Theil, L. A. Khawli, and M. W. Leach, "Pharmacokinetics and toxicology of therapeutic proteins: advances and challenges," *World Journal of Biological Chemistry*, vol. 3, no. 4, pp. 73–92, 2012.

[89] S. Hermeling, D. J. A. Crommelin, H. Schellekens, and W. Jiskoot, "Structure-immunogenicity relationships of therapeutic proteins," *Pharmaceutical Research*, vol. 21, no. 6, pp. 897–903, 2004.

[90] C. Sheridan, "Commercial interest grows in glycan analysis," *Nature Biotechnology*, vol. 25, no. 2, pp. 145–146, 2007.

[91] J.-Y. Park, M.-R. Park, D.-N. Kwon et al., "Alpha 1,3-galactosyltransferase deficiency in pigs increases sialyltransferase activities that potentially raise non-gal xenoantigenicity," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 560850, 8 pages, 2011.

[92] U. Galili, "The α-gal epitope and the anti-Gal antibody in xenotransplantation and in cancer immunotherapy," *Immunology and Cell Biology*, vol. 83, no. 6, pp. 674–686, 2005.

[93] V. Padler-Karavani and A. Varki, "Potential impact of the non-human sialic acid *N*-glycolylneuraminic acid on transplant rejection risk," *Xenotransplantation*, vol. 18, no. 1, pp. 1–5, 2011.

[94] K. A. van Schie, G.-J. Wolbink, and T. Rispens, "Cross-reactive and pre-existing antibodies to therapeutic antibodies—effects on treatment and immunogenicity," *mAbs*, vol. 7, no. 4, pp. 662–671, 2015.

[95] J. Qian, T. Liu, L. Yang, A. Daus, R. Crowley, and Q. Zhou, "Structural characterization of N-linked oligosaccharides on monoclonal antibody cetuximab by the combination of orthogonal matrix-assisted laser desorption/ionization hybrid quadrupole-quadrupole time-of-flight tandem mass spectrometry and sequential enzymatic digestion," *Analytical Biochemistry*, vol. 364, no. 1, pp. 8–18, 2007.

[96] X. Xu, H. Nagarajan, N. E. Lewis et al., "The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line," *Nature Biotechnology*, vol. 29, no. 8, pp. 735–741, 2011.

[97] D. Ghaderi, R. E. Taylor, V. Padler-Karavani, S. Diaz, and A. Varki, "Implications of the presence of *N*-glycolylneuraminic acid in recombinant therapeutic glycoproteins," *Nature Biotechnology*, vol. 28, no. 8, pp. 863–867, 2010.

[98] E. Maeda, S. Kita, M. Kinoshita, K. Urakami, T. Hayakawa, and K. Kakehi, "Analysis of nonhuman N-glycans as the minor constituents in recombinant monoclonal antibody pharmaceuticals," *Analytical Chemistry*, vol. 84, no. 5, pp. 2373–2379, 2012.

[99] A. M. Goetze, M. R. Schenauer, and G. C. Flynn, "Assessing monoclonal antibody product quality attribute criticality through clinical studies," *mAbs*, vol. 2, no. 5, pp. 500–507, 2010.

[100] A. M. Goetze, Y. D. Liu, Z. Zhang et al., "High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans," *Glycobiology*, vol. 21, no. 7, pp. 949–959, 2011.

[101] D. Reusch and M. L. Tejada, "Fc glycans of therapeutic antibodies as critical quality attributes," *Glycobiology*, vol. 25, no. 12, pp. 1325–1334, 2015.

[102] F. Sallusto, M. Cella, C. Danieli, and A. Lanzavecchia, "Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products," *Journal of Experimental Medicine*, vol. 182, no. 2, pp. 389–400, 1995.

[103] L. Runkel, W. Meier, R. B. Pepinsky et al., "Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-β (IFN-β)," *Pharmaceutical Research*, vol. 15, no. 4, pp. 641–649, 1998.

[104] A. J. Chirino and A. Mire-Sluis, "Characteristics biological products and assessing comparability following manufacturing changes," *Nature Biotechnology*, vol. 22, no. 11, pp. 1383–1391, 2004.

[105] R. J. Harris, "Heterogeneity of recombinant antibodies: linking structure to function," *Developments in Biologicals*, vol. 122, pp. 117–127, 2005.

[106] M. C. Manning, D. K. Chou, B. M. Murphy, R. W. Payne, and D. S. Katayama, "Stability of protein pharmaceuticals: an update," *Pharmaceutical Research*, vol. 27, no. 4, pp. 544–575, 2010.

[107] R. Torosantucci, O. Mozziconacci, V. Sharov, C. Schöneich, and W. Jiskoot, "Chemical modifications in aggregates of recombinant human insulin induced by metal-catalyzed oxidation: covalent cross-linking via michael addition to tyrosine oxidation products," *Pharmaceutical Research*, vol. 29, no. 8, pp. 2276–2293, 2012.

[108] W. Chen, N. J. Ede, D. C. Jackson, J. McCluskey, and A. W. Purcell, "CTL recognition of an altered peptide associated with asparagine bond rearrangement. Implications for immunity and vaccine design," *The Journal of Immunology*, vol. 157, no. 3, pp. 1000–1005, 1996.

[109] J. L. Cleland, M. F. Powell, and S. J. Shire, "The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation," *Critical Reviews in Therapeutic Drug Carrier Systems*, vol. 10, no. 4, pp. 307–377, 1993.

[110] H. A. Doyle, J. Zhou, M. J. Wolff et al., "Isoaspartyl post-translational modification triggers anti-tumor T and B lymphocyte immunity," *Journal of Biological Chemistry*, vol. 281, no. 43, pp. 32676–32683, 2006.

[111] A. S. Rosenberg, "Effects of protein aggregates: an immunologic perspective," *AAPS Journal*, vol. 8, no. 3, pp. E501–E507, 2006.

[112] H. Schellekens, "Biosimilar therapeutics—what do we need to consider?" *NDT Plus*, vol. 2, supplement 1, pp. i27–i36, 2009.

[113] M. Sauerborn, V. Brinks, W. Jiskoot, and H. Schellekens, "Immunological mechanism underlying the immune response to recombinant human protein therapeutics," *Trends in Pharmacological Sciences*, vol. 31, no. 2, pp. 53–59, 2010.

[114] S. Noguchi, "Structural changes induced by the deamidation and isomerization of asparagine revealed by the crystal structure of *Ustilago sphaerogena* ribonuclease U2B," *Biopolymers*, vol. 93, no. 11, pp. 1003–1010, 2010.

[115] R. Torosantucci, V. S. Sharov, M. van Beers, V. Brinks, C. Schöneich, and W. Jiskoot, "Identification of oxidation sites and covalent cross-links in metal catalyzed oxidized interferon Beta-1a: potential implications for protein aggregation and immunogenicity," *Molecular Pharmaceutics*, vol. 10, no. 6, pp. 2311–2322, 2013.

[116] A. Basu, K. Yang, M. Wang et al., "Structure-function engineering of interferon-β-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation," *Bioconjugate Chemistry*, vol. 17, no. 3, pp. 618–630, 2006.

[117] J. S. Bee, S. A. Nelson, E. Freund, J. F. Carpenter, and T. W. Randolph, "Precipitation of a monoclonal antibody by soluble tungsten," *Journal of Pharmaceutical Sciences*, vol. 98, no. 9, pp. 3290–3301, 2009.

[118] P. Hong, S. Koza, and E. S. P. Bouvier, "A review size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates," *Journal of Liquid Chromatography and Related Technologies*, vol. 35, no. 20, pp. 2923–2950, 2012.

[119] G. Shankar, E. Shores, C. Wagner, and A. Mire-Sluis, "Scientific and regulatory considerations on the immunogenicity of biologics," *Trends in Biotechnology*, vol. 24, no. 6, pp. 274–280, 2006.

[120] J. Wang, J. Lozier, G. Johnson et al., "Neutralizing antibodies to therapeutic enzymes: considerations for testing, prevention and treatment," *Nature Biotechnology*, vol. 26, no. 8, pp. 901–908, 2008.

[121] V. Brinks, W. Jiskoot, and H. Schellekens, "Immunogenicity of therapeutic proteins: the use of animal models," *Pharmaceutical Research*, vol. 28, no. 10, pp. 2379–2385, 2011.

[122] S. Hermeling, H. Schellekens, D. J. A. Crommelin, and W. Jiskoot, "Micelle-associated protein in epoetin formulations: a risk factor for immunogenicity?" *Pharmaceutical Research*, vol. 20, no. 12, pp. 1903–1907, 2003.

[123] M. M. C. van Beers, M. Sauerborn, F. Gilli, V. Brinks, H. Schellekens, and W. Jiskoot, "Aggregated recombinant human interferon beta induces antibodies but no memory in immune-tolerant transgenic mice," *Pharmaceutical Research*, vol. 27, no. 9, pp. 1812–1824, 2010.

[124] A. S. De Groot and W. Martin, "Reducing risk, improving outcomes: bioengineering less immunogenic protein therapeutics," *Clinical Immunology*, vol. 131, no. 2, pp. 189–201, 2009.

[125] S. Hermeling, L. Aranha, J. M. A. Damen et al., "Structural characterization and immunogenicity in wild-type and immune tolerant mice of degraded recombinant human interferon alpha2b," *Pharmaceutical Research*, vol. 22, no. 12, pp. 1997–2006, 2005.

[126] M. M. C. van Beers, M. Sauerborn, F. Gilli, V. Brinks, H. Schellekens, and W. Jiskoot, "Oxidized and aggregated recombinant human interferon beta is immunogenic in human interferon beta transgenic mice," *Pharmaceutical Research*, vol. 28, no. 10, pp. 2393–2402, 2011.

[127] P. Gaitonde and S. V. Balu-Iyer, "In vitro immunogenicity risk assessment of therapeutic proteins in preclinical setting," in *Drug Design and Discovery*, vol. 716 of *Methods in Molecular Biology*, pp. 267–280, Springer, Berlin, Germany, 2011.

[128] K. D. Ratanji, J. P. Derrick, R. J. Dearman, and I. Kimber, "Immunogenicity of therapeutic proteins: influence of aggregation," *Journal of Immunotoxicology*, vol. 11, no. 2, pp. 99–109, 2014.

[129] M. K. Joubert, M. Hokom, C. Eakin et al., "Highly aggregated antibody therapeutics can enhance the in vitro innate and late-stage T-cell immune responses," *The Journal of Biological Chemistry*, vol. 287, no. 30, pp. 25266–25279, 2012.

[130] T. W. Tobery, S. A. Dubey, K. Anderson et al., "A comparison of standard immunogenicity assays for monitoring HIV type 1 gag-specific T cell responses in Ad5 HIV type 1 gag vaccinated human subjects," *AIDS Research and Human Retroviruses*, vol. 22, no. 11, pp. 1081–1090, 2006.

[131] M. G. Tovey, J. Legrand, and C. Lallemand, "Overcoming immunogenicity associated with the use of biopharmaceuticals," *Expert Review of Clinical Pharmacology*, vol. 4, no. 5, pp. 623–631, 2011.

[132] E. Koren, A. S. De Groot, V. Jawa et al., "Clinical validation of the 'in silico' prediction of immunogenicity of a human recombinant therapeutic protein," *Clinical Immunology*, vol. 124, no. 1, pp. 26–32, 2007.

[133] X. Wang, T. K. Das, S. K. Singh, and S. Kumar, "Potential aggregation prone regions in biotherapeutics: a survey of commercial monoclonal antibodies," *mAbs*, vol. 1, no. 3, pp. 254–267, 2009.

[134] R. Jefferis, "Aggregation, immune complexes and immunogenicity," *mAbs*, vol. 3, no. 6, pp. 503–504, 2011.

[135] S. Maier, C. H. Chung, M. Morse et al., "A retrospective analysis of cross-reacting cetuximab IgE antibody and its association with severe infusion reactions," *Cancer Medicine*, vol. 4, no. 1, pp. 36–42, 2015.

[136] Y. H. Messinger, N. J. Mendelsohn, W. Rhead et al., "Successful immune tolerance induction to enzyme replacement therapy in CRIM-negative infantile Pompe disease," *Genetics in Medicine*, vol. 14, no. 1, pp. 135–142, 2012.

[137] C. Ma, V. Huang, D. K. Fedorak et al., "Adalimumab dose escalation is effective for managing secondary loss of response in Crohn's disease," *Alimentary Pharmacology and Therapeutics*, vol. 40, no. 9, pp. 1044–1055, 2014.

[138] S.-L. Wang, S. Hauenstein, L. Ohrmund et al., "Monitoring of adalimumab and antibodies-to-adalimumab levels in patient

serum by the homogeneous mobility shift assay," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 78-79, pp. 39–44, 2013.

[139] R. Jefferis, "Posttranslational modifications and the immunogenicity of biotherapeutics," *Journal of Immunology Research*, vol. 2016, Article ID 5358272, 15 pages, 2016.

[140] H. C. Ebbers, S. A. Crow, A. G. Vulto, and H. Schellekens, "Interchangeability, immunogenicity and biosimilars," *Nature Biotechnology*, vol. 30, no. 12, pp. 1186–1190, 2012.

[141] G. M. Peterson, "Generic substitution: a need for clarification," *British Journal of Clinical Pharmacology*, vol. 71, no. 6, pp. 966–968, 2011.

[142] G. Walsh, "Post-translational modifications of protein biopharmaceuticals," *Drug Discovery Today*, vol. 15, no. 17-18, pp. 773–780, 2010.

[143] A. Colbert, A. Umble-Romero, S. Prokop et al., "Bioanalytical strategy used in development of pharmacokinetic (PK) methods that support biosimilar programs," *mAbs*, vol. 6, no. 5, pp. 1178–1189, 2014.

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Research Article

Immunogenicity Assessment of Lipegfilgrastim in Patients with Breast Cancer Receiving Chemotherapy

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Lipegfilgrastim is a long-acting, once-per-cycle, glycopegylated recombinant granulocyte colony-stimulating factor (G-CSF) used to prevent neutropenia in patients receiving myelosuppressive chemotherapy. This integrated analysis examined the immunogenicity of lipegfilgrastim and its potential clinical impact in two double-blind randomized studies (phases II and III) of patients with breast cancer receiving chemotherapy. Serum samples were analyzed using sequential assays for screening, confirmation, antibody titer, and characterization of antidrug antibodies (ADA). Neutropenia-related efficacy measures were reviewed for each ADA-positive patient. Among 255 patients receiving lipegfilgrastim (154 in phase II, 101 in phase III) and 155 patients receiving pegfilgrastim (54 in phase II, 101 in phase III), the incidence of treatment-emergent ADA was low and similar between the lipegfilgrastim (phase II: 1.3%; phase III: 1.0%) and pegfilgrastim (phase II: 1.9%; phase III: 1.0%) arms. None of the treatment-emergent ADA-positive samples exhibited neutralizing activity against lipegfilgrastim, pegfilgrastim, or glycosylated G-CSF in a cell-based neutralizing antibody assay. No changes were observed in neutropenia-related efficacy measures among ADA-positive patients, and no treatment-related hypersensitivity or anaphylaxis occurred. These results indicate that there is no apparent impact of ADA on lipegfilgrastim efficacy and safety.

1. Introduction

Granulocyte colony-stimulating factor (G-CSF) is an endogenous growth factor that promotes neutrophil production, maturation, survival, and activity [1]. Recombinant G-CSFs, such as filgrastim and pegfilgrastim, are used commonly for the prevention and treatment of neutropenia in patients receiving myelosuppressive chemotherapy [2–4].

Filgrastim requires daily administration to maintain therapeutic levels because of its relatively short half-life. Conjugating filgrastim to polyethylene glycol (PEG; pegylation yielding pegfilgrastim) reduces renal clearance and extends the drug's half-life such that it need be administered only once per chemotherapy treatment cycle, with efficacy and safety comparable to those of daily filgrastim [5–7].

Lipegfilgrastim (Lonquex; Teva Pharmaceuticals Ltd.) is a recombinant human G-CSF that is glycopegylated in a site-specific manner, resulting in greater structural homogeneity, with pharmacological properties slightly different from those of pegfilgrastim in healthy volunteers. Specifically, lipegfilgrastim provided a longer-lasting increase in absolute neutrophil count (ANC) compared with pegfilgrastim at an equivalent dose, without increasing the peak ANC values [8]. The noninferiority of lipegfilgrastim to pegfilgrastim in the treatment of severe neutropenia was demonstrated in a randomized, double-blind, active-controlled, phase III trial evaluating the efficacy and safety of lipegfilgrastim in 202 chemotherapy naive patients with breast cancer [9]. Lipegfilgrastim was approved in the European Union in 2013 as once-per-cycle, fixed-dose prophylaxis for severe neutropenia.

Immunogenicity is a potential concern for any biological product, and its assessment is one of the most critical elements for the development of such products. Antidrug antibody (ADA) production, as an unwanted immune response

due to product immunogenicity, may lead to serious safety consequences that manifest as hypersensitivity responses such as anaphylaxis and development of cross-reactive neutralizing antibodies (NAbs) to endogenous proteins [10, 11]. Recombinant G-CSFs, including filgrastim and pegfilgrastim, have been shown to elicit ADA in a minority of patients [12, 13].

The objective of this analysis was to assess the immunogenicity of lipegfilgrastim and its potential clinical impact using data from phase II dose-finding trial and phase III noninferiority trial conducted with patients with breast cancer receiving chemotherapy.

2. Methods

2.1. Study Design and Treatments. Immunogenicity assessments were performed on blood samples collected during two independent clinical studies [9, 14]. The first study was a phase II, double-blind, randomized, dose-optimization study that evaluated the efficacy, safety, pharmacokinetics, and immunogenicity of drug treatments in 208 breast cancer patients undergoing myelosuppressive chemotherapy. Patients were assigned 1 : 1 : 1 : 1 to receive lipegfilgrastim (3.0, 4.5, or 6.0 mg administered via subcutaneous [SC] injection) or pegfilgrastim (6.0 mg SC) once per cycle while undergoing chemotherapy with intravenous doxorubicin 60 mg/m^2 and docetaxel 75 mg/m^2 [14]. The second study was a phase III, double-blind, randomized, noninferiority study in which 202 patients with breast cancer received either lipegfilgrastim (6.0 mg SC) or pegfilgrastim (6.0 mg SC) once per cycle while undergoing the same chemotherapy regimen [9].

In both studies, patients received intravenous doxorubicin/docetaxel administered on day 1 of four 21-day cycles. Lipegfilgrastim or pegfilgrastim was administered on day 2 of each cycle (i.e., 24 hours after chemotherapy was administered).

Blood samples were collected at several time points in each study: at baseline, prior to each chemotherapy cycle, at the end of treatment (day 85), and on posttreatment follow-up days 180 and 360.

2.2. Study Populations. Eligible patients (≥18 years of age) had a diagnosis of stage II, III, or IV breast cancer, were chemotherapy naive, had a baseline ANC of at least 1.5 × 10^9/L and a platelet count of 100 × 10^9/L or greater, and had an Eastern Cooperative Oncology Group performance status of 2 or less [9, 14]. Exclusion criteria included known hypersensitivity to filgrastim or pegfilgrastim or exposure to those agents prior to randomization, prior malignancy within 5 years, radiation therapy within 4 weeks of randomization, or long-term use of oral corticosteroids.

The overall population comprised 208 patients from the phase II study (54 pegfilgrastim, 154 lipegfilgrastim) and 202 patients from the phase III study (101 in each treatment arm), for a total of 410 patients. Patient demographics and baseline clinical characteristics were matched between treatment groups within each study and have been reported elsewhere [9, 14]. The mean ages were similar between the lipegfilgrastim (51.4 years) and pegfilgrastim (50.5 years) groups. All patients were white, and all but three were female.

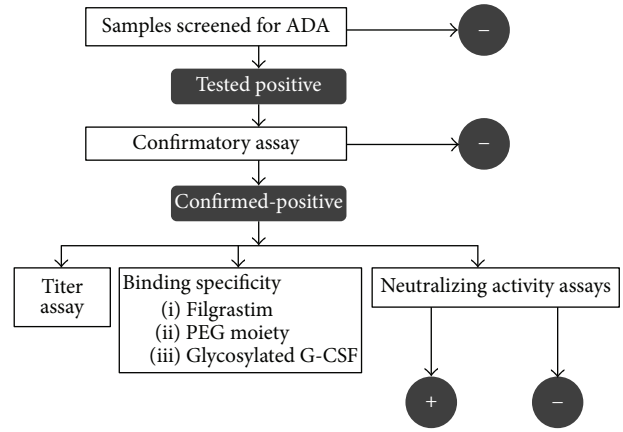

Figure 1: Sequential approach to assessing immunogenicity.

2.3. Immunogenicity Assays. A sequential cascade of validated assays was used to analyze ADA against lipegfilgrastim in patient serum samples (Figure 1). All samples were first tested in the screening assay. Samples screening positive were then analyzed using a confirmatory assay. After ADA confirmation, samples were characterized to determine binding specificity, antibody titers, and neutralizing activity.

2.3.1. Screening Assay. The screening assay used a ligand-binding principle in an electrochemiluminescent bridging format (Figure 2). Briefly, the bivalent property of ADA allows for simultaneous binding of a capture reagent (biotin-labeled lipegfilgrastim or pegfilgrastim) and a detection reagent (ruthenium-labeled lipegfilgrastim or pegfilgrastim). In the presence of a read buffer containing tripropylamine, an electrical current causes the captured ruthenium to emit measureable light. Signal was measured using a Sector Imager 6000 Analyzer (Meso Scale Discovery). The relative sensitivity of the screening assay was 10 ng/mL ADA to lipegfilgrastim and 45 ng/mL ADA to pegfilgrastim, determined using affinity-purified rabbit anti-lipegfilgrastim and rabbit anti-pegfilgrastim antibodies, respectively, as surrogate positive controls. Assay signals from a panel of samples from study drug-naive breast cancer patients were used for the determination of the assay cut-point factor, which in conjunction with the negative control was used to calculate the cut-point for each assay run. Cut-point factors with a false-positive rate set at 5% were determined using statistical methodology described elsewhere [15, 16]. A sample with an assay signal at or above the cut-point was considered as having screened positive.

2.3.2. Confirmatory Assay. Samples that tested positive in the screening assay were subsequently analyzed in an immunocompetition assay for confirmation. Binding was measured with and without the study drug (lipegfilgrastim or pegfilgrastim) in solution phase, and the ratio of binding signal was

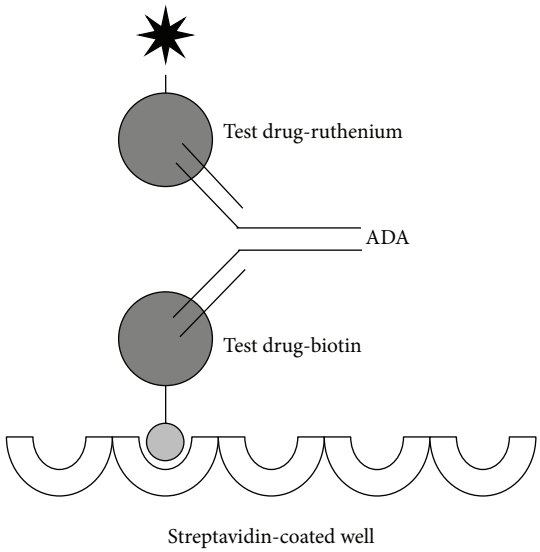

Figure 2: Schematic presentation of the electrochemiluminescent bridging immunoassay. Patient samples were diluted at the minimum required dilution, mixed with biotin- and ruthenium-conjugated test drug (lipegfilgrastim or pegfilgrastim) and the complex formed by antidrug antibodies (ADA). The drug conjugates were captured on a streptavidin-coated assay plate. In the presence of a read buffer containing tripropylamine and upon application of an electrical potential, the ruthenium tag emits light.

calculated and expressed as a percentage of signal inhibition. A cut-point with a false-positive rate set at 1% was determined for each drug using commercially available serum samples from treatment-naive cancer patients in accordance with statistical methods [15, 16]. Samples were identified as confirmed-positive if the percentage of signal inhibition was greater than or equal to the confirmatory cut-point for lipegfilgrastim (28.5%) or pegfilgrastim (28.2%).

2.3.3. Characterization Assays. Confirmed-positive samples were subsequently characterized to evaluate the ADA binding specificity. Binding signals were determined in the presence of unlabeled filgrastim, the PEG portion of lipegfilgrastim (cPEG), or glycosylated G-CSF (glycoG-CSF; Granocyte®; Chugai Pharma), which is an analog of endogenous G-CSF. The cut-points (% signal inhibition) for binding specificity were determined statistically (with false-positive rate set at 1%), using commercially available serum samples from treatment-naive cancer patients in accordance with statistical methodology [15, 16], to be 22.0% for filgrastim, 19.4% for cPEG, and 17.5% for glycoG-CSF.

All confirmed-positive serum samples were also subjected to a semiquantitative titer assay. The titer was defined as the logarithm-transformed highest dilution factor resulting in a signal at the screening cut-point.

2.3.4. NAb Assays and Clinical Assessment. Neutralizing activity was assessed in the confirmed-positive ADA samples using a cell-based proliferation assay that tested the ability of the serum samples to inhibit various G-CSF (glycoG-CSF, lipegfilgrastim, or pegfilgrastim) stimulated proliferation of NSF-60 cells in vitro measured using WST-1 reagent. Samples were also tested in the absence of any G-CSF inducer to detect the presence of nonspecific cell growth that could result in false-negative neutralizing activity. Samples that inhibited any G-CSF inducers in the NAb assay underwent specificity testing in which proliferation was stimulated by murine interleukin-3, an inducer not specific to G-CSF activity; neutralization of this proliferation indicated nonspecific inhibition.

The cut-point for positivity was defined as an optical density (OD) ratio (OD sample : OD viability control) less than or equal to 0.824 for lipegfilgrastim inducer, 0.761 for pegfilgrastim inducer, and 0.821 for glycoG-CSF inducer. These cut-points were established statistically with the false-positive rate set at 5%. A cut-point with a multiplicative correction factor of 1.402 (1% false-positive rate) was established in accordance with statistical methods [15, 16] using commercially available serum samples from treatment-naive cancer patients when samples were tested in the absence of inducer.

Clinical measures were examined for all patients with confirmed-positive samples of ADA to lipegfilgrastim or pegfilgrastim for a possible correlation between the presence of ADA and the potential clinical impact of immunogenicity.

2.4. Efficacy Measurements. The primary efficacy measure was the duration of severe neutropenia (DSN), defined as the number of days with grade 4 neutropenia (ANC $< 0.5 \times 10^9$/L) in each treatment cycle. A secondary efficacy measure was the incidence of febrile neutropenia, defined as axillary body temperature greater than 38.5°C for more than 1 hour and ANC less than 0.5×10^9/L across all cycles. Additional measures included ANC area under the concentration-time curve, maximum ANC, and mean depth of the ANC nadir.

3. Results

3.1. ADA Incidence. A total of 208 patients (54 pegfilgrastim and 154 lipegfilgrastim) were investigated in the phase II study and 202 patients in the phase III study. Results from the patients with confirmed ADA are summarized in Table 1. In the phase II study, 2 of the 154 lipegfilgrastim-treated patients (Table 1, patients 1 and 2) had treatment-emergent ADA, representing an incidence of 1.3%. These patients had confirmed-positive samples at a single postdose time point (day 85 or day 360), indicating that the response was transient. There were seven patients with predose ADA, including three with positive samples at both baseline and postdose time points (Table 1, patients 3–5) and four with positive samples at baseline only (Table 1, patients 6–9). One of 54 pegfilgrastim-treated patients (Table 1, patient 10) had treatment-emergent ADA, representing an incidence of 1.9%. In this patient, the ADA-positive sample occurred only at day 85. There were two patients in the pegfilgrastim group with positive ADA samples observed at baseline only (Table 1, patients 11 and 12).

In the phase III study, 1 of the 101 lipegfilgrastim-treated patients (Table 1, patient 13) had treatment-emergent ADA, with positive samples at days 180 and 360. This reflects

Table 1: Summary of patients with ADA-positive samples.

Phase II study									
Patient	Treatment group	Visit							
		BL	C2D1	C3D1	C4D1	D85	D180	D360	ET
1	*Lipegfilgrastim*	*Neg*	*Neg*	*Neg*	*Neg*	*Pos*	*NA*	*NA*	*NA*
2	*Lipegfilgrastim*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*Pos*	*NA*
3	Lipegfilgrastim	Pos	Pos	Pos	Pos	Pos	Pos	Pos	NA
4	Lipegfilgrastim	Pos	Pos	Pos	Pos	Pos	Pos	Neg	NA
5	Lipegfilgrastim	Pos	Pos	NA	NA	NA	Pos	Pos	Neg
6	Lipegfilgrastim	Pos	NA	NA	NA	NA	NA	NA	NA
7	Lipegfilgrastim	Pos	NA	NA	NA	NA	NA	NA	NA
8	Lipegfilgrastim	Pos	NA	NA	NA	NA	NA	Neg	NA
9	Lipegfilgrastim	Pos	NA	NA	NA	NA	NA	NA	NA
10	*Pegfilgrastim*	*NA*	*NA*	*NA*	*NA*	*Pos*	*NA*	*NA*	*NA*
11	Pegfilgrastim	Pos	NA	NA	NA	NA	NA	NA	NA
12	Pegfilgrastim	Pos	NA	NA	NA	NA	NA	NA	NA

Phase III study								
Patient	Treatment group	Visit						
		S	BL	C2D1	C3D1	C4D1	D85	D180	D360
13	*Lipegfilgrastim*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*Pos*	*Pos*
14	Lipegfilgrastim	NA	Pos	Pos	Pos	Pos	Neg	Neg	Neg
15	Lipegfilgrastim	NA	Pos	Pos	Neg	Neg	Neg	Neg	Neg
16	Lipegfilgrastim	NA	Pos	NA	NA	NA	NA	NA	NA
17	*Pegfilgrastim*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*Pos*
18	Pegfilgrastim	NA	Pos	NA	NA	NA	NA	NA	NA
19	Pegfilgrastim	Pos	NA	NA	NA	NA	NA	NA	NA
20	Pegfilgrastim	NA	Pos	NA	NA	NA	NA	NA	NA
21	Pegfilgrastim	NA	Pos	NA	NA	NA	NA	NA	NA
22	Pegfilgrastim	NA	Pos	Neg	NA	NA	NA	NA	NA
23	Pegfilgrastim	NA	Pos	NA	NA	NA	NA	NA	NA

Note: table includes only patients with ADA-positive samples.
ADA: antidrug antibody; BL: baseline; C2D1: cycle 2 day 1; C3D1: cycle 3 day 1; C4D1: cycle 4 day 1; D85: day 85; D180: day 180; D360: day 360; NA: screened negative sample; Neg: confirmed-negative sample; Pos: confirmed-positive sample; S: screening (predose) time point.
Italic font indicates patient with treatment-emergent ADA.

an ADA incidence of 1.0%. There were three patients with predose ADA, including one who had a positive sample at baseline only (Table 1, patient 16) and two who had positive samples at both baseline and at least a postdose time point (Table 1, patients 14 and 15). Among 101 pegfilgrastim-treated patients in this study, one had treatment-emergent ADA, with a positive postdose sample at day 360 only (Table 1, patient 17). This also represents an ADA incidence of 1.0%. Six patients (Table 1, patients 18–23) had predose ADA, with positive samples at baseline only.

3.2. ADA Characterization and Titer. The binding specificity and titer of ADA were determined in samples from lipegfilgrastim-treated patients with predose or treatment-emergent ADA. Twenty-two confirmed-positive samples from nine lipegfilgrastim-treated patients in the phase II trial were tested using filgrastim, cPEG, or glycoG-CSF competitors to identify ADA binding specificity for the G-CSF moiety of lipegfilgrastim, the PEG moiety of lipegfilgrastim, and endogenous G-CSF, respectively (Table 2).

As noted above, only two patients in the lipegfilgrastim-treated group exhibited treatment-emergent ADA. One had a positive sample on day 85, with an antibody titer of 0.6 against cPEG only (Table 2). The other patient had the positive sample on day 360. The antibody titer was undetectable in this sample and showed no recognition of filgrastim, glycoG-CSF, or cPEG. Five of the seven remaining patients showed predose ADA; two of these five had positive postdose ADA samples as well. One of these two patients had antibodies recognizing filgrastim and glycoG-CSF without detectable titer throughout the duration of the study. The other patient had antibodies recognizing filgrastim and cPEG whose titer diminished over time from 0.9 to 0.3. The remaining three of these five patients had predose antibodies recognizing filgrastim and cPEG but no detectable postdose antibody titer.

From the phase III study, nine confirmed-positive samples from four patients underwent the same characterization and titer assays. One lipegfilgrastim-treated patient with a confirmed-positive sample had possible treatment-emergent

Table 2: ADA titer and binding specificity of ADA-positive samples from lipegfilgrastim-treated patients.

Patient	Competitor/titer	Time point						
		BL	C2D1	C3D1	C4D1	D85	D180	D360
Phase II study								
1	*Filgrastim*	*NA*	*NA*	*NA*	*NA*	*Neg*	*NA*	*NA*
	glycoG-CSF	*NA*	*NA*	*NA*	*NA*	*Neg*	*NA*	*NA*
	cPEG	*NA*	*NA*	*NA*	*NA*	*Pos*	*NA*	*NA*
	Titer	*NA*	*NA*	*NA*	*NA*	*0.6*	*NA*	*NA*
2	*Filgrastim*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*Neg*
	glycoG-CSF	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*Neg*
	cPEG	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*Neg*
	Titer	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*0*
3	Filgrastim	Pos	Pos	Pos	Pos	Pos	Pos	Pos
	glycoG-CSF	Pos	Pos	Pos	Pos	Pos	Pos	Pos
	cPEG	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Titer	0	0	0	0	0	0	0
4	Filgrastim	Pos	Pos	Pos	Pos	Neg	Pos	NA
	glycoG-CSF	Neg	Neg	Neg	Pos	Pos	Pos	NA
	cPEG	Pos	Pos	Pos	Pos	Pos	Pos	NA
	Titer	0.3	0.9	0.6	0.6	0.6	0.3	NA
5	Filgrastim	NSQ	Neg	NA	NA	NA	Neg	Neg
	glycoG-CSF	NSQ	Neg	NA	NA	NA	Neg	Neg
	cPEG	NSQ	Pos	NA	NA	NA	Pos	Pos
	Titer	NSQ	0	NA	NA	NA	0	0
6	Filgrastim	Neg	NA	NA	NA	NA	NA	NA
	glycoG-CSF	Neg	NA	NA	NA	NA	NA	NA
	cPEG	Neg	NA	NA	NA	NA	NA	NA
	Titer	0.6	NA	NA	NA	NA	NA	NA
7	Filgrastim	Pos	NA	NA	NA	NA	NA	NA
	glycoG-CSF	Neg	NA	NA	NA	NA	NA	NA
	cPEG	Pos	NA	NA	NA	NA	NA	NA
	Titer	1.5	NA	NA	NA	NA	NA	NA
8	Filgrastim	Pos	NA	NA	NA	NA	NA	NA
	glycoG-CSF	Neg	NA	NA	NA	NA	NA	NA
	cPEG	Pos	NA	NA	NA	NA	NA	NA
	Titer	0.3	NA	NA	NA	NA	NA	NA
9	Filgrastim	Pos	NA	NA	NA	NA	NA	NA
	glycoG-CSF	Neg	NA	NA	NA	NA	NA	NA
	cPEG	Pos	NA	NA	NA	NA	NA	NA
	Titer	0	NA	NA	NA	NA	NA	NA
Phase III study								
13	*Filgrastim*	*NA*	*NA*	*NA*	*NA*	*NA*	*Pos*	*Pos*
	glycoG-CSF	*NA*	*NA*	*NA*	*NA*	*NA*	*Pos*	*Pos*
	cPEG	*NA*	*NA*	*NA*	*NA*	*NA*	*Neg*	*Neg*
	Titer	*NA*	*NA*	*NA*	*NA*	*NA*	*1.2*	*2.1*
14	Filgrastim	Neg	Neg	Neg	Neg	NA	NA	NA
	glycoG-CSF	Neg	Neg	Neg	Neg	NA	NA	NA
	cPEG	Pos	Pos	Pos	Pos	NA	NA	NA
	Titer	0.6	1.2	0.9	0.6	NA	NA	NA
15	Filgrastim	Pos	Neg	NA	NA	NA	NA	NA
	glycoG-CSF	Pos	Pos	NA	NA	NA	NA	NA
	cPEG	Neg	Neg	NA	NA	NA	NA	NA
	Titer	2.1	1.8	NA	NA	NA	NA	NA
16	Filgrastim	Pos	NA	NA	NA	NA	NA	NA
	glycoG-CSF	Neg	NA	NA	NA	NA	NA	NA
	cPEG	Pos	NA	NA	NA	NA	NA	NA
	Titer	0.9	NA	NA	NA	NA	NA	NA

ADA: antidrug antibody; BL: baseline; C2D1: cycle 2 day 1; C3D1: cycle 3 day 1; C4D1: cycle 4 day 1; cPEG: PEG portion of lipegfilgrastim; D85: day 85; D180: day 180; D360: day 360; glycoG-CSF: glycosylated granulocyte colony-stimulating factor; NA: screened negative sample or confirmed-negative one (not analyzed in characterization assay); Neg: confirmed-negative sample; NSQ: insufficient sample quantity for analysis; Pos: confirmed-positive sample.
Italic font indicates patient with treatment-emergent ADA.

Table 3: Evaluation of the potential impact of ADA on efficacy among patients with treatment-emergent ADA.

Treatment	Patient	ADA + time point		DSN (days)	ANC AUC	ANC maximum	ANC nadir
Phase II study							
Pegfilgrastim	10	D85	Cycle 1	0	160.25	9.60	6.8
			Cycle 2	0	212.95	14.90	7.9
			Cycle 3	0	116.25	8.50	3.6
			Cycle 4	0	91.85	7.50	2.4
Lipegfilgrastim	2	D360	Cycle 1	1	154.24	20.39	0.18
			Cycle 2	1	170.20	21.40	0.0
			Cycle 3	0	225.40	27.64	1.25
			Cycle 4	0	202.26	28.42	1.72
	1	D85	Cycle 1	0	127.80	17.00	0.8
			Cycle 2	0	147.25	9.60	2.2
			Cycle 3	0	121.60	8.30	0.7
			Cycle 4	0	179.55	16.70	1.3
Phase III study							
Pegfilgrastim	17	D85	Cycle 1	0	219.88	47.99	0.89
			Cycle 2	0	198.15	13.73	2.2
			Cycle 3	0	302.98	68.32	2.16
			Cycle 4	0	315.63	57.42	4.06
Lipegfilgrastim	13	D360	Cycle 1	2	160.16	26.14	0.25
			Cycle 2	0	257.95	51.92	1.25
			Cycle 3	0	300.24	58.91	1.2
			Cycle 4	0	291.36	68.75	1.45

ADA: antidrug antibody; ANC: absolute neutrophil count; AUC: area under the curve; D85: day 85; D360: day 360; DSN: duration of severe neutropenia.

antibody induction, recognizing filgrastim and glycoG-CSF, but not cPEG, on both day 180 and day 360 (Table 2). The sample also showed antibody titers of 1.2 and 2.1 for days 180 and 360, respectively. The remaining three patients had confirmed-positive samples at baseline (i.e., predose ADA). One of these patients had antibodies recognizing cPEG at baseline, with a titer of 0.6 that increased to 1.2 in cycle 2 but diminished to undetectable levels by day 85. Another patient had antibodies recognizing filgrastim and glycoG-CSF at baseline, with a titer of 2.1; the antibodies for glycoG-CSF persisted to cycle 2, with a titer of 1.8. The third patient had antibodies against filgrastim and cPEG, with a titer of 0.9 and no detectable postdose antibodies.

3.3. Neutralizing Activity of ADA. Among patients identified as having treatment-emergent ADA, no postdose sample from either treatment group in each study tested positive for NAb activity against lipegfilgrastim, pegfilgrastim, or glycoG-CSF in the cell-based neutralizing antibody assay.

3.4. Clinical Impact Assessments. Clinical efficacy measures for patients with confirmed-positive, treatment-emergent ADA are summarized in Table 3. Neither ANC nor DSN values changed in these patients after initiation of chemotherapy, and no patient experienced febrile neutropenia. No drug-related events with the Medical Dictionary for Regulatory Activities preferred term "drug hypersensitivity" or "hypersensitivity" and no anaphylactic reactions were reported.

The effect of ADA on the pharmacokinetics of lipegfilgrastim was investigated in a pooled analysis of data from patients with breast cancer and patients with non-small cell lung cancer in the phase III study. Only two patients for whom pharmacokinetic data are available tested positive for ADA, and no decrease in exposure, as indicated by the predicted area under the curve data, to lipegfilgrastim was observed in these patients (data not shown). A pharmacodynamics analysis with a CD34+ endpoint conducted with adult patients for all lipegfilgrastim doses found only two patients with positive ADA response. The CD34+ values for these two patients were similar to those from ADA-negative subjects (data not shown).

4. Discussion

The objective of this analysis was to assess the immunogenicity of lipegfilgrastim in patients receiving chemotherapy for breast cancer. In both phase II and phase III studies, the incidence of treatment-emergent ADA was low and was similar between the lipegfilgrastim (phase II: 2/154 = 1.3%; phase III: 1/101 = 1.0%) and pegfilgrastim (phase II: 1/54 = 1.9%; phase III: 1/101 = 1.0%) groups.

Among lipegfilgrastim-treated patients with treatment-emergent ADA, none of the postdose positive samples from either study exhibited NAb activity. Furthermore, there was no apparent impact of treatment-emergent ADA on key measures of clinical efficacy in these patients, including the duration of severe neutropenia or incidence of febrile neutropenia. The limited pharmacokinetic/pharmacodynamic data available for patients positive for ADA suggested no correlation between lipegfilgrastim exposure and ADA positivity.

The results in this study are consistent with literature reporting the immunogenicity of pegfilgrastim, in which a small number (4/521; 0.77%) of pegfilgrastim-treated patients developed treatment-emergent ADA [13]. Similar to the current study, none of these ADA-positive patients showed evidence of NAb activity.

In the current analysis of ADA data from two clinical studies in patients with breast cancer, 10 of 255 (3.9%) lipegfilgrastim-treated patients had positive samples at baseline, a percentage similar to that among pegfilgrastim-treated patients (7/155; 4.5%). Moreover, these patients did not experience an increase in their preexisting antibody response, maintaining relatively low titer levels throughout the course of treatment. Previous studies, again possibly using different assay methodologies, detected anti-pegfilgrastim antibodies in baseline samples from nearly 6% of pegfilgrastim-treated patients with metastatic breast cancer [13]. Therefore, it is not unexpected that some preexisting anti-lipegfilgrastim antibodies were observed at baseline in the current analysis.

This analysis found a low incidence of treatment-emergent ADA in both lipegfilgrastim- and pegfilgrastim-treated patients with breast cancer and treated with doxorubicin and docetaxel. The presence of treatment-emergent ADA did not appear to impact the clinical efficacy of either treatment; this was expected, because none of the detected antibodies in patients with possible treatment-emergent antibodies were neutralizing. Similarly, 11 of 333 patients (3%) developed ADA following filgrastim treatment in clinical studies, and no neutralizing response was observed in the 11 patients [12].

Competing Interests

Linglong Zou, Anton Buchner, and Patrick M. Liu are employees of Teva Pharmaceuticals and declare financial interests regarding the publication of this paper; Martin Roberge reports employment from CIRION BioPharma Research Inc., a contract research laboratory providing services to Teva Pharmaceuticals.

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References

[1] A. W. Roberts, "G-CSF: a key regulator of neutrophil production, but that's not all!," *Growth Factors*, vol. 23, no. 1, pp. 33–41, 2005.

[2] K. L. Cooper, J. Madan, S. Whyte, M. D. Stevenson, and R. L. Akehurst, "Granulocyte colony-stimulating factors for febrile neutropenia prophylaxis following chemotherapy: systematic review and meta-analysis," *BMC Cancer*, vol. 11, article 404, 2011.

[3] J. Crawford, C. Caserta, and F. Roila, "Hematopoietic growth factors: ESMO Clinical Practice Guidelines for the applications," *Annals of Oncology*, vol. 21, supplement 5, pp. v248–v251, 2010.

[4] D. C. Dale, "Colony-stimulating factors for the management of neutropenia in cancer patients," *Drugs*, vol. 62, supplement 1, pp. 1–15, 2002.

[5] T. Arvedson, J. O'Kelly, and B.-B. Yang, "Design rationale and development approach for pegfilgrastim as a long-acting granulocyte colony-stimulating factor," *BioDrugs*, vol. 29, no. 3, pp. 185–198, 2015.

[6] M. D. Green, H. Koelbl, J. Baselga et al., "A randomized double-blind multicenter phase III study of fixed-dose single-administration pegfilgrastim versus daily filgrastim in patients receiving myelosuppressive chemotherapy," *Annals of Oncology*, vol. 14, no. 1, pp. 29–35, 2003.

[7] F. A. Holmes, S. E. Jones, J. O'Shaughnessy et al., "Comparable efficacy and safety profiles of once-per-cycle pegfilgrastim and daily injection filgrastim in chemotherapy-induced neutropenia: a multicenter dose-finding study in women with breast cancer," *Annals of Oncology*, vol. 13, no. 6, pp. 903–909, 2002.

[8] A. Buchner, A. Lammerich, A. Abdolzade-Bavil, U. Müller, and P. Bias, "Lipegfilgrastim: pharmacodynamics and pharmacokinetics for body-weight-adjusted and 6 mg fixed doses in two randomized studies in healthy volunteers," *Current Medical Research and Opinion*, vol. 30, no. 12, pp. 2523–2533, 2014.

[9] I. Bondarenko, O. A. Gladkov, R. Elsaesser, A. Buchner, and P. Bias, "Efficacy and safety of lipegfilgrastim versus pegfilgrastim: a randomized, multicenter, active-control phase 3 trial in patients with breast cancer receiving doxorubicin/docetaxel chemotherapy," *BMC Cancer*, vol. 13, article 386, 2013.

[10] H. Frost, "Antibody-mediated side effects of recombinant proteins," *Toxicology*, vol. 209, no. 2, pp. 155–160, 2005.

[11] A. S. Rosenberg, "Immunogenicity of biological therapeutics: a hierarchy of concerns," *Developments in Biologicals*, vol. 112, pp. 15–21, 2003.

[12] *Neupogen [Prescribing Information]*, Amgen, Thousand Oaks, Calif, USA, 2015.

[13] *Neulasta [Prescribing Information]*, Amgen, Thousand Oaks, Calif, USA, 2016.

[14] A. Buchner, R. Elsässer, and P. Bias, "A randomized, double-blind, active control, multicenter, dose-finding study of lipegfilgrastim (XM22) in breast cancer patients receiving myelosuppressive therapy," *Breast Cancer Research and Treatment*, vol. 148, no. 1, pp. 107–116, 2014.

[15] G. Shankar, V. Devanarayan, L. Amaravadi et al., "Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 48, no. 5, pp. 1267–1281, 2008.

[16] H. W. Smith, M. Moxness, and R. Marsden, "Summary of confirmation cut point discussions," *AAPS Journal*, vol. 13, no. 2, pp. 227–229, 2011.

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Research Article

Preexisting Antibodies to an F(ab′)$_2$ Antibody Therapeutic and Novel Method for Immunogenicity Assessment

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Anti-therapeutic antibodies (ATAs) may impact drug exposure and activity and induce immune complex mediated toxicity; therefore the accurate measurement of ATA is important for the analysis of drug safety and efficacy. Preexisting ATAs to the hinge region of anti-Delta like ligand 4 (anti-DLL4) F(ab′)$_2$, a potential antitumor therapeutic, were detected in cynomolgus monkey serum, which presented a challenge in developing assays for detecting treatment induced ATA. A total ATA assay was developed using a bridging ELISA that detected both anti-CDR and anti-framework ATA including anti-hinge reactivity. A competition assay that could detect 500 ng/mL of anti-CDR ATA in the presence of preexisting ATA was also developed to determine ATA specific to the anti-DLL4 F(ab′)$_2$ CDR using anti-DLL4 F(ab′)$_2$ and a control F(ab′)$_2$. We used these assay methods in a cynomolgus monkey in vivo study to successfully evaluate total and anti-CDR ATA. The preexisting anti-hinge reactivity was also observed to a lesser extent in human serum, and a similar approach could be applied for specific immunogenicity assessment in clinical trials.

1. Introduction

The administration of large molecule protein drugs can result in the development of antibodies against the therapeutic protein, which may lead to loss of efficacy [1] and alteration of clearance or induction of immune-mediated toxicities. Assessment of these anti-therapeutic antibodies (ATAs) responses is important for interpretation of relevant endpoints including pharmacokinetics, pharmacodynamics, safety, and/or efficacy of the molecule [2–4]. ATA can affect drug responses by decreasing drug exposure through clearance of large protein : ATA complexes [5]. Conversely, clearance can be decreased for proteins that are contained in immune complexes, leading to accumulation of total protein [6]. In addition, an Fab or F(ab′)$_2$ antibody that does not itself have effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) may have this function reconstituted through the Fc portion of ATA that is present in the drug : ATA complex [7]. These have potential safety implications such as induction of immune-complex toxicities such as vasculitis. Finally, drug activity may be neutralized by anti-complementarity determining region (anti-CDR) ATA reactivity that blocks the bind of drug to its target [8].

Delta like ligand 4 (DLL4) is a member of the Notch signaling pathway [9, 10]. DLL4 inhibition impairs tumor growth by disrupting the balance of tip and stalk cells of sprouting endothelium and thus promoting nonproductive angiogenesis [11, 12]. Although anti-DLL4 full length antibody showed potent antitumor activity, nonclinical in vivo testing resulted in unmanageable toxicity, with vascular and liver toxicities [13]. Therefore a rapidly cleared F(ab′)$_2$ form of a humanized anti-DLL4 monoclonal antibody was generated to ameliorate toxicity while maintaining efficacy [14] by reducing drug exposure but maintaining sufficient target engagement. During development of an ATA assay for this F(ab′)$_2$ molecule, we observed a high prevalence of

preexisting reactivity to anti-DLL4 $F(ab')_2$ in cynomolgus monkey serum samples from drug naïve animals. These sera did not react with the precursor full length antibody molecule but did react with both the anti-DLL4 $F(ab')_2$ and an $F(ab')_2$ prepared from a different humanized monoclonal antibody with the same framework residues but with a different CDR sequence. This indicates that the preexisting reactivity is directed to the hinge epitope that is exposed when the Fc fragment is enzymatically removed to generate the $F(ab')_2$ from the full length antibody. It has been reported that anti-hinge antibody reactivity can be highly specific to the exact IgG cleavage site [15, 16]. The observed cross reactivity of the cynomolgus monkey preexisting anti-hinge antibodies to the human $F(ab')_2$ molecule suggests that there is a high degree of homology between the human and cynomolgus monkey hinge epitopes.

Fab and $F(ab')_2$ fragments are known to be generated in vivo by certain bacterial proteases, probably as a survival mechanism by preventing anti-bacterial antibodies from utilizing effector activities [17]. Anti-hinge antibodies have been reported by other researchers and have been linked to various in vitro and in vivo effects including reconstitution of effector activity [7, 18, 19]. In a study of several therapeutic drugs where $F(ab')_2$ fragments were used to avoid rheumatoid factor interference, an increase in ATA assay background was observed due to anti-hinge IgG in human serum reacting with drug $F(ab')_2$ [20]. A therapeutic $F(ab')_2$ anti-glycoprotein IIb/IIIa drug intended to prevent platelet aggregation unexpectedly resulted in a decrease in platelets in treated cynomolgus monkeys, probably due to reconstitution of Fc effector function by anti-hinge antibodies [21]. Higher preexisting anti-hinge antibody activity has also been correlated with kidney transplant survival [22]. Stimulation of complement activation by complexes of anti-hinge antibodies with $F(ab')_2$ has also been reported [23].

In this study, we describe methods to evaluate both ATA to the entire $F(ab')_2$ molecule and to also evaluate anti-CDR ATA. Use of these methods can potentially enable interpretation and analysis of various mechanistic effects due to ATA development.

2. Materials and Methods

2.1. Materials. Anti-DLL4 $F(ab')_2$ was prepared by pepsin cleavage as described in Couch et al. [14]. Anti-DLL4 Fab was prepared by standard papain digestion methods at Genentech. Herceptin® $F(ab')_2$ was prepared by standard pepsin digestion methods at Genentech. Affinity purified anti-CDR antibody to anti-DLL4 $F(ab')_2$ was prepared by immunizing goats with recombinant human anti-DLL4 antibody Fab fragments on days 0, 14, 28, 42, and 56, followed by serum collection on day 66. The immunized goat antiserum was affinity-enriched for anti-CDR antibodies using immobilized full length anti-DLL4 coupled via primary amines to an agarose column followed by elution with 0.1 M glycine pH 2.5. Remaining anti-framework antibody was removed by adsorbing the eluate over a column coupled with a framework control antibody. Cynomolgus monkey serum samples from untreated animals were purchased from BioreclamationIVT (Hicksville, NY) and Covance (Westbury, NY). Detection conjugate for the PK assay was prepared using 10C4, a mouse monoclonal antibody that recognizes anti-DLL4 in the presence of human IgG [24], coupled to horseradish peroxidase (HRP) as described by the manufacturer (Pierce Plus Activated Peroxidase, ThermoFisher Scientific, Waltham, MA).

2.2. Direct ATA Assay. Anti-DLL4 $F(ab')_2$ diluted to 1 μg/mL in carbonate buffer pH 9.6 was added to a high binding polystyrene ELISA plate (Nunc ThermoFisher, Waltham, MA) and incubated overnight at 4°C. The plate was washed with wash buffer (PBS/0.05% polysorbate 20) and remaining binding sites were blocked using assay diluent (PBS, 0.5% BSA, 0.05% polysorbate 20, 0.05% ProClin 300, pH 7.4).

After incubation and washing, samples diluted in assay diluent were added and incubated for two hours. The plate was washed, and captured ATA was detected by adding donkey anti-human IgG Fc-specific HRP conjugate (Jackson, West Grove, PA). After incubation and washing, signal was generated by adding tetramethylbenzidine (TMB; Moss, Pasadena, MD) and stopping the reaction with phosphoric acid. Absorbance was measured at 450 nm using 650 nm reference (Molecular Devices, Sunnyvale, CA).

2.3. Bridging ATA Assay. Anti-DLL4 $F(ab')_2$ was conjugated to biotin using sulfo-NHS-LC-biotin (Pierce/ThermoFisher, Waltham, MA) or digoxigenin using 3-amino-3-deoxydigoxigenin hemisuccinamide succinimidyl ester (Invitrogen, Carlsbad, CA). Master mix was prepared as a mixture of both conjugates, each at 2 μg/mL, in assay buffer. Samples were diluted 1/20 in assay diluent and then titered with seven subsequent 1/5 dilutions. Equal volumes of master mix and diluted sample were mixed and incubated overnight.

This reaction mixture was then incubated in a washed high binding streptavidin plate (Roche, Indianapolis, IN). After washing, bound antibody : conjugate complexes were detected by adding mouse monoclonal anti-DIG HRP conjugate (Jackson ImmunoResearch, West Grove, PA) to the streptavidin plate. After washing, signal was generated by adding TMB (Kirkegaard & Perry, Gaithersburg, MD), stopping the reaction with 1 M phosphoric acid. Absorbance was measured at 450 nm using a reference wavelength of 630 nm using an Infinite 200 spectrophotometer (Tecan, Switzerland).

Goat anti-DLL4 $F(ab')_2$ CDR purified antibody was used as a positive control. Assay buffer was used as a negative control due to the preexisting anti-$F(ab')_2$ antibodies observed in cynomolgus monkey serum. In the final bridging assay, concentrations as low as 500 ng/mL of positive control antibody could be detected in the presence of up to 2 μg/mL anti-DLL4 $F(ab')_2$.

2.4. Bridging ATA Assay Using Competitive Molecules. Molecules for competition were prepared at 100 μg/mL in assay diluent. Cynomolgus monkey serum samples were first diluted 1/10 into assay diluent and then mixed in a 1 : 1 ratio with either diluted competition molecule solution or assay diluent, resulting in a final sample dilution of 1/20 serum with 50 μg/mL final competitor concentration. The mixture

was incubated with agitation for at least one hour at room temperature to allow complex formation before addition of the Master Mix. These dilutions were assayed using the bridging ATA assay format.

2.5. PK Assay. Recombinant human DLL4 extracellular domain diluted to 1 μg/mL in pH 9.6 carbonate buffer was added to a high binding polystyrene ELISA plate (Nunc ThermoScientific, Waltham, MA) and incubated overnight at 4°C. The plate was washed and assay buffer was added to block any remaining binding sites.

After incubation and washing, the diluted serum sample was added to capture anti-DLL4 F(ab′)$_2$ in the sample.

The plate was washed, and captured anti-DLL4 F(ab′)$_2$ was detected by adding 10C4-HRP conjugate, a mouse monoclonal antibody that recognizes anti-DLL4 in the presence of human IgG [24] coupled to HRP. After incubation and washing, signal was generated by adding TMB (KPL), stopping the reaction with phosphoric acid. Absorbance was measured at 450 nm using 620 nm reference wavelength on a Tecan Infinite ELISA plate reader (Tecan).

3. Results and Discussion

During development of an ATA assay for a full length therapeutic antibody, assay signals from untreated animal sera are generally used to set the ATA positive threshold (cutpoint), which differentiates ATA negative and positive samples, by calculating the signal variance using a target 5% false positive ATA rate [25]. Because we observed that almost all cynomolgus monkey serum samples from treatment-naïve animals gave high and variable responses during the development of the anti-DLL4 F(ab′)$_2$ ATA assay, this approach could not be used. Therefore, we developed a new approach based on setting individual cutpoints for each animal. First we determined that the preexisting reactivity was specific to the neo-epitope at the F(ab′)$_2$ hinge region. We then evaluated the expected temporal changes in this reactivity in the absence of any drug treatment. We developed robust methods using individual cutpoints for determining both changes in titer to the entire molecule and methods for assessing changes in ATA binding epitopes on anti-DLL4 (i.e., hinge neo-epitope versus anti-CDR specific ATA).

3.1. Preexisting Reactivity Is Specific for Anti-Hinge of F(ab′)$_2$ but Not for Full Length Molecule as Shown by Competition. The specificity of the preexisting ATA antibodies found in cynomolgus monkey serum was mapped using the strategy shown in Figure 1. Serum samples were tested using a competition assay that contained either no competitor, anti-DLL4 F(ab′)$_2$, or full length anti-DLL4. In most samples, the reactivity was reduced by anti-DLL4 F(ab′)$_2$ but not by the full length antibody, indicating anti-hinge specificity. Binding of cynomolgus monkey ATA in the bridging ATA assay is blocked by F(ab′)$_2$ fragments from anti-DLL4 and from another control monoclonal antibody but is not blocked by anti-DLL4 full length antibody, indicating that the reactivity is specific for the hinge region of anti-DLL4 F(ab′)$_2$ (Figure 2).

	Specificity		
	Anti-CDR	Hinge	Framework
Anti-DLL4 F(ab′)$_2$ competition	+	+	+
Herceptin F(ab′)$_2$ competition	–	+	+
Full length anti-DLL4 competition	+	–	+

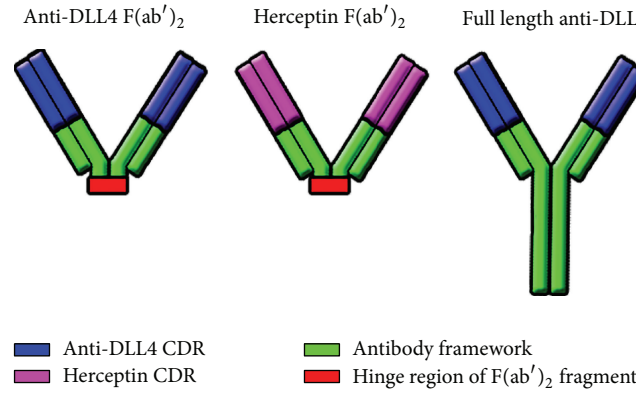

Figure 1: Specificity of signal reduction in the anti-DLL4 F(ab′)$_2$ ATA assay by different competitor molecules. Anti-DLL4 F(ab′)$_2$ competitor will reduce all reactivity in the ATA assay. Control F(ab′)$_2$ will not reduce anti-DLL4 F(ab′)$_2$ CDR reactivity but will reduce F(ab′)$_2$ framework and hinge reactivity. Anti-DLL4 full length antibody will reduce anti-DLL4 CDR and framework reactivity, but not hinge reactivity.

3.2. Preexisting Reactivity Is Specific for the Hinge Neo-Epitope on the F(ab′)$_2$, Since Little Reactivity Is Seen for Fab as Shown by Direct Binding Assay. Direct detection of preexisting anti-hinge IgG in a panel of 20 individual cynomolgus monkey serum samples was tested using anti-DLL4 F(ab′)$_2$ or anti-DLL4 Fab coated onto ELISA wells, with detection using anti-human IgG Fc specific HRP labeled conjugate. Papain-generated Fab fragment is 10 amino acids shorter in the hinge region than pepsin-generated F(ab′)$_2$ fragment and is monomeric rather than dimeric [17]. Sample reactivity to the F(ab′)$_2$ coat was readily observed while little reactivity was seen when plates were coated with the Fab fragment, thus confirming reactivity against the hinge epitope on the F(ab′)$_2$ fragment (Figure 3).

3.3. Preexisting Reactivity Is Stable within Individual Animals over 14 Days but Varies Considerably between Animals. In order to determine whether the overall reactivity to the drug is changed after the animal is treated, a qualitative assessment of the temporal response variability within an animal without any drug treatment was done. In this way the magnitude of an induced change in assay signal could be distinguished from longitudinal variation in the preexisting signal. A series of three samples was collected from each of ten treatment-naïve cynomolgus monkeys over 14 days and the samples were titered in the bridging ATA assay. Overall consistent titers were observed within animal week to week, but in contrast the titers varied widely between animals at the same time points. Representative data are shown below. Thus,

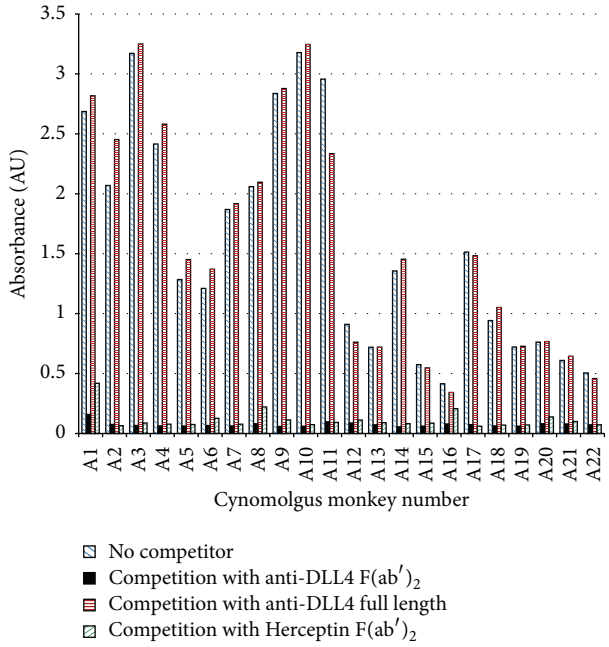

Figure 2: Preexisting ATAs are directed primarily at the $F(ab')_2$ hinge, as shown by the reactivity reduction by competition with anti-DLL4 $F(ab')_2$, Herceptin $F(ab')_2$, or anti-DLL4 full length antibody in the bridging ELISA format. In the bridging ATA assay using 50 μg/mL competitor (in-well concentration) and a 1/20 sample dilution, full length antibody causes very little signal reduction; however the $F(ab')_2$ form of the same antibody reduces the signal almost to background. An $F(ab')_2$ form of Herceptin, which has the same framework as anti-DLL4 but with a different CDR, also reduces the signal, indicating that the signal is due to anti-hinge antibodies.

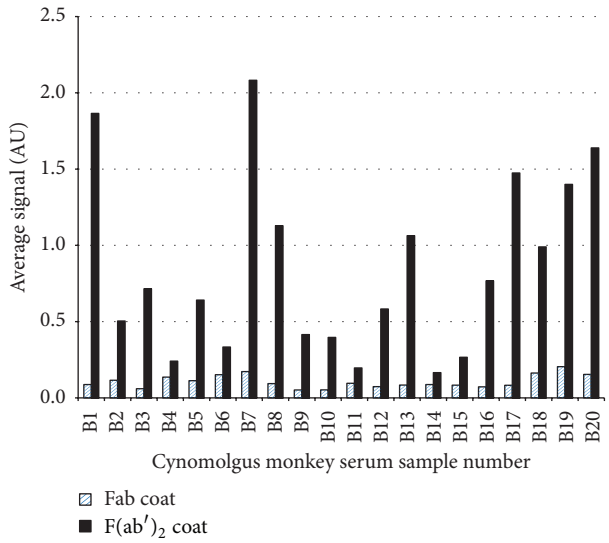

Figure 3: Comparative reactivity of cynomolgus monkey serum samples with anti-DLL4 Fab or anti-DLL4 $F(ab')_2$ directly coated on a well (direct binding format). More sample reactivity is observed with the $F(ab')_2$ coat.

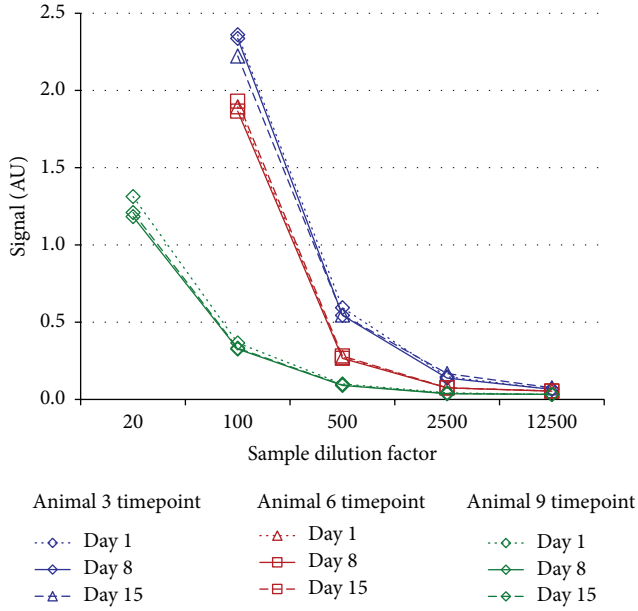

Figure 4: Representative anti-DLL4 $F(ab')_2$ bridging ATA assay titer curves from three cynomolgus monkeys sampled weekly, three samples per animal total. The signals were consistent within animal but varied widely between animals.

preexisting reactivity is stable within individual animals over 14 days but varies considerably between animals (Figure 4).

In this study we used the two pretreatment samples from each animal for individual cutpoint calculation, but the use of the control group variation in antibody titers over the course of the study to define the pooled standard deviation could also be used to refine the estimated antibody temporal variation that is unrelated to treatment.

3.4. Anti-CDR Can Be Detected in the Presence of Preexisting Reactivity Using Competition with Full Length Antibody or $F(ab')_2$, but Competition with $F(ab')_2$ Has Better Dynamic Range. Given the robust preexisting ATA responses seen in cynomolgus monkey samples, the challenge was confirming a small anti-DLL4-specific anti-CDR signal on top of a large anti-DLL4-nonspecific assay signal generated by preexisting anti-hinge ATA. Two assay formats were explored for the detection of anti-CDR antibody in the presence of high levels of preexisting anti-hinge antibodies. We evaluated these formats for their ability to detect a low concentration of anti-CDR antibodies in the presence of high levels of anti-hinge antibody. We found that anti-CDR can be detected in the presence of preexisting reactivity using competition with full length or $F(ab')_2$, and that competition with $F(ab')_2$ gave assay signals with a better dynamic range.

We used a competitive molecule concentration of 50 μg/mL in a 1/20 sample dilution. This concentration was shown during assay development to reduce most of the signal in samples with preexisting reactivity.

The first assay format used competition with full length anti-DLL4 in the bridging assay. Anti-CDR antibodies are detected by calculating the change in signal between sample

tested with buffer and sample tested with full length anti-DLL4. Full length anti-DLL4 contains all epitopes seen in anti-DLL4 $F(ab')_2$ except for the hinge neo-epitope which is formed by Fc cleavage and thus will deplete anti-CDR and anti-framework but not anti-hinge antibodies. Affinity-purified anti-CDR antibody was added at a concentration of 500 ng/mL to a panel of 14 serum samples from untreated cynomolgus monkeys. These sera were tested using different diluents to evaluate the effect of either no competition or competition with 50 μg/mL of competitive molecule. Each sample was diluted either with assay diluent alone (no competition) or with diluent containing full length anti-DLL4 or with anti-DLL4 $F(ab')_2$. The diluted samples were then tested in the bridging ATA assay, resulting in average signals of 1.656, 1.388, and 0.260 absorbance units (AU), respectively. Thus competition with full length anti-DLL4 only reduced average signal by 16%.

The second assay format used a dual competition with anti-DLL4 $F(ab')_2$ and with Herceptin $F(ab')_2$ in the bridging assay. Anti-CDR antibodies are detected by calculating the change in signal between sample tested with anti-DLL4 $F(ab')_2$ and sample tested with Herceptin $F(ab')_2$. The same sample panel to which 500 ng/mL anti-CDR antibody had been added was competed with either Herceptin $F(ab')_2$, which will compete with anti-hinge but not anti-CDR ATA, or anti-DLL4 $F(ab')_2$, which competed with both anti-hinge and anti-CDR ATA. The average signals from the anti-DLL4 $F(ab')_2$ competition and the Herceptin $F(ab')_2$ competition were 0.250 and 0.653 AU, respectively, resulting in a 62% reduction in average signal using anti-DLL4 $F(ab')_2$ compared to Herceptin $F(ab')_2$.

Thus we were able to show clear signal drops in both competitive assays for 500 ng/mL anti-CDR antibody in the presence of a high preexisting ATA response, but the $F(ab')_2$ dual competition provided a more robust approach for the detection of anti-CDR antibodies in the presence of high levels of anti-hinge ATA. This differential competition method was therefore used to determine the presence or absence of anti-CDR ATA.

3.5. Final Method: ATAs to Whole Molecule. A titer method was used to evaluate ATA response to the entire anti-DLL4 $F(ab')_2$ molecule. Serum was diluted 1/20 and then titered in 1/5 dilution steps, eight dilutions in total. The titer cutpoint was set as 2 times the average signal of assay diluent wells. A similar method has been described [26].

Because every animal had a preexisting ATA (anti-hinge) response, including some animals with very high titers, the ability to detect a postdose titer change was problematic. Therefore, a method to set an individual cutpoint for each animal was also developed to determine if the ATA response to the whole molecule changed upon treatment. As shown earlier, preexisting ATA responses vary considerably between animals, but the responses over time within individual animals are more consistent. A pooled CV calculation method was used to compensate for this interanimal variability. This is based on the pooled variance method, which estimates the variance when the mean response may vary between animals, but where repeated samples from an animal are expected to have similar variability.

Two pretreatment samples separated by one week were obtained for each animal and titered in the assay. The standard deviation of the two pretreatment sample titers for each animal was calculated. The pooled standard deviation was calculated by averaging the individual prestudy standard deviations.

The range of titers that would be expected from normal variability was set for each animal using the pooled standard deviation by multiplying the pooled standard deviation by 2.33, the 99th percentile of the normal distribution, and then adding and subtracting this factor from each animal's average predose titer. Posttreatment sample titers that fell outside that range were thus considered to have been increased or decreased due to drug treatment.

3.6. Final Method: ATAs to CDR. Anti-CDR ATA responses were detected by testing each sample at a 1/20 dilution using the dual $F(ab')_2$ competition method. The sample drop score is the difference of the signals relative to the Herceptin $F(ab')_2$ signal. The drop score for each sample was computed as follows:

H = signal when 50 μg/mL Herceptin $F(ab')_2$ is added to sample.

D = signal when 50 μg/mL anti-DLL4 $F(ab')_2$ is added to sample.

$$\text{Drop score} = [H - D]/H \times 100\%.$$

The cutpoint for anti-CDR positivity was set for each animal individually by adding 2.33 (the 99th percentile of the standard normal distribution) times the estimated standard deviation (SD) to each animal's baseline sample signal. The SD is estimated by pooling individual SDs across animals. The steps in computing and applying the anti-CDR cutpoint are the following:

(i) For each animal, two predose samples were taken and tested in the two competitive assays.

(ii) For each animal, the individual mean as well as variance of the two pretreatment sample drop scores was calculated.

(iii) The pooled SD was calculated by taking the square root of the average of the individual variances.

(iv) Each animal's individual cutpoint was calculated by adding 2.33 times the pooled SD times to the individual mean.

(v) Any posttreatment sample that had a signal above its individual cutpoint was positive for anti-CDR.

Some animals had signals for one or both of the $F(ab')_2$ competitions that were above the accuracy limit of the spectrophotometer. For these samples, the drop score could not be computed and therefore the result was reported as indeterminate.

3.7. Detection, Titration, and Anti-CDR Assay Signal Changes in Samples. The titer and anti-CDR methods were used to analyze serum samples from cynomolgus monkeys that were dosed weekly with either vehicle (control group) or 5, 15, or 50 mg/kg anti-DLL4 $F(ab')_2$ [14], with five males and five females in each dose group. Nine doses in total per animal were given over eight weeks.

The changes seen in ATA titer for the study animals are summarized by dose group and by day in Table 1.

In the control group, 27 of 28 samples in the control group showed no significant titer change. One sample at day 29 showed a significant titer change; this may be a false positive due to the statistical basis of the method.

In the 5 mg/kg low dose group, the majority of samples showed an increase in titer posttreatment. The 50 mg/kg high dose group showed the opposite pattern, with most of the samples showing a decrease in titer. This may be due to drug interference in the ATA assay; however measured drug concentrations were below the drug interference level in most cases. This result could also be due to high dose tolerance [27, 28]. The 15 mg/kg mid dose group was split between decreasing, unchanged, and increasing titer.

3.8. Development of Anti-CDR ATA Could Be Detected in Some Animals Treated with Anti-DLL4 $F(ab')_2$. Anti-CDR antibodies were detected in all dose groups, as shown in Figures 5(a)–5(d). In the control group, three of the 28 posttreatment samples were anti-CDR positive, probably due to the statistical method of setting the cutpoints resulting in false positive outcomes. The cutpoint factor is chosen to give a predicted 5% false positive rate to ensure that more true positive samples are detected, but higher pretreatment rates may be seen in the actual study samples due to the small number of samples used to set the cutpoint, assay variation, or differences between samples used to set the cutpoint and the study samples. In the anti-DLL4 $F(ab')_2$ treatment groups, positive anti-CDR responses were seen in 18 of the 25 treated animals with interpretable results.

Table 1: Changes in titer by study day and by dose group.

Group	Change in titer	Number of animals/total number of animals			
		Day 29	Day 57	Day 85	Day 112
Control	Decreased	0/10	0/10	0/4	0/4
	Unchanged	9/10	10/10	4/4	4/4
	Increased	1/10	0/10	0/4	0/4
5 mg/kg	Decreased	1/10	1/10	0/4	0/4
	Unchanged	2/10	2/10	1/4	1/4
	Increased	7/10	7/10	3/4	3/4
15 mg/kg	Decreased	5/10	3/10	0/4	0/4
	Unchanged	2/10	4/10	2/4	2/4
	Increased	3/10	3/10	2/4	2/4
50 mg/kg	Decreased	8/10	7/9	1/4	3/4
	Unchanged	1/10	1/9	2/4	0/4
	Increased	1/10	1/9	1/4	1/4

4. Conclusions

Accurate measurement and appropriate interpretation of data on the immunogenicity of a therapeutic protein are important to its successful development due to the potential impact of immunogenicity on safety and efficacy [2, 3]. During the development of $F(ab')_2$ therapeutic we encountered preexisting anti-$F(ab')_2$ antibodies at high titers in virtually every cynomolgus monkey serum tested.

These high preexisting ATA levels could potentially confound our ability to detect a drug induced ATA response (similar results were observed with a small set of human serum samples; data not shown). Here we report methods to assess both total and anti-CDR specific ATA reactivity in the presence of preexisting anti-$F(ab')_2$ antibodies to enable immunogenicity assessment. Cutpoints for each individual animal were used with both a titration method for total ATA reactivity and a dual competition method to detect anti-CDR specific ATA.

This dual approach enabled a more detailed assessment of the nature of treatment induced ATA responses, since ATA response both to the entire molecule and to the CDR could be evaluated, even in the presence of high titers of anti-hinge antibodies. These are informative for interpretation of toxicology data, since anti-CDR antibodies may neutralize the drug and thus reduce any on target effect; conversely, anti-drug antibody complexes with either anti-CDR or anti-framework antibodies may alter drug clearance or induce off-target toxicological effects via immune complexes.

In this investigation we used pretreatment samples to derive cutpoints for use as decision thresholds for individuals. Some apparently false positive anti-CDR antibodies were detected in the control animals, which we attribute to the statistical method for setting the cutpoint. This may also be due to variation in antibody titers over the course of the study unrelated to treatment, since the study duration was longer than our evaluation of antibody variation. An approach that could potentially mitigate this issue would be to use control animal samples taken over the entire course of the study to calculate the pooled standard deviation and thus account for this variability. This method could also be extended by defining cutpoints that support further sample dilution to reduce the number of indeterminate samples.

Future work may include the analysis of neutralizing activity of anti-CDR specific ATA and study of mechanism and significance of preexisting anti-hinge antibodies and application of this method to human samples and clinical trials. We and other authors [20, 22] have also observed preexisting anti-$F(ab')_2$ reactivity in human serum samples from untreated subjects. This approach should be applicable to human studies, where either multiple pretreatment samples, study control group samples, or a historical control from a study in a comparable population could potentially be used to establish the pooled standard deviation.

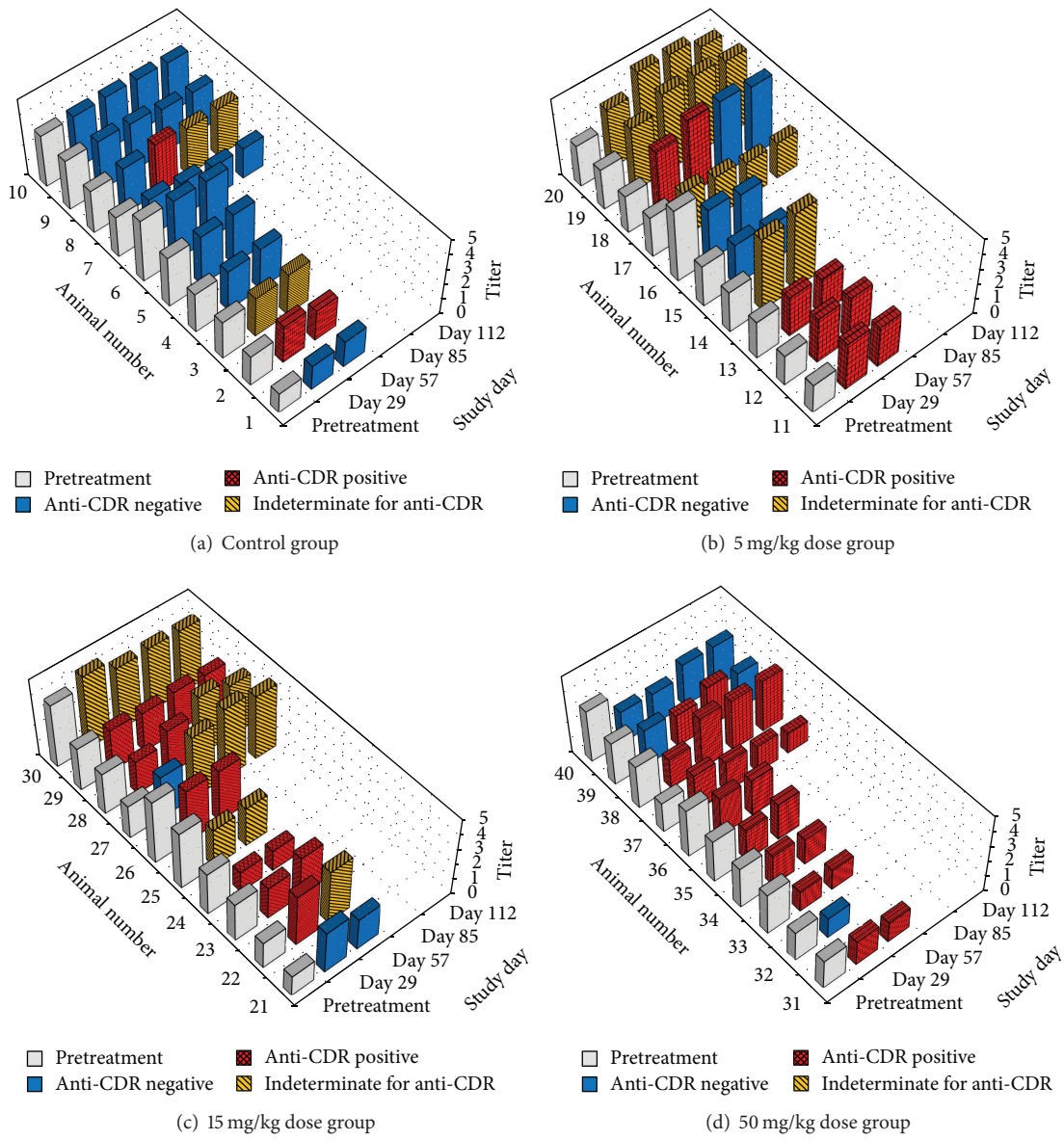

FIGURE 5: Response-time plots of ATA titer to the whole $F(ab')_2$ molecule and anti-CDR ATA positivity status by dose group. Solid light gray bar: pretreatment titer (average of two samples). Solid blue bar: posttreatment titer for anti-CDR negative sample. Horizontal hatched red: posttreatment titer for anti-CDR positive sample. Diagonal hatched yellow bar: posttreatment titer for anti-CDR indeterminate sample.

Competing Interests

The authors are employees of Genentech, Inc., and own stock in F. Hoffman-La Roche, Ltd.

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References

[1] F. Baert, M. Noman, S. Vermeire et al., "Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease," *New England Journal of Medicine*, vol. 348, no. 7, pp. 601–608, 2003.

[2] U.S. Department of Health and Human Services, Food and Drug Administration, CDER, and CBER, *Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products,*, 2014.

[3] European Medicines Agency. Committee for Medicinal Products for Human Use, "Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins," Doc. Ref. EMEA/CHMP/BMWP/14327/2006, 2007.

[4] European Medicines Agency and Committee for Medicinal Products for Human Use, "Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins intended for in-vivo clinical use," Doc. Ref. EMA/CHMP/BMWP/86289/2010, 2012.

[5] C. V. Pastuskovas, W. Mallet, S. Clark et al., "Effect of immune complex formation on the distribution of a novel antibody to the ovarian tumor antigen CA125," *Drug Metabolism and Disposition*, vol. 38, no. 12, pp. 2309–2319, 2010.

[6] J. A. Fox, T. E. Hotaling, C. Struble, J. Ruppel, D. J. Bates, and M. B. Schoenhoff, "Tissue distribution and complex formation with IgE of an anti-IgE antibody after intravenous administration in cynomolgus monkeys," *Journal of Pharmacology and Experimental Therapeutics*, vol. 279, no. 2, pp. 1000–1008, 1996.

[7] R. J. Brezski, J. L. Luongo, D. Petrone et al., "Human anti-IgG1 hinge autoantibodies reconstitute the effector functions of proteolytically inactivated IgGs," *Journal of Immunology*, vol. 181, no. 5, pp. 3183–3192, 2008.

[8] M. Keiserman, C. Codreanu, R. Handa et al., "The effect of antidrug antibodies on the sustainable efficacy of biologic therapies in rheumatoid arthritis: practical consequences," *Expert Review of Clinical Immunology*, vol. 10, no. 8, pp. 1049–1057, 2014.

[9] P. K. Rao, M. Dorsch, T. Chickering et al., "Isolation and characterization of the notch ligand Delta4," *Experimental Cell Research*, vol. 260, no. 2, pp. 379–386, 2000.

[10] J. R. Shutter, S. Scully, W. Fan et al., "D114, a novel Notch ligand expressed in arterial endothelium," *Genes and Development*, vol. 14, no. 11, pp. 1313–1318, 2000.

[11] J. Ridgway, G. Zhang, Y. Wu et al., "Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis," *Nature*, vol. 444, no. 7122, pp. 1083–1087, 2006.

[12] L. Jakobsson, C. A. Franco, K. Bentley et al., "Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting," *Nature Cell Biology*, vol. 12, no. 10, pp. 943–953, 2010.

[13] M. Yan, C. A. Callahan, J. C. Beyer et al., "Chronic DLL4 blockade induces vascular neoplasms," *Nature*, vol. 463, no. 7282, pp. E6–E7, 2010.

[14] J. A. Couch, G. Zhang, J. C. Beyer et al., "Balancing efficacy and safety of an anti-DLL4 antibody through pharmacokinetic modulation," *Clinical Cancer Research*, vol. 22, no. 6, pp. 1469–1479, 2016.

[15] R. J. Brezski, M. Kinder, K. D. Grugan et al., "A monoclonal antibody against hinge-cleaved IgG restores effector function to proteolytically-inactivated IgGs in vitro and in vivo," *mAbs*, vol. 6, no. 5, pp. 1265–1273, 2014.

[16] K. A. van Schie, G.-J. Wolbink, and T. Rispens, "Cross-reactive and pre-existing antibodies to therapeutic antibodies-effects on treatment and immunogenicity," *mAbs*, vol. 7, no. 4, pp. 662–671, 2015.

[17] R. J. Brezski and R. E. Jordan, "Cleavage of IgGs by proteases associated with invasive diseases: an evasion tactic against host immunity?" *mAbs*, vol. 2, no. 3, pp. 212–220, 2010.

[18] P. I. Terness, D. Navolan, C. Dufter, M. Welschof, and G. Opelz, "Immunosuppressive anti-immunoglobulin autoantibodies: specificity, gene structure and function in health and disease," *Cellular and Molecular Biology*, vol. 48, no. 3, pp. 271–278, 2002.

[19] B. Gorovits, A. Clements-Egan, M. Birchler et al., "Pre-existing antibody: biotherapeutic modality-based review," *The AAPS Journal*, vol. 18, no. 2, pp. 311–320, 2016.

[20] T. Rispens, H. de Vrieze, E. de Groot et al., "Antibodies to constant domains of therapeutic monoclonal antibodies: anti-hinge antibodies in immunogenicity testing," *Journal of Immunological Methods*, vol. 375, no. 1-2, pp. 93–99, 2012.

[21] S. Yano, S. Kaku, K.-I. Suzuki et al., "Natural antibodies against the immunoglobulin $F(ab')_2$ fragment cause elimination of antigens recognized by the $F(ab')_2$ from the circulation," *European Journal of Immunology*, vol. 25, no. 11, pp. 3128–3133, 1995.

[22] C. Süsal, J. Groth, H.-H. Oberg, P. Terness, G. May, and G. Opelz, "The association of kidney graft outcome with pretransplant serum IgG-Anti-$F(ab')_{2\gamma}$," *Transplantation*, vol. 54, no. 4, pp. 632–635, 1992.

[23] H. U. Lutz and S. Fumia, "Stimulation of complement amplification by F(ab′)2-containing immune complexes and naturally occurring anti-hinge antibodies, possible role in systemic inflammation," *Autoimmunity Reviews*, vol. 7, no. 6, pp. 508–513, 2008.

[24] X. Wang, V. Quarmby, C. Ng et al., "Generation and characterization of a unique reagent that recognizes a panel of recombinant human monoclonal antibody therapeutics in the presence of endogenous human IgG," *mAbs*, vol. 5, no. 4, pp. 540–554, 2013.

[25] A. R. Mire-Sluis, Y. C. Barrett, V. Devanarayan et al., "Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products," *Journal of Immunological Methods*, vol. 289, no. 1-2, pp. 1–16, 2004.

[26] E. Wakshull, R. Hendricks, C. Amaya, and D. Coleman, "Proposal for a new protein therapeutic immunogenicity titer assay cutpoint," *Bioanalysis*, vol. 3, no. 23, pp. 2627–2636, 2011.

[27] R. H. Schwartz, "Historical overview of immunological tolerance," *Cold Spring Harbor Perspectives in Biology*, vol. 4, no. 4, pp. 1–14, 2012.

[28] R. S. Liblau, R. Tisch, K. Shokat et al., "Intravenous injection of soluble antigen induces thymic and peripheral T-cell apoptosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 7, pp. 3031–3036, 1996.

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Research Article

Identification of Novel Vaccine Candidates against *Campylobacter* through Reverse Vaccinology

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Campylobacteriosis is the most prevalent bacterial foodborne gastroenteritis affecting humans in the European Union. Human cases are mainly due to *Campylobacter jejuni* or *Campylobacter coli*, and contamination is associated with the handling and/or consumption of poultry meat. In fact, poultry constitutes the bacteria's main reservoir. A promising way of decreasing the incidence of campylobacteriosis in humans would be to decrease avian colonization. Poultry vaccination is of potential for this purpose. However, despite many studies, there is currently no vaccine available on the market to reduce the intestinal *Campylobacter* load in chickens. It is essential to identify and characterize new vaccine antigens. This study applied the reverse vaccinology approach to detect new vaccine candidates. The main criteria used to select immune proteins were localization, antigenicity, and number of B-epitopes. Fourteen proteins were identified as potential vaccine antigens. *In vitro* and *in vivo* experiments now need to be performed to validate the immune and protective power of these newly identified antigens.

1. Introduction

Campylobacter is the leading cause of human bacterial gastroenteritis in Europe [1]. It has been estimated that 9 million people are affected each year, costing around €2.4 billion. *C. jejuni* is responsible for approximately 90% of cases, and *C. coli* is responsible for 10%. Other species can cause human campylobacteriosis but are more rarely involved [2]. Most infections are not severe, leading to gastroenteritis symptoms, but they can cause extraintestinal manifestations such as reactive arthritis, Guillain-Barré syndrome (GBS), or inflammatory bowel disease (IBD) [3]. In some cases, infection can even lead to death. Human contaminations are mainly associated with handling and/or consuming poultry meat [1]. Domestic and wild birds constitute the bacteria's main reservoir, carrying up to 10^9 CFU·g^{-1} of *Campylobacter* intestinally. In poultry flocks, natural colonization occurs in 2- to 3-week-old chicks by horizontal contamination from the environment [4], and birds remain infected until slaughter.

Decreasing avian colonization would appear to be an effective strategy for reducing the incidence of human campylobacteriosis. In 2013, Romero-Barrios et al. estimated that a reduction in *Campylobacter* cecal colonization from 2 to 3 $\log_{10}$ units could lead to a 100% reduction in the risk of human disease [5]. Along with the implementation of biosecurity, hygiene, and nutritional measures in flocks, poultry vaccination is one way of reducing avian intestine colonization by *Campylobacter* [6]. Several vaccine prototypes have already been tested with variable results. These include whole-cell, subunit, or microorganism-vectored vaccines. Globally, whole-cell vaccines have not been efficient in decreasing *Campylobacter* intestinal loads despite the induction of a specific immune response [7–10]. Among subunit vaccines, flagellin—described as the immunodominant antigen of

Campylobacter—has been tested and proved to be able to induce an immune response but this was not necessarily correlated with any decrease in chicken gut colonization [9, 11–13]. Furthermore, because of its weak homology across *Campylobacter* strains, flagellin-based vaccines do not induce cross-protection, making these vaccines inefficient in combatting all *C. jejuni* strains [14]. Other antigens such as CjaA [15]—a periplasmic protein—or CadF, FlpA, CmeC [16], and Dsp proteins [17] involved in *Campylobacter* adherence during colonization have also been trialed as subunit vaccines. In the same way, total outer membrane proteins [18] or fusion proteins [16] have also been tested. Another strategy is to deliver vaccine antigens by vectors such as attenuated bacterial strains. *Salmonella enterica* serovar Typhimurium [15, 19–21] and *Eimeria tenella* [22] have been evaluated as a vector for *C. jejuni* CjaA delivery. For example, in 2004, Wyszyńska et al. [19] indicated that chickens orally immunized with a virulent *Salmonella* strain carrying the *Campylobacter* CjaA gene develop a strong specific antibody response, and birds were protected from colonization after a homologous *C. jejuni* challenge. Recently, the same team was unable to confirm these results [21]. Other antigens were tested in the same way, including Omp18/CjaD, ACE393 [20], Dsp [17], Peb1A, GlnH, and ChuA [15]. Some of these experimental studies gave promising results, combining both the induction of a humoral immune response and a decrease in *Campylobacter* intestinal colonization in poultry, but experimentation has not yet been followed up. So, despite much research, no anti-*Campylobacter* vaccine aiming to reduce bacterial colonization in the poultry gut is yet available.

Identifying new potential vaccine antigens is one way of speeding up the development of new vaccines. Reverse vaccinology—a recent approach first described by Rappuoli in the early 2000s [23]—is used to predict antigens through the development of genomics and bioinformatic tools such as genome sequencing. This strategy is different from Hoppe et al. approach, where they identified novel immunodominant proteins by *in vitro* screening of mRNA of *C. jejuni* [24]. The following selection criteria are of particular importance for the reverse vaccinology approach. To be potentially good candidates, the selected proteins must be surface-exposed and able to be recognized by the immune system. Proteins with adhesin capacities are known to be involved in bacterial pathogenicity and invasion, so adhesins or adhesin-like proteins appear as good vaccine targets. The transmembrane helix number is also an important criterion. Indeed, it is difficult to purify proteins with more than one transmembrane helix, and it seems wise to exclude these proteins from the selection process [25]. Individual antigenicity and B-epitope density (the ratio between the number of B-epitopes and the protein length) need to be assessed as described by Oprea's study aimed at developing a vaccine against *S. aureus* endocarditis [26]. Although a few studies are describing innate intestinal inflammations and gut mucosa lesions upon *Campylobacter jejuni* infection (like in [27]), these bacteria are mostly described as a commensal organism for poultry [28]. In the avian intestinal tract, intensive *Campylobacter* multiplication occurs in the mucus layer of the epithelial cells. In this way, antigens need to induce a humoral immune response to neutralize and eliminate *Campylobacter* from the avian intestinal gut. The induction of a cytotoxic cellular response may not be a selection criterion since *Campylobacter* multiplication in intestinal epithelial cells of chickens was not clearly highlighted [28]. Anyway, bioinformatic tools aiming at predicting T epitopes for avian vaccines are still poorly developed, limiting the reverse vaccinology analysis in this goal. Finally, to provide cross-protection and avoid autoimmune response, it is essential that vaccine candidates are common to many pathogenic strains and do not mimic host proteins [25].

Our research identifies new potential vaccine antigens against *Campylobacter* using the reverse vaccinology strategy to develop an avian vaccine which could impact the incidence of human campylobacteriosis.

2. Material and Methods

2.1. Bacterial Strain. The highly virulent *Campylobacter jejuni* subsp. *jejuni* 81-176 strain was chosen for this *in silico* analysis. Its genome is available on the NCBI website under accession number CP000538.1 and listed in the Vaxign program used below.

2.2. OMP and Extracellular Protein Preselection. Vaxign (http://www.violinet.org/vaxign/index.php) was used to shortlist proteins with potential as vaccine candidates due to their cellular localization, probability of having adhesin-like characteristics, and number of transmembrane helixes [25]. Vaxign is a web-based pipeline dedicated to vaccine design and integrating several bioinformatic programs. Subcellular localization is predicted using PSORTb2.0 [29]. The probability of adhesin characteristics is predicted by SPAAN software [30] and the transmembrane helix topology is predicted by HMMTOP [31] using a hidden Markov model.

Campylobacter jejuni subsp. *jejuni* 81-176 is available in the Vaxign database of over 350 listed genomes, along with nine other *Campylobacter* genomes. Extracellular and outer membrane proteins having an adhesin probability score > 0.51 and either 1 or 0 transmembrane helixes were preselected.

2.3. Protein Antigenicity. VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) was used to predict protein antigenicity. This software uses the physicochemical properties of proteins to predict their antigenicity from FASTA-submitted amino acid sequences. This feature is characterized according to an antigenic score. Proteins with an antigenic score > 0.5 were selected as described by Doytchinova and Flower [32].

2.4. Epitope B Prediction. BCPreds software (http://ailab.ist.psu.edu/bcpred/) was used to identify B-cell epitopes in FASTA-submitted amino acid sequences. This program provides two methods based on different algorithms: the amino acid pair (AAP) antigenicity method [33] and the BCPreds method using string kernels [34]. These methods predict antigenic linear nonoverlapping 20-mer epitopes from the whole antigen. Each preselected protein was analyzed and B-cell epitopes with a score >0.8 were accepted (specificity > 80%).

The selected epitopes were again submitted to VaxiJen software to check their individual antigenicity and those having an antigenic score >0.5 were selected. Furthermore, for each protein and each algorithm, the ratio of B-epitopes to protein length was calculated to assess B-epitope density.

2.5. BLAST. In order to assess conservation of the selected proteins in the different Campylobacter strains, tblastn analyses were performed for each amino acid sequence against both *C. jejuni* and *C. coli* whole genomes available on the NCBI site on the day of analysis (February 9, 2016): 93 for *C. jejuni* and nine for *C. coli*. The identity percentage was set to 80% and the minimum query coverage was set to 50%. The amount and percentage of sharing among the available genomes were determined. The proteins with a sharing percentage lower than 80% (i.e., about the value for the flagellin) were eliminated from the protein shortlist.

A blastp analysis was also performed to ensure that the host *Gallus gallus* does not express the selected proteins. The identity percentage was set to 50% and the minimum query coverage was set to 50%.

3. Results

The reverse vaccinology protocol applied here and results are summarized in Figure 1.

3.1. Protein Preselection. The Vaxign server was used to preselect vaccine candidates. Of the 1758 ORFs encoded by the *C. jejuni* 81-176 genome, only 24 were identified as potential vaccine antigens according to the applied criteria (localization, adhesion features, and number of transmembrane helixes) (Table 1). Of these 24 identified ORFs, we found the two known flagellins A and B, which means that 22 new potential antigens were selected at this step.

3.2. Protein Selection according to Antigenicity and Number of B-Epitopes. To refine the selection, the 22 preselected proteins were submitted to the VaxiJen server for antigenicity prediction. Antigenicity scores ranged from 0.4511 to 0.7827. This step allowed the elimination of two proteins with an antigenicity score lower than 0.5 (YP_001000503.1 and YP_001000297.1) (Table 1). The VaxiJen software indicated that all other candidates were antigenic (score > 0.5).

Each antigenic protein was assessed in terms of B-epitopes using BCPreds and AAP algorithms, and each B-epitope was studied for its antigenicity. Table 1 summarizes the number of B-epitopes predicted for each protein and each algorithm as well as the ratio between the number of B-epitopes and protein length.

3.3. Conservation of the Selected Proteins in the Sequenced C. jejuni and C. coli Strains. tblastn analyses were performed in order to assess the individual sharing of the preselected proteins among *C. jejuni* and *C. coli* strains. As shown in Table 2, all the proteins were shared with available *C. coli* strains except YP_001001027.1, which was also poorly shared with the available *C. jejuni* strains (6%). This protein was therefore removed from the list of potential vaccine antigens. Of

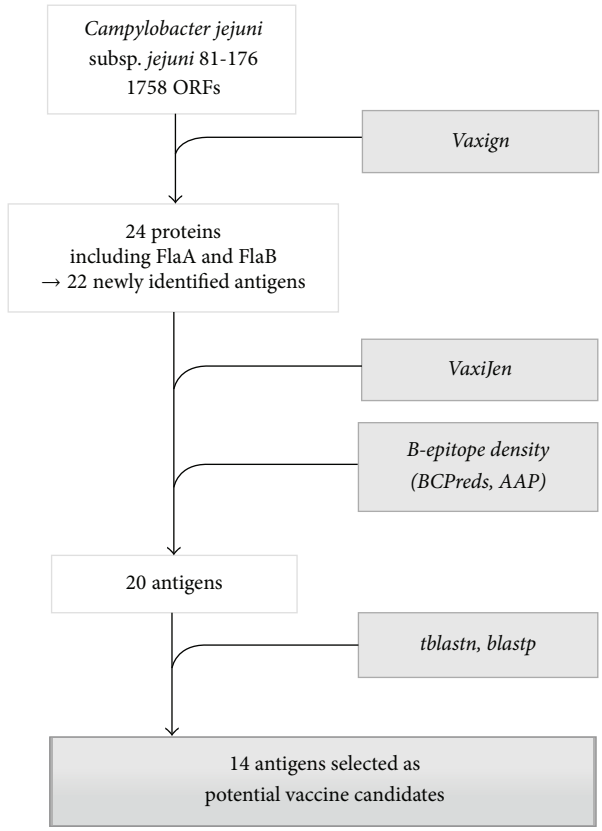

Figure 1: Summary of the reverse vaccinology protocol applied to *Campylobacter jejuni* for the selection of vaccine candidates.

the remaining 19 shortlisted proteins, five—YP_001001371.1, YP_001000248.1, YP_001000204.1, YP_001000654.1 and YP_001000615.1—were removed from the candidate list because of poor sharing among *C. jejuni* strains (<80%).

Table 2 also shows that none of the proteins are expressed by *Gallus gallus*.

3.4. Final Selection. Table 3 shows the final selection of potential vaccine candidates after the whole bioinformatic analysis process. Fourteen candidates were selected. Of these, three are extracellular proteins whereas the others are outer membrane proteins. Four flagellar proteins were identified and several were not characterized and designated as hypothetical proteins.

4. Discussion

In the last decades, advances in genomics, genome sequencing, and annotation, coupled with the development of bioinformatic tools has revolutionized vaccine development strategy. Reverse vaccinology allows vaccines to be designed even for noncultivable pathogens; genome availability is the only factor enabling *in silico* analysis or not. All the proteins are targeted even if only transiently expressed or scarce during infection. Furthermore, this strategy considerably reduces the time needed to develop new vaccines [35]. Reverse vaccinology was first successfully applied to the development

Table 1: Potential vaccine candidates selected by the Vaxign program. Localization and length were obtained by the Vaxign program, antigenic score was obtained by the VaxiJen program, and the number of B-cell epitopes was obtained from both BCPreds and AAP methods. The calculated ratio between the number of B-epitopes and protein length is also shown. The two candidates eliminated because of a low antigenic score are shown in italics.

Protein accession	Description	ID	Localization	Length (aa)	Vaxijen score	BCPreds B-epitopes		AAP B-epitopes	
						N	Ratio *N*/length	*N*	Ratio *N*/length
YP_001000996.1	Flagellin B	FlaB	Extracellular	576	0.7650	10	0.017	11	0.019
YP_001000997.1	Flagellin A	FlaA	Extracellular	576	0.8185	11	0.019	12	0.021
YP_001001371.1	Flagellar hook protein	FlgE	Extracellular	838	0.7659	16	0.019	18	0.021
YP_001000562.1	Flagellin protein family		Extracellular	750	0.6965	15	0.020	15	0.020
YP_001000248.1	Flagellar capping protein	FliD	Extracellular	642	0.7021	11	0.017	11	0.017
YP_001000204.1	Putative periplasmic protein		OMP	553	0.6702	11	0.021	11	0.021
YP_001000654.1	Putative periplasmic protein		OMP	553	0.6702	11	0.021	11	0.021
YP_999769.1	Flagellar hook protein	FlgE-1	Extracellular	545	0.6567	10	0.018	12	0.022
YP_001001115.1	Flagellar hook-associated protein	FlgK	Extracellular	608	0.5836	11	0.018	10	0.016
YP_001000153.1	TonB-dependent receptor, putative, degenerate		OMP	704	0.5437	12	0.017	9	0.013
YP_001000945.1	N-Acetylmuramoyl-L-alanine amidase		OMP	659	0.6475	9	0.014	11	0.017
YP_001001027.1	Serine protease		OMP	1121	0.5268	9	0.008	11	0.010
YP_001000437.1	Putative OMP		OMP	508	0.6122	9	0.018	6	0.012
YP_999838.1	Hypothetical protein		OMP	400	0.6809	5	0.013	9	0.023
YP_999817.1	Hypothetical protein		OMP	315	0.7827	6	0.019	7	0.022
YP_001000383.1	Flagellar basal body L-ring protein	FlgH	OMP	232	0.6978	6	0.026	4	0.017
YP_001000935.1	Major OMP	PorA	OMP	424	0.6051	5	0.012	5	0.012
YP_001001008.1	Phospholipase A	PldA	OMP	329	0.5819	4	0.012	3	0.009
YP_001001257.1	TonB-dependent heme receptor	ChuA	OMP	702	0.6213	7	0.010	5	0.007
YP_001000615.1	Hypothetical protein		Extracellular	294	0.5498	3	0.010	3	0.010
YP_001000663.1	Surface-exposed lipoprotein	JlpA	OMP	372	0.6642	2	0.005	3	0.008
YP_001000261.1	Hypothetical protein		OMP	309	0.5149	2	0.006	3	0.010
YP_001000503.1	*Hypothetical protein*		*Extracellular*	*444*	*0.4603*	/	/	/	/
YP_001000297.1	*Major antigenic peptide*	*PEB4*	*OMP*	*273*	*0.4511*	/	/	/	/

Table 2: Potential vaccine candidates selected after blast analysis. tblastn analyses were performed for each amino acid sequence against both *C. jejuni* and *C. coli* whole genomes available on the NCBI site. The amount and percentage of sharing among the available genomes were determined. A blastp analysis was also performed against the host *Gallus gallus*. The six candidates eliminated because of poor sharing among *Campylobacter* strains are shown in italics.

Protein accession	Description	ID	Sharing among *C. jejuni* strains		Sharing among*C. coli* strains		Similarity in *Gallus gallus*
			N/93	%	*N*/9	%	
YP_001000996.1	Flagellin B	FlaB	77	83	9	100	No
YP_001000997.1	Flagellin A	FlaA	75	81	9	100	No
YP_001001371.1	*Flagellar hook protein*	*FlgE*	*15*	*16*	*8*	*89*	*No*
YP_001000562.1	Flagellin protein family		93	100	9	100	No
YP_001000248.1	*Flagellar capping protein*	*FliD*	*38*	*41*	*9*	*100*	*No*
YP_001000204.1	*Putative periplasmic protein*		*2*	*2*	*9*	*100*	*No*
YP_001000654.1	*Putative periplasmic protein*		*2*	*2*	*9*	*100*	*No*
YP_999769.1	Flagellar hook protein	FlgE-1	93	100	9	100	No
YP_001001115.1	Flagellar hook-associated protein	FlgK	93	100	9	100	No
YP_001000153.1	TonB-dependent receptor, putative, degenerate		90	97	9	100	No
YP_001000945.1	N-Acetylmuramoyl-L-alanine amidase		93	100	9	100	No
YP_001001027.1	*Serine protease*	*PEB4*	*6*	*6*	*1*	*11*	*No*
YP_001000437.1	Putative OMP		89	96	6	67	No
YP_999838.1	Hypothetical protein		93	100	9	100	No
YP_999817.1	Hypothetical protein		92	99	9	100	No
YP_001000383.1	Flagellar basal body L-ring protein	FlgH	93	100	9	100	No
YP_001000935.1	Major OMP	PorA	81	87	9	100	No
YP_001001008.1	Phospholipase A	PldA	92	99	9	100	No
YP_001001257.1	TonB-dependent heme receptor	ChuA	93	100	9	100	No
YP_001000615.1	*Hypothetical protein*		*64*	*69*	*9*	*100*	*No*
YP_001000663.1	Surface-exposed lipoprotein	JlpA	93	100	9	100	No
YP_001000261.1	Hypothetical protein		92	99	9	100	No

Table 3: Potential vaccine candidates selected after the whole bioinformatic analysis process including Vaxign and VaxiJen programs, BCPreds and AAP algorithms, and blast analyses. Of 1758 ORFs encoded by *C. jejuni*, strain 81-176 genome, 14 proteins were selected as vaccine candidates.

Protein accession	Description	Localization	ID
YP_001000562.1	Flagellin protein family	Extracellular	
YP_999769.1	Flagellar hook protein	Extracellular	FlgE-1
YP_001001115.1	Flagellar hook-associated protein	Extracellular	FlgK
YP_001000153.1	TonB-dependent receptor, putative, degenerate	OMP	
YP_001000945.1	N-Acetylmuramoyl-L-alanine amidase	OMP	
YP_001000437.1	Putative OMP	OMP	
YP_999838.1	Hypothetical protein	OMP	
YP_999817.1	Hypothetical protein	OMP	
YP_001000383.1	Flagellar basal body L-ring protein	OMP	FlgH
YP_001000935.1	Major OMP	OMP	PorA
YP_001001008.1	Phospholipase A	OMP	PldA
YP_001001257.1	TonB-dependent heme receptor	OMP	ChuA
YP_001000663.1	Surface-exposed lipoprotein	OMP	JlpA
YP_001000261.1	Hypothetical protein	OMP	

of a vaccine against B serogroup *Neisseria meningitidis* [36]. Despite available prophylactic vaccines based on capsular polysaccharides (CPS) for four *N. meningitidis* serogroups (A, C, W, and Y), the development of a capsular vaccine against serogroup B was not possible because of CPS mimicry of polysialic acid in human cells. *In silico* analysis identified three proteins (fHbp, NadA, and NHBA) which were combined with outer membrane vesicles containing known antigen PorA and led to the European licensure of the 4CMenB vaccine in 2013 [37]. This strategy was then applied to several other pathogens such as herpes simplex viruses using the Vaxign program [38], *Staphylococcus aureus* for the *in silico* characterization of ten surface-exposed proteins [26], *Mycobacterium tuberculosis* with the identification of six novel antigen candidates to improve the tuberculosis vaccine [39], or *Streptococcus pneumonia* with the bioinformatic assessment of 13 protein targets [40]. The antigenicity and efficiency of the potential candidates selected in these last *in silico* studies have not yet been tested *in vitro* or *in vivo*.

Until now, and despite many studies, conventional development of a vaccine against *Campylobacter* in poultry has not led to an efficient vaccine in terms of immunogenicity and protection. Since 2005, *Campylobacter* has been and remains today the leading cause of bacterial foodborne gastroenteritis in Europe [1]. As poultry vaccination is one of the potential ways of reducing the incidence of human campylobacteriosis, it is important to pursue efforts to test new vaccine antigens. Reverse vaccinology is a suitable strategy to this end.

This *in silico* study predictively identified new vaccine antigens against *Campylobacter*. The reference *C. jejuni* ATCC (American Type Culture Collection) strain 81-176 was chosen for antigen prediction because of its high virulence in human diseases (namely, the chicken vaccine strategy is to prevent human infections). Even if this strain is not a good colonizer for chickens, this strain has been successfully used in several poultry experiments with high colonization levels [18]. Moreover, the reverse vaccinology aims to identify shared proteins among many *Campylobacter* strains (here more than 100 strains). Thereby, other more avian colonizer *Campylobacter* strains should be used for *in vivo* challenge experiments to evaluate the effectiveness of the proteins found by the bioinformatics analysis of the *C. jejuni* 81-176 genome.

Based on their cellular localization, adhesin-like properties, antigenicity, B-epitope density, and conservation among *Campylobacter* strains, 14 proteins were selected. It was decided to eliminate proteins with a sharing percentage lower than the flagellin sharing percentage. It has already been observed that flagellin could not be used as a vaccine candidate because of poor sharing among *Campylobacter* strains and the lack of cross-protection [14]. The known vaccine antigens of flagellins A and B were also identified alongside potential antigens using the same criteria. This strengthens the validity of the bioinformatic protocol used, because the flagellin has already been described and used as the immune-dominant antigen of *Campylobacter* [9, 11, 12]. However, it is important to keep in mind that the identified proteins were selected on the basis of predictions by various algorithms. Only *in vitro* and more *in vivo* experiments will confirm or refute the proteins' immune power. In terms of antigen ranking, proteins with a high antigenicity score and B-epitope density seem to be the best vaccine candidates and should therefore be evaluated for *in vivo* immunogenicity as a priority. Indeed, it has already been demonstrated that a high epitope density significantly enhances antigenicity and immunogenicity [41]. This strategy, being based on genome analysis, does not take into consideration lipid and saccharide antigens, which could also have immune properties. Concerning *Campylobacter*, capsule polysaccharides are not targeted through the reverse vaccinology protocol, although they could be immunogenic [42].

Several of the identified proteins had already been characterized and were mainly associated with *Campylobacter* virulence [43]. This is the case for three selected flagellar

proteins—FlgE-1, FlgK, and FlgH—involved in *Campylobacter* motility, essential for bacteria survival in the gastrointestinal tract. These proteins were recently tested *in vitro* along with other flagellar proteins [44]. The first two were immunostained by more than 70% of tested sera from chickens older than 5-6 weeks; the third one was immunostained by 50% of the tested sera. The present *in silico* analysis is in line with these *in vitro* results, leading us to consider these three flagellar proteins as a potential vaccine antigen. However, no *in vivo* assessment is available yet. The FliD flagellar protein was similarly tested *in vitro* and was observed to react strongly to sera from chickens over 4 weeks of age [45]. In the present analysis, this flagellar protein was eliminated from the shortlist because of poor sharing with other *Campylobacter* strains (41%). Moreover, the ChuA protein—involved in the iron uptake system—had already been tested in an avian vaccine experiment using attenuated *Salmonella* as a vector [15] and did not significantly reduce cecal *Campylobacter* counts. Furthermore, the major PorA outer membrane protein was tested *in vivo* in a mouse model [46]. Mice vaccinations led to significantly higher antibody levels in serum and intestinal lavage fluids. A decrease in *C. jejuni* colonization levels was also observed after a heterologous challenge. Phospholipase A (PldA) and lipoprotein JlpA are involved in *Campylobacter* adhesion since it has been demonstrated that mutations of *pldA* impair the ability of *C. jejuni* to colonize cecum [47] and since Jin et al. highlighted the interaction of JlpA with a surface-exposed protein of epithelial cells [48].

To conclude, reverse vaccinology—a powerful tool for identifying new vaccine antigens—allowed 14 candidates to be selected for the development of a vaccine against *Campylobacter* in poultry. Several antigens identified as potential vaccine candidates are currently under *in vitro* and *in vivo* investigations to evaluate their immunogenicities and protective potentials against *Campylobacter* in chickens.

Competing Interests

The authors declare that there is no conflict of interests regarding publication of this paper.

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References

[1] EFSA, "The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013," *EFSA Journal*, vol. 13, no. 1, p. 3991, 2015.

[2] I. A. Gillespie, S. J. O'Brien, J. A. Frost et al., "A case-case comparison of *Campylobacter coli* and *Campylobacter jejuni* infection: a tool for generating hypotheses," *Emerging Infectious Diseases*, vol. 8, no. 9, pp. 937–942, 2002.

[3] R. Janssen, K. A. Krogfelt, S. A. Cawthraw, W. van Pelt, J. A. Wagenaar, and R. J. Owen, "Host-pathogen interactions in Campylobacter infections: the host perspective," *Clinical Microbiology Reviews*, vol. 21, no. 3, pp. 505–518, 2008.

[4] O. Sahin, N. Luo, S. Huang, and Q. Zhang, "Effect of Campylobacter-specific maternal antibodies on Campylobacter jejuni colonization in young chickens," *Applied and Environmental Microbiology*, vol. 69, no. 9, pp. 5372–5379, 2003.

[5] P. Romero-Barrios, M. Hempen, W. Messens, P. Stella, and M. Hugas, "Quantitative microbiological risk assessment (QMRA) of food-borne zoonoses at the European level," *Food Control*, vol. 29, no. 2, pp. 343–349, 2013.

[6] M. Meunier, M. Guyard-Nicodème, D. Dory, and M. Chemaly, "Control strategies against *Campylobacter* at the poultry production level: biosecurity measures, feed additives and vaccination," *Journal of Applied Microbiology*, vol. 120, no. 5, pp. 1139–1173, 2016.

[7] B. E. Rice, D. M. Rollins, E. T. Mallinson, L. Carr, and S. W. Joseph, "Campylobacter jejuni in broiler chickens colonization and humoral immunity following oral vaccination and experimental infection," *Vaccine*, vol. 15, no. 17-18, pp. 1922–1932, 1997.

[8] G. Glünder, N. Spiering, and K. Hinz, "Investigations on parenteral immunization of chickens with a Campylobacter mineral oil vaccine," in *Proceedings of the International Congress of the World Veterinary Poultry Association*, B. Nagy and R. Mulder, Eds., pp. 247–253, Budapest, Hungary, 1997.

[9] P. R. Widders, L. M. Thomas, K. A. Long, M. A. Tokhi, M. Panaccio, and E. Apos, "The specificity of antibody in chickens immunised to reduce intestinal colonisation with *Campylobacter jejuni*," *Veterinary Microbiology*, vol. 64, no. 1, pp. 39–50, 1998.

[10] R. L. Ziprin, M. E. Hume, C. R. Young, and R. B. Harvey, "Inoculation of chicks with viable non-colonizing strains of *Campylobacter jejuni*: evaluation of protection against a colonizing strain," *Current Microbiology*, vol. 44, no. 3, pp. 221–223, 2002.

[11] C. A. Khoury and R. J. Meinersmann, "A genetic hybrid of the *Campylobacter jejuni* flaA Gene with LT-B of *Escherichia coli* and assessment of the efficacy of the hybrid protein as an oral chicken vaccine," *Avian Diseases*, vol. 39, no. 4, pp. 812–820, 1995.

[12] J.-L. Huang, Y.-X. Yin, Z.-M. Pan et al., "Intranasal immunization with chitosan/pCAGGS-flaA nanoparticles inhibits *Campylobacter jejuni* in a White Leghorn model," *Journal of Biomedicine and Biotechnology*, vol. 2010, Article ID 589476, 8 pages, 2010.

[13] M. Meunier, M. Guyard-Nicodème, E. Vigouroux, T. Poezevara, and V. Beven, *Sequential Optimization of an Avian Vaccine Protocol Against Campylobacter*, 2015.

[14] M. R. de Zoete, J. P. M. van Putten, and J. A. Wagenaar, "Vaccination of chickens against Campylobacter," *Vaccine*, vol. 25, no. 30, pp. 5548–5557, 2007.

[15] A. M. Buckley, J. Wang, D. L. Hudson et al., "Evaluation of live-attenuated Salmonella vaccines expressing Campylobacter antigens for control of C. jejuni in poultry," *Vaccine*, vol. 28, no. 4, pp. 1094–1105, 2010.

[16] J. M. Neal-McKinney, D. R. Samuelson, T. P. Eucker, M. S. Nissen, R. Crespo, and M. E. Konkel, "Reducing campylobacter jejuni colonization of poultry via vaccination," *PLoS ONE*, vol. 9, no. 12, Article ID e114254, 2014.

[17] J. R. Theoret, K. K. Cooper, B. Zekarias et al., "The *Campylobacter jejuni* dps homologue is important for *In vitro* biofilm

formation and cecal colonization of poultry and may serve as a protective antigen for vaccination," *Clinical and Vaccine Immunology*, vol. 19, no. 9, pp. 1426–1431, 2012.

[18] T. Annamalai, R. Pina-Mimbela, A. Kumar et al., "Evaluation of nanoparticle-encapsulated outer membrane proteins for the control of *Campylobacter jejuni* colonization in chickens," *Poultry Science*, vol. 92, no. 8, pp. 2201–2211, 2013.

[19] A. Wyszyńska, A. Raczko, M. Lis, and E. K. Jagusztyn-Krynicka, "Oral immunization of chickens with avirulent Salmonella vaccine strain carrying C. jejuni 72Dz/92 cjaA gene elicits specific humoral immune response associated with protection against challenge with wild-type Campylobacter," *Vaccine*, vol. 22, no. 11-12, pp. 1379–1389, 2004.

[20] S. L. Layton, M. J. Morgan, K. Cole et al., "Evaluation of Salmonella-vectored Campylobacter peptide epitopes for reduction of Campylobacter jejuni in broiler chickens," *Clinical and Vaccine Immunology*, vol. 18, no. 3, pp. 449–454, 2011.

[21] P. Łaniewski, M. Kuczkowski, K. Chrzastek et al., "Evaluation of the immunogenicity of *Campylobacter jejuni* CjaA protein delivered by *Salmonella enterica* sv. Typhimurium strain with regulated delayed attenuation in chickens," *World Journal of Microbiology and Biotechnology*, vol. 30, no. 1, pp. 281–292, 2014.

[22] J. D. Clark, R. D. Oakes, K. Redhead et al., "Eimeria species parasites as novel vaccine delivery vectors: anti-*Campylobacter jejuni* protective immunity induced by *Eimeria tenella*-delivered CjaA," *Vaccine*, vol. 30, no. 16, pp. 2683–2688, 2012.

[23] R. Rappuoli, "Reverse vaccinology," *Current Opinion in Microbiology*, vol. 3, no. 5, pp. 445–450, 2000.

[24] S. Hoppe, F. F. Bier, and M. von Nickisch-Rosenegk, "Rapid identification of novel immunodominant proteins and characterization of a specific linear epitope of *Campylobacter jejuni*," *PLoS ONE*, vol. 8, no. 5, Article ID e65837, 2013.

[25] Y. He, Z. Xiang, and H. L. Mobley, "Vaxign: the first web-based vaccine design program for reverse vaccinology and applications for vaccine development," *Journal of Biomedicine and Biotechnology*, vol. 2010, Article ID 297505, 15 pages, 2010.

[26] M. Oprea and F. Antohe, "Reverse-vaccinology strategy for designing T-cell epitope candidates for *Staphylococcus aureus* endocarditis vaccine," *Biologicals*, vol. 41, no. 3, pp. 148–153, 2013.

[27] S. Humphrey, G. Chaloner, K. Kemmett et al., "*Campylobacter jejuni* is not merely a commensal in commercial broiler chickens and affects bird welfare," *mBio*, vol. 5, no. 4, Article ID e01364-14, 2014.

[28] K. Van Deun, F. Pasmans, R. Ducatelle et al., "Colonization strategy of *Campylobacter jejuni* results in persistent infection of the chicken gut," *Veterinary Microbiology*, vol. 130, no. 3-4, pp. 285–297, 2008.

[29] J. L. Gardy, M. R. Laird, F. Chen et al., "PSORTb v.2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis," *Bioinformatics*, vol. 21, no. 5, pp. 617–623, 2005.

[30] G. Sachdeva, K. Kumar, P. Jain, and S. Ramachandran, "SPAAN: a software program for prediction of adhesins and adhesin-like proteins using neural networks," *Bioinformatics*, vol. 21, no. 4, pp. 483–491, 2005.

[31] L. Käll, A. Krogh, and E. L. L. Sonnhammer, "Advantages of combined transmembrane topology and signal peptide prediction—the Phobius web server," *Nucleic Acids Research*, vol. 35, no. 2, pp. W429–W432, 2007.

[32] I. A. Doytchinova and D. R. Flower, "VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines," *BMC Bioinformatics*, vol. 8, article 4, 2007.

[33] J. Chen, H. Liu, J. Yang, and K.-C. Chou, "Prediction of linear B-cell epitopes using amino acid pair antigenicity scale," *Amino Acids*, vol. 33, no. 3, pp. 423–428, 2007.

[34] Y. El-Manzalawy, D. Dobbs, and V. Honavar, "Predicting linear B-cell epitopes using string kernels," *Journal of Molecular Recognition*, vol. 21, no. 4, pp. 243–255, 2008.

[35] A. Sette and R. Rappuoli, "Reverse vaccinology: developing vaccines in the era of genomics," *Immunity*, vol. 33, no. 4, pp. 530–541, 2010.

[36] M. Pizza, V. Scarlato, V. Masignani et al., "Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing," *Science*, vol. 287, no. 5459, pp. 1816–1820, 2000.

[37] S. M. Andrews and A. J. Pollard, "A vaccine against serogroup B *Neisseria meningitidis*: dealing with uncertainty," *The Lancet Infectious Diseases*, vol. 14, no. 5, pp. 426–434, 2014.

[38] Z. Xiang and Y. He, "Genome-wide prediction of vaccine targets for human herpes simplex viruses using Vaxign reverse vaccinology," *BMC Bioinformatics*, vol. 14, supplement 4, article S2, 2013.

[39] G. P. Monterrubio-López, J. A. González-Y-Merchand, and R. M. Ribas-Aparicio, "Identification of novel potential vaccine candidates against tuberculosis based on reverse vaccinology," *BioMed Research International*, vol. 2015, Article ID 483150, 16 pages, 2015.

[40] A. P. C. Argondizzo, F. F. da Mota, C. P. Pestana et al., "Identification of proteins in *Streptococcus pneumoniae* by reverse vaccinology and genetic diversity of these proteins in clinical isolates," *Applied Biochemistry and Biotechnology*, vol. 175, no. 4, pp. 2124–2165, 2015.

[41] W. Liu and Y.-H. Chen, "High epitope density in a single protein molecule significantly enhances antigenicity as well as immunogenicity: a novel strategy for modern vaccine development and a preliminary investigation about B cell discrimination of monomeric proteins," *European Journal of Immunology*, vol. 35, no. 2, pp. 505–514, 2005.

[42] P. Guerry, F. Poly, M. Riddle, A. C. Maue, Y.-H. Chen, and M. A. Monteiro, "Campylobacter polysaccharide capsules: virulence and vaccines," *Frontiers in Cellular and Infection Microbiology*, vol. 2, p. 7, 2012.

[43] D. J. Bolton, "Campylobacter virulence and survival factors," *Food Microbiology*, vol. 48, pp. 99–108, 2015.

[44] H.-Y. Yeh, K. L. Hiett, and J. E. Line, "Reactions of chicken sera to recombinant *Campylobacter jejuni* flagellar proteins," *Archives of Microbiology*, vol. 197, no. 2, pp. 353–358, 2015.

[45] H.-Y. Yeh, K. L. Hiett, J. E. Line, and B. S. Seal, "Characterization and antigenicity of recombinant campylobacter jejuni flagellar capping protein FliD," *Journal of Medical Microbiology*, vol. 63, no. 4, pp. 602–609, 2014.

[46] A. Islam, R. Raghupathy, and M. J. Albert, "Recombinant porA, the major outer membrane protein of *Campylobacter jejuni*, provides heterologous protection in an adult mouse intestinal colonization model," *Clinical and Vaccine Immunology*, vol. 17, no. 11, pp. 1666–1671, 2010.

[47] R. L. Ziprin, C. R. Young, J. A. Byrd et al., "Role of Campylobacter jejuni potential virulence genes in cecal colonization," *Avian Diseases*, vol. 45, no. 3, pp. 549–557, 2001.

[48] S. Jin, Y. C. Song, A. Emili, P. M. Sherman, and V. L. Chan, "JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90α and triggers signalling pathways leading to the activation of NF-κB and p38 MAP kinase in epithelial cells," *Cellular Microbiology*, vol. 5, no. 3, pp. 165–174, 2003.

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Review Article

Understanding the Supersensitive Anti-Drug Antibody Assay: Unexpected High Anti-Drug Antibody Incidence and Its Clinical Relevance

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Numbers of biotherapeutic products in development have increased over past decade. Despite providing significant benefits to patients with unmet needs, almost all protein-based biotherapeutics could induce unwanted immunogenicity, which result in a loss of efficacy and/or increase the risk of adverse reactions, such as infusion reactions, anaphylaxis, and even life-threatening response to endogenous proteins. Recognizing these possibilities, regulatory agencies request that immunogenicity be assessed as part of the approval process for biotherapeutics. Great efforts have been made to reduce drug immunogenicity through protein engineering. Accordingly the immunogenicity incidence has been reduced from around 80% in murine derived products to 0–10% in fully human products. However, recent improvements in immunogenicity assays have led to unexpectedly high immunogenicity rates, even in fully human products, leading to new challenges in assessing immunogenicity and its clinical relevance. These new immunogenicity assays are becoming supersensitive and able to detect more of anti-drug antibodies (ADA) than with earlier assays. This paper intends to review and discuss our understanding of the supersensitive ADA assay and the unexpected high ADA incidence and its potential clinical relevance.

1. Introduction

The approvals of the first recombinant human protein (insulin, 1982) and the first therapeutic monoclonal antibody (Muromonab-CD3, OKT3, 1985) symbolized the start of the new biotherapeutic era. Since then, development of biotherapeutics (i.e., biologics, biopharmaceuticals, biological products, and biological medicinal products/drugs) has increased. Today, more than 250 approved biotherapeutics are available for unmet medical needs and there are estimated >500 biotherapeutics at various stages of development [1, 2].

One of the major differences between large molecule-based biotherapeutics and traditional small molecule drugs is the potential of biotherapeutics to produce unwanted immunogenicity. The patient's immune system recognizes the administered biotherapeutic as foreign and produces ADA against the foreign molecule. While new technologies have greatly reduced the primary sequence of the protein to be essentially human the immune system can still recognize differences due to both the production lines (i.e., grown in NS0, CHO, or insect cell lines) which may lead to alterations in secondary and tertiary structures (i.e., glycosylation patterns and protein misfolding) and formulation as foreign. In case of therapeutic fusion protein, bispecific antibody, PEGylated antibody and antibody drug conjugate (ADC), and so forth the region where the two molecules join may form a neoantigen (foreignness), and immune responses to this region may arise. In addition, the regions in which these proteins are added (i.e., subcutaneous or intramuscular) also increase the foreignness of the protein. Although a cellular immune response is also involved in drug immunogenicity, the regulatory agencies usually do not request assessment of cellular immunogenicity as the formation of class switched antibodies is highly dependent on the cellular immune response. Therefore this review will not cover cellular immunogenicity.

Almost all protein-based biotherapeutics have the potential to induce immunogenicity and large efforts have been

made to reduce drug immunogenicity, particularly in the therapeutic monoclonal antibody (mAb) field. One result of these efforts has been to facilitate the transition from murine and chimeric mAb to humanized and fully human monoclonal antibodies. Accordingly, the 84% ADA positive rate for murine products was reduced to 40% for chimeric products, to 9% for humanized products, and to 0–12% for fully human mAbs [1, 3–10]. Although it is inappropriate to compare the ADA positive rate between different products and different assays, the cited ADA incidences serve to illustrate the downward trend of ADA incidence that has occurred with the increased progress in therapeutic protein engineering.

An ADA response can result in a loss of efficacy and/or increase the risk of adverse reactions (e.g., infusion reactions, anaphylaxis, or immune-complex-mediated diseases) [3, 11–13]. In rare cases, the ADA response is directed not only to the administrated biotherapeutic but also to its endogenous counterpart protein and may elicit a life-threatening response in particular if the endogenous protein is unique and nonredundant and has a vital life function [6, 14]. In recognition of these possibilities, the regulatory agencies request that a biotherapeutic's immunogenicity be assessed and a determination of its characteristics relative to any induced clinical consequences be done as part of the approval process for biotherapeutics.

Without an appropriate immunogenicity assessment data package for a biotherapeutic, Biologic License Application (BLA) or New Drug Application (NDA) will have a much more difficult time being approved by the regulatory agencies. The immunogenicity assessment, in turn, highly depends on appropriate immunogenicity assays. In the past decade, drug developers, medical device industries, academics, and regulatory agencies have worked together to provide more technology platforms, different assay formats, and multiple important white papers [15, 16] and regulatory guidance (US Food and Drug Administration (FDA) and European Medicines Agency (EMA)) [17–21] that improved the ways to assess immunogenicity. Because some of our assays are now supersensitive and have better drug tolerance, they are able to detect more ADA, which has resulted in sharp increases in ADA positive rates, thereby generating new challenges and new issues for the drug developer. When a high ADA positive rate is unexpectedly seen, it should be asked as to why the ADA positive rate is so high and if the development of the drug should be put on hold. The regulatory agencies may ask for additional testing and characterization to ensure patient safety. Physicians may simply choose another drug with a lower ADA positive rate when there is more than one drug on the market for the same or similar indications, which would impact drug sales. This paper intends to review and understand the supersensitive ADA assay and examine the unexpected high ADA positive incidence and the potential clinical relevance of a high ADA positive incidence.

2. The Supersensitive ADA Assay

The sensitivity of the anti-drug antibody (ADA) assay is defined as the lowest concentration of ADA the assay method can reliably differentiate from background or the level of ADA response that is equal to or above the assay cut point (CP). The original FDA draft guidance has recommended the ADA screening assay sensitivity be around 250–500 ng/mL to be able to pick up clinically relevant immunogenicity; however, recent FDA draft guidance has now lowered this to 100 ng/mL as they have observed clinically relevant responses at this level [17, 21]. A supersensitive ADA assay is defined as an assay that is able to detect single to low double digit ng per mL of ADA in the testing sample. The development of supersensitive ADA assays has been possible because of better technologies and more experience in assay development and validation.

To evaluate the ADA sensitivity, we must know the assay drug tolerance limit. Without knowing the drug tolerance limit, the assay sensitivity is almost meaningless because biotherapeutics usually have a long half-life and are almost always present in the testing sample at most sampling time points. If the ADA assay drug tolerance level is lower than the drug concentration in the testing samples, the ADA assay is not able to detect ADA present in the sample due to interference of the drug and the ADA incidence would be underestimated. Although the regulatory guidance does not recommend an acceptable drug tolerance level, ideally we want the ADA assay to be able to tolerate higher than the trough level of the drug as ADA samples are most often taken during the drug trough period.

Unlike a quantitative assay that utilizes an appropriate reference standard curve to differentiate a positive response from background noise and to calculate the analyte concentrations in the study samples, there is no reference standard available in an ADA assay. The ADA assay is a qualitative or quasi-quantitative assay in which the CP is applied to differentiate ADA positives samples from negative samples and the surrogate positive control (PC) generated from immunized animals is used to assess the ADA assay sensitivity. The surrogate positive control cannot be expected to represent the spectrum of immune responses observed in individuals treated with the drug in clinical studies and the actual assay sensitivity and the drug tolerance level may also be different in the clinical sample testing. However, using the surrogate ADA positive control from immunized animals is considered to be the best practice in the industry and is accepted by regulatory agencies (FDA, EMA, etc.).

Previously, enzyme linked immunosorbent assay (ELISA) methods were used in most ADA assays and were associated with a higher background noise that resulted in a higher minimum required dilution (MRD) and lower assay sensitivity. It has also been reported that the drug tolerance levels in some of the earlier ADA assays were lower than the drug concentration present in the clinical testing samples [22]. Wang et al. [22] reviewed 28 FDA-approved biotherapeutics (10 proteins, 2 Fab products, 4 Fc fusion proteins, and 12 monoclonal antibodies) during 2005–2011 and found many FDA-approved biotherapeutics had higher steady-state drug concentrations than the drug tolerance of ADA assays by 1.2- to 800-fold. Consequently, the reported immunogenicity rates for the 28 biotherapeutics may have been underestimated.

Their survey showed that drug tolerance of the ADA assay for 19 products spanned the range between 1 ng/mL and 50 μg/mL whereas the steady-state trough drug concentrations of 22 products ranged from 0.3 ng/mL to nearly 400 μg/mL. They found that the ADA assays of more than half of approved products (13 out of 22 with appropriate data for evaluation) had a drug tolerance level lower than the steady-state trough drug concentration. These consisted of 9 out of 12 monoclonal antibody products, 2 out of 4 Fc fusion protein products, 1 out of 10 protein products, and 1 out of 2 Fab products, which suggested that the ADA assays for monoclonal antibody molecules were more susceptible to drug interference. Interestingly, many FDA-approved monoclonal antibody products (12 products as of March 2012) had postmarketing requirements/commitments (PMR/PMC) to develop improved immunogenicity assays and to assess the impact of immunogenicity after the new and improved assays were implemented.

For the monoclonal antibody biotherapeutic Humira (adalimumab), it was reported that the drug tolerance level for the anti-adalimumab antibody assay was less than 2 μg/mL, while the adalimumab trough levels were 5 μg/mL and 8 to 9 μg/mL. According to the drug insert, the ADA positive rate for rheumatoid arthritis (RA) patients is 5% [23], which might be underestimated due to the drug tolerance issue. In the plaque psoriasis clinical study, only approximately 40% of subjects had less than 2 μg/mL of adalimumab in their ADA testing samples.

In the past decade many new technologies have become available to detect immunogenicity, such as Meso Scale Discovery (MSD) electrochemiluminescence (ECL), Gyros, ImmunoCAP, and SQI Diagnostics. In addition, new sample pretreatment approaches, such as acid dissociation and SPEAD, to improve drug tolerance have also been developed and incorporated into the new generation of ADA assays [3, 6, 7, 24–32]. These methodology advances have increased assay sensitivity dramatically. Today, most ADA assays are ECL technology with bridging format and acid dissociation, which has made the assay background clean (less noise), the drug tolerance better, and the assay more sensitive. With more sensitive or supersensitive ADA assays, a higher ADA incidence is expected. However, if the new ADA positive rate is much higher (e.g., a 5–10-fold increase) than previous ADA reports or historical ADA reports or expected rate, it becomes imperative to interpret and report the results based on clinical relevance rather than total positive rates [33].

3. Are All ADA Positives True Positives?

When a high ADA positive rate is unexpectedly observed, first, it should be asked if all of the ADA positives are true positives. Does being equal to or just above the screening assay and confirmatory cut points mean they are a true positive? The following questions must be answered before accepting that the positives are true positives. Were the screening and confirmatory cut points set correctly? If outliers were removed, is it certain that the data point removed is not the part of the study population and the data still accurately reflects the biological variability? Was enough of the normal range of biological and analytical background noise included into the screening CP and confirmatory CP to ensure the CPs are not set too low? Does the drug have a soluble target in the clinical testing sample? If it does, has it been determined during assay development and validation if the soluble drug target has a positive interference on the assay? If it does have a positive interference, has an approach to remove the interference in the assay been developed? All of these questions should be answered during assay development and assay validation. However, sometimes the well-validated assay in prestudy phase does not work as expected during the study phase. This may occur as a result of the following: (1) in the prestudy stage (when assay development and validation are done), the CP is determined with commercially available serum lots, either normal healthy or disease state serum lots, that might not represent the serum of the study population and the CP may not be appropriate; (2) the assay developer lacks knowledge about the drug's mechanism of action (MOA) and the assay format is not chosen appropriately; (3) potential interference factors presented in the testing samples are not investigated thoroughly during assay development; (4) statistical tools are not appropriately used in the CP determination (e.g., too many outliers might be removed); (5) during the assay development, the interrelations of assay sensitivity, drug tolerance, and CP are not balanced appropriately [15]; and (6) the assay background noise is so low that the response of drug naive serum is near or at the relative lower limits of the instruments. In the following section, examples will be discussed in detail to explain how these factors impact the ADA positive rate.

3.1. Drug Soluble Target May Cause False High Positive ADA Rate. It has been well accepted that the bridging ADA screening ECL assay with acid dissociation is one of the most popular and effective ADA screening assays. In this assay format, a biotin-labeled drug is used as capture and a Sulfo-TAG™-labeled drug is used as detection. ADA present in the testing sample can be detected because ADA can bridge both the labeled capture and the detection drug. The advantages of the bridging ADA screening assay are that it is species independent, it is able to detect all isotypes (except IgG4 due to Fab arm exchange), and it is easy and convenient. The weakness of this assay format is that it is prone to interference by free drug in the testing sample, which may cause a false negative [30]. To overcome the drug interference, acid dissociation and/or drug removing approaches have been incorporated into the bridging assay format, allowing ADA to be successfully detected in the presence of excess free drug in samples, and have almost become the universal approach in preclinical and clinical ADA screening [26, 27, 31].

However, due to the fact that drug conjugates are utilized as the capture and detection molecules, this type of approach is also susceptible to soluble drug target interference. Bivalent soluble drug target (homodimer or multimer) can produce a false positive signal due to its ability to bridge the labeled capture and detection drugs in the assay system. In the ADA testing samples, the soluble drug target or ligand forms complexes with the drug while soluble drug forms complex with ADA. The acid dissociation not only dissociates ADA

from the drug/ADA complex but will also dissociate drug target from the drug/drug target complexes to make more drug target available. If the soluble drug target is bivalent, it could bridge the labeled drugs in the assay system and produce a false ADA positive result. If monovalent, soluble drug targets could also cause a false negative result.

Dai et al. [31] recently reported an unexpected high ADA incidence (>60%) in fulranumab phase I clinical study samples using the bridging ADA screening assay format with acid dissociation. Fulranumab is a human IgG2 monoclonal antibody that neutralizes nerve growth factor (NGF) and currently it is in development for the treatment of pain. They speculated that NGF produced a false positive signal because of its ability to bridge fulranumab molecules in their assay system. Thus, they then revised the assay, which involved a two-step specificity confirmation assay that first used anti-NGF antibody-coated beads to selectively remove NGF (both free and bound to fulranumab), followed by competitive inhibition with fulranumab. Using the revised method, they confirmed that the high apparent anti-fulranumab antibody incidence (>60%) in the clinical study samples was in fact due to fulranumab-bound NGF released during the acid dissociation step of the ADA testing method. Analysis of samples from four phase 2 clinical studies showed that ADA positive rates were >50% in the screening assay but only 1.3% or less were confirmed true positives using their "double" confirmatory assay. The majority of the false ADA positives were because of NGF positive interference.

Zou [32] also observed an unexpected high ADA incidence (>70%) in a phase I clinical study of a therapeutic humanized antibody (TA), which had a soluble target (ST) present in the serum. After investigation, it turned out that the majority (~90%) of TA-confirmed samples were proved to be false positive due to ST interference. They developed a double competitive confirmatory assay, with which they were able to eliminate false positives caused by ST and were able to identify a true ADA positive of only 6.6%–7.8%.

It is recommended that when soluble targets are found in the testing sample as a divalent molecule or potentially forming a divalent molecule (dimer or multimer), the drug target interference be assessed during assay development and an appropriate approach to overcome the drug target interference be developed and validated. Developing an immunogenicity assay without consideration of the MOA should be avoided. As the assay developer or assay scientist, it is essential to communicate to the R&D team early in the drug development phase and understand the MOA, which will help the analyst choose the appropriate assay technology platform, assay format, and reagents.

The ADA screening bridging assay format with acid dissociation should not be used as the universal approach in immunogenicity testing. For example, as stated above, when the drug has a divalent soluble target the acid pretreatment procedure included in the screening assay may make target interference worse by releasing additional target from drug/target immune complexes and may result in a higher false ADA positive rate. In the past, it was underappreciated that the drug target can cause a high false ADA positive in immunogenicity testing. This point should be kept in mind when ADA assays are developed and when the clinical ADA data are analyzed, particularly when the ADA positive rate is unexpectedly high.

3.2. Unnecessary Outlier Removal May Cause a Lower CP and a Higher ADA Positive Rate. Identification and removal of outliers is an important step in the CP determination because outliers can change the value of the CP. In most cases, the CP raw data do not result in a normal (bell shape) distribution; instead, the majority of data points are low values distributed to the left side of the mean, while high values are distributed on the right side of the mean and form a long tail (i.e., skewed to the right or a positive skew). The positive skew of ADA screening data is often a result of the values being at the low end of the instruments signal and therefore tending to "pile" at that end. Thus when considering outliers, the overall representative true variability must incorporate the variability caused by the sample and not just the instrument. In general these new assays have removed so much serum variability that often only the instrument and analytical variability are measured which may not be sufficient to capture patient variability in the clinical trial. Hence when considering outlier removal a highly conservative approach must be taken.

An outlier is a value that "lies outside" most of the other values in a set of data. As outliers, they can be either smaller or larger values and are distributed far from the major population. There are two kinds of outliers in an immunogenicity assay: biological outliers and analytical outliers. Generally, in immunogenicity data the values for outliers are much larger than those of the major population. Outliers should be removed during the CP determination. One of the most common statistical methods used to identify an outlier is the Outlier Box Plot.

The case study given here shows the challenge to identify outliers and illustrates which outliers should be removed. The graph (Figure 1) shows box plot analysis of 100 lots of normal drug naïve individual serums in an actual CP experiment. Seven outliers were identified and they were removed in the first run of outlier identification, followed by a second run in which 2 more outliers were identified and removed. After the 9 outliers were removed, a third run identified 2 more outliers. Should we continue to remove all outliers identified by an Outlier Box Plot?

The outliers identified may make sense from a statistical point of view, but do they make sense from a biological point of view? Obviously solely relying on statistical analysis for this determination would not suffice and therefore the data must be judged biologically and statistically. In general, in the authors' experience, removing outliers after only one run works the best. If the data fit a normal distribution (Gaussian distribution) after the removal of the outliers identified in the first run, the CP can be calculated using a parametric approach. However, if the data do not fit a normal distribution, transform all the data, including "outliers," to log scale and see if the data fit a log normal distribution with and without the outliers removal. If the transformed data fit a log normal distribution, either with or without the outliers, calculate the CP on log scale using a parametric

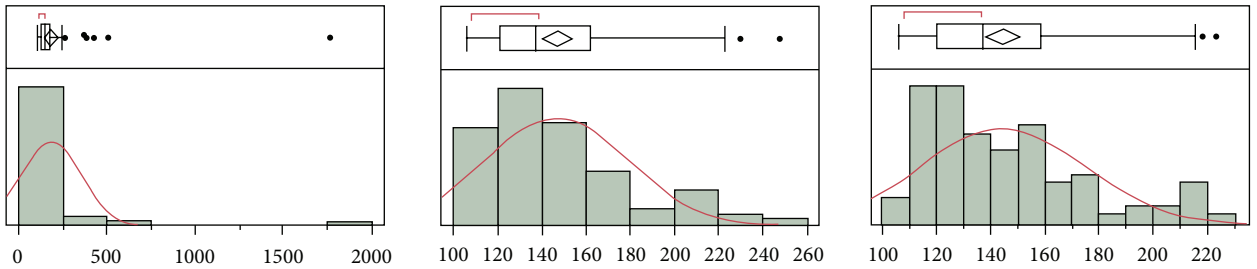

Figure 1: Outlier identification and distribution of the drug naïve matrix sample results using JMP Outlier Box Plot. The Outlier Box Plot is composed of the top and bottom parts. The bottom part is a histogram plot which indicates the data distribution. x-axis represents data values (assay response values) and y-axis represents data frequency. The red curve is the normal density curve. The top part is Quantile Box Plot (the Outlier Box Plot) and the disconnected points are potential outliers. A red bracket defines the shortest half of the data (the densest region). The results of the first, second, and third run of the outlier identification are displayed in each individual plot from left to right.

approach. If the data do not fit a log normal distribution after one run of outlier removal, then calculate the CP with the nontransformed data using a nonparametric approach.

The second run of outlier removal could be considered only if the situation meets the following criteria: (1) the data point(s) are very far from the major data population; (2) only a few of the outliers were removed in the first run; and (3) there is a biological reason to believe they are outliers. It can simply be the case that some observations happen to be a long way from the center of the data even though the data belong to the major population. It has been recognized that data have biological significance even though identified as outliers using the box plot. Elimination of those biologically significant data points would bias the value of the CP and further bias the results of the study.

When data points can be identified as resulting from analytical mistakes (a measurement mistake or a technique mistake), then a case can be made for elimination of the data points from the data set because they do not represent the population/experiment in question. Anytime a single outliner is rejected we run the risk of throwing away biologically significant data.

Outlier identification and exclusion may not be straightforward and sometimes can be problematic dependent on the population being tested. For example, in a rheumatoid arthritis (RA) population, many outliers may be identified using a box plot analysis because RA serum contains rheumatoid factor as well as other unknown interfering agents, which can generate higher signals. However by excluding these in the analysis, which results in a lower CP, the majority of the testing samples will become positive for ADA thus making drug specific information more difficult to ascertain. Therefore it is suggested to be very cautious in removing outliers in populations such as the RA patient population.

Whenever an outlier is removed, it should be asked if there is a valid reason to remove it. We cannot simply remove all outliers identified only by the statistical tool. If there is no reason to remove them, then the points should be kept. It is critical to take a conservative approach to remove outliers to set up the CP properly, which in turn will impact the ADA positive rate. When there is a very high ADA positive rate, one question that should be asked is, were too many "outliers" removed and is it certain that the deleted outliers do not belong to the study population? Considering the ADA assay today has much less background noise and the assay background range is very narrow, it is recommended not to exclude too many data points as "outliers" unless we are certain they are outliers from both statistical and biological standpoint.

3.3. Inappropriate CP Established in Prestudy Stage May Cause High ADA Positive Rate in Study Stage. Usually the CP is established during the ADA assay validation in the prestudy stage using a commercially available serum matrix, which can be either normal or disease state serum. Whether the CP established in the prestudy stage works in the study stage is mainly dependent on whether the commercially available serum represents that of the study population. Unfortunately, sometimes the commercially available serum does not react in the same or similar way to the serum samples from the study population and can result in either a false high or low positive rate. Based on our experience, there is a higher probability to cause a false higher positive rate than that of a false lower positive rate, particularly when the ADA assay is supersensitive. If an ADA positive rate of more than 15% is seen in the baseline samples, it should trigger a CP reassessment and the need to reset the CP using the clinical baseline samples.

Currently, most assays are developed with a 5% false positive rate built into the screening CP to ensure capture of all true positives. While a target of 5% false positive in screening is the goal, often the actual false positive rate for a screening CP is either lower or higher than 5%. In our experience, the screening CP was more often higher than 5% and up to 10% or above. The 2015 9th Workshop on Recent Issues in Bioanalysis (WRIB) recommended [34] that if the false positive rate of the in-study baseline samples is too low (<2%) or too high (>11%), the means and variances of the log-transformed ratio of individual subject sera to negative control from the validation (prestudy) and clinical study baseline (in-study) be compared first. If only the means of these ratios are significantly different, use the variance from the validation along with the mean of the ratios from the in-study baseline samples to adjust the CP factor accordingly. If the variances are different, the in-study baseline samples may be used to calculate a new study-specific CP correction factor.

This is reasonable to do as long as baseline data are available from at least 50 subjects, tested over at least two runs and by at least two analysts. More detailed information about the study-specific CP reassessment is available in [16, 34].

4. Unexpected High ADA Positive Rate and Its Clinical Relevance

The majority of high ADA positive rates are caused by the supersensitive ADA assay which is able to detect more ADA than the earlier assays. After ruling out a false high ADA positive due to any positive interferences or technical issues such as the CP setup issues, the high ADA positive rate must be accepted and appropriately interpreted to know what the clinical relevance of the high ADA positive rate means. It should be noted that clinical efficacy and adverse effects may not change much despite a dramatically increased ADA positive rate. For example, the ADA positive rate of Tysabri increased from 4.5% to 58%, but only 25% out of the 58% of the patients with high ADA titers also had adverse effects and a loss in drug efficacy [35]. There are numerous reports which indicate that persistent ADA positives rates with high titers are closely related to adverse effects and a loss in clinical efficacy, while transient ADA positives rates with low titer often have little or no loss of clinical efficacy or safety issues [3, 11, 12, 33, 35–38]. Therefore it is very important to break down the total ADA positive population into ADA positive subpopulations to identify which subpopulation of ADA positive is clinically relevant. The clinically relevant ADA is defined as the ADA which impacts safety, efficacy, or pharmacokinetics regardless of persistence or transience or titer level. However, often the clinical relevant ADA is associated with the persistent ADA with moderate to high titers. On the other hand, the nonclinically relevant ADA is the ADA which has no or little impact on safety, efficacy, or pharmacokinetics regardless of persistence or transience or titer level, but the nonclinically relevant ADA often is associated with the transient ADA and low titer. It is speculated that a dramatic increase in ADA positive rate is mainly because of an increase in the nonclinical relevant ADA fraction, which usually occurs in a low assay signal. This kind of low assay signal is easily picked up by the supersensitive ADA assay, while it was often missed by the older ADA assays used previously.

In 2013, Vennegoor et al. reported [35] on the clinical relevance of serum natalizumab (Tysabri) concentration and anti-natalizumab antibodies in multiple sclerosis. Their results showed that patients with a high antibody titer had a 10.5 times higher odds ratio (OR) ($p = 0.02$) to develop gadolinium positive (Gd+) lesions and 10.9 times higher odds ($p = 0.008$) to have a relapse compared to patients with no antibodies. They found the low concentration drug was associated with high titer of ADA and had a 14.5 times higher OR ($p = 0.006$) to develop gadolinium positive Gd+ lesions and a nine times higher OR ($p = 0.01$) to have a relapse compared to normal serum natalizumab concentrations.

Their data showed that only 25% of all patients had high antibody titers (range 110–260,000 AU/mL) at least at one time point during the study and demonstrated the clinical relevance of the persistent ADA with high titer. The authors did not state the clinical relevance of transient ADA with low titer; however, it was noted that patients with low antibody titer had similar Gd+ lesions as those with no antibody titer. Presumably, the low antibody titers, possibly in combination with a relatively low affinity, were insufficient to significantly affect natalizumab concentrations. They concluded that both low natalizumab serum concentration and high antibody titers were associated with a lack of efficacy of natalizumab.

Interestingly, the authors found a substantially higher percentage (58%) of patients with anti-natalizumab antibody positive results than had been previously reported (58% versus 4.5–14.1%) [36–39]. They postulated the difference was most likely due to the differences in the assay methods used. The RIA method they used was better suited than the ELISA method to detect anti-natalizumab antibodies in serum when free natalizumab is also present in the serum [35]. In addition, the possibility that the ELISA assay did not have as good drug tolerance capability as the newer ADA assay also may have been a factor.

The majority of the reports on the clinical relevance of ADA incidence are short-term studies that show the development of ADA is associated with diminished drug serum levels and a diminished treatment response. However, little is known about the clinical relevance of ADA against these drugs during a long-term follow-up.

Baert et al. [11] reported the influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. Infliximab (Remicade), used to treat autoimmune diseases, is a chimeric monoclonal IgG1 antibody against tumor necrosis factor. In a cohort of 125 consecutive patients with Crohn's disease treated with infliximab infusions, the authors evaluated the concentrations of infliximab and of antibodies against infliximab, clinical data, and side effects (including infusion reactions) before and 4, 8, and 12 weeks after each infusion. A mean of 3.9 infusions (range, 1 to 17) per patient were administered over a mean period of 10 months. The median follow-up was 36 months (range, 25 to 48). After the fifth infusion, 76 patients (61%) had detectable antibodies and the incidence did not increase further with repeated infusions. But only 46 out of the 125 patients (37%) had a high titer of ADA (the author used 8.0 μg ADA per milliliter as the threshold to differentiate high titer of ADA from low titer of ADA (see [12] for the detailed information)). The presence of high titer ADA before an infusion predicted a shorter duration of response (35 days, as compared with 71 days among patients with low titer of ADA ($p < 0.001$) and a higher risk of infusion reactions ($p < 0.001$) and a lower concentration of infliximab). Based on their results, the authors concluded that the development of high titer antibodies against infliximab was associated with an increased risk of infusion reactions and a reduced duration of response to treatment.

Bartelds et al. [12] reported the development of anti-drug antibodies against adalimumab (Humira) and its clinical relevance during a 3-year follow-up in 272 patients with rheumatoid arthritis (RA). In their study, the CP was set as the ADA concentration that exceeded 12 AU/mL (AU, arbitrary units, 1 AU = 12 ng/mL ADA) and the adalimumab concentration was 5 mg/L or less. The mean cutoff value was

derived from 100 healthy donors and set at 12 AU/mL. They divided the patients with ADA positives into two groups: low ADA titer group (≤100 AU/mL) and high ADA titer group (>100 AU/mL).

After 3 years, 76 of 272 patients (28%) developed anti-adalimumab antibodies (ADA). Patients without ADA had much higher adalimumab concentrations compared with patients with ADA. Forty-five of 76 patients (72.4%) had low ADA titer at all time points and 31 patients (27.6%) had high ADA titer at one or more time points. The median adalimumab concentration for patients without antibodies was 12 mg/L, for patients with low ADA titers median adalimumab concentration was 5 mg/L, and for patients with high ADA titer median adalimumab concentration was 0–3 mg/L. However, among the total ADA positive group, only 27.6% patients, who had high ADA titer, had very low drug concentration, lower minimal disease activity, and less often achieved remission. Their data showed the majority (72.4%) of the ADA positive were low titer and only the minority of ADA positive population (27.6%), who had high ADA titer, had obvious lower adalimumab concentration and lower minimal disease activity or clinical remission.

5. Conclusions

The very high ADA positive rate seen in recent reports relative to the low ADA positive rate seen in earlier reports suggests the ADA positive rate in earlier studies may have been underestimated (false negative) due to the low assay sensitivity and low drug tolerance. In contrast, the supersensitive ADA assay is able to pick up a low assay signal very close to the assay background noise and while it is usually a nonclinically relevant ADA, it may be a major contributor to the high ADA positive rate. In addition, the positive interference that can result from free drug target interference may also contribute to a very high ADA positive rate (false positive) in some of the cases. False negative and false positive data resulting from inaccurate test methods can lead to flawed correlations of ADA with clinical safety, pharmacokinetics, and efficacy results. It is, therefore, recommended that the first step of analysis and interpretation of ADA data when an unexpectedly very high ADA rate is observed is to rule out possible positive or negative interference and to check the appropriateness of the CP setup and then interpret ADA data in context with PK/PD, efficacy, and safety profiles. It has been reported that high titer of persistent ADA is usually associated with safety issues and the loss of efficacy in the clinic, while low titer of transient ADA usually has no or little clinical impact.

Improvement of bioanalytical methods and advancement of technology platforms that continue through the life cycles of drug development and the bioanalytical method itself not only benefit drug development, but eventually benefit the patients as well. With supersensitive ADA assays, we are able to detect ADA not previously detected by the earlier ADA assays and this level of sensitivity not only helps us better understand the drug immunogenicity risk, but also helps ensure patient safety. However it must also be recognized that with supersensitive ADA assays the total ADA incidence may increase significantly and that the total ADA incidence might be misleading if the clinical relevance is not clear. It is necessary to dissect the ADA positive population to identify the clinical relevant ADA positive subpopulation in order to provide clinically meaningful immunogenicity information to the physicians so they know how to assess and manage immunogenicity in the clinic.

Competing Interests

The authors declare that they have no competing interests.

References

[1] I. N. Foltz, M. Karow, and S. M. Wasserman, "Evolution and emergence of therapeutic monoclonal antibodies what cardiologists need to know," *Circulation*, vol. 127, no. 22, pp. 2222–2230, 2013.

[2] G. Shankar, C. Pendley, and K. E. Stein, "A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs," *Nature Biotechnology*, vol. 25, no. 5, pp. 555–561, 2007.

[3] N. Chirmule, V. Jawa, and B. Meibohm, "Immunogenicity to therapeutic proteins: impact on PK/PD and efficacy," *The AAPS Journal*, vol. 14, no. 2, pp. 296–302, 2012.

[4] C. Hay, M. Recht, M. Carcao, and B. Reipert, "Current and future approaches to inhibitor management and aversion," *Seminars in Thrombosis and Hemostasis*, vol. 32, no. 2, pp. 15–21, 2006.

[5] P. G. Swann, M. Tolnay, S. Muthukkumar, M. A. Shapiro, B. L. Rellahan, and K. A. Clouse, "Considerations for the development of therapeutic monoclonal antibodies," *Current Opinion in Immunology*, vol. 20, no. 4, pp. 493–499, 2008.

[6] R. Thorpe and S. J. Swanson, "Assays for detecting and diagnosing antibody-mediated pure red cell aplasia (PRCA): an assessment of available procedures," *Nephrology Dialysis Transplantation*, vol. 20, no. 4, pp. 16–22, 2005.

[7] W. Y. K. Hwang and J. Foote, "Immunogenicity of engineered antibodies," *Methods*, vol. 36, no. 1, pp. 3–10, 2005.

[8] R. Ponce, L. Abad, L. Amaravadi et al., "Immunogenicity of biologically-derived therapeutics: assessment and interpretation of nonclinical safety studies," *Regulatory Toxicology and Pharmacology*, vol. 54, no. 2, pp. 164–182, 2009.

[9] M. Wadhwaa, C. Birda, P. Dilgera, R. Gaines-Dasb, and R. Thorpea, "Strategies for detection, measurement and characterization of unwanted antibodies induced by therapeutic biological," *Journal of Immunological Methods*, vol. 278, no. 1-2, pp. 1–17, 2003.

[10] K. E. Stein, "Immunogenicity: concepts/issues/concerns," *Developments in Biologicals*, vol. 109, pp. 15–23, 2002.

[11] F. Baert, M. Noman, S. Vermeire et al., "Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease," *The New England Journal of Medicine*, vol. 348, no. 7, pp. 601–608, 2003.

[12] G. M. Bartelds, C. L. M. Krieckaert, M. T. Nurmohamed et al., "Development of antidrug antibodies against Adalimumab and association with disease activity and treatment failure during long-term follow-up," *The Journal of the American Medical Association*, vol. 305, no. 14, pp. 1460–1468, 2011.

[13] B. A. Baldo, "Adverse events to monoclonal antibodies used for cancer therapy focus on hypersensitivity responses," *OncoImmunology*, vol. 2, no. 10, article e26333, 2013.

[14] K.-U. Eckardt and N. Casadevall, "Pure red-cell aplasia due to anti-erythropoietin antibodies," *Nephrology Dialysis Transplantation*, vol. 18, no. 5, pp. 865–869, 2003.

[15] A. R. Mire-Sluis, Y. C. Barrett, V. Devanarayan et al., "Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products," *Journal of Immunological Methods*, vol. 289, no. 1-2, pp. 1–16, 2004.

[16] G. Shankar, V. Devanarayan, L. Amaravadi et al., "Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 48, no. 5, pp. 1267–1281, 2008.

[17] US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research, and Center for Biologics Evaluation and Research, *Draft Guidance for Industry. Assay Development for Immunogenicity Testing of Therapeutic Proteins*, 2009, http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm338856.pdf.

[18] US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research, and Center for Biologics Evaluation and Research, "Guidance for industry, Immunogenicity assessment for therapeutic protein products," 2014, http://www.fda.gov/downloads/%20drugs/guidancecomplianceregulatoryinformation/guidances/ucm338856.pdf.

[19] European Medicines Agency, *Guideline on Immunogenicity Assessment of Monoclonal Antibodies Intended for In Vivo Clinical Use*, European Medicines Agency, London, UK, 2012.

[20] European Medicines Agency, "Guideline on Immunogenicity assessment of biotechnology-derived therapeutic proteins (Draft)," 2015, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2015/10/WC500194507.pdf.

[21] US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research, and Center for Biologics Evaluation and Research, "Draft guidance for industry. Assay development and validation for immunogenicity testing of therapeutic proteins, Revision 1," 2016, http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm192750.pdf.

[22] Y.-M. C. Wang, L. Fang, L. Zhou, J. Wang, and H.-Y. Ahn, "A survey of applications of biological products for drug interference of immunogenicity assays," *Pharmaceutical Research*, vol. 29, no. 12, pp. 3384–3392, 2012.

[23] AbbVie: Humira Insert, 2013, http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/125057s310lbl.pdf.

[24] A. Patton, M. C. Mullenix, S. J. Swanson, and E. Koren, "An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen," *Journal of Immunological Methods*, vol. 304, no. 1-2, pp. 189–195, 2005.

[25] H. W. Smith, A. Butterfield, and D. Sun, "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," *Regulatory Toxicology and Pharmacology*, vol. 49, no. 3, pp. 230–237, 2007.

[26] Meso Scale Discovery, "Immunogenicity Assay from Meso Scale Discovery," 2012, http://www.mesoscale.com/.

[27] Meso Scale Discovery, *MSD Technology Platform*, 2013, http://www.mesoscale.com.

[28] Gyros, "Automated immunoassays at nanoliter-scale," 2016, http://www.gyros.com/why-gyros/technology/.

[29] Genalyte, "Detection of isotype and IgG subclass anti-adalimumab antibodies with a multiplex photonic ring immunoassay," 2014, http://www.info@genalyte.com.

[30] M. H. Hart, H. de Vrieze, D. Wouters et al., "Differential effect of drug interference in immunogenicity assays," *Journal of Immunological Methods*, vol. 372, no. 1-2, pp. 196–203, 2011.

[31] S. Dai, A. Schantz, A. Clements-Egan, M. Cannon, and G. Shankar, "Development of a method that eliminates false-positive results due to nerve growth factor interference in the assessment of fulranumab immunogenicity," *AAPS Journal*, vol. 16, no. 3, pp. 464–477, 2014.

[32] L. Zou, "Overcome drug and soluble target interferences in immunogenicity sample analysis for antibody therapy," in *Proceedings of the IBC's 16th Annual Immunogenicity for Biotherapeutics*, Reston, Va, USA, 2015.

[33] G. Shankar, S. Arkin, L. Cocea et al., "Assessment and reporting of the clinical immunogenicity of therapeutic proteins and peptides—harmonized terminology and tactical recommendations," *The AAPS Journal*, vol. 16, no. 4, pp. 658–673, 2014.

[34] L. Amaravadi, A. Song, H. Myler et al., "2015 White paper on recent issues in bioanalysis: focus on new technologies and biomarkers (part 3—LBA, biomarkers and immunogenicity)," *Bioanalysis*, vol. 7, no. 24, pp. 3107–3124, 2015.

[35] A. Vennegoor, T. Rispens, E. M. M. Strijbis et al., "Clinical relevance of serum natalizumab concentration and anti-natalizumab antibodies in multiple sclerosis," *Multiple Sclerosis Journal*, vol. 19, no. 5, pp. 593–600, 2013.

[36] P. A. Calabresi, G. Giovannoni, C. Confavreux et al., "The incidence and significance of anti-natalizumab antibodies: results from AFFIRM and SENTINEL," *Neurology*, vol. 69, no. 14, pp. 1391–1403, 2007.

[37] B. Oliver, Ó. Fernández, T. Órpez et al., "Kinetics and incidence of anti-natalizumab antibodies in multiple sclerosis patients on treatment for 18 months," *Multiple Sclerosis*, vol. 17, no. 3, pp. 368–371, 2011.

[38] P. S. Sørensen, P. E. Hyldgaard Jensen, A. Haghikia et al., "Occurrence of antibodies against natalizumab in relapsing multiple sclerosis patients treated with natalizumab," *Multiple Sclerosis Journal*, vol. 17, no. 9, pp. 1074–1078, 2011.

[39] Biogen Idec and Élan, Tysabri Insert, 2013, http://www.fda.gov/safety/medwatch/safetyinformation/%20safety-relateddrugla-belingchanges%20/ucm106674.htm.

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Research Article

Evaluation of Multiple Immunoassay Technology Platforms to Select the Anti-Drug Antibody Assay Exhibiting the Most Appropriate Drug and Target Tolerance

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The aim of this study was, at the assay development stage and thus with an appropriate degree of rigor, to select the most appropriate technology platform and sample pretreatment procedure for a clinical ADA assay. Thus, ELISA, MSD, Gyrolab, and AlphaLISA immunoassay platforms were evaluated in association with target depletion and acid dissociation sample pretreatment steps. An acid dissociation step successfully improved the drug tolerance for all 4 technology platforms and the required drug tolerance was achieved with the Gyrolab and MSD platforms. The target tolerance was shown to be better for the ELISA format, where an acid dissociation treatment step alone was sufficient to achieve the desired target tolerance. However, inclusion of a target depletion step in conjunction with the acid treatment raised the target tolerance to the desired level for all of the technologies. A higher sensitivity was observed for the MSD and Gyrolab assays and the ELISA, MSD, and Gyrolab all displayed acceptable interdonor variability. This study highlights the usefulness of evaluating the performance of different assay platforms at an early stage in the assay development process to aid in the selection of the best fit-for-purpose technology platform and sample pretreatment steps.

1. Introduction

Monoclonal antibodies have been successfully used as therapeutic agents for the treatment of diseases including breast cancer, leukemia, asthma, arthritis, psoriasis, Crohn's disease, and transplant rejection [1–5]. As part of a therapeutic antibody clinical development program it is necessary to evaluate the immunogenic potential of the antibody. This is measured as an anti-drug antibody (ADA) response and if it occurs it can cause undesired effects ranging from loss of drug exposure and loss of efficacy to serious adverse events. Therefore, immunogenicity assessment is a regulatory requirement of clinical studies [6–10].

Testing of ADA to therapeutic proteins is typically performed using a tiered approach [7–12]. The samples are initially screened for their ability to bind the therapeutic drug, screened positive samples are then confirmed in a second assay, and their isotype and neutralizing capacity can also be evaluated. In recent times, the most common ADA assay format is a bridging assay and the traditional enzyme-linked immunosorbent assay (ELISA) is often a suitable option. However, new immunoassay platforms have been developed including MSD, Gyrolab, and AlphaLISA with improved sensitivity, accuracy, variability, reduced assay time, and reduced sample volume requirements.

Figure 1(a) is a schematic representation of a typical bridging assay format where the ADA bridges two molecules of therapeutic drug labeled with different tags and elicits a signal (e.g., fluorescence and electrochemiluminescence) that can be measured by a reader. With this assay format, the most common form of interference is from the therapeutic protein itself. Therapeutic drug in the clinical sample is able to bind ADA and prevents it from forming a complex with the capture and detection reagents and thus can lead to a false negative result during the clinical sample analysis (Figure 1(b)). The ability of the assay to detect ADA in

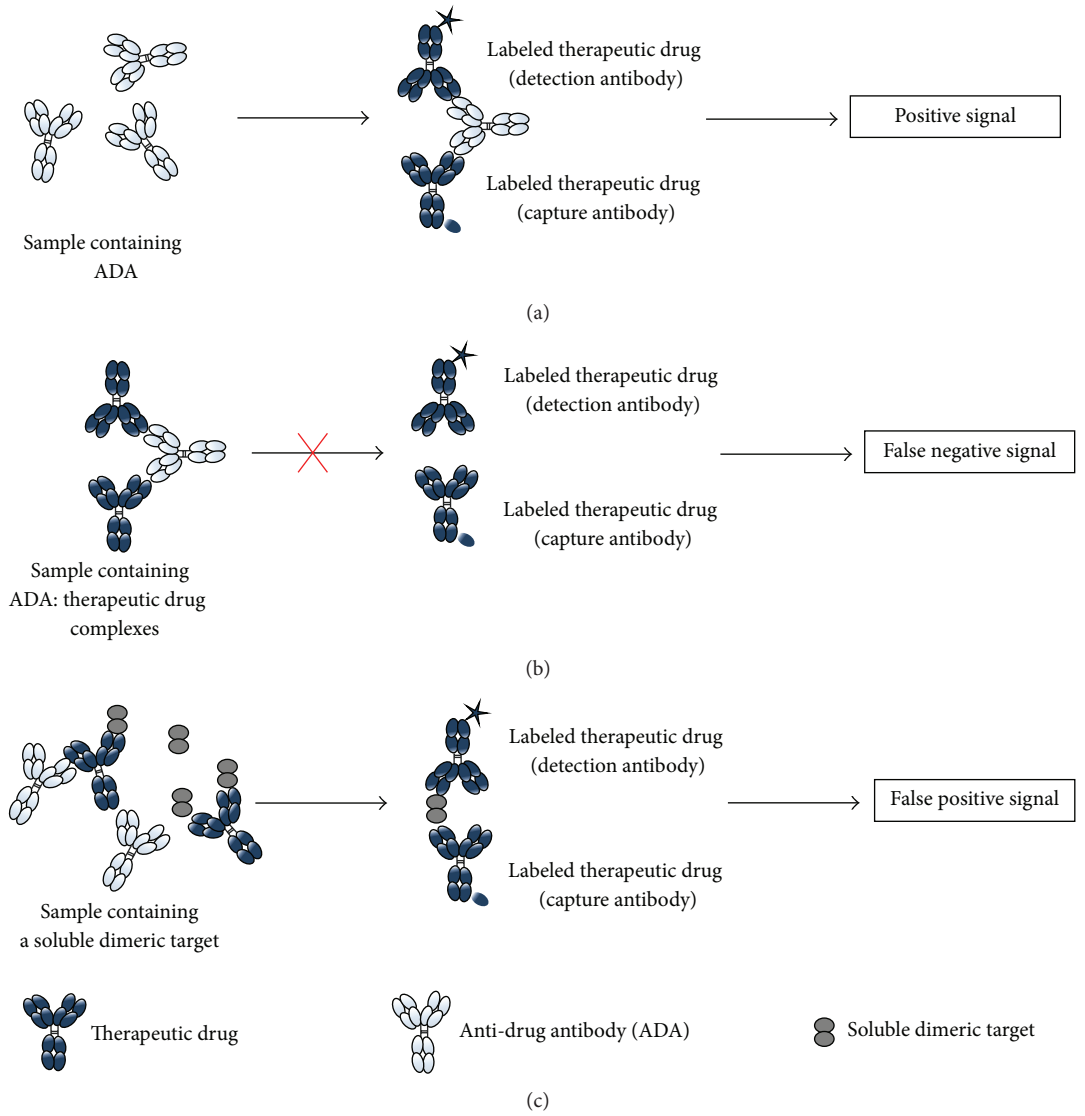

Figure 1: Schematic representation of different scenarios that can occur in the bridging assay. (a) Presence of ADA leading to a positive signal. (b) Drug interference leading to false negative signal. (c) Target interference leading to false positive signal.

the presence of therapeutic drug is called drug tolerance and this parameter needs to be addressed during assay development. Drug interference is common in preclinical toxicology studies and in multiple dose clinical trials where high therapeutic drug concentrations are reached. Drug interference can be mitigated by collecting samples for ADA testing at late time points in the clinical trial when the concentration of the therapeutic is expected to be lower, for example, after the wash-out period or at the end of the clinical trial. However, in order to make ADA assessments at appropriate time points in relation to the underlying disease, drug interference often has to be addressed methodologically. Over the last few years, the challenge of drug interference in ADA assays has often been overcome by performing an acid dissociation of the therapeutic drug-ADA complex step, followed by a neutralization step in the presence of the capture and detection reagents [13–18].

A second challenge observed during immunogenicity assessment with a bridging assay format is interference due to the target. The presence of dimeric or multimeric forms of soluble target in a clinical sample may lead to the bridging of the capture and detection reagent and can lead to a false positive result during the clinical sample analysis (Figure 1(c)). Pretreatment with blocking antibodies to the target, blocking target-binding proteins, and immunodepletion of the target can eliminate this type of interference [19].

In our study, we describe the development of an ADA assay for a therapeutic antibody directed against a soluble dimeric target (Novimab) with evaluation of different technology platforms (ELISA, MSD, Gyrolab, and AlphaLISA) and different additional steps (±acid dissociation, ±target depletion) in order to achieve an assay with an appropriate sensitivity, drug tolerance, and target tolerance for our clinical trial population. Appropriate sensitivity was defined

as 50 ng/mL reference ADA, somewhat more sensitive than the 250–500 ng/mL level suggested in industry guidelines. As the sensitivity parameter was defined using a polyclonal reference ADA purified from immunized rabbit serum, likely to differ in affinity, avidity, and antigenic specificity to a real ADA response in treated subjects, it was felt that an assay with greater sensitivity would be more appropriate to detect low level immunogenicity events. Appropriate drug tolerance and target tolerance were defined as 20 μg/mL Novimab and 50 ng/mL target, respectively, levels defined with the clinical pharmacologist based on PK-PD modeling of the likely accumulation of therapeutic drug and target in the clinical population with the proposed study dosing regimen. Sensitivity, interdonor variability, drug tolerance, and target tolerance were used as the key parameters to assess during this assay development and technology evaluation study. The goal of this work was not to validate an assay on each platform but to evaluate the critical parameters on each platform in order to select the most appropriate assay for subsequent validation and clinical sample analysis.

2. Materials and Methods

2.1. Reagents, Assay Materials, and Serum. The fully human IgG1 therapeutic monoclonal antibody (Novimab) was produced at Novimmune. The anti-drug polyclonal antibody (reference ADA) purified from immunized rabbit serum was supplied by Agro-Bio (La Ferté-Saint-Aubin, France). The soluble dimeric target was purchased from R&D Systems (Minneapolis, MN, United States). The biotinylated nonblocking anti-target mouse monoclonal antibody was purchased from Mabtech (Stockholm, Sweden). Streptavidin magnetic beads were supplied by Thermo Fisher Scientific (Waltham, MA, United States). Horseradish Peroxidase-(HRP-) conjugated mouse-anti-FITC IgG Fraction Monoclonal was purchased from Jackson Immunoresearch (West Grove, PA, United States). 30 individual human sera and a pooled human serum were purchased from SeraLab (West Sussex, United Kingdom). Tetramethylbenzidine (TMB) substrate and the Stop Solution for TMB substrate were supplied by Sigma-Aldrich (Postfach, Switzerland). 96-well round bottomed polypropylene plates and 96-skirt PCR plates were purchased from Fisher Scientific (Wohlen, Switzerland). 96-well streptavidin plates were purchased from Roche Diagnostics (Basel, Switzerland). High binding streptavidin plates and the 4x MSD read buffer were supplied by Meso Scale Discovery (Rockville, MD, United States). Gyrolab Bioaffy 200 CDs, Gyrolab ADA CDs, Rexxip F, and Rexxip ADA were purchased from Gyros AB (Uppsala, Sweden). 96-well 1/2 area white plates, acceptor beads, and donor beads were purchased from PerkinElmer (Lausanne, Switzerland). SULFO-TAG-, FITC-, and acceptor beads-labeled Novimab were prepared at Novimmune according to the recommendations from Meso Scale Discovery (Rockville, MD, United States), Thermo Fisher Scientific (Waltham, MA, United States), and PerkinElmer (Lausanne, Switzerland), respectively. Biotinylated- and Alexa Fluor 647-labeled Novimab were prepared by R&D Biotech (Besançon, France).

2.2. Equipment. Different readers were required for the different assay formats as follows.

2.2.1. ELISA. Absorbance was measured on the Synergy HT instrument (Biotek, Luzern, Switzerland).

2.2.2. MSD. Electrochemiluminescence was measured on the Sector Imager 6000 instrument (Meso Scale Discovery, Rockville, MD, United States).

2.2.3. Gyrolab. Fluorescence was measured on the Gyrolab Workstation with the Gyrolab Control software (v5.4.0) (Gyros AB, Uppsala, Sweden).

2.2.4. AlphaLISA. Fluorescence was measured on the Synergy NEO instrument (Biotek, Luzern, Switzerland).

2.3. Sample Preparation. All samples were prepared on the same day, aliquoted into 96-well polypropylene plates, and stored at −80°C.

2.3.1. Standard Samples. A reference ADA standard (from 0.34 to 20 ng/mL) was prepared in neat pooled human serum. The negative control (NC) sample consisted of neat pooled human serum alone. For the comparison of the different platforms, relative quantitation against this reference ADA was performed. In the final assay format, a tiered approach was used with the reference ADA being used to evaluate system suitability.

2.3.2. PC and Drug Interference Samples. Positive Control (PC) samples were prepared to final concentrations of 2, 5, 20, 50, 100, 250, 500, 1000, and 10000 ng/mL, by diluting the reference ADA in pooled human serum. Novimab samples were prepared to final concentrations of 0, 20, and 100 μg/mL in pooled human serum. Samples were incubated at 22°C for 1 h with agitation (450 rpm) to allow Novimab: ADA complex formation.

2.3.3. Target Interference Samples. Target interference samples were prepared by diluting the soluble dimeric target to final concentrations of 5, 20, 50, and 200 ng/mL in pooled human serum.

2.3.4. Selectivity. Thirty individual human sera and one pooled human serum were tested. The pooled human serum was also used for the preparation of the standard, NC, PC, drug interference, and target interference samples.

2.4. Sample Pretreatment

2.4.1. Target Depletion. For each technology evaluated, the samples spiked with the soluble dimeric target were tested under two conditions: nondepleted or depleted. The nondepleted samples were diluted at 1 : 3 in PBS-1% BSA 0.05% tween. The depleted samples were first diluted at 1 : 2 with a biotinylated noncompetitive anti-target mouse monoclonal

antibody and incubated at 22°C for 1 h with agitation (450 rpm). Two volumes of this solution were then mixed with one volume of prewashed streptavidin magnetic beads. After 1 h incubation at 22°C with agitation (450 rpm) the plate was placed on a magnet for 2 minutes. The supernatant was then removed with a pipette and used in the subsequent steps of the assay.

2.4.2. Acid Treatment. For each technology evaluated, the samples were tested under two conditions: nonacidified or acidified. No pretreatment was applied to the nonacidified samples. The acid dissociation method was optimized on the ELISA platform by testing various parameters. Several acid solutions were compared including 100 mM, 300 mM, and 600 mM acetic acid, 50 mM and 100 mM hydrochloric acid, and 0.5 M Glycine at pH 2.2 and 2.6. Sample : acid ratios at 1 : 2, 1 : 3, 1 : 5, and 1 : 10 (volume : volume) were tested and acidification time of 20 and 40 minutes was compared. Similarly the neutralization step was optimized by preparing Tris solutions at different molarities (from 0.1 to 2 M), at different pH (from 7.5 to 9.0) and neutralization time of 2 hours and 16 hours for comparison. All of these optimizations were conducted on samples containing reference ADA concentrations ranging from 0 to 10000 ng/mL complexed with 0 or 20 μg/mL of Novimab. Based on these experiments 1 in 5 dilutions of sample in 300 mM acetic acid was selected as the most suitable, combining appropriate sensitivity, drug tolerance, and sample usage. The suitability of this sample acidification procedure was then confirmed on the other plate-based platforms (i.e., MSD and AlphaLISA). For the Gyrolab platform, a specific optimization of the acid treatment was performed due to the use of the Gyrolab Mixing CDs, where a sample : acid ratio of 1 : 1 (volume : volume) was used due to the volume definition of the CD microstructures. The samples that required an acidification were treated as follows.

(i) ELISA, MSD, and AlphaLISA. 20 μL of each sample was mixed with 80 μL of 300 mM acetic acid in a 96-well round-bottom polypropylene plate. The acidified samples were then incubated at 22°C for 40 minutes with agitation (450 rpm).

(ii) Gyrolab. A Gyrolab Mixing CD was used in order to have a fully automated acid treatment step. 200 nL of sample was transferred into the reacting chamber of the Mixing CD and mixed with 200 nL of 0.5 M Glycine-HCl pH 2.6. This was then incubated for 10 minutes at room temperature.

2.5. ADA Screening Assay

2.5.1. ELISA

(i) For the Nonacidified Samples. 2x Master Mix containing 2 μg/mL biotin- and 2 μg/mL FITC-labeled drug was prepared in PBS-1% BSA 0.05% tween. One volume of Master Mix was added to one volume of sample and incubated for 16–24 h at 22°C, with agitation (450 rpm).

(ii) For the Acidified Samples. 3x Master Mix containing 3 μg/mL biotin- and 3 μg/mL FITC-labeled drug was prepared in PBS-1% BSA 0.05% tween. One volume of Master Mix, one volume of acidified samples, and one volume of 1 M Tris pH 8.0 were mixed together and incubated for 2 h at 22°C, with agitation (450 rpm).

(iii) For Both Treatments. A 96-well streptavidin plate was blocked with 200 μL/well of PBS-3% BSA for 1 to 4 h and washed three times with PBS-0.05% tween. The sample/conjugate mix (45 μL/well) was pipetted into the blocked streptavidin plate and incubated at 22°C for 1 h with agitation (450 rpm). The plate was washed three times using PBS-0.05% tween followed by the addition of 50 μL/well of 1/8000 diluted HRP-conjugated mouse-anti-FITC IgG Fraction Monoclonal. The plate was incubated at 22°C for 1 h with agitation (450 rpm) and then washed three times with PBS-0.05% tween. The TMB substrate was added to each well (50 μL/well) and incubated at 22°C for 5 minutes. The reaction was stopped by the addition of 50 μL/well of Stop Solution for TMB substrate and absorbance was measured at 450 nm on the Synergy plate reader.

2.5.2. MSD

(i) For the Nonacidified Samples. 2x Master Mix containing 4 μg/mL biotin- and 4 μg/mL SULFO-TAG-labeled drug was prepared in PBS-1% BSA 0.05% tween. One volume of Master Mix was added to one volume of sample and incubated for 16–24 h at 22°C, with agitation (450 rpm).

(ii) For the Acidified Samples. 3x Master Mix containing 6 μg/mL biotin- and 6 μg/mL SULFO-TAG-labeled drug was prepared in PBS-1% BSA 0.05% tween. One volume of Master Mix, one volume of acidified samples, and one volume of 1 M Tris pH 8.0 were mixed together and incubated for 2 h at 22°C, with agitation (450 rpm).

(iii) For Both Treatments. A MSD high binding avidin plate was blocked with 200 μL/well of PBS-3% BSA for 1 to 4 h and washed three times with PBS-0.05% tween. The sample/conjugate mix (45 μL/well) was pipetted into the blocked plate and incubated at 22°C for 1 h, with agitation (450 rpm). The plate was then washed three times using PBS-0.05% tween and 150 μL of the 2x MSD Read Buffer was transferred into each well. The electrochemiluminescence signal was then measured on the Sector Imager 6000 instrument.

2.5.3. AlphaLISA

(i) For the Nonacidified Samples. 2.5x Master Mix containing 5 nM biotin-labeled drug and 50 μg/mL drug coated acceptor beads was prepared in PBS-1% BSA 0.05% tween. Two volumes of Master Mix were added to one volume of sample and incubated for 16–24 h at 22°C, with agitation (450 rpm). Streptavidin donor beads were prepared at 50 μg/mL in PBS-1% BSA 0.05% tween and diluted at 1 : 2.5 with the sample/acceptor beads mix. This solution was incubated at 22°C for 1 h, with agitation (450 rpm).

(ii) For the Acidified Samples. 6x Master Mix containing 12 nM biotin-labeled drug and 120 μg/mL drug coated acceptor

beads was prepared in PBS-1% BSA 0.05% tween. One volume of Master Mix, two volumes of acidified samples, and one volume of 1 M Tris pH 8.0 were mixed together and incubated for 16 to 24 h at 22°C, with agitation (450 rpm). Streptavidin donor beads were prepared at 60 μg/mL in PBS-1% BSA 0.05% tween and diluted at 1 : 3 with the sample/acceptor beads mix. This solution was incubated at 22°C for 1 h with agitation (450 rpm).

(iii) For Both Treatments. 50 μL of the sample/beads mix was transferred into a 96-well 1/2 area white plate and the plates were measured on the Synergy plate reader.

2.5.4. Gyrolab

(i) For the Nonacidified Samples. 2x Master Mix containing 4 μg/mL biotin- and 4 μg/mL Alexa Fluor 647-labeled drug was prepared in Rexxip F. One volume of Master Mix was added to one volume of sample and incubated for 16–24 h at 22°C, with agitation (450 rpm) and transferred into a 96-well skirted PCR plate. The plate was loaded into the Gyrolab platform together with a Gyrolab Bioaffy 200 CD. The run was designed with the Gyrolab Client v5.4.0 software and the data were analyzed at the 5% photomultiplier tube setting using the Gyrolab Evaluator v3.3.7.171 software.

(ii) For the Acidified Samples. 3x Master Mix containing 6 μg/mL biotin- and 6 μg/mL Alexa Fluor 647-labeled drug was prepared in a 2 M Tris pH 8.0 : Rexxip ADA buffer (1 : 1 vol) solution. The samples, the Master Mix, and 0.5 M Glycine pH 2.6 solution were transferred into a 96-well skirted PCR plate. The plate was loaded into the Gyrolab platform together with a Gyrolab Mixing CD. The run was designed with the Gyrolab Client software in order to achieve an acidification and a neutralization time of 10 minutes each. The acidification was performed by mixing one volume of sample with one volume of 0.5 M Glycine pH 2.6 solution. One volume of Master Mix was then added to the acidified samples for the neutralization. The data were analyzed at the 5% photomultiplier tube setting using the Gyrolab Evaluator software.

2.6. Data Analysis

2.6.1. Run Specific Cut-Point Determination. ADA assays require the determination of a cut-point to evaluate whether a sample is considered as positive (signal of the sample at or above the cut-point) or negative (signal of the sample below the cut-point). The calculation of this cut-point requires assessment of a large population of individual matrices and statistical analysis of the results. As the primary aim of this study was to evaluate and compare different technology platforms and respective methods, a statistical method using a floating cut-point was employed by calculating a run specific cut-point (RSCP) for each analysis plate/CD.

A limited number of naïve individual matrices (n = 30) were screened on each method. The outliers were then excluded using a BoxPlot analysis. The assay cut-point (ACP) was determined as follows:

$$\text{ACP} = \text{Mean individual matrix} + 1.645 \times \text{Standard deviation}. \quad (1)$$

A normalization factor (NF) was calculated using the ACP and the NC from the 30 donor screening runs:

$$\text{NF} = \text{ACP} - \text{Mean NC of the runs used to calculate the ACP}. \quad (2)$$

Finally, the RSCP was calculated for each run using the run NC and the NF:

$$\text{RSCP} = \text{Mean NC of the run} + \text{NF}. \quad (3)$$

2.6.2. Threshold Determination. Due to issues encountered on the ELISA and the AlphaLISA platforms, the RSCP determination was found to be biased and of limited value for the technology evaluation. For the ELISA platform, signals very close to the lower detection limit of the reader (optical density below 0.05) were obtained for the individual donors leading to an underestimation of the interdonor variability. On the contrary, for the AlphaLISA technology, a high interdonor variability was observed. Whilst extended optimization work may have addressed these issues to some degree, for the comparison work performed as part of the assay development phase, these were viewed as limitations of the platforms. In addition, the evaluation performed with individual donors was limited during this development phase as compared to the comprehensive balanced design assessment that is typical of the assay validation phase, a further source of bias for the determination of the cut-point. For these reasons a statistical cut-point, from a limited individual population evaluation, was deemed inappropriate because it would not have permitted a fair comparison of the different methods.

Thus an arbitrary threshold (Th) was calculated, according to the following equation:

$$\text{Th} = \text{Mean NC of the run} \times 1.25. \quad (4)$$

The value was set at 25% above the background, a level that would robustly distinguish a real change in signal response as a result of different experimental parameters to those that were due to method precision, thus multiplication factor of 1.25. Using the threshold method still allowed normalization for run-to-run variation and applied a consistent factor to all of the runs and across each of the different platforms. In this study, samples containing the same matrix (pool of sera) were used. Consequently, an approach based on the background of this matrix rather than on the interdonor variability was found to be more relevant.

2.7. Analysis Software and Parameters. All of the raw data generated on ELISA, MSD, and AlphaLISA were imported into the Gen5 analysis software (v1.6). The curves were generated using a 4-parameter analysis and weight $1/y$. The raw data generated on Gyrolab were analyzed on the Gyrolab Evaluator software (v3.3.7.171) using the Quantification

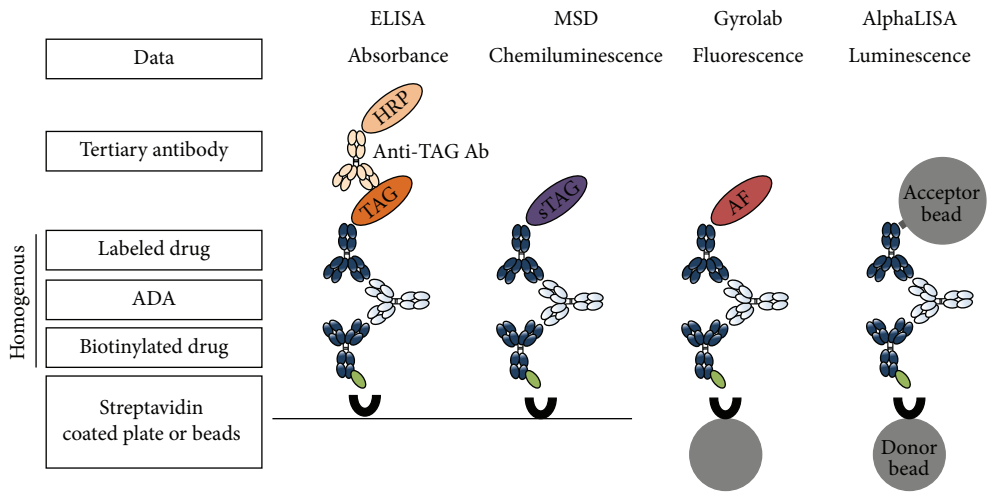

Figure 2: Assay formats and platforms. For each platform, a bridging format was used with the biotinylated drug (Novimab) as the capture antibody. The drug (Novimab) was also used as the detection antibody and was conjugated to a specific labeling molecule appropriate to each of the different platforms. Only the ELISA assay was developed using a tertiary antibody directed against the detection antibody TAG. HRP: Horseradish Peroxidase, sTAG: SULFO-TAG, and AF: Alexa Fluor 647.

module (v3.3.7.171) and the ADA Analysis module (v3.3.7.171). The curves were generated using a 5-parameter analysis and weight $1/y^2$.

2.8. Acceptance Criteria. The goal of this study was to select a fit-for-purpose method for further assay development. This study was conducted as a preliminary evaluation and acceptance criteria were set according to the individual project requirements.

2.8.1. Sensitivity. Industry guidance recommends the development of an immunogenicity assay with a sensitivity of approximately 250–500 ng/mL [11, 12, 20]. However, the choice of the reference ADA used in the assay has a significant impact on the sensitivity achieved. Purified polyclonal rabbit anti-drug antibodies are commonly used in the development of immunogenicity assays at Novimmune and with this reference ADA it was felt that a lower sensitivity would be more appropriate in order to detect low level immunogenicity events. Consequently, the sensitivity acceptance criteria were set at 50 ng/mL in this study.

2.8.2. Drug Tolerance. An ADA assay with an inappropriate drug tolerance leads to false negative samples. Based on PK-PD modeling of the likely drug accumulation with the clinical dosing regimen, the drug tolerance acceptance criteria were set at 20 μg/mL in this study.

2.8.3. Target Tolerance. In this ADA assay, lack of target tolerance would lead to the generation of false positive results. Based on the soluble dimeric target level in a disease population and its potential accumulation [21] during the treatment period, a target tolerance up to 50 ng/mL was considered as acceptable for this ADA assay.

2.8.4. Interassay and Interdonor Variability. These two parameters are studied during an ADA assay validation. Consequently it was important to evaluate them in the assay development step to ensure they would not create any major issues during the assay validation phase. As a result, the data obtained from these parameters was only used for information but was not considered as critical as the drug and the target tolerance.

The interassay precision based on the PC signal was calculated over 3 runs and performed on 3 different days, for each method. The number of donors considered as positive (with a signal above the threshold) was analyzed to evaluate the interdonor variability. The method showing minimal interassay and interdonor variability would be selected.

3. Results and Discussion

The same bridging format immunogenicity assay using a labeled Novimab as a capture and detection reagent was developed and optimized in a homogenous manner on 4 different technology platforms: ELISA, MSD, Gyrolab, and AlphaLISA. The optimization included the determination of the capture/detection reagent concentrations, the minimum required dilution, and the incubation time. The different assay formats for the platforms are described in Figure 2.

In the evaluation study, samples were subjected to different steps (±acid dissociation, ±target depletion), as shown in Figure 3, and analyzed on each of the 4 platforms. The objective was then to select the best fit-for-purpose method (combination of platform and additional steps). As elevated drug and target levels were expected in the clinical population, it was crucial to select a method able to tolerate these levels to expose true ADA positive events. The sensitivity of the assay was assessed to achieve a suitable level for the clinical sample testing phase and evaluate the concentration

Table 1: Sample dilution factors according to the different methods (platforms and additional steps).

Technology used	ELISA				MSD				Gyrolab				AlphaLISA			
Acid dissociation step	No		Yes		No		Yes		No		Yes		No		Yes	
Target depletion step	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
Dilution induced by the target depletion step[(1)]	1 in 3		1 in 3		1 in 3		1 in 3		1 in 3		1 in 3		1 in 3		1 in 3	
Dilution induced by the acidification step	NA		1 in 5		NA		1 in 5		NA		1 in 2		NA		1 in 5	
Dilution induced by the neutralization step/Master Mix addition	1 in 2		1 in 3		1 in 2		1 in 3		1 in 2		1 in 1.5		1 in 5		1 in 3	
Final dilution applied before platform analysis	*1 in 6*		*1 in 45*		*1 in 6*		*1 in 45*		*1 in 6*		*1 in 9*		*1 in 15*		*1 in 45*	

[(1)]Samples not target depleted were diluted at 1 in 3 in PBS-1% BSA 0.05% tween.

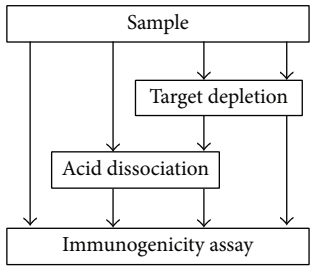

Figure 3: Samples (standard, NC, PC, drug, and target interference samples) were subjected to different treatments and analyzed in the ADA screening assay.

of the low PC concentration that would be used for system suitability purposes. The assessment of interassay and interdonor variability was also an important consideration.

During the optimization experiments of the bridging assay on the different platforms, different minimum required dilutions were obtained. Consequently, according to the pretreatment (additional step) and the platform used, the final dilutions applied to the samples varied and are summarized in Table 1. The samples submitted to a target depletion step were diluted at 1 in 3 due to the addition of the reagents used for the immunodepletion. To allow a direct comparison of the efficiency of the target depletion procedure, equivalent 1 in 3 dilutions in buffer was applied to samples not submitted to the target depletion step. For acid dissociation, samples were diluted at 1 in 5 with a low pH buffer for all technologies, except Gyrolab. For Gyrolab the volumes for the different sample and buffer addition steps are defined within the CD microstructure and fixed at 200 nL allowing only a sample : acid ratio of 1 : 1 (volume : volume). No additional dilution was applied to the samples requiring no acid dissociation treatment. The nonacidified samples were diluted at 1 in 2 with the Master Mix solution, except for the AlphaLISA platform where 1 in 5 dilutions was performed, as recommended by the provider. One in 3 dilutions was applied to the acidified samples as 1 volume of neutralization buffer had to be added in addition to the Master Mix solution. The Gyrolab technology was an exception as the Master Mix had to be prepared directly in the neutralization buffer and 1 volume of this solution added to 2 volumes of acidified sample.

3.1. Analysis Strategy. The goal of this evaluation was to select a fit-for-purpose method to support clinical development of Novimab. In the ADA assay, the determination of a cut-point is a key parameter because it defines the limit above which a result is considered as "positive" during the clinical sample testing phase. In this study, to allow evaluation of the different technologies, a relative-qualitative approach was taken, using a reference ADA to describe key assay attributes such as sensitivity, drug, and target tolerance and interdonor variability and interassay precision. Increasing concentrations of PCs were prepared and analyzed in the different methods to check whether the signal obtained was positive or negative (signal above or below the cut-point value, resp.). In the final method validation and sample analysis, the reference ADA would instead be used for system suitability assessment, ADA positivity of samples being evaluated following the tiered approach with semiquantitation by serial dilution titration.

Consequently, the cut-point parameter is critical and has to be carefully calculated. As explained in Section 2, a statistical approach based on a limited number of donors (30) was initially used to calculate the cut-point. Using this approach, a bias was observed for two methods, ELISA and AlphaLISA, without an acid dissociation step leading to a misinterpretation of the data. There are different reasons to explain this observation. For the ELISA platform, the signals given by the different donors were very close to the lower detection limit of the reader (optical density below 0.05), leading to an underestimation of the interdonor variability. On the contrary, for the AlphaLISA technology, a high interdonor variability was observed. Moreover, the number of donors used for the determination of the cut-point may have been too low to provide a robust value. For these reasons a statistical cut-point was deemed inappropriate because it would not have permitted a fair comparison of all of the methods. Furthermore, the determination of a robust cut-point requires the assessment of multiple donors (80 or more). This intensive approach is not normally required during the assay development phase.

Table 2: Sensitivity of the different methods determined as the lowest PC concentration exhibiting a response above the threshold.

Technology used	ELISA				MSD				Gyrolab				AlphaLISA			
Acid dissociation step	No		Yes		No		Yes		No		Yes		No		Yes	
Target depletion step	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
Final dilution applied before platform analysis	1 in 6		1 in 45		1 in 6		1 in 45		1 in 6		1 in 9		1 in 15		1 in 45	
Sensitivity ng/mL of reference ADA	5		100		2		20		2		5		20		20	

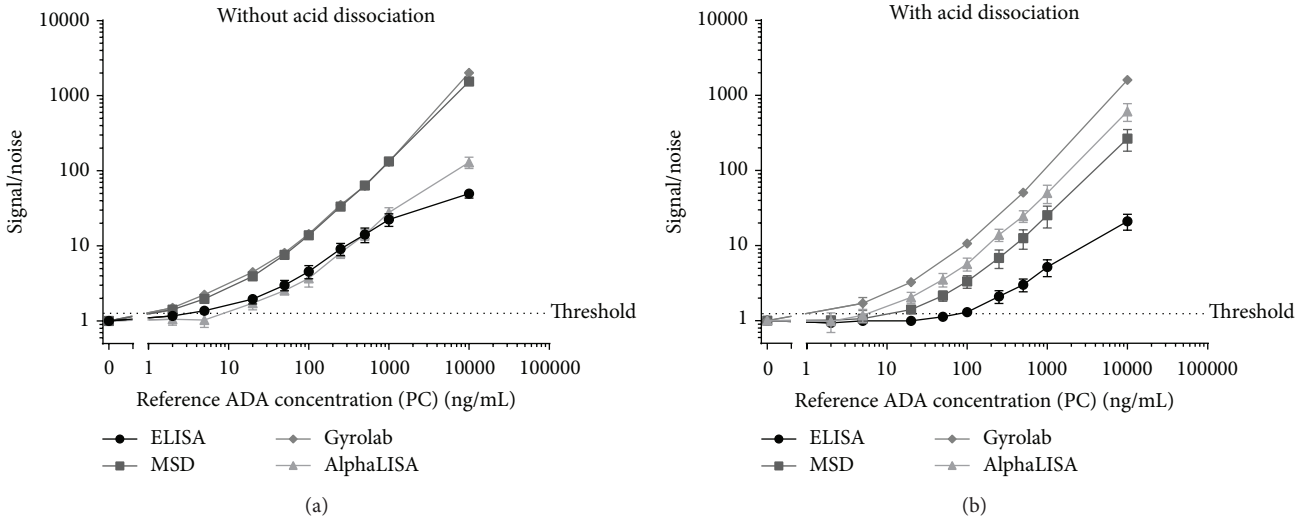

Figure 4: (a) PC data obtained for the interassay assessment on the different platforms without acid dissociation. The data are presented as the mean PC signal obtained divided by the mean NC signal (signal/noise). (b) PC data obtained for the interassay assessment on the different platforms with acid dissociation. The data are presented as the mean PC signal obtained divided by the mean NC signal (signal/noise).

Therefore, a second approach was used to set a limit above which a result is considered "positive." For this approach, an arbitrary threshold was defined. It was calculated by applying a multiplication factor of 1.25 to the mean negative control signal observed in each assay run. This approach gave a fairer comparison of all the methods as different interdonor variability did not affect the value of the threshold. The value was set at 25% above the background, a level that would robustly distinguish a real change in signal response as a result of different experimental parameters to those that could be the result of method precision. Using the threshold method still allowed normalization for run-to-run variation, as the threshold value was calculated from the mean signal of the negative control samples included in each run. Only the threshold value approach was used to evaluate the different parameters for each method.

3.2. Sensitivity. A NC and nine PC samples were analyzed across three different days, on each of the four platforms with the two steps (with or without an acid dissociation step) (Figure 4(a)). The PC concentrations tested were 2, 5, 20, 50, 100, 250, 500, 1000, and 10000 ng/mL of reference ADA. The assay sensitivity was determined based on the results obtained across the 3 interassay runs as the lowest PC concentration consistently returning a signal above the threshold value.

All of the methods exhibited a good level of sensitivity below 100 ng/mL of reference ADA (Table 2). In the absence of an acid dissociation step, the highest sensitivities were obtained for the ELISA, Gyrolab, and MSD platforms, where the determined sensitivities were equal to 5 ng/mL, 2 ng/mL, and 2 ng/mL of reference ADA, respectively. The AlphaLISA platform also achieved a sensitivity equal to 20 ng/mL, well within target criteria, but a little lower than the other platforms. Two reasons could explain this lower sensitivity. Firstly, the final dilution factor required in all of the other technologies was 1 in 6 (1 in 3 for the target depletion and 1 in 2 for the assay) compared to 1 in 15 (1 in 3 for the target depletion and 1 in 5 for the assay) for the AlphaLISA technology (Table 2). This 1 in 5 dilutions is recommended by the provider. Secondly, the NC in the AlphaLISA assay gave a higher signal response than expected (compared to other immunoassays runs on the platform), resulting in a lower signal-to-noise ratio for samples. Further investigation work would be required such as evaluating different buffers or sample dilution parameters to address this issue. However, this would be a time-consuming step and may be viewed as a limitation of the platform in this particular assay development context.

For the methods including an acid dissociation step, the Gyrolab platform was the most sensitive (equal to 5 ng/mL of reference ADA), followed by a similar sensitivity of 20 ng/mL

of reference ADA for the MSD and the AlphaLISA platforms (Table 2 and Figure 4(b)). The ELISA platform exhibited sensitivity equal to 100 ng/mL of reference ADA. The loss in sensitivity observed for most of the technologies arose from the fact that a higher dilution factor was applied when an acid dissociation step was implemented. Moreover, the dilution factor increase was not the same for all of the platforms. For the MSD and ELISA platforms, the samples were 7.5-fold more diluted (final dilution of 1 in 45 compared to 1 in 6 without acid dissociation; Table 2) whereas the final sample dilution factor was only increased from 1 in 6 to 1 in 9 for the Gyrolab technology. The Gyrolab technology defines the sample and buffer volumes within the CD microstructure, limiting the technology to a sample : acid ratio of 1 : 1. For the other 3 technologies, the sample : acid ratio was optimized as a 1 : 5 ratio, combining appropriate sensitivity, drug tolerance, and sample usage. The inability to modify the sample : acid volume ratio is seen as a small limitation of the Gyrolab assay; however, the number of samples which can be tested on each CD (48 microstructures allowing only 24 samples in duplicate) represents a bigger limitation for the use of this technology. It is noted that runs can be performed with multiple Mixing CDs (up to 5 per run), but sample numbers are still limited if appropriate controls are included on each CD and result in an increase in the assay time, though the "hands-off" benefits of automation are still retained. The only technology that showed no difference in terms of sensitivity with the acid dissociation step, despite the increased dilution factor of the samples from 1 in 15 to 1 in 45, was the AlphaLISA. This may be explained by the fact that the acid dissociation method was less sensitive to a matrix effect. Indeed for the AlphaLISA the NC signal obtained with the acid dissociation was lower than the NC signal obtained without the acid dissociation step. This suggested that the high NC signal observed without the dissociation step may have been due to the presence of a component in the matrix (serum) which was altered by the acidification process.

The dynamic range and the saturation level (hook effect) are additional parameters that can be taken into consideration for the final selection of the methods. A method exhibiting a high saturation level for anti-drug antibody concentrations is preferred. In our study, the saturation level, shown in Figures 4(a) and 4(b) for all of the methods with and without an acid dissociation step, was reached earlier on the ELISA platform in comparison to the MSD, Gyrolab, and AlphaLISA platforms. The saturation level shown for the AlphaLISA platform in the absence of an acid dissociation step is not a true representative of the assay performance due to the high background observed in the blank samples.

The sensitivity was one of the key parameters for the selection of the fit-for-purpose method but drug and target tolerance were considered of higher priority. Looking only at the sensitivity, the MSD and Gyrolab technologies displayed better results without an acid dissociation step, but the ELISA and AlphaLISA were both viable options. In the presence of the acid dissociation step, MSD and Gyrolab technologies again displayed better results. The AlphaLISA platform exhibited a similar level of performance, whilst the ELISA displayed a lower, but adequate, level of sensitivity. However, it is important to remember that the ADA assay sensitivity and saturation level are relative to the choice of the reference ADA used as a surrogate.

3.3. Interassay Variability. Nine different PC concentrations of 2, 5, 20, 50, 100, 250, 500, 1000, and 10000 ng/mL were tested across three different days with or without acid dissociation treatment. Due to the lower throughput on the Mixing CD (i.e., 48 sample microstructures compared to 112 microstructures on a Bioaffy CD200), only five concentrations of PCs were tested per run on Gyros. Instead, the five PC concentrations of 5, 20, 100, 500, and 10000 ng/mL were tested across 5 different runs. The precision (CV%) obtained for the different runs performed for each method was calculated on the signal value obtained at each PC level and the results are presented in Table 3.

For this stage of development, all of the methods demonstrated an acceptable interassay precision. For the eight methods, the PCs with a concentration above the threshold achieved a precision below 27% (Table 3). However, differences were observed between the different methods. Without acid dissociation, the better interassay precision was obtained on the Gyrolab and MSD platforms with a precision below 10% for all of the PC samples. For the two other platforms (ELISA and AlphaLISA), precision between 4% and 27% was obtained for the methods without an acid dissociation step. For ELISA and AlphaLISA platforms, the precisions were similar (below 19%) with the addition of an acid dissociation step. For the MSD platform, an increase in imprecision from 10% to 26% was clearly observed with the method containing an acid dissociation step. For the Gyrolab method only the 5 ng/mL PC sample produced a CV greater than 10%; this was almost certainly due to the automation of the Gyrolab platform that reduced the manual handling work and thus the variability. As this work corresponds to a preliminary assay evaluation, the precision obtained with the acid dissociation step might be improved in the future with a refinement of the method.

The interassay precision was assessed only on samples containing free ADA with and without an acid dissociation treatment. It was not tested whether the presence of Novimab in the samples and the target depletion step would affect the interassay precision. There is indeed a risk that the additional treatment steps might increase the interassay variability. Whilst this has not been assessed for all the methods, interdonor variability has been assessed for the MSD method with the inclusion of target depletion and acid dissociation steps and resulted in no noticeable increase in the precision.

3.4. Interdonor Variability. Thirty naïve individual sera were tested to assess the interdonor variability on the different platforms with or without an acid dissociation step. The results are shown in Figure 5.

During this study, thirty naïve individual sera were tested in order to establish a RSCP and to evaluate the interdonor variability. As explained previously in Analysis Strategy, the approach based on a statistical cut-point was not appropriate

Table 3: Interassay precision obtained on the different methods for nine levels of PCs (five levels of PCs were assessed on the Gyrolab platform with acid dissociation due to the lower number of structures present on the Gyrolab Mixing CDs). The precision is presented in %CV and is calculated from three independent runs performed on different days (except for the Gyrolab assay with acid dissociation where five independent runs were performed). Each run contained two sets of independently prepared PCs.

	Interassay precision (CV%)							
Technology used	ELISA		MSD		Gyrolab		AlphaLISA	
Acid dissociation step	No	Yes	No	Yes	No	Yes	No	Yes
Reference ADA concentration (PC) in ng/mL								
2	<*BLQ*	<*BLQ*	5.51	<*BLQ*	8.39	NT	<*BLQ*	<*BLQ*
5	14.83	<*BLQ*	6.00	<*BLQ*	6.03	16.07	<*BLQ*	<*BLQ*
20	20.14	<*BLQ*	4.99	4.03	1.67	3.88	14.49	17.11
50	21.94	<*BLQ*	5.45	8.96	3.11	NT	9.46	14.43
100	24.67	18.89	3.09	11.57	2.58	5.04	10.34	13.83
250	23.81	5.66	4.62	21.46	4.97	NT	12.91	14.67
500	26.77	7.30	5.31	22.47	3.61	6.07	16.23	9.37
1000	23.56	2.50	4.73	25.98	2.89	NT	11.44	18.99
10000	13.41	1.73	6.37	25.95	6.87	7.21	4.19	4.76

"NT" was used for the PCs not tested due to the limited number of structures on Gyrolab Mixing CDs. "<BLQ" refers to a result below the sensitivity of the method.

in this study. Consequently, only the interdonor variability is presented in Figure 5. For all of the methods except for the AlphaLISA, the signal response of the serum matrix pool used for the negative controls and preparation of the positive controls was representative of the signal response of the individual matrices. Similar signals were obtained for the blanks and for the 30 naïve individual sera. Consequently, the second approach for the calculation of the cut-point based on a threshold was used to determine the number of donors that would be considered as false positives. For the AlphaLISA platform using a method without an acid dissociation step, a high background was observed with the serum matrix pool. As previously explained, more investigations would be required to understand the origin of this high background.

A very low interdonor variability was observed for the ELISA and MSD platforms in the presence or absence of an acid dissociation step. For both technologies, a maximum of one individual, which represents less than 5% of the donors, exhibited a signal above the threshold. A higher variability was observed with the Gyrolab platform. Indeed, 3 and 2 naïve individuals out of the 30 naïve individuals tested produced a signal above the threshold, for the method without or with acid dissociation, respectively. In some cases, it was due to an outlier in the duplicate data leading to a sample precision greater than 30%. This phenomenon is occasionally observed with this technology. As it is an instrument based on microfluidics the matrix should be free of particulate matter that could interfere in the assay and result in high signal for one of the replicates. The AlphaLISA platform showed a very high interdonor variability, especially without the acid dissociation step where almost 50% (14 out 30) of the naïve individuals were above the threshold and considered as false positives. The results were improved with the acid dissociation step with 20% (6 out 30) of the individuals above the threshold but the data still remained out of criteria.

As a conclusion, the ELISA, MSD, and Gyrolab platforms displayed an acceptable interdonor variability. No selectivity issues would be anticipated for assay validation on the ELISA and MSD platforms. For the Gyrolab platform, additional care may be required with sample handling to avoid outliers and thus this can be seen as a limitation of this platform. For the AlphaLISA technology, investigation work would be required such as evaluating different buffers or sample dilution parameters to address this issue. However, this would represent a time-consuming step and therefore may be viewed as a limitation of the platform in this particular assay development context.

3.5. Drug Tolerance. Based on our previous clinical data, Novimab levels approaching 20 μg/mL were anticipated in clinical samples during the ADA assessment. As previously described this can lead to false negative results during the clinical sample testing phase if the assay does not have sufficient tolerance. Acid dissociation is a well-documented approach that can be used to overcome drug interference problems. In order to evaluate the impact of an acid dissociation step, the comparison study was performed on the 4 platforms, with and without an acid dissociation step. The acid dissociation step was optimized on each platform and the optimal conditions were used. The results are presented in Table 4.

Nine PC samples (5, 20, 50, 100, 250, 500, 1000, 2500, and 10000 ng/mL) were analyzed in the presence of 0, 20, and 100 μg/mL concentrations of Novimab. Table 4 shows the drug tolerance data obtained for two concentrations of Novimab (20 and 100 μg/mL) for both treatments tested on the 4 different platforms.

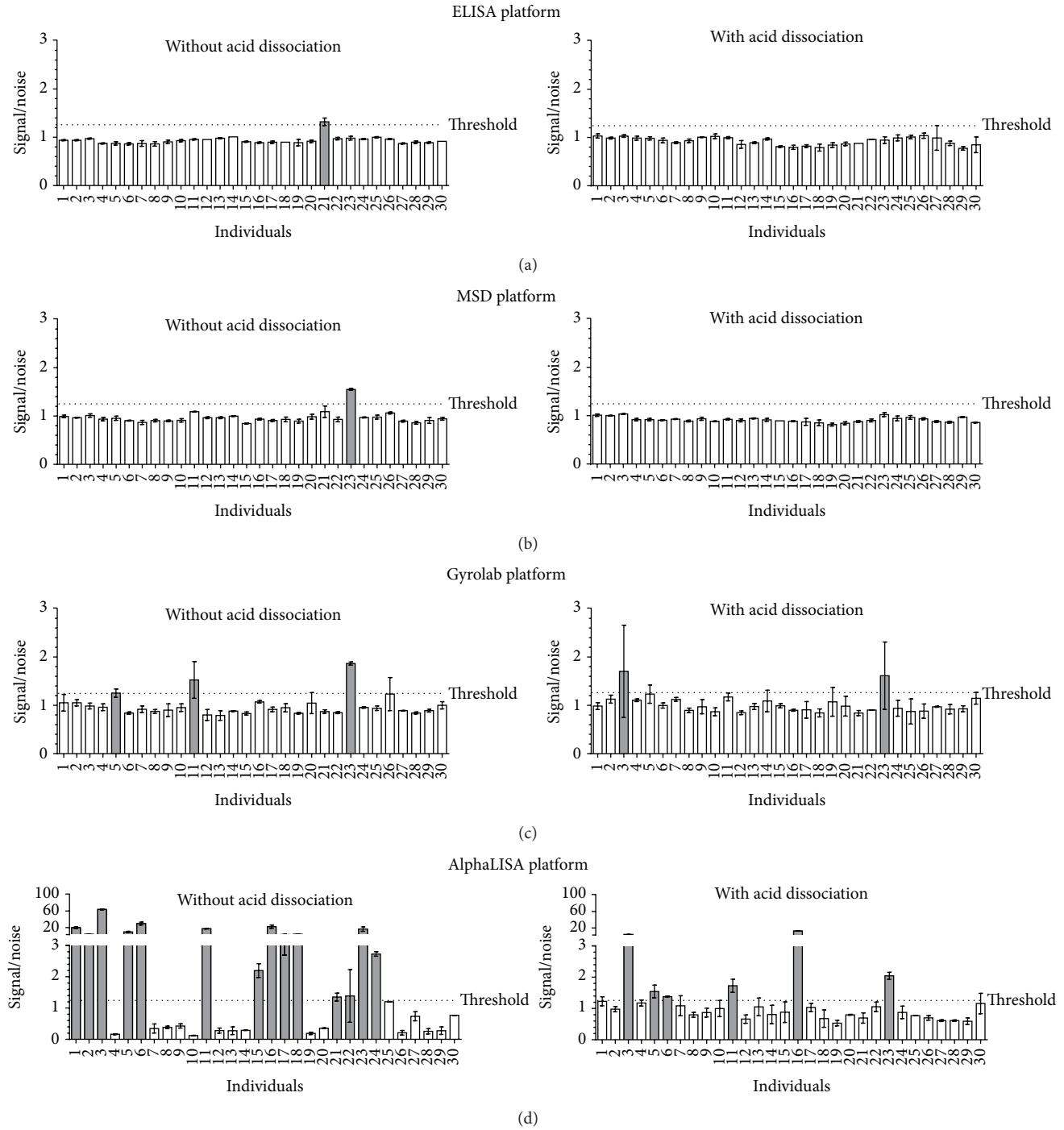

Figure 5: Interdonor variability according to the platform used and the treatment applied to the samples. The signals obtained for the 30 donors were normalized in signal/noise values where the noise represents the mean NC signal measured in each method. The dotted line represents the threshold fixed at a signal/noise value equal to 1.25. The individual sera giving a signal/noise above the threshold were identified and the corresponding bars in the graphs were colored grey.

In the absence of an acid treatment step, the Gyrolab platform showed the highest level of drug tolerance where 100 ng/mL and 500 ng/mL of reference ADA could be detected in the presence of 20 μg/mL and 100 μg/mL of Novimab, respectively. The MSD platform also performed well because 250 ng/mL and 1000 ng/mL of reference ADA could be detected in presence of 20 μg/mL and 100 μg/mL of Novimab, respectively. The ELISA and the AlphaLISA assays showed a critical loss of sensitivity in the presence of Novimab when no acid dissociation was performed.

Despite the decrease in sensitivity for the acidified samples containing no Novimab, except for the AlphaLISA

Table 4: Sensitivity observed for each assay in the presence of 0, 20, and 100 μg/mL of Novimab. The sensitivity was calculated as the lowest PC concentration where the signal obtained was above the threshold value.

	Sensitivity (ng/mL of reference ADA)							
Technology used	ELISA		MSD		Gyrolab		AlphaLISA	
Acid dissociation step	No	Yes	No	Yes	No	Yes	No	Yes
Novimab concentration (μg/mL)								
0	5	100	2	20	2	5	20	20
20	1000	100	250	50	100	5	10000	1000
100	10000	250	1000	50	500	50	>10000	10000

where it is equivalent with and without acid dissociation, the drug tolerance level was generally improved when an acid treatment was performed, consistent with previous publications [6–10, 13]. The acid treatment step dissociates the ADA-therapeutic drug complexes, thus increasing the accessibility of the capture and detection reagents to the ADA. However, whilst the drug tolerance was improved by the acid treatment on all of the platforms, the performances still varied across them. Thus when considering the 20 μg/mL Novimab level, the Gyrolab platform exhibited a higher level of drug tolerance than the MSD, the ELISA, and the AlphaLISA, as these platforms allowed detection of 5, 50, 100, and 1000 ng/mL of reference ADA, respectively. With 100 μg/mL of Novimab, the Gyrolab and the MSD platforms still exhibited the highest level of drug tolerance because 50 ng/mL of reference ADA could be detected.

Taken together, these data suggest that the acid dissociation treatment is efficient in increasing the drug tolerance level by a factor 5- to 40-fold. Despite this increase some platforms still exhibited a poor level of drug tolerance (e.g., AlphaLISA). In this particular study, the Gyrolab and the MSD were the two technologies where a sensitivity of 50 ng/mL of reference ADA could be achieved in the presence of 20 μg/mL of Novimab, when an acid dissociation step was performed.

3.6. Target Tolerance. A major challenge encountered in immunogenicity assays is the level of target tolerance. The presence of dimeric or multimeric forms of a soluble target may induce a signal in a bridging immunogenicity assay and lead to a false positive result. In our example, the soluble target has the capacity to form dimers that can interfere in the assay.

In some disease populations, the basal level of soluble dimeric target can be considerably higher than the level measured in a healthy population. It is also known that injection of an antibody directed against a soluble dimeric target can lead to the accumulation of the latter in the serum due to the different clearance rate of the therapeutic drug-soluble target complex [21]. Based on the anticipated soluble dimeric target level in the disease population and its expected accumulation during the treatment period, it was important to improve the target tolerance of our assay format.

Methods to improve the target tolerance can include addition of a high concentration of a competitive anti-target antibody to the samples to compete with the labeled drugs for the binding to the target. This approach was tested with several competing anti-target antibodies and whilst the target interference was improved even a 300-fold molar excess of competing anti-target antibody compared to the target was not enough to reduce the interference of the target below the threshold of the assay, leading to the generation of false positive results (data not shown). Consequently, immunodepletion of the target was implemented as described in Section 2. This is important to keep in mind that such additional sample processing should be applied only if needed because it may cause a loss of the low affinity ADA (e.g., IgM) due to the additional processing step. The impact of sample processing on detection of low affinity ADA was not investigated in the context of this work.

In the comparison study, in order to evaluate the efficiency of the target depletion procedure, samples containing increasing concentrations of soluble dimeric target were split into 2 groups, with and without a target depletion step. The samples not depleted were diluted in PBS-1% BSA 0.05% tween to mimic the depletion step dilution and to maintain the appropriate final dilution. To identify the optimal method to enhance the target tolerance in the ADA assay, samples containing four different concentrations of soluble dimeric target (5, 20, 50, and 200 ng/mL) were analyzed across the different technologies. The samples that had undergone different treatments, that is, with/without acid dissociation and with/without target depletion, were finally compared.

With no acid dissociation and no target depletion, even a low level of soluble dimeric target of 5 ng/mL generated a false positive signal on all of the platforms (Table 5 and Figure 6). The soluble dimeric target interfering in the assay is acid-labile and the addition of an acid dissociation treatment step alone was sufficient to achieve the desired target tolerance of 50 ng/mL in the ELISA assay. This was a clear advantage compared to the other technologies where further sample processing in the form of a target depletion step was required to reach the required target tolerance level. However, the ELISA assay with acid treatment did have lower albeit adequate sensitivity.

Indeed across all of the platforms and with both methods (i.e., with or without acid dissociation), the target depletion procedure enhanced the levels of target tolerance (Table 5). However, in the absence of acid dissociation, the MSD, the Gyrolab, and the AlphaLISA were only tolerant to 5 ng/mL of soluble dimeric target with target depletion alone. This level of target could be expected in clinical samples and thus remained problematic. The ELISA displayed the highest level of target tolerance, up to 20 ng/mL, with target depletion

Table 5: Results obtained on the different methods according to the levels of soluble dimeric target.

Technology used	ELISA				MSD				Gyrolab				AlphaLISA			
Acid dissociation step	No		Yes		No		Yes		No		Yes		No		Yes	
Depleted samples	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
Soluble Target Concentration (ng/mL)																
5	+	−	−	−	+	−	−	−	+	−	+	−	+	−	+	−
20	+	−	−	−	+	+	+	−	+	+	+	−	+	+	+	−
50	+	+	−	−	+	+	+	−	+	+	+	−	+	+	+	−
200	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	−

The symbols "+" and "−" refer to sample exhibiting a signal above or below the threshold value, respectively.

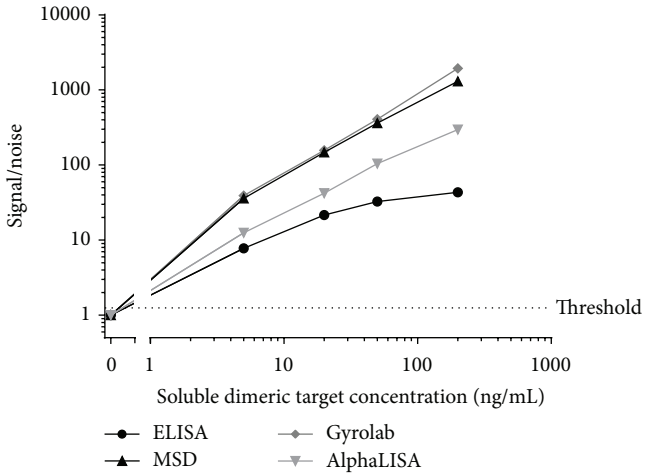

Figure 6: Evaluation of target interference for each assay in the presence of 0, 5, 20, 50, and 200 ng/mL of soluble dimeric target evaluated in the method without acid dissociation or target depletion. The noise represents the mean NC signal measured in each method. The dotted line represents the threshold fixed at a signal/noise value equal to 1.25.

alone (Table 5) and also with acid dissociation alone, thus showing clear advantages for the target tolerance evaluation parameter.

For all of the 4 technologies, the highest level of target tolerance was obtained with a combination of the target depletion and acid dissociation steps. With these combined sample treatments the target tolerance was increased to 200 ng/mL of soluble dimeric target for all of the platforms assessed.

4. Conclusion

The aim of this study was to develop an ADA assay on different technology platforms (ELISA, MSD, Gyrolab, and AlphaLISA) with different additional steps (±acid dissociation, ±target depletion) in order to achieve an assay with an appropriate sensitivity, interdonor variability, drug, and target tolerance level.

All of the 4 platforms were tested either with or without acid dissociation and with or without target depletion. The data obtained in this study showed that the acid dissociation step improved the drug tolerance by a factor of 5 to 40 depending on the technology used and the drug concentration. For the expected Novimab concentration in the clinical samples, that is, 20 μg/mL, the Gyrolab and MSD platforms showed a drug tolerance level equal to 5 ng/mL and 50 ng/mL of reference ADA, respectively. These values satisfied our 50 ng/mL sensitivity criteria for the reference ADA. The drug tolerance for the ELISA and AlphaLISA platforms was also improved by the acid dissociation treatment but the level remained below our target criteria (100 ng/mL and 1000 ng/mL of reference ADA in presence of 20 μg/mL of Novimab, resp.). The acid dissociation treatment also had a positive impact on the target tolerance as the soluble dimeric target is acid-labile. For a soluble dimeric target concentration of 50 ng/mL this treatment alone was sufficient to lower the signal below the threshold value for the ELISA platform. This is a clear advantage for the ELISA platform where less sample processing was required to meet our requirements in terms of drug and target tolerance, whilst achieving adequate sensitivity. For the MSD technology, inclusion of an acid dissociation step improved target tolerance a little; for Gyrolab and AlphaLISA no marked improvement was noted. For these latter 3 technologies the signal remained above the threshold value at the required target tolerance level of 50 ng/mL, thus producing false positive results. This interference was overcome by combining a target depletion step with the acid treatment. For all platforms this was efficient to raise the target tolerance to 200 ng/mL of soluble dimeric target. Despite all of the advantages in terms of drug and target tolerance obtained by the inclusion of acid treatment, it did have a negative impact on sensitivity and the interassay variability parameters. The sensitivity was indeed reduced due to a higher sample dilution required by the acid treatment. The increase in interassay variability observed with the acid dissociation treatment is likely a result of the additional sample processing steps which had to be performed during the assay. However, despite this these two parameters still remained within our acceptance criteria.

There are a number of different technologies available for the development of ADA assays. In this study the ELISA, Gyrolab, MSD, and AlphaLISA were evaluated. The MSD, the ELISA, and the Gyrolab appeared to be the 3 platforms that gave the better results in terms of sensitivity,

interassay precision, interdonor variability, drug tolerance, and target tolerance. Each platform has particular advantages. The ELISA platform exhibited a relative sensitivity slightly lower than MSD and Gyrolab technology and when acid dissociation treatment was applied, the sensitivity was reduced to 100 ng/mL, less than our target criteria, although adequate in terms of industry expectations. In achieving our requirements in terms of drug and target tolerance, with less sample pretreatment steps, the ELISA represents an attractive option. Additionally, from a practical perspective, implementation of this methodology in the laboratory environment requires no specialist immunoassay platforms. However, a disadvantage of the ELISA was that the signal response of the NC was often close to the lower limit of detection of the reader with a consequent decreased interdonor variability which could be problematic for the selectivity and cut-point assessments during the assay validation phase. This parameter could probably be improved by optimizing the incubation times, reagent concentrations, or sample dilutions to slightly increase the background signal.

With the MSD and the Gyrolab technologies, for the key parameters of drug tolerance and target tolerance, the best performance was obtained when the samples were subjected to both acid dissociation and target depletion procedures. In this case the interassay variability was higher when using the MSD platform compared to the Gyrolab. This almost certainly comes from the automation of this latter technology which provides a better reproducibility between each run. Additionally, the sample volume requirements are lower for the Gyrolab than the MSD. However, the Gyrolab requires a more regular and time-consuming maintenance than the MSD reader and is subject to more technical challenges. For the present ADA assay application the throughput is limited by the availability of only 48 microstructures on the Gyrolab Mixing CD used to perform the acid dissociation treatment. Specific care is required during the sample handling and assay preparation to avoid the presence of outliers. The matrix should be free of particulate matter that could block the microstructures on the CDs and the stickiness of the Alexa Fluor conjugated detection antibodies requires specific handling to avoid aggregate formation, both of which can result in anomalous fluorescence peaks.

The AlphaLISA platform cannot be considered a suitable technology for further development of this particular assay because a lower drug tolerance and a higher interdonor variability were observed compared to the other platforms. Other technology comparison studies have reached different conclusions concerning their preferred choice of technology [18]; however, as demonstrated in this study, it is important to consider the individual requirements of each particular clinical study to select the most appropriate assay and platform. Factors that also need to be considered in addition to assay performance are available sample volume, assay run time, automation, reagent costs, and platform availability at Contract Research Organizations (CROs). Therefore, the testing of multiple technology platforms, in parallel, during method development, is recommended to facilitate the selection of the most appropriate ADA screening assay format to support a clinical trial program.

Abbreviations

ACP:	Assay cut-point
ADA:	Anti-drug antibody
AF:	Alexa Fluor 647
AlphaLISA:	Amplified luminescent proximity homogeneous assay developed by PerkinElmer Company
BLQ:	Below level of quantification
CRO:	Contract Research Organization
ELISA:	Enzyme-linked immunosorbent assay
FDA:	Food and Drug Administration
Gyrolab:	Automated microfluidics immunoassay developed by Gyros AB
HRP:	Horseradish Peroxidase
MSD:	Electrochemiluminescent immunoassay developed by Meso Scale Discovery
NC:	Negative control
NF:	Normalization factor
Novimab:	Fully human therapeutic monoclonal antibody
NT:	Not tested
PC:	Positive control reference ADA
RPM:	Rotations per minute
RSCP:	Run specific cut-point
sTAG:	SULFO-TAG
Th:	Threshold.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Justine Collet-Brose and Pierre-Jean Couble contributed equally to this work.

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References

[1] J.-F. Colombel, W. J. Sandborn, P. Rutgeerts et al., "Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial," *Gastroenterology*, vol. 132, no. 1, pp. 52–65, 2007.

[2] F. Schnitzler, H. Fidder, M. Ferrante et al., "Long-term outcome of treatment with infliximab in 614 patients with Crohn's disease: results from a single-centre cohort," *Gut*, vol. 58, no. 4, pp. 492–500, 2009.

[3] L. Moja, L. Tagliabue, S. Balduzzi et al., "Trastuzumab containing regimens for early breast cancer," *Cochrane Database of Systematic Reviews*, vol. 4, Article ID CD006243, 2012.

[4] L. H. Kircik and J. Q. Del Rosso, "Anti-TNF agents for the treatment of psoriasis," *Journal of Drugs in Dermatology*, vol. 8, no. 6, pp. 546–559, 2009.

[5] A. Seki, M. Iwamuro, M. Yoshioka et al., "Primary duodenal follicular lymphoma treated with rituximab monotherapy and followed-up for 15 years," *Acta Medica Okayama*, vol. 69, no. 5, pp. 301–306, 2015.

[6] M. Deehan, S. Garcês, D. Kramer et al., "Managing unwanted immunogenicity of biologicals," *Autoimmunity Reviews*, vol. 14, no. 7, pp. 569–574, 2015.

[7] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), and Center for Biologics Evaluation and Research (CBER), "Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products," August 2014, http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm338856.pdf.

[8] European Medicines Agency, Science Medicines Health, and Committee for Medicinal Products for Human Use (CHMP), *Guideline on Immunogenicity Assessment of Monoclonal Antibodies Intended for In Vivo Clinical Use*, EMA/CHMP/BMWP/86289/2010, 2012, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/06/WC500128688.pdf.

[9] Y.-M. C. Wang, V. Jawa, and M. Ma, "Immunogenicity and PK/PD evaluation in biotherapeutic drug development: scientific considerations for bioanalytical methods and data analysis," *Bioanalysis*, vol. 6, no. 1, pp. 79–87, 2014.

[10] E. Koren, H. W. Smith, E. Shores et al., "Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products," *Journal of Immunological Methods*, vol. 333, no. 1-2, pp. 1–9, 2008.

[11] G. Shankar, V. Devanarayan, L. Amaravadi et al., "Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 48, no. 5, pp. 1267–1281, 2008.

[12] A. R. Mire-Sluis, Y. C. Barrett, V. Devanarayan et al., "Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products," *Journal of Immunological Methods*, vol. 289, no. 1-2, pp. 1–16, 2004.

[13] D. Sickert, K. Kroeger, C. Zickler et al., "Improvement of drug tolerance in immunogenicity testing by acid treatment on Biacore," *Journal of Immunological Methods*, vol. 334, no. 1-2, pp. 29–36, 2008.

[14] Z. D. Zhong, S. Dinnogen, M. Hokom et al., "Identification and inhibition of drug target interference in immunogenicity assays," *Journal of Immunological Methods*, vol. 355, no. 1-2, pp. 21–28, 2010.

[15] P. A. van Schouwenburg, G. M. Bartelds, M. H. Hart, L. Aarden, G. J. Wolbink, and D. Wouters, "A novel method for the detection of antibodies to adalimumab in the presence of drug reveals "hidden" immunogenicity in rheumatoid arthritis patients," *Journal of Immunological Methods*, vol. 362, no. 1-2, pp. 82–88, 2010.

[16] A. Patton, M. C. Mullenix, S. J. Swanson, and E. Koren, "An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen," *Journal of Immunological Methods*, vol. 304, no. 1-2, pp. 189–195, 2005.

[17] H. W. Smith, A. Butterfield, and D. Sun, "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," *Regulatory Toxicology and Pharmacology*, vol. 49, no. 3, pp. 230–237, 2007.

[18] A. Mikulskis, D. Yeung, M. Subramanyam, and L. Amaravadi, "Solution ELISA as a platform of choice for development of robust, drug tolerant immunogenicity assays in support of drug development," *Journal of Immunological Methods*, vol. 365, no. 1-2, pp. 38–49, 2011.

[19] D. K. Weeraratne, J. Lofgren, S. Dinnogen, S. J. Swanson, and Z. D. Zhong, "Development of a biosensor-based immunogenicity assay capable of blocking soluble drug target interference," *Journal of Immunological Methods*, vol. 396, no. 1-2, pp. 44–55, 2013.

[20] US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), and Center for Biologics Evaluation and Research (CBER), *Guidance for Industry: Assay Development for Immunogenicity Testing of Therapeutic Proteins*, 2009, http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm192750.pdf.

[21] J. J. Haringman, D. M. Gerlag, T. J. M. Smeets et al., "A randomized controlled trial with an anti-CCL2 (anti–monocyte chemotactic protein 1) monoclonal antibody in patients with rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 54, no. 8, pp. 2387–2392, 2006.

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Review Article

Posttranslational Modifications and the Immunogenicity of Biotherapeutics

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Whilst the amino acid sequence of a protein is determined by its gene sequence, the final structure and function are determined by posttranslational modifications (PTMs), including quality control (QC) in the endoplasmic reticulum (ER) and during passage through the Golgi apparatus. These processes are species and cell specific and challenge the biopharmaceutical industry when developing a production platform for the generation of recombinant biologic therapeutics. Proteins and glycoproteins are also subject to chemical modifications (CMs) both *in vivo* and *in vitro*. The individual is naturally tolerant to molecular forms of self-molecules but nonself variants can provoke an immune response with the generation of anti-drug antibodies (ADA); aggregated forms can exhibit enhanced immunogenicity and QC procedures are developed to avoid or remove them. Monoclonal antibody therapeutics (mAbs) are a special case because their purpose is to bind the target, with the formation of immune complexes (ICs), a particular form of aggregate. Such ICs may be removed by phagocytic cells that have antigen presenting capacity. These considerations may frustrate the possibility of ameliorating the immunogenicity of mAbs by rigorous exclusion of aggregates from drug product. Alternate strategies for inducing immunosuppression or tolerance are discussed.

1. Introduction

The modern era of biologic therapeutics may be identified with the FDA approval of recombinant insulin (Humulin) in 1982, produced in *E. coli*, and recombinant erythropoietin (EPO) in 1989 (Epogen); since glycosylation of EPO is essential to its function it was, necessarily, produced in a mammalian cell line (a Chinese hamster ovary (CHO) cell line). Despite extensive clinical experience adverse reactions to these recombinant molecules are still encountered, ~2% for insulin [1] and rarer, but more devastating, for EPO [2]. These incidences are frequently due to the patient developing antibodies that are specific for the therapeutic and neutralising its activity (anti-drug antibodies (ADA): anti-therapeutic antibodies (ATA)). The development of ADA suggests that the therapeutic is being recognized as "foreign" (nonself) by the patient's immune system, due to the presence of molecules that exhibit structural features different to those of the endogenous protein/glycoprotein (P/GP). Ironically, a high incidence of ADA is encountered for recombinant antibody therapeutics (mAb/s); this is due, in part, to the fact that each mAb therapeutic is selected for unique epitope specificity and consequently exhibits unique structure.

The starting point for the generation of recombinant P/GPs requires determination of the primary, secondary, and tertiary structure of the endogenous (natural) molecule. This is not a trivial exercise since whilst the gene sequence determines the primary amino acid sequence it does not provide a guide to the precise structure of the active molecule. The nascent polypeptide chain may be subject to cotranslational modifications (CTMs) as it is extruded from the ribosome tunnel, for example, the addition of oligosaccharide; editing for correct folding and initial oligosaccharide processing take place within the endoplasmic reticulum and further posttranslational modifications (PTMs) are effected during passage through the Golgi apparatus. The P/GPs that exit the Golgi may be trafficked within the cell, inserted in the plasma membrane, or secreted into the extracellular environment. The functional activity of a P/GP may be dependent on subsequent chemical modifications (CMs), for example, phosphorylation, and further CMs that constitute its "aging" and subsequent catabolism [3]. It is important to emphasise,

therefore, that endogenous (native!) P/GPs exhibit structural heterogeneity. These heterogeneities are compounded when establishing the structure of a purified form of a P/GP because additional heterogeneities are introduced during its isolation, purification, and characterization; in addition the purer the isolated protein is, the lower the yield is and the less certain one can be that it is representative of the active endogenous molecule *in vivo*.

2. Overview of Co- and Posttranslational Modifications

Variations in protein structure from that predicted by open reading frame gene sequences may be introduced during transcription and/or translation, by misincorporation at the DNA, RNA, and amino acid level, and the introduction of CTMs [4–10]. Commonly encountered CTMs/PTMs and CMs include glycosylation, phosphorylation, sulphation, glycation, deamidation, and deimination [4, 5]. Additionally, the structural profile, *in vivo*, may vary with age, sex, health, and disease. The human genome contains ~21,000 genes encoding expressed proteins; however, it is estimated that the human proteome may be comprised of 1-2 million protein entities, due to the PTMs/CTMs that are essential to physiological function, systemically, and/or microenvironments [4, 5]. Each human individual should, in theory, be immunologically tolerant to all molecules within their proteome, including all isoforms exhibiting natural PTMs and CMs; however, the exquisite sensitivity of the current generation of assay technologies allows the detection of low affinity antibody to many self-antigens in healthy individuals. Paradoxically, antibodies having the same, or similar, specificity may be amplified in disease states and be a diagnostic marker for individual disease entities [11]. Developments in qualitative and quantitative mass spectrometry during the past decade have "revolutionized" the enumeration of PTMs and CMs generating P/GP heterogeneity and defined >300 structural CTM/PTMs [12–15]. Analysis of 530,264 sequences in the Swiss-Prot database was shown to yield 87,308 experimentally identified PTMs and 234,938 putative PTMs [15]. The potential for structural and functional complexity can be appreciated from the fact that the human genome encodes 518 protein kinases and 200 phosphatases [16, 17]. The second most frequent CTM/PTM is glycosylation. Oligosaccharides may be attached to asparagine residues to generate *N*-linked glycoproteins or to the hydroxyl groups of serine, threonine, or tyrosine to generate *O*-linked glycoforms. The *N*-linked repertoire contains >500 different oligosaccharide structures that may be differentially attached at multiple glycosylation sites to generate >1000 different types of glycan, a consequence of the activities of >250 glycosyltransferases [5, 6, 18, 19].

The potential for complexity/heterogeneity can be illustrated for recombinant mAbs. The full length sequence of an IgG molecule includes ~40 asparagine/glutamine residues; therefore, random deamidation of one of these residues will generate 40 structural variants (isoforms); deamidation of two residues may generate $40 \times 40 = 1{,}560$ variants; three: $40 \times 1{,}560 = 59{,}280$ variants; and so forth. When all potential PTMs are considered it has been calculated that a full length IgG molecule may exhibit a heterogeneity embracing 10^8 isoforms [20]. Recombinant mAbs present a particular challenge since each exhibit a unique structure and must be evaluated on a "case-by-case" basis. Fortunately, current technologies allow for early screening and selection of clones that do not have amino acid residues susceptible to PTM/CTMs within their complimentary determining regions (CDRs); other criteria may be selected to optimise solubility, stability, and so forth. The constant region sequence can also be selected, that is, Ig class and subclass, to define the final drug substance/product developed. A biosimilar candidate must be demonstrated to be structurally and functionally comparable to the innovator product [21, 22].

The starting point for development of a recombinant P/GP therapeutic is the consensus structure of the "wild-type" (WT) molecule. It is required that a candidate recombinant P/GP therapeutic exhibits PTMs comparable to those of the consensus WT structure and an absence of unnatural PTMs introduced by the production process. Structural identity with the WT P/GP may not be possible since production platforms employ nonhuman tissues (CHO, NS0, Sp2/0 cells, etc.) and the secreted P/GP is exposed to the culture medium and products of intact and effete producer cells over an extended period, prior to rigorous downstream purification, formulation, and storage conditions. Any departure from the consensus WT structure may be perceived as nonself by the immune system and result in the generation of ADA. Production in a prokaryotic system (e.g., *E. coli*) may result in a protein being recovered as an inclusion body that has to be solubilized and refolded *in vitro* to yield a product that may lack natural PTMs or bear unnatural ones. This system may be purposely exploited to develop novel therapeutics, for example, aglycosylated full length antibody molecules that may act as agonists or antagonists but not provoke downstream effector activities. Comparison of the structure of a candidate antibody therapeutic with a WT counterpart is not possible due to the unique structure of its variable regions; however, the amino acid sequence of the constant regions and potential glycoform profiles are established.

Approval of a candidate P/GP therapeutic is dependent on the demonstration of clinical efficacy for a product that has been structurally characterized employing multiple orthogonal physicochemical techniques [23, 24]. The physicochemical characteristics established define the drug substance and drug product and must be maintained throughout the life cycle of an approved drug. Critical Quality Attributes (CQAs) that define drug efficacy are defined [25] and maintained within the production platform developed [26]. These data are the undisclosed intellectual property of an innovator company and it is deemed essentially impossible to produce an identical product employing a similar or alternative platform within another facility; that is, in principle, it is not possible to develop generic biopharmaceuticals. It is possible to introduce improvements in the production process that result in changes in structural parameters if it is demonstrated not to compromise drug efficacy and patient benefit. Importantly, each drug will be assigned a "shelf-life," that is, a period of time after which physicochemical changes may be

evident which impact biologic activity and limit its efficacy and/or prejudice patient benefit. Accelerated storage studies under varying conditions establish structural and functional stability and guide formulation to provide an acceptable shelf-life.

3. Immunogenicity

As previously stated endogenous P/GPs may be present *in vivo* in multiple structural isoforms and it may be possible to demonstrate the presence of self-reactive antibodies in serum; however, a healthy individual is functionally tolerant, that is, asymptomatic. Within a disease state the quantitative and qualitative nature of the PTM/CTM repertoire may be amplified with consequent generation of immune complexes and/or aggregated forms that are engulfed by phagocytic cells that have the capacity to process and present antigens, with consequent induction or amplification of an anti-self-response [11, 27–30].

A "casebook" example that may be cited is the anti-citrullinated protein response, accepted as the most specific biomarker for rheumatoid arthritis (RA). Citrullinated proteins are generated by the action of peptidylarginine deiminases (PADs), which convert arginine into citrulline in a process called citrullination or deimination [31–33]. This is a natural process; however, in RA, it is amplified and several citrullinated proteins are present within inflamed synovial tissue. It is possible that within the milieu of inflammation some proteins may be denatured and arginine residues that are not normally exposed become accessible to citrullination and may be "seen" as nonself by the immune system. The specificity of this response is reflected in the fact that the diagnostic assay employs a cyclic citrullinated peptide as antigen: the anti-citrullinated peptide antibody (ACPA) response [34, 35]. Importantly, increased levels of ACPA may be detected in advance of clinical manifestations. More recently the presence of anti-carbamylated protein (anti-CarP) autoantibodies have shown specificity for RA and their presence to overlap, at least in part, with ACPA activity: however, anti-CarP-positive and ACPA-negative patients have been described [36–38]. Carbamylated protein arises from the action of cyanate on the epsilon amino groups of lysine residues to generate homocitrulline; cyanate is generated *in vivo* by several routes and its production is enhanced in inflammation.

The original recombinant erythropoietin (EPO) drug (Epogen) was introduced in 1988 and has been used successfully worldwide; however, incidences of neutralising anti-EPO ADA have been reported with the development of pure red cell aplasia (PRCA). With the expiry of the original patent various alternative EPOs have been approved and additional incidences of PRCA reported. A meta-analysis published in 2008 identified 215 cases, worldwide, of ADA and consequent PRCA; 189 of the patients were exposed to Eprex only [39, 40]. In 1998 a "cluster" of PRCA incidences was reported in Europe and investigation revealed variations in formulation of the EPO associated with the absence of human serum albumin (HSA), subcutaneous administration, and capping with an uncoated rubber stopper [40, 41]. It was posited that the net result was likely to be chemical modification and/or aggregation of a critical proportion of the therapeutic; aggregation is considered to be a principle PTM/CM resulting in immunogenicity and the initiation of ADA responses.

Anticipating the patent expiry date of an approved biologic therapeutic the innovator company may develop a variant having enhanced properties and submit for approval and further patent protection. Other biopharmaceutical companies may similarly anticipate patent expiry and seek to develop a copy of the original innovator product, not claimed to be the same but biosimilar, or comparable [25, 26]. Numerous EPO biosimilars have been approved in Europe and the USA and, due to their competitive pricing, have gained market penetration. Copies of EPO have also been generated outside of Europe/USA; however, not all have been rigorously examined by a regulatory authority; others that have not been submitted to a regulatory authority for approval may be marketed as a biosimilar. Cumulative incidences of PRCA have been reported from Thailand where investigations identified EPO products that did not meet international standards and/or were illegal, having been smuggled into the country [41–43].

4. Protein Aggregation and Immunogenicity

Early studies investigating the capacity of the immune system to produce specific antibody responses employed potential immunogens of differing molecular weight (MW) and size, from small molecule chemicals to protein antigens and their denatured aggregated forms. These studies suggested a threshold for immunogenicity of ~10 kDa; below this MW immune responses could be elicited if the molecule is presented conjugated to a macromolecule, in which case the chemical is termed a hapten. High MW proteins (e.g., bovine serum albumin; MW ~60 kDa) proved to be immunogenic when delivered together with an adjuvant or in aggregated form. These principles have been validated and extended particularly in relation to vaccine research and development [44].

The biotherapeutics considered in this review are P/GPs having an overall structure (conformation) that confers solubility in aqueous solutions. The integrity (stability) of a P/GP molecule is contributed to by a core of hydrophobic amino acid side chains buried within the internal space of the molecule; however, some such side chains may be only partially buried and form hydrophobic patches on the surface of the molecule. Similarly, most hydrophilic amino acid side chains are exposed on the surface of a molecule but may also be partially buried. Maintenance of structural integrity is essential to function and it is posited that all soluble P/GPs are susceptible to the formation of insoluble fibrillar aggregates under specific denaturing conditions. However, the individual is normally protected by an essential editing function performed within the endoplasmic reticulum (ER) that allows only correctly folded protein to transfer to the Golgi apparatus; misfolded protein activates the unfolded protein response (UPR) and enzymatic degradation within the proteasome [45]. Any disturbance (denaturation) of the native conformation, after secretion, is liable to expose hydrophobic "patches" to aqueous solvent and is compensated for by hydrophobic

protein/protein intermolecular interactions, manifest as aggregation [46–48]. The phenomenon of conversion from soluble to insoluble protein may be illustrated by lysozyme, a highly soluble protein that has been studied as a structural model for ~100 years; in spite of its intrinsic solubility it is readily denatured, under nonphysiologic conditions, to form insoluble fibrils (amyloidosis). A more extreme example is the phenomenon of prion disease in which the prion proteins undergo a conformational change, *in vivo*, with the α helix portion (54%) of its structure converting to a β sheet structure that renders it both insoluble and infectious; that is, it can induce native prion protein to convert to the insoluble/infectious form [48].

From the above it follows that if a P/GP proves to be immunogenic, with the formation of ADA, it is likely due to the presence of nonself forms resulting from denaturation, the presence of inappropriate PTMs/CMs, and/or aggregation. Regulatory authorities have focused particularly on aggregation as being the "bête noire." Following extensive studies a biologic therapeutic is formulated with excipients selected to minimize denaturation and aggregate formation over the shelf-life of the drug product; it is essential, therefore, to physiochemically characterize the molecular size of drug substance, drug product, and any aggregates present in the approved material and to further monitor these parameters over time [47, 49–55].

5. Potential Immunogenicity of Antibody Therapeutics

Clinical experience has shown that even "fully" human mAbs are immunogenic, at least in a proportion of patients [56–64]. An obvious explanation lies with the unique structure of the mAb variable regions that comprise the paratope, formed by the complementarity determining regions (CDRs) that confer unique specificity. The variable regions of recombinant mAb therapeutics are selected from libraries of antibody genes expressed in (i) mice (chimeric); (ii) humanized chimeric mAbs; (iii) phage display libraries generated from an outbred human population; (iv) humanized mice, and so forth. In each case an approved mAb will present unique structural features to an individual recipient (patient) and may be recognized as foreign, with the generation of ADA. Additional nonself structures may be present due to the extensive polymorphism of genes encoding the constant regions of IgG heavy chains and the constant regions of kappa light chains that differs widely in their distribution between ethnic populations [62–65]. To date only one polymorphic form of each therapeutic mAb has been generated and approved; therefore, there will be a high frequency mismatch between the polymorphic variant of a given mAb therapeutic and a proportion of patients. Additionally, since mAbs are manufactured in xenogeneic tissue (hamster, CHO, mouse, NS0, Sp20, etc.), drug substance and drug product may lack the expression of some human CTMs and PTMs whilst expressing others that are not present on human IgGs and may contribute to immunogenicity [11, 56–66].

For the above reasons regulatory authorities demand stringent postapproval pharmacovigilance and reporting of adverse events [67–70]. A considerable literature suggests that the immunogenicity of approved biologics may differ between (i) individual biologics; (ii) manufacturer of the same biologic; (iii) the physical form of the biologic; (iv) patient population; (v) the disease entity; (vi) comorbidities; (vii) doe and route of administration; (vii) period of administration, and so forth. Therefore, each approved mAb therapeutic has to be evaluated on a case-by-case basis. It is of interest to compare clinical experiences from administration of the anti-TNF therapeutics currently approved for the treatment of inflammatory diseases, for example, rheumatoid arthritis, Lupus, and Crohn's disease [59–62]. Whilst the percentage of patients developing ADA may differ between reported studies and disease entities there is general agreement that neutralising antibodies predominate [57, 58, 71]. The presence and specificity of neutralising ADA may be demonstrated by the ability of a patient's serum to inhibit the binding of the mAb to TNF, for example, infliximab; the same patient serum does not inhibit the binding of another anti-TNF mAb, for example, adalimumab mAb, to TNF. Thus if a patient becomes refractive to a given anti-TNF mAb therapeutic, due to the generation of ADA, it may be possible to effect disease remission by switching to an alternative anti-TNF biologic. The formation of ADA may be ameliorated, in a proportion of patients, by coadministration of a mild immunosuppressant, for example, methotrexate [57, 58, 61, 71].

6. Aggregation of IgG Antibodies

Monoclonal human IgG antibodies have been available for many decades as myeloma proteins, the products of plasma cell tumours; however, their antigen specificities have not been determined. The plentiful availability of these proteins allowed for antigenic and structural definition of the IgG subclasses, elucidation of Fc receptor recognition specificities, complement activation, and so forth. The first full length sequence of an IgG molecule was determined for an IgG1 myeloma protein [72]. The lack of known antigen specificity did not allow for the generation of antigen/antibody immune complexes (IC); therefore, aggregated forms of IgG were generated as surrogate IC; common protocols included heating at 60–70°C, followed by high speed centrifugation to yield a slightly opalescent solution [73] or cross-linking by bifunctional small molecules 78. Rabbit antisera produced to heat aggregated IgG revealed no new antigenic determinants, compared to normal IgG, but responses to some determinants were heightened [74]. These early findings were corroborated by a recent study of heat aggregated IgGs binding to each of the FcγR types, expressed as transmembrane molecules on the surface of CHO cells [51].

The demonstration that aggregated forms of IgG could function as surrogate IC has been interpreted in the modern era to suggest that aggregated forms of biologic therapeutics and mAbs in particular may be the principle immunogen triggering the generation of ADA responses. Subsequently, techniques and technologies for qualitative and quantitative characterization of aggregated forms of IgG have been developed and applied to analysis of IgG mAbs subjected

to multiple protocols that each mimics conditions experienced by a mAb throughout its production, formulation, storage, and preparation for administration [75–78]. A mouse mAb that was exposed to similar protocols proved to be immunogenic when administered to a naive mouse; the most immunogenic forms were relatively large, insoluble aggregates that exhibited structural features of denatured molecules [79]. It is presumed that aggregates are removed from the circulation by phagocytic cells that degrade them to generate peptides that may be presented by MHC Class II molecules to B and T cells [50].

The propensity for a mAb to aggregate may be determined by the unique structure of the variable regions superimposed on an intrinsic susceptibility of the selected heavy and light chain isotypes. An analysis of the constant regions of an IgG1/kappa molecule identified partially exposed hydrophobic side chains that when in proximity to other hydrophobic residues constitute aggregation prone regions (APRs) ("spatial-aggregation-propensity (SAP)") [80]. APRs were identified within the CH1, hinge, CH2, and CH3 domains of the IgG1 heavy chain and the constant regions of both kappa and lambda light chains; substitution of targeted hydrophobic amino acids with selected hydrophilic residues generated more stable proteins with a diminished tendency to aggregate. The APRs identified in IgG1 molecules are present also in the IgG2, IgG3, and IgG4 subclass proteins [80–82]. The presence of "open" or "free" cysteine residues is another parameter shown to result in the generation of aggregates, through intermolecular disulphide bond formation, under differing stress conditions, and is particularly relevant for IgG2 subclass proteins [83]. As expected the individual variable sequences can have a profound impact on susceptibilities to aggregation. The germline sequences for VH, VK, and VL have been comprehensively reviewed [84] and analysed for the aggregation potential of their protein products [85]. These criteria may now be applied to the selection of clones producing potential IgG therapeutics. Close interrogation of the immunogenicity of currently approved mAbs and biologics may reveal further parameters that contribute to immunogenicity.

Recent studies have shown that the structure/form of a mAb therapeutic must be considered beyond that of the formulated drug product received by the pharmacy. Instructions to pharmacists for resuspension of the mAb therapeutics Herceptin and Avastin, for intravenous administration, specified the use of 0.9% saline and specifically excluded of the use of 5% dextrose solutions; no reason was given [86, 87]. The consequences for contravening this instruction demonstrated that titration of either Herceptin or Avastin in 5% dextrose into human plasma or serum resulted in the formation of insoluble aggregates [87]; possibly resulting in adverse reactions on administration to patients and/or sensitising them to later production of ADA. The phenomenon was further investigated and it was shown that addition of a 5% dextrose solution, at pH 6.0–6.2, to plasma/serum resulted in the formation of aggregates of complement components that then bound the mAb [88]. It was noted that instructions for resuspension of Remicade specified 0.9% saline, pH 7.2, conditions that did not result in the formation of complexes; similarly, neither did Herceptin or Avastin; however, these mAbs exhibit other instabilities at higher pH values [87–89]; for example, Asn 30 of Herceptin deamidates at pH > 5.0, which lowers product bioactivity [4, 88].

7. Formation and Removal of Immune Complexes (ICs)

It is axiomatic that an IgG antibody binds its target antigen (pathogen!) to form an antigen/antibody immune complex (IC). Similarly, it is axiomatic that the IC has to be removed and destroyed. This is accomplished by cells that bear receptors specific for the Fc region (FcγR) of the IgG molecule (IgG-Fc) resulting in uptake and consequent destruction within lysosomes. The peptides generated may bind to MHC Class II molecules and subsequently be expressed on the cell surface for presentation to helper CD4 T cells. This promotes and amplifies a humoral immune response [90]. The integrity of the individual is dependent on tolerance to self-molecules; however, tolerance has been shown to be a dynamic ongoing process with activation/anergy being dependent on the strength of binding of peptide bearing MHC II molecules and helper T cells. Consequently, self-reactive T cells and antibodies, usually of IgM isotype, can be enumerated in normal healthy individuals.

The above summarizes the body's protective response to "foreign bodies" (pathogens) that gain access to tissue or vascular sites. The aim of delivering a mAb therapeutic to a patient is for it to bind its target antigen (self-molecule) with the formation of IC that will activate mechanism of removal and destruction similar to those activated by aggregated P/GPs. I pose the following question therefore.

What Is the Difference between Aggregates and Immune Complexes? An early study employed a matches set of recombinant mAbs to evaluate the ability IC of each human antibody class and subclass to trigger the neutrophil respiratory burst; presumed to act through IgG-Fc receptors (FcγRI, FcγRIIA, and FcγRIIIB), in both the presence and absence of complement [91, 92]. This and other studies demonstrated different outcomes for each antibody isotype depending on the epitope density and the antibody/antigen ratio at which ICs were formed [28, 29, 93]. A further refinement has monitored the binding of ICs, formed with antigens having differing epitope densities, with a panel of CHO cells each expressing a single FcγR type. This study also demonstrated that the avidity of binding to FcγR increased with epitope density and revealed a different order of FcγR binding from that reported for studies of monomeric mAb binding to FcγR, [51]; that is, the "received" FcγR binding specificities of the IgG subclasses widely reported that the IgG subclasses are not an accurate guide to the specificities of their IC *in vivo*. The fine epitope specificity of a mAb can also have a determining impact on the structure of the IC formed and resulting MoA, as illustrated by approved anti-CD20 mAbs. Anti-CD20 mAbs are classified as Type I or Type II depending on whether or not they trigger redistribution of cell surface CD20 into lipid rafts; Type I do so and as a result are able to activate the complement cascade whilst Type II bind to individual CD20 tetramers only and do not activate complement [94, 95].

The extracellular domain of the CD20 molecule is comprised of only ~40 amino acids residues and crystallography demonstrates that Type I and Type II antibodies bind overlapping nonidentical epitopes.

A further example is provided by anti-TNF mAbs. Soluble TNF exists as a trimer and is potentially trivalent for mAb binding and able to form three-dimensional immune complexes with divalent antibody. A study of the size distribution of immune complex formed between TNF and the approved anti-TNF biologics infliximab and etanercept, at differing antigen/antibody ratios, showed that each antibody generated immune complexes having a unique size profile [96]. It may be presumed therefore that they will differ in their FcγR activating properties. It has been suggested that a fundamental difference exits between IgG-ICs and aggregated IgG in that the CDRs of the former are engaged but they are exposed in the latter; however, X-ray crystal structural analysis of Fab-antigen complexes shows that, for the majority of complexes analysed, not all CDRs are engaged in antigen binding [97]. The above parameters may be compounded by the fact that, in contrast to most recombinant biologics, mAbs are delivered at high doses (~400 mg); therefore, an unnatural or degraded nonself component present at a level of 0.001% can constitute a viable immunogenic dose [9, 98].

8. Chemical Modifications (CMs) of Amino Acid Side Chains

N- and C-Terminal Residues. Unique N-terminal sequence may be obtained for the heavy and light chains of most monoclonal IgG paraprotein; however, for some, the N-terminal amino acid yield may not be quantitative or may appear to be entirely "blocked" [99]. This results when a gene encodes for the incorporation of N-terminal glutamic acid or glutamine residues that may subsequently cyclize, *in vivo* and/or *in vitro*, with the generation of pyroglutamic acid (pGlu) [99–104]. The formation of pGlu in antibodies [101] and therapeutic proteins is a concern for the biopharmaceutical industry since it introduces charge heterogeneity and variations may be considered to be evidence for lack of process control [18]. Importantly, N-terminal pGlu is also implicated in Alzheimer's disease and dementia since it increases the tendency for proteins to form insoluble fibrils; light chains are particularly prone to the formation of pGlu fibrils [101, 105, 106]. As there is no evidence of benefit attached to the presence of N-terminal pGlu, to either the heavy or light chain, it may be best to select against its presence, where possible, during clone selection for a potential mAb therapeutic.

Sequencing studies reported the C-terminal residue of serum derived IgG heavy chains to be glycine; however, the IgG subclass genes encode a C-terminal lysine residue. It was later shown that the lysine residue is cleaved, *in vivo*, by an endogenous carboxypeptidase B. Recombinant IgG molecules produced in mammalian cells exhibit mixed populations of molecules with lysine present or absent on each heavy chain and subsequent charge heterogeneity [107]. A concept paper produced by the EBE (European Biopharmaceutical Enterprises), a specialized group of EFPIA (European Federation of Pharmaceutical Industries and Associations), included the statements: "A number of scientific publications suggest that C-terminal lysine truncation has no impact on biological activity, PK/PD, immunogenicity and safety." And elsewhere in the document: "Lysine truncation does not appear to adversely affect product potency or safety. However, taking a conservative approach potential C-terminal lysine effects on all antibodies cannot be ruled out. Thus, lysine truncation should be characterized, and process consistency should be demonstrated during product development; regulatory agencies suggest that C-terminal lysine content should be reported for both the characterization and development phases" [108]. Removal of C-terminal lysine results in the presence of a C-terminal glycine residue that, when produced in CHO cells, may be subject to amidation, introducing further structural and charge heterogeneity [109]. A recent report demonstrated that this has been circumnavigated by genetically engineering CHO cells to "knock-down" expression of the peptidylglycine α-amidating monooxygenase (PAM) enzyme [110]. The above comments and recommendations are contradicted by a recent study that claimed that IgG with C-terminal lysine constitutes a preform of the molecule that prevents the formation of IgG hexamers that can activate the complement cascade [111].

9. Cysteine and Disulphide Bond Formation

The gene sequence for the human IgG1 subclass protein Eu encodes for 5 light chain and 9 heavy chain cysteine residues, that is, 28 for the H2L2 heterodimer. The standard structural cartoon for the human IgG1 protein (Eu) exhibits 12 intrachain and four interchain disulphide bridges. This general pattern of intrachain disulphide bridge formation is maintained for each of the IgG subclasses; however, the number of interchain bridges and their architecture vary between and within the IgG subclasses [72, 112–118]. Heterogeneity of disulphide bridge formation has been reported for normal serum derived IgG, myeloma proteins, and recombinant mAbs. Formation of the H2L2 dimer occurs following release of heavy and light chains into the endoplasmic reticulum (ER), with evidence that binding of the constant region of the light chain (C_L) to the heavy chain C_H1 domain "catalyzes" the generation of a correctly folded H2L2 structure [112]. This nascent form explores multiple dynamic structures, with the formation of native and nonnative disulphide bonds that are transiently formed and reduced until a low energy conformation is achieved [112, 113]; it should be noted that little or no processing of the high mannose oligosaccharide will have occurred at this point; therefore, the conformation of the secreted IgG-Fc will not be achieved until oligosaccharide processing is completed.

The IgG1 molecule establishes the "standard" pattern with two inter-heavy chain disulphide bridges and a single light-heavy chain bridge; IgG2, IgG3, and IgG4 express 3, 11, and 2 inter-heavy chain bridges, respectively. The cysteine residues that form interchain disulphide bridges are clustered within the hinge region and may be subject to reduction and reformation when present in a reducing environment. Heterogeneity in disulphide bond formation in IgG2 was first reported for recombinant IgG2 proteins but, later, observed

for normal serum derived IgG2 also [114–118]. The interconversion of these isoforms is dynamic and promoted by a reducing environment provided by the presence of thioredoxin reductase, released into culture media by effete cells; it can be ameliorated by control of dissolved oxygen levels [114–118]. An *in vitro* model revealed that susceptibility to reduction/oxidation differed between IgG subclasses and light chain types with sensitivity being in the order IgG1λ > IgG1κ > IgG2λ > IgG2κ [116].

A core hinge region sequence of -Cys-Pro-Pro-Cys-, present in IgG1, IgG2, and IgG3, forms a partial helical structure that does not allow for intra-heavy chain disulphide bridge formation; however, the homologous sequence in the IgG4 subclass is -Cys-Pro-Ser-Cys- and this does allow for intra-heavy chain disulphide bridge formation. Consequently, natural and recombinant IgG4 antibody populations are a mixture of molecules exhibiting inter- and intrahinge heavy chain disulphide bridge isoforms [118–122]. The IgG4 form having intrahinge heavy chain bridges is susceptible to dissociation into half-molecules (HL) that may reassociate randomly to generate bispecific molecules; that is, a molecule that is monovalent for two nonidentical antigens (epitopes); this phenomenon is referred to as "Fab arm exchange." The exchange is also facilitated by presence of an arginine residue at position 409 (R409) in the IgG4 heavy chain that reduces noncovalent CH3/CH3 interactions, relative to the presence of lysine 409 (K409) present in IgG1, IgG2, and IgG3 molecules. Lateral noncovalent interactions between the two C_H3 domains of R409 IgG4 are reduced such that, under physiologic conditions and in the absence of hinge region intra-heavy chain disulphide bridges, they dissociate to form HL heteromonomers; a polymorphic variant of IgG4 exists that has K409 residue and is not subject to Fab arm exchange [120–122].

10. Oxidation of Methionine

Methionine residues exposed, or partially exposed, on the surface of native or denatured proteins may be susceptible to oxidation. Methionine residues within variable region framework sequences of mAbs have not been reported to be vulnerable to oxidation but residues exposed within CDRs have been [123–127]. Consequently, sequencing of prospective clones is advised to inform selection and rejection of clones having methionine within CDRs. It has been shown that methionine residues M252 and M428 of IgG1 and IgG2 subclass proteins are susceptible to oxidation [123]. Although these residues are distant from each other in linear sequence, they are conformationally proximal, at the C_H2/C_H3 interface. The interaction site for the neonatal Fc receptor (FcRn), SpA, and SpG is similarly localised to the C_H2/C_H3 interface and M252 and M428 oxidation has been shown to reduce the affinity of binding to these ligands and to reduce catabolic half-life [124–127]. Minimal levels of M252 oxidation (2–5%) are reported for IgG mAbs held in formulation buffers, whilst lower levels of oxidation are reported for M428; however, oxidation of these residues increases under conditions of accelerated stability testing and on prolonged storage [123]. Analysis of Herceptin, obtained from a pharmacy, and a potential biosimilar demonstrated that care has to be exercised when resuspending this mAb therapeutic since a discrepancy was observed for the level of M252 oxidation between the innovator product (4.39%) and the proposed biosimilar (10.33%) [128].

11. Deamidation: Asparagine and Glutamine

Deamidation of asparagine and glutamine residues generates aspartic acid, isoaspartic acid, or glutamic acid, respectively, and is a frequently encountered PTM [9, 10, 129, 130]. Deamidation of asparagine residues is influenced by adjacent amino acid residues, particularly the presence of a glycine residue C-terminal to the asparagine [-N-G-] and the degree of exposure to external environments. Studies of IgG1 and IgG2 proteins, *in vitro* and *in vivo*, have shown that asparagine residues 315 and 384 are susceptible to deamidation with the formation of isoaspartic and aspartic acid residues, respectively [129–134]. The relative susceptibility to deamidation at these sites varied between studies; however, the significance may be ameliorated by the finding that ~23% of asparagine 384 residues of normal polyclonal IgG are deaminated to aspartic acid; thus it may be assumed that healthy humans are constantly exposed to IgG bearing this PTM and that it might be considered to be a "self" structure. These studies did not identify asparagine deamidation within the constant region of the kappa light chains.

By contrast deamidation within variable regions, particularly within CDRs, of recombinant antibodies has been shown to compromise antibody specificity and/or binding affinity [131–134]. Interestingly, the approved blockbuster antibody therapeutic Trastuzumab (Herceptin) has asparagine residues in light chain CDR1 (Asn 30) and heavy chain CDR2 (Asn 55) that were shown to be susceptible to deamidation on accelerated degradation studies [4, 13]; the approved drug substance did not exhibit deamidation of these residues; therefore, their presence or absence could be used as a lot release criterion [134]. As previously discussed the levels of deamidation of asparagine residues, both within variable and constant regions, of a proposed Herceptin biosimilar were higher than that reported for the innovator molecule [128] underlining the susceptibility to deamidation of these residues and the care that has to be exercised when resuspending this antibody therapeutic.

Glutamine residues are relatively resistant to deamidation and no glutamine residues were reported to be subject to deamidation under nondenaturing conditions [8, 13]. Under conditions of accelerated degradation six Gln residues of a mAb were shown to be susceptible to deamination: four in variable regions and residues 295 and 418 in the IgG-Fc [8]. The cyclization of N-terminal glutamine to form pyroglutamic acids has been discussed, above.

12. γ-Carboxylation and β-Hydroxylation

The function of proteins of the blood coagulation system may be dependent on γ-carboxylation and β-hydroxylation [135–138]. Both PTMs contribute to the binding of calcium ions

and are important, and in some cases essential, for blood factors VII, IX, and X, activated protein C, and protein S of the anticoagulant system. These proteins are comprised of structurally distinct domains with the N-terminal Gla (gamma-carboxyglutamic acid-rich) domain providing γ-carboxylation sites and the EGF (epidermal growth factor-like) domains the β-hydroxylation sites. Typically Gla domains are approximately 45 amino acids long and contain 9–12 Gla residues. Carboxylation of Gla domain glutamate residues is not dependent upon occurrence within a specific consensus sequence, but carboxylase binding is mediated by an immediately adjacent propeptide region, which is subsequently removed by proteolysis [135–137].

Hydroxylation of target EGF domain aspartate or asparagine residues is catalyzed by a β-hydroxylase located in the ER. EGF domains are ~45 amino acids long and contain one potential hydroxylation site. Hydroxylation is consensus sequence dependent and is usually partial, with only a fraction of target molecules being hydroxylated. Full carboxylation and hydroxylation, on the other hand, are essential to maintaining biological activity of protein C [136–139]. The native molecule displays nine carboxylation and one hydroxylation sites. Such stringent PTM requirements could not be met by CHO cells, forcing the developers of the recombinant proteins of the coagulation system to develop a modified human cell line (HEK 293) for their manufacture [138].

13. Sulphation

Sulphation is a PTM predominantly associated with secretory and membrane proteins [140]. The attachment of a sulphate (SO^{3-}) group to an oxygen atom of tyrosine, serine, or threonine residues is effected by a sulfotransferases enzyme present in the *trans*-Golgi network [141]. In the context of biopharmaceuticals native hirudin (a leech-derived anticoagulant) and blood factors VIII and IX are usually sulphated. Neither of the approved recombinant forms of hirudin are sulphated although it has been shown that sulphated hirudin (at Tyr63) displays 10-fold tighter affinity for thrombin than do unsulphated analogues [142]. Whilst over 90% of native factor IX molecules are sulphated, less than 15% of the approved recombinant form are, with apparently little if any difference in product efficacy [143]. Sulphation of factor VIII is required for optimal binding to its plasma carrier protein (von Willebrand's factor) and, interestingly, people inheriting a factor VIII Tyr1680 → Phe mutation often display mild haemophilia [140]. Several hormone cell surface receptors are known to be tyrosine sulphated, and sulphation is required for high affinity ligand binding and subsequent receptor activation [144, 145].

14. Glycosylation: Immunogenic Glycoforms Produced by Rodent Cell Lines

The impact of glycosylation on secretion, stability, function, and immunogenicity of recombinant GPs remains a focal point within the biopharmaceutical industry. The glycoform profile of a GP may be species, cell type, and, possibly, sex specific. Recombinant EPO (rEPO) when first produced in CHO cells exhibited increased biologic activity compared to the natural product *in vitro*; however, *in vivo* studies showed a very low level of biological function. It was shown that this was due to rapid clearance in the liver, via the asialo glycoprotein receptor, due to the oligosaccharide chains not being "capped" with terminal sialic acid residues. Successful generation of appropriately sialylated rEPO was achieved and Epogen was approved in 1989 [2]. Since that time many improvements have been introduced, including protein engineering to introduce additional glycosylation sites [146, 147]. The nonhuman production platforms employed, for example, CHO, NS0, and Sp2/0 cells which may add sugar residues that are foreign to humans and consequently confer immunogenicity.

The prototype IgG antibody molecule bears oligosaccharide N-linked to asparagine residue 297 of the IgG-Fc heavy chain. Although the presence or absence of IgG-Fc oligosaccharide does not affect antigen binding specificity, it has been reported to modulate binding affinity. Its main impact is to modulate activation of downstream effector functions that eliminate and destroy antibody/pathogen ICs; mAb therapeutics may be "customised" to activate the same effector mechanisms for the elimination of cancer cells, and so forth [11, 148]. The oligosaccharide released from normal polyclonal IgG-Fc is heterogeneous and essentially comprised of the core heptasaccharide with the variable addition of fucose, galactose, bisecting N-acetylglucosamine, and N-acetylneuraminic (sialic) acid residues [9, 11, 148–150]. Early analytical studies revealed variations in the content of galactose residues and G0 (zero galactose), G1, and G2 glycoforms were enumerated; however, it was later shown that whilst a majority of IgG-Fc oligosaccharides bore fucose residues, a significant proportion did not; therefore a revision of glycoform designations was required; thus G0, G1, and G2 refer to IgG-Fc oligosaccharides that do not include fucose, whilst G0F, G1F, and G2F refer to oligosaccharides bearing both fucose and galactose; when bisecting N-acetylglucosamine is present a B is added, for example, G0B, G0BF, and G1BF; sialylation at the galactose residues is designated as G1FS, G2FBS, and so forth. The approximate composition of neutral oligosaccharides released from normal polyclonal human IgG-Fc is G0: 3%; G1: 3%; G2: 6%; G0F: 23%; G1F: 30%; G2F: 24%; G0BF: 3%; G1BF: 4%; and G2BF: 7% [9, 11, 22–24, 148–151]. It is important to define the glycoform of the intact IgG molecule, for example, [G0/G1F] and [G1F/G2BF], since it has been shown that individual IgG molecules may be comprised of symmetrical or asymmetrical heavy chain glycoform pairs [152, 153]. Recombinant mAbs expressing each of the naturally occurring IgG-Fc glycoforms have been generated to determine their respective abilities to activate a range of effector mechanisms; in addition truncated, aglycosylated, and novel glycoforms have been generated that have contributed to our understanding of the role of glycosylation in the interactions of IgG-Fc with effector ligands [11, 22, 148–155].

The glycosylation profile of each approved mAb therapeutic is identified as a CQA, whether the aim is to optimise or minimize effector function potential, that is, the MoA. The first criterion is therefore to produce a mAb having

either 100% or 0% oligosaccharide occupancy. The CHO, NS0, and Sp2/0 cell lines used for the production of mAbs produce predominantly G0F heavy chain glycoforms with relatively low levels of galactosylated and nonfucosylated IgG-Fc, relative to normal polyclonal IgG-Fc; however, precise culture conditions may impact the glycoform profile of the product [22, 156–159]. Unfortunately, these production cell lines may also add sugars that are not expressed on human glycoproteins and may be immunogenic. Thus, whilst CHO cell lines may add N-acetylneuraminic acid residues they do so in α(2-3) linkage, rather than the α(2–6) linkage present in human IgG-Fc. A particular concern is that the addition, by NS0 and Sp2/0 cells, of galactose in α(1–3) linkage to galactose linked β(1–4) to the N-acetylglucosamine residues [160–163]. Humans and higher primates do not have a functional gene encoding the transferase that adds galactose in α(1–3) linkage; however, due to continual environmental exposure to the gal α(1–3) gal epitope, for example, in red meats, humans develop IgG antibodies specific to this antigen; a proportion of individuals develop IgE antibodies and incidences of immediate hypersensitivity reactions have been reported, some resulting in death [160, 161]. The gal α(1–3) gal epitope is widely expressed on hamster cells and some derived CHO cell lines have been shown to be capable of (gal α(1–3) gal) addition [163]. Similarly, CH0, NS0, and Sp2/0 cells may add an N-glycolylneuraminic acid, in α(2-3) linkage that also may be immunogenic in humans [156–163]. A significant population of normal human IgG-Fc bears a bisecting N-acetylglucosamine residue that is absent from IgG-Fc produced in CHO, NS0, or Sp2/0 cells. Studies of homogeneous IgG-Fc glycoforms, generated *in vitro*, have shown qualitative and quantitative differences in effector function activities between the IgG subclasses and for differing glycoforms within each subclass [11, 64, 164–166]. It has not proved possible to manipulate culture medium conditions to generate predetermined homogeneous mAb glycoform profiles; however, significant "tweaking" of the profile can be achieved during a production run [158, 159] and cellular engineering has been employed to enhance production of particular human IgG-Fc glycoforms.

15. Summary and Conclusions

The thrust of this review is that natural P/GPs are structurally heterogeneous and comprised of multiple isoforms, due to variations in CTMs, PTMs, and CMs. The isoform composition of the proteome may differ between individuals and, over time, within individuals; however, the integrity of the individual is maintained by functional immunological tolerance. The production of a recombinant P/GP in a heterologous system will inevitably result in the generation of isoforms structurally different to endogenous P/GPs that may be perceived by the immune system as nonself and result in an immune response with the production of ADA that compromise therapeutic benefit and/or induce adverse events. Recombinant mAbs presents a particular challenge because an endogenous molecule is not available for structural comparison; that is, they are, by definition, structurally and functionally unique. Multiple parameters that may impact on immunogenicity have been discussed with a particular emphasis on denaturation leading to the formation of immunogenic aggregates. Whilst this has validity for recombinant P/GP therapeutics in general, it may not be so evident for mAbs since their MoA depends on the formation of ICs that are themselves aggregates. The size and architecture of IC aggregates (ICA) formed are dependent on multiple parameters, including affinity and antigen/mAb ratio.

It is held that on chronic exposure all recombinant P/GPs are immunogenic, at least in a proportion of patients; however, the incidence and consequences of immunogenicity may vary depending on the disease treated. Treatments for cancer include drugs that target dividing cells with the inevitable collateral consequence of compromising the immune system, that is, immunosuppression; thus short term exposure to a mAb in the treatment of cancers may not occasion the generation of ADA. In contrast treatment of chronic diseases with mAb may result in repeated episodes of remission and relapse over extended time periods and has been shown to increase the incidence of development of ADA. A palliative measure may be to induce a low level of immunosuppression. This has been realized for rheumatic diseases with exposure to the mild immunosuppressant methotrexate and anti-TNF mAb [167, 168]. Early studies established that immunological tolerance can be induced experimentally by exposure to aggregate free forms of a potential immunogen [169, 170]; this potential has been exploited clinically to reduce the incidence of ADA formation in patients receiving the mAb Alemtuzumab. A single amino acid mutant of the antibody was generated that resulted in loss of antigen binding activity 178. Exposure of patients to a high dose of this mutant prior to dosing with the active antibody reduced the incidence of immunogenicity from ~74% to 21% [29, 170, 171]. The crucial difference between Alemtuzumab and the mutant was that the latter is not able to form ICs, thus demonstrating the generation of ICs as a parameter contributing to immunogenicity; that is, the outcome of exposure to ICs and aggregates may be equivalent. This suggests that in addition to characterizing aggregated forms of mAb in drug product, assumed to be present at the time of administration, studies of the structure and function of ICs formed on administration of mAb should be investigated [86–88]; the necessary tools are now available [172–176].

Competing Interests

The author declares that they have no competing interests.

References

[1] M. K. Ghazavi and G. A. Johnston, "Insulin allergy," *Clinics in Dermatology*, vol. 29, no. 3, pp. 300–305, 2011.

[2] I. C. MacDougall, S. D. Roger, A. De Francisco et al., "Antibody-mediated pure red cell aplasia in chronic kidney disease patients receiving erythropoiesis-stimulating agents: new insights," *Kidney International*, vol. 81, no. 8, pp. 727–732, 2012.

[3] S. Welle, *Human Protein Metabolism*, Springer, New York, NY, USA, 1999.

[4] R. J. Harris, B. Kabakoff, F. D. Macchi et al., "Identification of multiple sources of charge heterogeneity in a recombinant antibody," *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 752, no. 2, pp. 233–245, 2001.

[5] X. Zhong and J. F. Wright, "Biological insights into therapeutic protein modifications throughout trafficking and their biopharmaceutical applications," *International Journal of Cell Biology*, vol. 2013, Article ID 273086, 19 pages, 2013.

[6] http://proteomics.cancer.gov/whatisproteomics.

[7] R. Jefferis, "Isotype and glycoform selection for antibody therapeutics," *Archives of Biochemistry and Biophysics*, vol. 526, no. 2, pp. 159–166, 2012.

[8] A. M. Goetze, Y. D. Liu, T. Arroll, L. Chu, and G. C. Flynn, "Rates and impact of human antibody glycation in vivo," *Glycobiology*, vol. 22, no. 2, pp. 221–234, 2012.

[9] L. A. Khawli, S. Goswami, R. Hutchinson et al., "Charge variants in IgG1: isolation, characterization, in vitro binding properties and pharmacokinetics in rats," *mAbs*, vol. 2, no. 6, pp. 613–624, 2010.

[10] W. Wang, S. Singh, D. L. Zeng, K. King, and S. Nema, "Antibody structure, instability, and formulation," *Journal of Pharmaceutical Sciences*, vol. 96, no. 1, pp. 1–26, 2007.

[11] A. N. Burska, L. Hunt, M. Boissinot et al., "Autoantibodies to posttranslational modifications in rheumatoid arthritis," *Mediators of Inflammation*, vol. 2014, Article ID 492873, 19 pages, 2014.

[12] N. Chicooree, R. D. Unwin, and J. R. Griffiths, "The application of targeted mass spectrometry-based strategies to the detection and localization of post-translational modifications," *Mass Spectrometry Reviews*, vol. 34, no. 6, pp. 595–626, 2015.

[13] F. Lanucara and C. E. Eyers, "Top-down mass spectrometry for the analysis of combinatorial post-translational modifications," *Mass Spectrometry Reviews*, vol. 32, no. 1, pp. 27–42, 2013.

[14] N. Farriol-Mathis, J. S. Garavelli, B. Boeckmann et al., "Annotation of post-translational modifications in the Swiss-Prot knowledge base," *Proteomics*, vol. 4, no. 6, pp. 1537–1550, 2004.

[15] G. A. Khoury, R. C. Baliban, and C. A. Floudas, "Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database," *Scientific Reports*, vol. 1, article 90, 2014.

[16] G. Manning, D. B. Whyte, R. Martinez, T. Hunter, and S. Sudarsanam, "The protein kinase complement of the human genome," *Science*, vol. 298, no. 5600, pp. 1912–1934, 2002.

[17] F. Sacco, L. Perfetto, L. Castagnoli, and G. Cesareni, "The human phosphatase interactome: an intricate family portrait," *FEBS Letters*, vol. 586, no. 17, pp. 2732–2739, 2012.

[18] P. Stanley, "Golgi glycosylation," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 4, Article ID a005199, 2011.

[19] J. D. Marth and P. K. Grewal, "Mammalian glycosylation in immunity," *Nature Reviews Immunology*, vol. 8, no. 11, pp. 874–887, 2008.

[20] S. Kozlowski and P. Swann, "Current and future issues in the manufacturing and development of monoclonal antibodies," *Advanced Drug Delivery Reviews*, vol. 58, no. 5-6, pp. 707–722, 2006.

[21] http://www.fda.gov/Drugs/DevelopmentApprovalProcess/%20HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/ucm113522.htm.

[22] EMA, Guideline on Development, Production, Characterisation and Specifications for Monoclonal Antibodies and Related Products, January 2016, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003074.pdf.

[23] http://www.fda.gov/downloads/Drugs/.../Guidances/ucm073507.pdf.

[24] http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/document_listing/document_listing_000162.jsp.

[25] FDA, *Guidance for Industry: Scientific Considerations in Demonstrating Biosimilarity to a Reference Product*, 2014, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM397017.pdf.

[26] http://www.ema.europa.eu/ema/index.jsp?curl=pages/special_topics/document_listing/document_listing_000318.jsp.

[27] E. Gatti and P. Pierre, "Understanding the cell biology of antigen presentation: the dendritic cell contribution," *Current Opinion in Cell Biology*, vol. 15, no. 4, pp. 468–473, 2003.

[28] G. Walsh and R. Jefferis, "Post-translational modifications in the context of therapeutic proteins," *Nature Biotechnology*, vol. 24, no. 10, pp. 1241–1252, 2006.

[29] R. Jefferis, "Aggregation, immune complexes and immunogenicity," *mAbs*, vol. 3, no. 6, pp. 503–504, 2011.

[30] A. Mastrangelo, T. Colasanti, C. Barbati et al., "The role of posttranslational protein modifications in rheumatological diseases: focus on rheumatoid arthritis," *Journal of Immunology Research*, vol. 2015, Article ID 712490, 10 pages, 2015.

[31] G. J. M. Pruijn, "Citrullination and carbamylation in the pathophysiology of rheumatoid arthritis," *Frontiers in Immunology*, vol. 6, article 192, 2015.

[32] M. A. Shelef, J. Sokolove, L. J. Lahey et al., "Peptidylarginine deiminase 4 contributes to tumor necrosis factor α-induced inflammatory arthritis," *Arthritis and Rheumatology*, vol. 66, no. 6, pp. 1482–1491, 2014.

[33] X. Zhao, N. L. Okeke, O. Sharpe et al., "Circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis," *Arthritis Research and Therapy*, vol. 10, no. 4, article R94, 2008.

[34] M. P. M. van der Linden, D. van der Woude, A. Ioan-Facsinay et al., "Value of anti-modified citrullinated vimentin and third-generation anti-cyclic citrullinated peptide compared with second-generation anti-cyclic citrullinated peptide and rheumatoid factor in predicting disease outcome in undifferentiated arthritis and rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 60, no. 8, pp. 2232–2241, 2009.

[35] A. A. Jilani and C. G. Mackworth-Young, "The role of citrullinated protein antibodies in predicting erosive disease in rheumatoid arthritis: a systematic literature review and meta-analysis," *International Journal of Rheumatology*, vol. 2015, Article ID 728610, 8 pages, 2015.

[36] M. Brink, M. K. Verheul, J. Rönnelid et al., "Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage," *Arthritis Research and Therapy*, vol. 17, no. 1, article 25, 2015.

[37] M. S. Chimenti, P. Triggianese, M. Nuccetelli et al., "Autoreactions, autoimmunity and psoriatic arthritis," *Autoimmunity Reviews*, vol. 14, no. 12, pp. 1142–1146, 2015.

[38] A. Willemze, R. E. M. Toes, T. W. J. Huizinga, and L. A. Trouw, "New biomarkers in rheumatoid arthritis," *Netherlands Journal of Medicine*, vol. 70, no. 9, pp. 392–399, 2012.

[39] I. C. Macdougall, N. Casadevall, F. Locatelli et al., "Incidence of erythropoietin antibody-mediated pure red cell aplasia: the

Prospective Immunogenicity Surveillance Registry (PRIMS)," *Nephrology Dialysis Transplantation*, vol. 30, no. 3, pp. 451–460, 2015.

[40] F. Locatelli, L. Del Vecchio, and P. Pozzoni, "Pure red-cell aplasia 'epidemic'—mystery completely revealed?" *Peritoneal Dialysis International*, vol. 27, supplement 2, pp. S303–S307, 2007.

[41] J. B. Wish, "Erythropoiesis-stimulating agents and pure red-cell aplasia: you can't fool Mother Nature," *Kidney International*, vol. 80, no. 1, pp. 11–13, 2011.

[42] L. A. Halim, V. Brinks, W. Jiskoot et al., "How bio-questionable are the different recombinant human erythropoietin copy products in Thailand?" *Pharmaceutical Research*, vol. 31, no. 5, pp. 1210–1218, 2014.

[43] F. Fotiou, S. Aravind, P.-P. Wang, and O. Nerapusee, "Impact of illegal trade on the quality of epoetin alfa in Thailand," *Clinical Therapeutics*, vol. 31, no. 2, pp. 336–346, 2009.

[44] M. F. Bachmann and G. T. Jennings, "Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns," *Nature Reviews Immunology*, vol. 10, no. 11, pp. 787–796, 2010.

[45] M. Wang and R. J. Kaufman, "The impact of the endoplasmic reticulum protein-folding environment on cancer development," *Nature Reviews Cancer*, vol. 14, no. 9, pp. 581–597, 2014.

[46] E. De Genst, A. Messer, and C. M. Dobson, "Antibodies and protein misfolding: from structural research tools to therapeutic strategies," *Biochimica et Biophysica Acta*, vol. 1844, no. 11, pp. 1907–1919, 2014.

[47] C. J. Roberts, "Protein aggregation and its impact on product quality," *Current Opinion in Biotechnology*, vol. 30, pp. 211–217, 2014.

[48] G. V. Barnett, W. Qi, S. Amin, E. Neil Lewis, and C. J. Roberts, "Aggregate structure, morphology and the effect of aggregation mechanisms on viscosity at elevated protein concentrations," *Biophysical Chemistry*, vol. 207, pp. 21–29, 2015.

[49] January 2016, http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm338856.pdf.

[50] A. S. Rosenberg, "Effects of protein aggregates: an immunologic perspective," *The AAPS Journal*, vol. 8, no. 3, pp. E501–E507, 2006.

[51] P. Bruhns, B. Iannascoli, P. England et al., "Specificity and affinity of human Fcγ receptors and their polymorphic variants for human IgG subclasses," *Blood*, vol. 113, no. 16, pp. 3716–3725, 2009.

[52] A. Bajardi-Taccioli, A. Blum, C. Xu, Z. Sosic, S. Bergelson, and M. Feschenko, "Effect of protein aggregates on characterization of FcRn binding of Fc-fusion therapeutics," *Molecular Immunology B*, vol. 67, no. 2, pp. 616–624, 2015.

[53] T. Perevozchikova, H. Nanda, D. P. Nesta, and C. J. Roberts, "Protein adsorption, desorption, and aggregation mediated by solid-liquid interfaces," *Journal of Pharmaceutical Sciences*, vol. 104, no. 6, pp. 1946–1959, 2015.

[54] M. Phay, A. T. Welzel, A. D. Williams et al., "IgG Conformer's binding to amyloidogenic aggregates," *PLOS ONE*, vol. 10, no. 9, Article ID e0137344, 2015.

[55] W. Jelkmann, "Biosimilar recombinant human erythropoietins ("epoetins") and future erythropoiesis-stimulating treatments," *Expert Opinion on Biological Therapy*, vol. 12, no. 5, pp. 581–592, 2012.

[56] http://gabi-journal.net/wp-content/uploads/LatAM-SBP-2015-WS-V15B10-TP-FINAL1.pdf.

[57] F. B. Vincent, E. F. Morand, K. Murphy, F. Mackay, X. Mariette, and C. Marcelli, "Antidrug antibodies (ADAb) to tumour necrosis factor (TNF)-specific neutralising agents in chronic inflammatory diseases: a real issue, a clinical perspective," *Annals of the Rheumatic Diseases*, vol. 72, no. 2, pp. 165–178, 2013.

[58] D. Jullien, J. C. Prinz, and F. O. Nestle, "Immunogenicity of biotherapy used in psoriasis: the science behind the scenes," *Journal of Investigative Dermatology*, vol. 135, no. 1, pp. 31–38, 2015.

[59] A. Armuzzi, P. Lionetti, C. Blandizzi et al., "anti-TNF agents as therapeutic choice in immune-mediated inflammatory diseases: focus on adalimumab," *International journal of immunopathology and pharmacology*, vol. 27, supplement 1, pp. 11–32, 2014.

[60] J. S. Smolen, R. Landewé, F. C. Breedveld et al., "EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update," *Annals of Rheumatic Diseases*, vol. 73, no. 3, pp. 492–509, 2014.

[61] M. Wadhwa, I. Knezevic, H.-N. Kang, and R. Thorpe, "Immunogenicity assessment of biotherapeutic products: an overview of assays and their utility," *Biologicals*, vol. 43, no. 5, pp. 298–306, 2015.

[62] R. Jefferis and M.-P. Lefranc, "Human immunoglobulin allotypes: possible implications for immunogenicity," *mAbs*, vol. 1, no. 4, pp. 332–338, 2009.

[63] M.-P. Lefranc and G. Lefranc, "Human Gm, Km, and Am allotypes and their molecular characterization: a remarkable demonstration of polymorphism," *Methods in Molecular Biology*, vol. 882, pp. 635–680, 2012.

[64] G. Vidarsson, G. Dekkers, and T. Rispens, "IgG subclasses and allotypes: from structure to effector functions," *Frontiers in Immunology*, vol. 5, article 520, 2014.

[65] L. K. Hmiel, K. A. Brorson, and M. T. Boyne, "Post-translational structural modifications of immunoglobulin G and their effect on biological activity," *Analytical and Bioanalytical Chemistry*, vol. 407, no. 1, pp. 79–94, 2015.

[66] WHO, *Guidelines on the Quality, Safety, and Efficacy of Biotherapeutic Products Prepared by Recombinant DNA Technology*, 2013, http://apps.who.int/iris/bitstream/10665/129494/1/TRS_987_eng.pdf?ua=1&ua=1.

[67] EMA, *Guideline on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins*, EMA, 2007, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003946.pdf.

[68] EMA: guideline on immunogenicity assessment of monoclonal antibodies intended for in vivo clinical use has been released for consultation, 2012, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/06/WC500128688.pdf.

[69] FDA, *Assay Development for Immunogenicity Testing of Therapeutic Proteins*, 2009, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf.

[70] C. C. Mok, W. C. Tsai, Y. Chen et al., "Immunogenicity of anti-TNF biologic agents in the treatment of rheumatoid arthritis," *Expert Opinion in Biological Therapy*, vol. 2, pp. 1–11, 2015.

[71] T. Schaeverbeke, M. Truchetet, M. Kostine, T. Barnetche, B. Bannwarth, and C. Richez, "Immunogenicity of biologic agents in rheumatoid arthritis patients: lessons for clinical practice," *Rheumatology*, vol. 55, no. 2, pp. 210–220, 2016.

[72] G. M. Edelman, B. A. Cunningham, W. E. Gall, P. D. Gottlieb, U. Rutishauser, and M. J. Waxdal, "The covalent structure of

an entire gamma G immunoglobulin molecule. 1969," *Journal of Immunology*, vol. 173, no. 9, pp. 5335–5342, 2004.

[73] H. B. Dickler, "Studies of the human lymphocyte receptor for heat aggregated or antigen complexed immunoglobulin," *Journal of Experimental Medicine*, vol. 140, no. 2, pp. 508–522, 1974.

[74] I. M. Hunneyball and D. R. Stanworth, "Studies on immune tolerance to heat-aggregated human IgG in rabbits: its relevance to the production of rheumatoid factors," *Immunology*, vol. 37, no. 3, pp. 529–537, 1979.

[75] P. Gagnon, R. Nian, D. Leong, and A. Hoi, "Transient conformational modification of immunoglobulin G during purification by protein A affinity chromatography," *Journal of Chromatography A*, vol. 1395, pp. 136–142, 2015.

[76] S. N. Telikepalli, O. S. Kumru, C. Kalonia et al., "Structural characterization of IgG1 mAb aggregates and particles generated under various stress conditions," *Journal of Pharmaceutical Sciences*, vol. 103, no. 3, pp. 796–809, 2014.

[77] V. Filipe, R. Poole, O. Oladunjoye, K. Braeckmans, and W. Jiskoot, "Detection and characterization of subvisible aggregates of monoclonal igg in serum," *Pharmaceutical Research*, vol. 29, no. 8, pp. 2202–2212, 2012.

[78] T. W. Randolph, E. Schiltz, D. Sederstrom et al., "Do not drop: mechanical shock in vials causes cavitation, protein aggregation, and particle formation," *Journal of Pharmaceutical Sciences*, vol. 104, no. 2, pp. 602–611, 2015.

[79] A. J. Freitag, M. Shomali, S. Michalakis et al., "Investigation of the immunogenicity of different types of aggregates of a murine monoclonal antibody in mice," *Pharmaceutical Research*, vol. 32, no. 2, pp. 430–444, 2015.

[80] N. Chennamsetty, B. Helk, V. Voynov, V. Kayser, and B. L. Trout, "Aggregation-prone motifs in human immunoglobulin G," *Journal of Molecular Biology*, vol. 391, no. 2, pp. 404–413, 2009.

[81] X. Wang, T. K. Das, S. K. Singh, and S. Kumar, "Potential aggregation prone regions in biotherapeutics: a survey of commercial monoclonal antibodies," *mAbs*, vol. 1, no. 3, pp. 254–267, 2009.

[82] T. Skamris, X. Tian, M. Thorolfsson et al., "Monoclonal antibodies follow distinct aggregation pathways during production-relevant acidic incubation and neutralization," *Pharmaceutical Research*, vol. 33, no. 3, pp. 716–728, 2016.

[83] J. H. Huh, A. J. White, S. R. Brych, H. Franey, and M. Matsumura, "The identification of free cysteine residues within antibodies and a potential role for free cysteine residues in covalent aggregation because of agitation stress," *Journal of Pharmaceutical Sciences*, vol. 102, no. 6, pp. 1701–1711, 2013.

[84] R. Rouet, D. Lowe, and D. Christ, "Stability engineering of the human antibody repertoire," *FEBS Letters*, vol. 588, no. 2, pp. 269–277, 2014.

[85] S. Ewert, T. Huber, A. Honegger, and A. Plückthun, "Biophysical properties of human antibody variable domains," *Journal of Molecular Biology*, vol. 325, no. 3, pp. 531–553, 2003.

[86] US Food and Drug Administration, *Herceptin®, Avastin® and Remicade® Prescribing Information*, US Food and Drug Administration, Silver Spring, Md, USA, http://www.accessdata.fda.gov/scripts/cder/drugsatfda/.

[87] T. Arvinte, C. Palais, E. Green-Trexler et al., "Aggregation of biopharmaceuticals in human plasma and human serum: implications for drug research and development," *mAbs*, vol. 5, no. 3, pp. 491–500, 2013.

[88] S. Luo and B. Zhang, "Dextrose-mediated aggregation of therapeutic monoclonal antibodies in human plasma: implication of isoelectric precipitation of complement proteins," *mAbs*, vol. 7, no. 6, pp. 1094–1103, 2015.

[89] J. M. R. Moore, T. W. Patapoff, and M. E. M. Cromwell, "Kinetics and thermodynamics of dimer formation and dissociation for a recombinant humanized monoclonal antibody to vascular endothelial growth factor," *Biochemistry*, vol. 38, no. 42, pp. 13960–13967, 1999.

[90] S. I. van Kasteren, H. Overkleeft, H. Ovaa, and J. Neefjes, "Chemical biology of antigen presentation by MHC molecules," *Current Opinion in Immunology*, vol. 26, no. 1, pp. 21–31, 2014.

[91] W. Zhang, J. Voice, and P. J. Lachman, "A systematic study of neutrophil degranulation and respiratory burst in vitro by defined immune complexes," *Clinical and Experimental Immunology*, vol. 101, no. 3, pp. 507–514, 1995.

[92] J. K. Voice and P. J. Lachmann, "Neutrophil Fcγ and complement receptors involved in binding soluble IgG immune complexes and in specific granule release induced by soluble IgG immune complexes," *European Journal of Immunology*, vol. 27, no. 10, pp. 2514–2523, 1997.

[93] J. Steensgaard, C. Jacobsen, J. Lowe, N. R. Ling, and R. Jefferis, "Theoretical and ultracentrifugal analysis of immune complex formation between monoclonal antibodies and human IgG," *Immunology*, vol. 46, no. 4, pp. 751–760, 1982.

[94] W. Alduaij, A. Ivanov, J. Honeychurch et al., "Novel type II anti-CD20 monoclonal antibody (GA101) evokes homotypic adhesion and actin-dependent, lysosome-mediated cell death in B-cell malignancies," *Blood*, vol. 117, no. 17, pp. 4519–4529, 2011.

[95] G. Niederfellner, A. Lammens, O. Mundigl et al., "Epitope characterization and crystal structure of GA101 provide insights into the molecular basis for type I/II distinction of CD20 antibodies," *Blood*, vol. 118, no. 2, pp. 358–367, 2011.

[96] M.-S. Kim, S.-H. Lee, M.-Y. Song, T. H. Yoo, B.-K. Lee, and Y.-S. Kim, "Comparative analyses of complex formation and binding sites between human tumor necrosis factor-alpha and its three antagonists elucidate their different neutralizing mechanisms," *Journal of Molecular Biology*, vol. 374, no. 5, pp. 1374–1388, 2007.

[97] E. A. Padlan, "X-ray crystallography of antibodies," *Advances in Protein Chemistry*, vol. 49, pp. 57–133, 1996.

[98] D. Guo, A. Gao, D. A. Michels et al., "Mechanisms of unintended amino acid sequence changes in recombinant monoclonal antibodies expressed in Chinese Hamster Ovary (CHO) cells," *Biotechnology and Bioengineering*, vol. 107, no. 1, pp. 163–171, 2010.

[99] C. Nakajima, H. Kuyama, T. Nakazawa, O. Nishimura, and S. Tsunasawa, "A method for N-terminal de novo sequencing of Nα-blocked proteins by mass spectrometry," *Analyst*, vol. 136, no. 1, pp. 113–119, 2011.

[100] A. Kumar and A. K. Bachhawat, "Pyroglutamic acid: throwing light on a lightly studied metabolite," *Current Science*, vol. 102, no. 2, pp. 288–297, 2012.

[101] Y. D. Liu, A. M. Goetze, R. B. Bass, and G. C. Flynn, "N-terminal glutamate to pyroglutamate conversion in vivo for human IgG2 antibodies," *The Journal of Biological Chemistry*, vol. 286, no. 13, pp. 11211–11217, 2011.

[102] S. Yin, C. V. Pastuskovas, L. A. Khawli, and J. T. Stults, "Characterization of therapeutic monoclonal antibodies reveals differences between in vitro and in vivo time-course studies," *Pharmaceutical Research*, vol. 30, no. 1, pp. 167–178, 2013.

[103] R. Perez-Garmendia and G. Gevorkian, "Pyroglutamate-modified amyloid beta peptides: emerging targets for Alzheimer's

disease immunotherapy," *Current Neuropharmacology*, vol. 11, no. 5, pp. 491–498, 2013.

[104] H. Liu, G. Ponniah, H.-M. Zhang et al., "In vitro and in vivo modifications of recombinant and human IgG antibodies," *mAbs*, vol. 6, no. 5, pp. 1145–1154, 2014.

[105] G. Merlini, R. L. Comenzo, D. C. Seldin, A. Wechalekar, and M. A. Gertz, "Immunoglobulin light chain amyloidosis," *Expert Review of Hematology*, vol. 7, no. 1, pp. 143–156, 2014.

[106] J. Li, C. Menzel, D. Meier, C. Zhang, S. Dübel, and T. Jostock, "A comparative study of different vector designs for the mammalian expression of recombinant IgG antibodies," *Journal of Immunological Methods*, vol. 318, no. 1-2, pp. 113–124, 2007.

[107] L. Tang, S. Sundaram, J. Zhang et al., "Conformational characterization of the charge variants of a human IgG1 monoclonal antibody using H/D exchange mass spectrometry," *mAbs*, vol. 5, no. 1, pp. 114–125, 2013.

[108] http://www.ebe-biopharma.eu/uploads/Modules/Documents/ebe-concept-paper-%E2%80%93-considerations-in-setting-specifications.pdf.

[109] M. Tsubaki, I. Terashima, K. Kamata, and A. Koga, "C-terminal modification of monoclonal antibody drugs: amidated species as a general product-related substance," *International Journal of Biological Macromolecules*, vol. 52, no. 1, pp. 139–147, 2013.

[110] M. Škulj, D. Pezdirec, D. Gaser, M. Kreft, and R. Zorec, "Reduction in C-terminal amidated species of recombinant monoclonal antibodies by genetic modification of CHO cells," *BMC biotechnology*, vol. 14, p. 76, 2014.

[111] E. T. J. van den Bremer, F. J. Beurskens, M. Voorhorst et al., "Human IgG is produced in a pro-form that requires clipping of C-terminal lysines for maximal complement activation," *mAbs*, vol. 7, no. 4, pp. 672–680, 2015.

[112] H. Liu and K. May, "Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function," *mAbs*, vol. 4, no. 1, pp. 17–23, 2012.

[113] A. R. Aricescu and R. J. Owens, "Expression of recombinant glycoproteins in mammalian cells: towards an integrative approach to structural biology," *Current Opinion in Structural Biology*, vol. 23, no. 3, pp. 345–356, 2013.

[114] J. Wypych, M. Li, A. Guo et al., "Human IgG2 antibodies display disulfide-mediated structural isoforms," *The Journal of Biological Chemistry*, vol. 283, no. 23, pp. 16194–16205, 2008.

[115] T. M. Dillon, M. S. Ricci, C. Vezina et al., "Structural and functional characterization of disulfide isoforms of the human IgG2 subclass," *Journal of Biological Chemistry*, vol. 283, no. 23, pp. 16206–16215, 2008.

[116] K. L. Koterba, T. Borgschulte, and M. W. Laird, "Thioredoxin 1 is responsible for antibody disulfide reduction in CHO cell culture," *Journal of Biotechnology*, vol. 157, no. 1, pp. 261–267, 2012.

[117] K. M. Hutterer, R. W. Hong, J. Lull et al., "Monoclonal antibody disulfide reduction during manufacturing: untangling process effects from product effects," *mAbs*, vol. 5, no. 4, pp. 608–613, 2013.

[118] Y. H. Kao, M. W. Laird, M. T. Schmidt, R. L. Wong, and D. P. Hewitt, "Prevention of disulfide bond reduction during recombinant production of polypeptides," US Patent 8574869 B2, 2013.

[119] I. R. Correia, "Stability of IgG isotypes in serum," *mAbs*, vol. 2, no. 3, pp. 221–232, 2010.

[120] T. Rispens, J. Meesters, T. H. den Bleker et al., "Fc-Fc interactions of human IgG4 require dissociation of heavy chains and are formed predominantly by the intra-chain hinge isomer," *Molecular Immunology*, vol. 53, no. 1-2, pp. 35–42, 2013.

[121] A. M. Davies, T. Rispens, T. H. den Bleker et al., "Crystal structure of the human IgG4 C_H3 dimer reveals the role of Arg409 in the mechanism of Fab-arm exchange," *Molecular Immunology*, vol. 54, no. 1, pp. 1–7, 2013.

[122] T. Rispens, A. M. Davies, P. Ooijevaar-de Heer et al., "Dynamics of inter-heavy chain interactions in human immunoglobulin G (IgG) subclasses studied by kinetic Fab arm exchange," *The Journal of Biological Chemistry*, vol. 289, no. 9, pp. 6098–6109, 2014.

[123] C. Chumsae, G. Gaza-Bulseco, J. Sun, and H. Liu, "Comparison of methionine oxidation in thermal stability and chemically stressed samples of a fully human monoclonal antibody," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 850, no. 1-2, pp. 285–294, 2007.

[124] A. Bertolotti-Ciarlet, W. Wang, R. Lownes et al., "Impact of methionine oxidation on the binding of human IgG1 to FcRn and Fcγ receptors," *Molecular Immunology*, vol. 46, no. 8-9, pp. 1878–1882, 2009.

[125] W. Wang, J. Vlasak, Y. Li et al., "Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies," *Molecular Immunology*, vol. 48, no. 6-7, pp. 860–866, 2011.

[126] H. Pan, K. Chen, L. Chu, F. Kinderman, I. Apostol, and G. Huang, "Methionine oxidation in human IgG2 Fc decreases binding affinities to protein A and FcRn," *Protein Science*, vol. 18, no. 2, pp. 424–433, 2009.

[127] G. Gaza-Bulseco, S. Faldu, K. Hurkmans, C. Chumsae, and H. Liu, "Effect of methionine oxidation of a recombinant monoclonal antibody on the binding affinity to protein A and protein G," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 870, no. 1, pp. 55–62, 2008.

[128] H. Xie, A. Chakraborty, J. Ahn et al., "Rapid comparison of a candidate biosimilar to an innovator monoclonal antibody with advanced liquid chromatography and mass spectrometry technologies," *mAbs*, vol. 2, no. 4, pp. 379–394, 2010.

[129] D. Chelius, D. S. Render, and P. V. Bondarenko, "Identification and characterization of deamidation sites in the conserved regions of human immunoglobulin gamma antibodies," *Analytical Chemistry*, vol. 77, no. 18, pp. 6004–6011, 2005.

[130] Y. D. Liu, J. Z. van Enk, and G. C. Flynn, "Human antibody Fc deamidation in vivo," *Biologicals*, vol. 37, no. 5, pp. 313–322, 2009.

[131] S. Sinha, L. Zhang, S. Duan et al., "Effect of protein structure on deamidation rate in the Fc fragment of an IgG1 monoclonal antibody," *Protein Science*, vol. 18, no. 8, pp. 1573–1584, 2009.

[132] L. Huang, J. Lu, V. J. Wroblewski, J. M. Beals, and R. M. Riggin, "In vivo deamidation characterization of monoclonal antibody by LC/MS/MS," *Analytical Chemistry*, vol. 77, no. 5, pp. 1432–1439, 2005.

[133] K. Diepold, K. Bomans, M. Wiedmann et al., "Simultaneous assessment of Asp isomerization and Asn deamidation in recombinant antibodies by LC-MS following incubation at elevated temperatures," *PLoS ONE*, vol. 7, no. 1, Article ID e30295, 2012.

[134] J. Vlasak, M. C. Bussat, S. Wang et al., "Identification and characterization of asparagine deamidation in the light chain CDR1 of a humanized IgG1 antibody," *Analytical Biochemistry*, vol. 392, no. 2, pp. 145–154, 2009.

[135] K. Hansson and J. Stenflo, "Post-translational modifications in proteins involved in blood coagulation," *Journal of Thrombosis and Haemostasis*, vol. 3, no. 12, pp. 2633–2648, 2005.

[136] K. Fischer, A. Iorio, R. Lassila et al., "Inhibitor development in non-severe haemophilia across Europe," *Thrombosis and Haemostasis*, vol. 114, no. 4, pp. 670–675, 2015.

[137] J. S. Powell, "Lasting power of new clotting proteins," *Hematology*, vol. 2014, no. 1, pp. 355–363, 2014.

[138] B. W. Grinnell, S. B. Yan, and W. L. Macias, "Activated protein C," in *Directory of Therapeutic Enzymes*, B. McGrath and G. Walsh, Eds., pp. 69–95, CRC Press, Boca Raton, Fla, USA, 2006.

[139] J. Liu, A. Jonebring, J. Hagström, A.-C. Nyström, and A. Lövgren, "Improved expression of recombinant human factor IX by co-expression of GGCX, VKOR and furin," *Protein Journal*, vol. 33, no. 2, pp. 174–183, 2014.

[140] Y.-S. Yang, C.-C. Wang, B.-H. Chen, Y.-H. Hou, K.-S. Hung, and Y.-C. Mao, "Tyrosine sulfation as a protein post-translational modification," *Molecules*, vol. 20, no. 2, pp. 2138–2164, 2015.

[141] Z. Darula, R. J. Chalkley, P. Baker, A. L. Burlingame, and K. F. Medzihradszky, "Mass spectrometric analysis, automated identification and complete annotation of O-linked glycopeptides," *European Journal of Mass Spectrometry*, vol. 16, no. 3, pp. 421–428, 2010.

[142] S. Costagliola, V. Panneels, M. Bonomi et al., "Tyrosine sulfation is required for agonist recognition by glycoprotein hormone receptors," *The EMBO Journal*, vol. 21, no. 4, pp. 504–513, 2002.

[143] B. McGrath, "Factor IX (protease zymogen)," in *Directory of Therapeutic Enzymes*, B. McGrath and G. Walsh, Eds., pp. 209–238, CRC Press, Boca Raton, Fla, USA, 2006.

[144] J. P. Ludeman and M. J. Stone, "The structural role of receptor tyrosine sulfation in chemokine recognition," *British Journal of Pharmacology*, vol. 171, no. 5, pp. 1167–1179, 2014.

[145] H. Choe, W. Li, P. L. Wright et al., "Tyrosine sulfation of human antibodies contributes to recognition of the CCR5 binding region of HIV-1 gp120," *Cell*, vol. 114, no. 2, pp. 161–170, 2003.

[146] E. Llop, R. G. Gallego, V. Belalcazar et al., "Evaluation of protein N-glycosylation in 2-DE: erythropoietin as a study case," *Proteomics*, vol. 7, no. 23, pp. 4278–4291, 2007.

[147] B. Yin, Y. Gao, C.-Y. Chung et al., "Glycoengineering of Chinese hamster ovary cells for enhanced erythropoietin N-glycan branching and sialylation," *Biotechnology and Bioengineering*, vol. 112, no. 11, pp. 2343–2351, 2015.

[148] R. Jefferis, "Monoclonal antibodies: mechanisms of action," in *Current State of the Art & Emerging Technologies for the Characterisation of Monoclonal Antibodies, Volume 1. Monoclonal Antibody Therapeutics: Structure, Function, and Regulatory Space*, D. L. Davis, J. Schiel, and O. Borisov, Eds., vol. 1 of *ACS Symposium Series*, American Chemical Society, Washington, DC, USA, 2014.

[149] L. J. Brady, J. Velayudhan, D. B. Visone et al., "The criticality of high-resolution N-linked carbohydrate assays and detailed characterization of antibody effector function in the context of biosimilar development," *mAbs*, vol. 7, no. 3, pp. 562–570, 2015.

[150] D. Reusch and M. L. Tejada, "Fc glycans of therapeutic antibodies as critical quality attributes," *Glycobiology*, vol. 25, no. 12, pp. 1325–1334, 2015.

[151] L. Liu, "Antibody glycosylation and its impact on the pharmacokinetics and pharmacodynamics of monoclonal antibodies and Fc-fusion proteins," *Journal of Pharmaceutical Sciences*, vol. 104, no. 6, pp. 1866–1884, 2015.

[152] Y. Mimura, P. R. Ashton, N. Takahashi, D. J. Harvey, and R. Jefferis, "Contrasting glycosylation profiles between Fab and Fc of a human IgG protein studied by electrospray ionization mass spectrometry," *Journal of Immunological Methods*, vol. 326, no. 1-2, pp. 116–126, 2007.

[153] K. Masuda, T. Kubota, E. Kaneko et al., "Enhanced binding affinity for FcγRIIIa of fucose-negative antibody is sufficient to induce maximal antibody-dependent cellular cytotoxicity," *Molecular Immunology*, vol. 44, no. 12, pp. 3122–3131, 2007.

[154] T. S. Raju and S. E. Lang, "Diversity in structure and functions of antibody sialylation in the Fc," *Current Opinion in Biotechnology*, vol. 30, pp. 147–152, 2014.

[155] M. Thomann, T. Schlothauer, T. Dashivets et al., "In vitro glycoengineering of IgG1 and its effect on Fc receptor binding and ADCC activity," *PLoS ONE*, vol. 10, no. 8, Article ID e0134949, 2015.

[156] K. M. Heffner, D. B. Hizal, A. Kumar et al., "Exploiting the proteomics revolution in biotechnology: from disease and antibody targets to optimizing bioprocess development," *Current Opinion in Biotechnology C*, vol. 30, pp. 80–86, 2014.

[157] P. H. C. van Berkel, J. Gerritsen, G. Perdok et al., "N-linked glycosylation is an important parameter for optimal selection of cell lines producing biopharmaceutical human IgG," *Biotechnology Progress*, vol. 25, no. 1, pp. 244–251, 2009.

[158] P. Hossler, "Protein glycosylation control in mammalian cell culture: past precedents and contemporary prospects," *Advances in Biochemical Engineering/Biotechnology*, vol. 127, pp. 187–219, 2012.

[159] N. A. McCracken, R. Kowle, and A. Ouyang, "Control of galactosylated glycoforms distribution in cell culture system," *Biotechnology Progress*, vol. 30, no. 3, pp. 547–553, 2014.

[160] A. Daguet and H. Watier, "2nd Charles Richet et Jules Héricourt workshop: therapeutic antibodies and anaphylaxis; May 31-June 1, 2011; Tours, France," *mAbs*, vol. 3, no. 5, pp. 417–421, 2011.

[161] T. A. E. Platts-Mills, A. J. Schuyler, A. Tripathi, and S. P. Commins, "Anaphylaxis to the carbohydrate side chain alpha-gal," *Immunology and Allergy Clinics of North America*, vol. 35, no. 2, pp. 247–260, 2015.

[162] U. Galili, "Discovery of the natural anti-Gal antibody and its past and future relevance to medicine," *Xenotransplantation*, vol. 20, no. 3, pp. 138–147, 2013.

[163] C. J. Bosques, B. E. Collins, J. W. Meador et al., "Chinese hamster ovary cells can produce galactose-α-1,3-galactose antigens on proteins," *Nature Biotechnology*, vol. 28, no. 11, pp. 1153–1156, 2010.

[164] Y. Mimura, S. Church, R. Ghirlando et al., "The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms," *Molecular Immunology*, vol. 37, no. 12-13, pp. 697–706, 2001.

[165] Y. Mimura, P. Sondermann, R. Ghirlando et al., "Role of oligosaccharide residues of IgG1-Fc in FcγRIIb Binding," *The Journal of Biological Chemistry*, vol. 276, no. 49, pp. 45539–45547, 2001.

[166] S. Krapp, Y. Mimura, R. Jefferis, R. Huber, and P. Sondermann, "Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity," *Journal of Molecular Biology*, vol. 325, no. 5, pp. 979–989, 2003.

[167] C. Ha, J. Mathur, and A. Kornbluth, "Anti-TNF levels and anti-drug antibodies, immunosuppressants and clinical outcomes in inflammatory bowel disease," *Expert Review of Gastroenterology and Hepatology*, vol. 9, no. 4, pp. 497–505, 2015.

[168] J.-Y. Choe, N. Prodanovic, J. Niebrzydowski et al., "A randomised, double-blind, phase III study comparing SB2, an infliximab biosimilar, to the infliximab reference product Remicade in patients with moderate to severe rheumatoid arthritis despite methotrexate therapy," *Annals of the Rheumatic Diseases*, 2015.

[169] N. A. Mitchison, "Induction of immunological paralysis in two zones of dosage," *Proceedings of the Royal Society of London. Series B, Containing Papers*, vol. 161, pp. 275–292, 1964.

[170] J. Somerfield, G. A. Hill-Cawthorne, A. Lin et al., "A novel strategy to reduce the immunogenicity of biological therapies," *The Journal of Immunology*, vol. 185, no. 1, pp. 763–768, 2010.

[171] M. A. Tito, J. Miller, N. Walker et al., "Probing molecular interactions in intact antibody: antigen complexes, an electrospray time-of-flight mass spectrometry approach," *Biophysical Journal*, vol. 81, no. 6, pp. 3503–3509, 2001.

[172] C. Atmanene, E. Wagner-Rousset, M. Malissard et al., "Extending mass spectrometry contribution to therapeutic monoclonal antibody lead optimization: characterization of immune complexes using noncovalent ESI-MS," *Analytical Chemistry*, vol. 81, no. 15, pp. 6364–6373, 2009.

[173] C. Atmanene, E. Wagner-Rousset, N. Corvaïa, A. Van Dorsselaer, A. Beck, and S. Sanglier-Cianférani, "Noncovalent mass spectrometry for the characterization of antibody/antigen complexes," *Methods in Molecular Biology*, vol. 988, pp. 243–268, 2013.

[174] M. Oda, S. Uchiyama, M. Noda et al., "Effects of antibody affinity and antigen valence on molecular forms of immune complexes," *Molecular Immunology*, vol. 47, no. 2-3, pp. 357–364, 2009.

[175] N. J. Thompson, S. Rosati, and A. J. R. Heck, "Performing native mass spectrometry analysis on therapeutic antibodies," *Methods*, vol. 65, no. 1, pp. 11–17, 2014.

[176] N. J. Thompson, S. Rosati, R. J. Rose, and A. J. R. Heck, "The impact of mass spectrometry on the study of intact antibodies: from post-translational modifications to structural analysis," *Chemical Communications*, vol. 49, no. 6, pp. 538–548, 2013.

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Research Article

Application of a Plug-and-Play Immunogenicity Assay in Cynomolgus Monkey Serum for ADCs at Early Stages of Drug Development

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Immunogenicity assessment during early stages of nonclinical biotherapeutic development is not always warranted. It is rarely predictive for clinical studies and evidence for the presence of anti-drug antibodies (ADAs) may be inferred from the pharmacokinetic (PK) profile. However, collecting and banking samples during the course of the study are prudent for confirmation and a deeper understanding of the impact on PK and safety. Biotherapeutic-specific ADA assays commonly developed can require considerable time and resources. In addition, the ADA assay may not be ready when needed if the study of PK and safety data triggers assay development. During early stages of drug development for antibody-drug conjugates (ADCs), there is the added complication of the potential inclusion of several molecular variants in a study, differing in the linker and/or drug components. To simplify analysis of ADAs at this stage, we developed plug-and-play generic approaches for both the assay format and the data analysis steps. Firstly, the assay format uses generic reagents to detect ADAs. Secondly, we propose a cut point methodology based on animal specific baseline variability instead of a population data approach. This assay showed good sensitivity, drug tolerance, and reproducibility across a variety of antibody-derived biotherapeutics without the need for optimization across molecules.

1. Introduction

All biotherapeutics, including antibody-drug conjugates (ADCs), have the potential to elicit an immune response in humans that could impact their efficacy, pharmacokinetics, and safety. Hence, the assessment of immunogenicity is a key component during clinical development as well as a regulatory requirement [1–4]. ADCs for oncology indications are composed of a cytotoxic drug linked to a monoclonal antibody (mAb) that recognizes a tumor-associated antigen. Although ADCs contain structural motifs that may increase their immunogenicity, they can nevertheless follow the immunogenicity and assay strategies used for other biotherapeutics with some modifications [5–7].

In a nonclinical setting, it is expected that human protein therapeutics elicit an immune response in animal species. Differences in protein sequences between humans and nonclinical species together with other product related factors contribute to this immune response [8]. Immunogenicity in animals is generally not predictive of immunogenicity in humans and evaluations in nonclinical studies are not always warranted [9]. However, collecting and banking samples during the course of the study are recommended to ensure samples are available if future analysis is needed to explain the pharmacokinetics (PK), exposure, and/or safety data from the study.

Immunogenicity in animal species is normally evaluated by detecting anti-drug antibodies (ADAs) in circulation.

Immunoassay-based technologies are widely used for this purpose [8] with technologies such as mass spectrometry emerging in this arena [10]. Detection of ADAs requires the use of the biotherapeutic as a reagent, which for some immunoassay formats involves conjugation to specific labels (e.g., biotin, ruthenium, digoxigenin, and Alexa Fluor® dyes). Assay development, qualification, and validation require ADA surrogate controls to characterize the performance of the assay. ADA controls for nonclinical assays can be either biotherapeutic-specific or generic, anti-human IgG polyclonal, or monoclonal antibodies. The threshold to determine positivity for biotherapeutic-specific assays is usually established based on the population variability by the analysis of samples from nontreated naive individuals [11, 12].

Our nonclinical immunogenicity strategy for ADC lead candidates selected for preclinical development includes developing ADC-specific ADA assays to support PK and toxicity studies in cynomolgus monkeys [5]. However, there are some caveats with this approach when a program is at the discovery stage. Often a variety of candidate molecules may be evaluated in the same study. In the case of ADCs, these studies may include candidates with different linkers and/or small molecule drugs. In addition, a small number of animals may be used to evaluate each candidate. At this early stage of drug development, the development of molecule specific ADA assays for each candidate could be laborious and resource intensive. Moreover, if the samples are banked and the analysis is triggered by the need to understand PK and/or safety data, developing an assay at that time could impact the ability to make key decisions for the program in a timely manner.

For ADCs in research, our immunogenicity strategy for most PK and safety studies in cynomolgus monkeys is to collect and bank the samples. Having a nonclinical immunogenicity assay applicable across all ADCs would be beneficial to enabling streamlined ADA evaluation across all candidate molecules. The key requirements for such an assay would be readily available capture and detection reagents either in-house or from vendors, a universal assay positive control, ability to detect ADAs to all domains of an ADC, appropriate sensitivity, drug tolerance, and no need for assay optimization with each ADC molecule. In addition to the assay format, cut points or thresholds to determine ADA positivity should be the same for all molecules. Generic or universal assay formats to detect ADAs against mAb biotherapeutics in nonclinical species have been described by others [13, 14]. These types of assays could be also applied to ADCs.

In this paper, we present a generic ADA assay format in cynomolgus monkey serum modified from the one described by Stubenrauch et al. [13] to determine ADAs to ADCs. In addition, we describe a cut point methodology based on animal specific baseline variability instead of the commonly used population data approach. This assay relies on the detection of complexes formed between ADAs and the biotherapeutic. The specific biotherapeutic was added to the samples to ensure that ADAs not complexed to the biotherapeutic in the sample were captured. Capture and detection reagents as well as assay controls either were readily available in our laboratory, were purchased from vendors, or were easy to produce. In addition to generic reagents, the positivity of a sample was determined by a simple cut point methodology specific to each animal instead of a biotherapeutic-specific population approach. As there is no need for assay optimization across molecules, sample testing from programs in preclinical research can be quick. This is a desirable attribute when analysis of banked samples is only triggered by the need to understand PK and/or safety data. This assay was reproducible and showed appropriate sensitivity and drug tolerance. Samples from PK studies in cynomolgus monkeys with two ADCs were analyzed in the plug-and-play assay. The data were generally similar to those previously obtained using our default ADC-specific ELISA. The plug-and-play assay was superior in detecting more incidences of positive responses and earlier on.

2. Materials and Methods

2.1. Buffers, Reagents, Serum Samples, and Test Molecules. The capture reagent for the plug-and-play assay was a murine IgG1 monoclonal antibody R10Z8E9 [13, 15, 16] generated at Genentech, a member of the Roche group (South San Francisco, CA, USA), and is also available from various commercial sources. This antibody is directed against a conformation epitope on the CH2 domain of all four subclasses of human Fc gamma [17]. The detection reagent was a horseradish peroxidase- (HRP-) conjugated goat anti-monkey IgG polyclonal antibody against heavy and light chain with minimal reactivity to human IgG (Bethyl Laboratories Inc., Montgomery, TX, USA). The anticytotoxic drug ADA proof of concept assay used a sheep anti-human IgG (Binding Site, San Diego, CA, USA) as capture reagent and an HRP-conjugated goat anti-mouse IgG antibody (KPL Inc., Gaithersburg MD, USA) for detection.

All the test molecules were generated at Genentech (South San Francisco, CA, USA). These included 4 ADCs, a one-arm antibody produced using knob and hole technology (mAb 1), four recombinant humanized IgG1 mAbs (mAbs 2, 3, 5, and 6), and one recombinant humanized IgG4 mAb (mAb 4). ADCs A, B, C, and D were composed of humanized IgG1 mAbs binding to different targets and sharing the same cleavable peptide linker and cytotoxic drug.

Cynomolgus monkey serum pool and serum from individual animals were purchased from Bioreclamation IVT (Westbury, NY, USA). Baseline samples taken one week apart from 15 cynomolgus monkeys were obtained from a Genentech study conducted at Charles River Laboratories (Reno, NV, USA). Positive controls from three sources were used: human IgG-cyno IgG fusion molecule produced for Genentech at R&D Systems (Minneapolis, MN), purified cynomolgus monkey anti-human IgG1 polyclonal Ab from a cynomolgus monkey hyperimmunized with a Genentech mAb-derived biotherapeutic (Genentech Inc.), and two mouse anticytotoxic drug monoclonal antibodies also produced at Genentech.

Other reagents included biotin (Thermo Fisher Scientific, MA, USA); digoxigenin ([DIG] Invitrogen, NY, USA); bovine serum albumin ([BSA] Equitech-Bio Inc., Kerrville, TX, USA); HRP-conjugated mouse antidigoxin mAb (Jackson

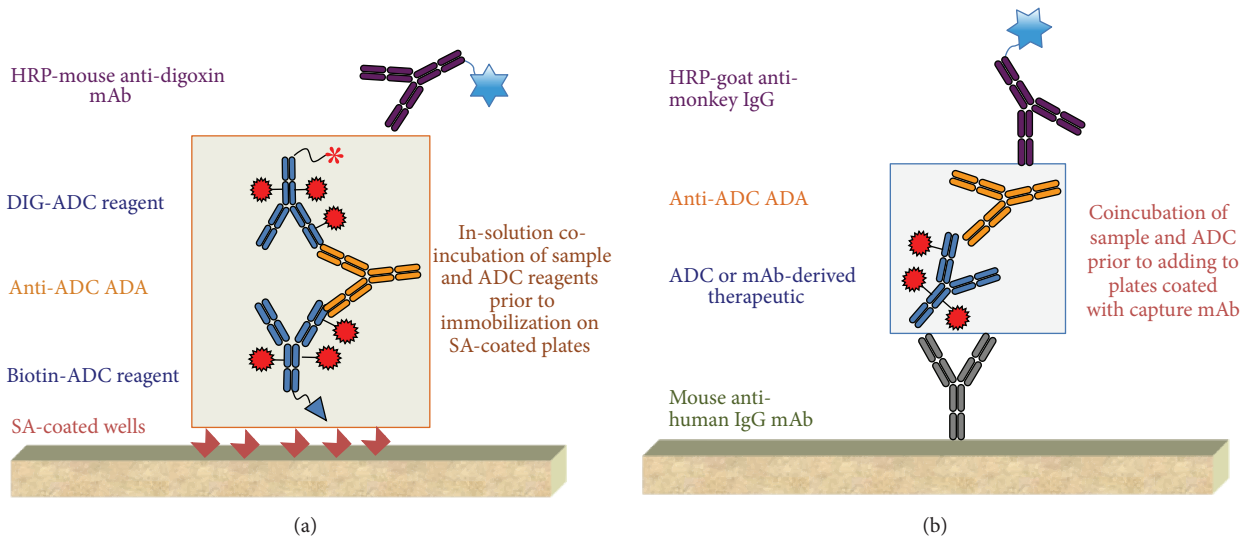

Figure 1: ADA assay formats used for cynomolgus monkeys. (a) Biotherapeutic-specific biotin-DIG ELISA used to screen ADAs in samples from two cynomolgus studies with different ADCs. In this assay, the specific ADC reagents conjugated to biotin or digoxigenin are incubated with the samples. The complexes are immobilized using streptavidin-coated plates and then detected using a mouse antidigoxin mAb conjugated to HRP. (b) Generic ADA assay format developed for ADCs and other mAb-derived biotherapeutics, also used for analysis of samples from two studies in cynomolgus monkey with different ADCs. In this generic assay, the samples are incubated with the specific biotherapeutic and the complexes immobilized onto plates coated with a mouse anti-human IgG mAb. A goat anti-monkey IgG polyclonal antibody conjugated to HRP is used for detection of the immune complexes. ADC = antibody-drug conjugate; ADAs = anti-drug antibodies; DIG = digoxigenin; ELISA = enzyme-linked immunosorbent assay; HRP = horseradish peroxidase; mAb = monoclonal antibody; SA = streptavidin.

ImmunoResearch Labs Inc., PA, USA); tetramethylbenzidine ([TMB] KPL Inc., Gaithersburg, MD, USA).

2.2. Cynomolgus Monkey Study Samples. Cynomolgus monkey serum study samples were obtained from Covance Laboratories (Madison, WI, USA). In Study 1, cynomolgus monkeys received ADC A or the corresponding monoclonal antibody A at a single dose of 3 mg/kg, with four monkeys in each arm. Samples for ADA analysis were collected at baseline and on study days 15 and 44 after baseline. In Study 2, cynomolgus monkeys received a single dose of ADC B (0.3 and 1 mg/kg) or the corresponding monoclonal antibody B (1 mg/kg), with three animals in each arm. Samples for ADA were collected at baseline and on study day 43 after baseline.

2.3. Biotherapeutic-Specific ADA ELISA. Our current platform for nonclinical ADA assays utilizing biotherapeutic-specific assay reagents is an in-solution bridging enzyme-linked immunosorbent assay (ELISA) [5, 18]. This assay was used to evaluate immune responses in the two studies selected for comparing with the plug-and-play assay. Figure 1(a) shows a representation of the assay. Briefly, a mixture of biotinylated-biotherapeutic and digoxigenin-(DIG-) biotherapeutic conjugates was diluted in assay diluent (phosphate-buffered saline (PBS), 0.5% BSA, 0.05% polysorbate 20, and 0.05% ProClin® 300 at pH 7.4) and added to each well of a 96-well round bottom polypropylene microtiter plate (Costar/Corning). Then an equal volume of the test samples and controls diluted in assay diluent were added to appropriate wells and incubated overnight while shaking at room temperature. A mixture of diluted samples or controls with the biotherapeutic-conjugated reagents was then added to prewashed streptavidin-coated plates and incubated for 2 hours. After a wash (PBS, 0.05% polysorbate 20 at pH 7.4), HRP-mouse antidigoxin mAb was added to each well and incubated for another hour at room temperature. The peroxidase substrate TMB was added to develop color. The enzymatic reaction was stopped with phosphoric acid and the plates were read on a plate reader at 450 nm to detect absorbance and at 630 nm for reference absorbance.

For each ADC, specific ADC-conjugated reagents were prepared. A floating screening cut point based on the population biological variation and using a negative control for normalization was established for each ADC targeting a 5% nontreated positive rate [12]. Samples that screened positive were serially diluted and analyzed in the assay to obtain ADA relative levels by titer. The titer was expressed as the log 10 of the sample dilution whose signal was equal to the cut point signal.

2.4. Generic ADA Assay in Cynomolgus Monkey Serum. In the generic assay represented in Figure 1(b), 100 μL of mouse IgG1 monoclonal antibody, R10Z8E9 at 2 μg/mL in coating buffer (PBS, pH 7.2), was added to the 96-well microtiter plates and incubated overnight at 4°C. Test samples were diluted to 10-fold in assay diluent (PBS/pH 7.4/0.5% BSA/0.05% polysorbate 20/0.05% ProClin 300/0.25% CHAPS/0.35 M NaCl). The test molecules (ADCs or mAb-derived

biotherapeutics) were diluted to 0.5 μg/mL in assay diluent. The diluted samples and the biotherapeutic were mixed in 1 : 1 ratio and incubated for 2 hours with a final 20-fold dilution of the test sample and 0.25 μg/mL in-well concentration of the biotherapeutic. The coated plates were blocked (PBS/pH 7.4/0.5% BSA/0.05% polysorbate 20/0.05% ProClin 300) and washed (PBS/pH 7.4/0.05% polysorbate 20). Then, the sample mixture was added at 100 μL per well in duplicate. The assay controls, human IgG-cyno IgG fusion positive control, and the pooled cynomolgus monkey serum negative control were directly diluted to 20-fold in assay diluent and added to the coated plates. In some experiments (e.g., sensitivity assessment), further 2-fold dilutions of the positive control were performed in the assay diluent supplemented with 5% pooled cynomolgus monkey serum. After 1-hour incubation followed by another wash step, wells were incubated with 100 μL of the HRP-conjugated goat anti-monkey IgG at 25 ng/mL in assay diluent. After washing, the peroxidase substrate, TMB, was added for the color to develop. The enzymatic reaction was stopped with phosphoric acid. The plates were read on a BioTek ELx405 plate reader (BioTek, Vt, USA) at 450 nm to detect absorbance and at 630 nm for reference absorbance.

The relative levels of ADAs for each animal with post-baseline positive signals were estimated from the screening run data by the ratio of the postbaseline signal to the animal baseline signal. With this approach, it is important to ensure that the sample signals are within the spectrophotometer range prior to calculating the ratios.

2.5. Determination of Generic Biotherapeutic Concentration Needed for Immune Complex Formation. To establish the level of biotherapeutic added to the samples for immune complex formation prior to analysis in the assay, 4 ADCs and 4 antibody-derived biotherapeutics were evaluated. The biotherapeutics were spiked at concentrations of 0, 1.25, 2.5, 5.0, 10, and 20 μg/mL in pooled cynomolgus monkey serum and were also supplemented with ADAs (purified cynomolgus monkey anti-human IgG antibody) at concentrations of 0, 0.5, 5, and 50 μg/mL. For each ADA concentration, the response curves and the signal-to-background ratios were evaluated across each biotherapeutic level range. The generic concentration of biotherapeutic was established based on the signal-to-background curves obtained for all the biotherapeutics tested in this experiment. As the generic biotherapeutic concentration would be added to each test sample, its effect on background signal was also assessed for each biotherapeutic.

2.6. Determination of Individual Cut Points. Background responses were measured in 30 biotherapeutic naive serum samples from 15 cynomolgus monkeys; two baseline samples from each animal were collected one week apart. Four biotherapeutic molecules were used in the generic cynomolgus ADA assay format for this evaluation: ADC C, mAb 1, mAb 2, and mAb 4. A total of 120 biotherapeutic spiked samples were tested in the assay in duplicate at the minimum dilution. The absorbance value for each sample replicate was reported for statistical analysis.

2.7. Statistical Methods to Determine Individual Cut Point. Background signal data from the two baseline samples obtained from all 15 cynomolgus monkeys with the 4 biotherapeutics were used to estimate the within monkey baseline variability. For each sample replicate, normalized scores were calculated as the log (sample signal/negative control signal). A one-way Analysis of Variance (ANOVA) was fit with normalized scores as the dependent variable and monkeys as the independent variable. The residual error of the model, denoted as SD, was used to estimate the within monkey variability. Separately, a one-way ANOVA was also fit for each subset of cynomolgus monkeys specific to each of the 4 molecules.

The generic cut point factor (CPF) was defined as

$$\mathrm{CPF} = \exp\left(Z_{1-\alpha}\mathrm{SD}\right), \tag{1}$$

where Z is standard score for the standard normal, α is the false positive rate, and SD is the overall estimate of the within monkey variability.

The individual optical density (OD) signal cut point (CP_i) for cynomolgus monkey i was determined by

$$\mathrm{CP}_i = \overline{x}_i * \mathrm{CPF}, \tag{2}$$

where $\overline{x}_i$ is the mean of monkey i's baseline signal measurements. A monkey was deemed ADA positive if a postbaseline measurement exceeds CP_i.

Individual OD cut points determined this way are only valid when a monkey baseline and postbaseline samples are run on the same assay plate.

2.8. Analysis of Study Samples from Cynomolgus Monkey Studies with Two ADCs. The ability of the plug-and-play assay to detect ADAs in study samples was evaluated by analyzing samples from PK studies in cynomolgus monkeys with two ADCs. Immunogenicity data from these two studies with the ADC-specific biotin-DIG ELISA were available. In Study 1, the cynomolgus was dosed with ADC A or the corresponding mAb A. Samples for immunogenicity evaluation were taken at the baseline and at postbaseline days 15 and 44. In Study 2, the cynomolgus monkeys were dosed with ADC B or the corresponding mAb B. Samples for immunogenicity evaluation were taken at baseline and postbaseline day 43.

The samples from the two studies were analyzed in the plug-and-play screening assay and if positive, the relative levels of ADAs were calculated. The results from the plug-and-play assay were compared to those obtained in the biotherapeutic-specific biotin-DIG ELISA in terms of ADA incidence and the relative levels. In the biotin-DIG ELISA, incidence is defined as the sum of enhanced responses and induced responses. In this assay, the cut point determination used a population approach with a 5% false positive rate so it was possible to detect positive signals at baseline. In such a situation, animals with positive signals at baseline could display increased signal at postbaseline time points, which would be considered enhanced by exposure to the biotherapeutic. In the case of animals with positive signals

only after treatment, the ADA responses were considered induced by the exposure to the biotherapeutic. However, in the plug-and-play assay, these types of responses are not distinguished as only signals above the individual animal baseline cut point are considered positive.

3. Results and Discussion

3.1. Generic ADA Screening Assay Format Selection. Several factors were considered in selecting the generic ADA screening assay format in cynomolgus monkey serum. The first consideration was the required data output for the analysis. For our purpose, screening samples to determine if a positive response was produced after exposing the animals to the biotherapeutic and, if positive, a measure of the relative levels of ADAs were the key goals. The potential of the assay format to be used for confirmation of positive responses or further characterization of the ADAs was not a part of the initial goals. The next consideration was the necessity for reagents to be easy to produce and available either in-house or commercially, including capture and detection reagents as well as assay controls. We also looked at operational factors that would enable the assay to be easily implemented in our laboratory using widely available technology and readily transferable across laboratories. Lastly, determining the positive or negative status of a sample by using a generic cut point/threshold was essential for a plug-and-play assay so no experimental work would be necessary to establish a cut point for any new molecule.

The assays described by Stubenrauch et al. [13, 17] and Bautista et al. [14, 19] used in-house produced reagents. The assay that Stubenrauch et al. developed could measure total ADAs by adding the biotherapeutic to the sample to ensure immune complex formation. A biotinylated-murine anti-human IgG Fc was used to capture the complexes after binding to streptavidin-coated plates. Following appropriate washing steps, a monoclonal digoxigenylated anticynomolgus monkey IgG and polyclonal anti-digoxigenin-HRP were used for detection. As an assay control, a fusion of human IgG and cyno IgG was used. Bautista et al. [14, 19] developed the UNISA (Universal Indirect Species-Specific Immunoassay), an electrochemiluminescence assay where the ADAs in the sample were captured by the biotherapeutic coated on MSD (Meso Scale Discovery) plates. A commercial antispecies IgG Fc ruthenium conjugate was used as the detection reagent. A mouse anti-human IgG/cynomolgus monkey IgG1 chimeric monoclonal antibody was used as a positive control for monkey studies and similar chimeras were produced for studies in rat.

The assay that Stubenrauch et al. developed presented most of the qualities for the assay format that we were looking for. Moreover, the number of reagents, labeling of reagents, and assay steps could be simplified. Although the UNISA is a simple ELISA, it required the production of a chimeric mouse-cynomolgus monkey IgG assay control. Our laboratory has a key reagent, a mouse monoclonal antibody that is reactive to many Genentech humanized mAb biotherapeutics [20] that could be used to produce similar assay control chimeras. However, a broader binding specificity was desirable as this antibody showed limited binding to human derived antibodies. Moreover, producing a new clone with broader specificity was not an option at the time.

Our generic assay format is represented in Figure 1(b). It uses murine IgG1 monoclonal antibody, R10Z8E9, as the capture reagent (same as Stubenrauch et al.) directly coated on polystyrene 96-well microtiter plates. Thus, there was no need for streptavidin-coated plates and biotin conjugation of the antibody. R10Z8E9 specificity is directed against a conformation epitope on the CH2 domain of all four subclasses of human Fc gamma [17] and is available at Genentech. Regarding the detection antibody, Stubenrauch et al. produced a specific murine anticynomolgus monkey IgG mAb that was labeled with digoxigenin. A secondary mouse anti-DIG-HRP was necessary for detection. In contrast, our assay uses only one detection reagent that is commercially available conjugated to HRP. For assay positive control, a human IgG-cyno IgG conjugate was produced and used for routine sample analysis similar as described previously [13]. Again, human IgG and cynomolgus monkey IgG are readily available materials that can be easily conjugated. Furthermore, the ADC or the biotherapeutic of interest is another reagent for this assay that is available prior to starting the animal study and does not need to be conjugated unlike our default biotherapeutic-specific biotin-DIG ELISA.

Similar to the assays that Stubenrauch et al. and Bautista et al. developed, our assay is versatile and could be applicable to species other than cynomolgus monkeys by preparing the appropriate human IgG-species IgG fusion assay control and acquiring the antispecies detection reagent.

3.2. The Same Concentration of Biotherapeutic Can Be Used across Biotherapeutics to Form Complexes with ADA. The generic assay format detects complexes formed by ADAs with the biotherapeutic, which can already be present in circulation and could be detected in the assay. However, for our purposes the assay should detect not only ADAs already complexed with the biotherapeutic but also not-complexed ADAs. In the absence of biotherapeutic, the ADAs in the sample would not be detected. Therefore to ensure measurement of total ADAs in the sample, the biotherapeutic has to be added to the sample for the complex formation prior to the analysis. Hence, once the reagents and the assay format were established, experiments were conducted to determine the appropriate amount of biotherapeutic that was needed to add to the test samples in order to detect the total ADAs.

As this is a generic assay format with potential application not only to ADCs but also to other antibody-derived biotherapeutics, several types of molecules were included in the evaluation. Cynomolgus monkey pooled serum samples were prepared by adding 0, 0.5, 5, and 50 μg/mL of ADA (purified cynomolgus monkey anti-human IgG) with 8 biotherapeutics at concentrations ranging from 0 to 20 μg/mL. With all the biotherapeutics, an increase in signal was observed with increasing levels of ADAs and increasing concentrations of biotherapeutics. As more complexes with the ADAs were formed, a plateau was reached where no significant changes in signal were observed with varying concentrations of both

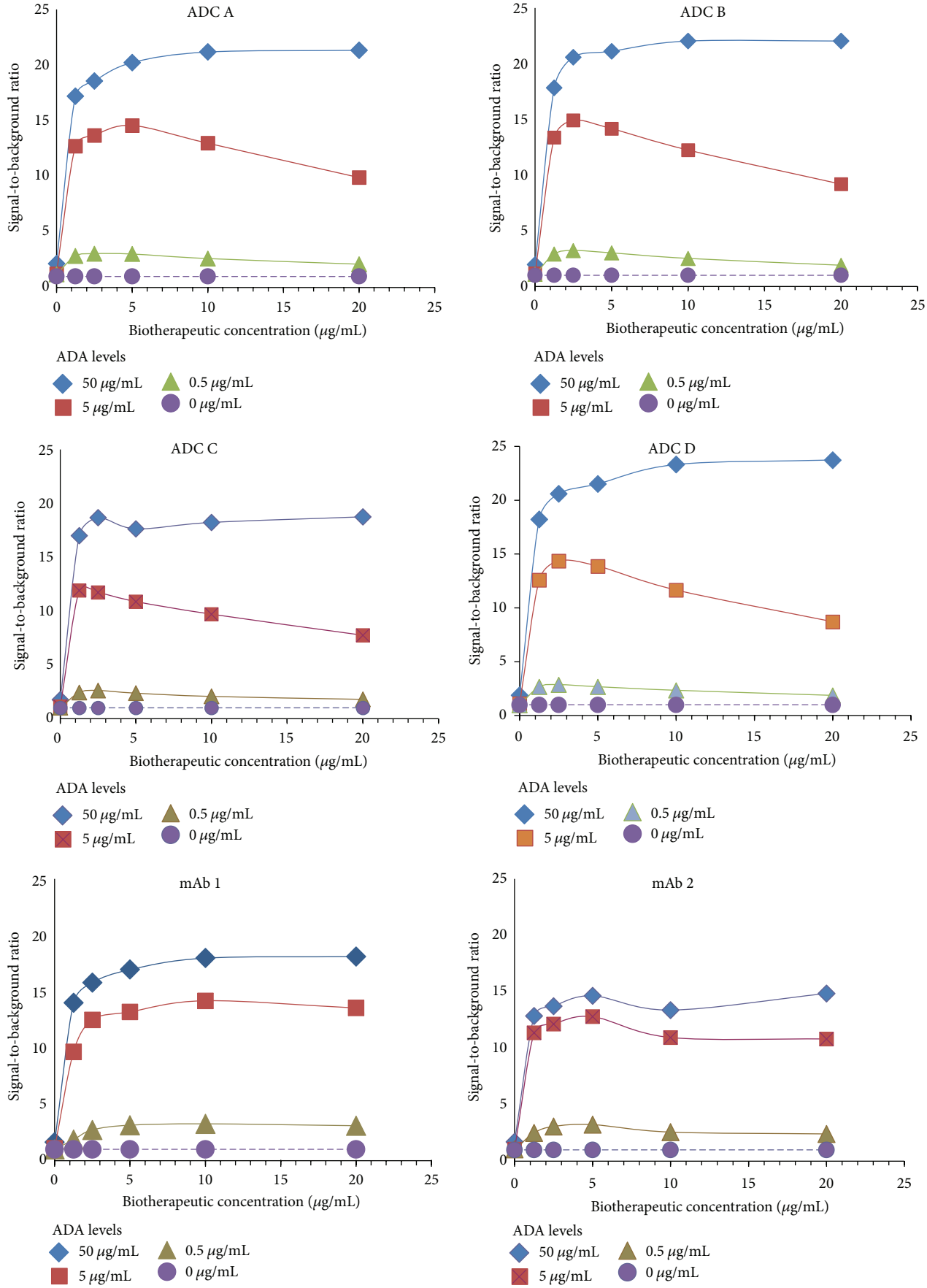

Figure 2: Continued.

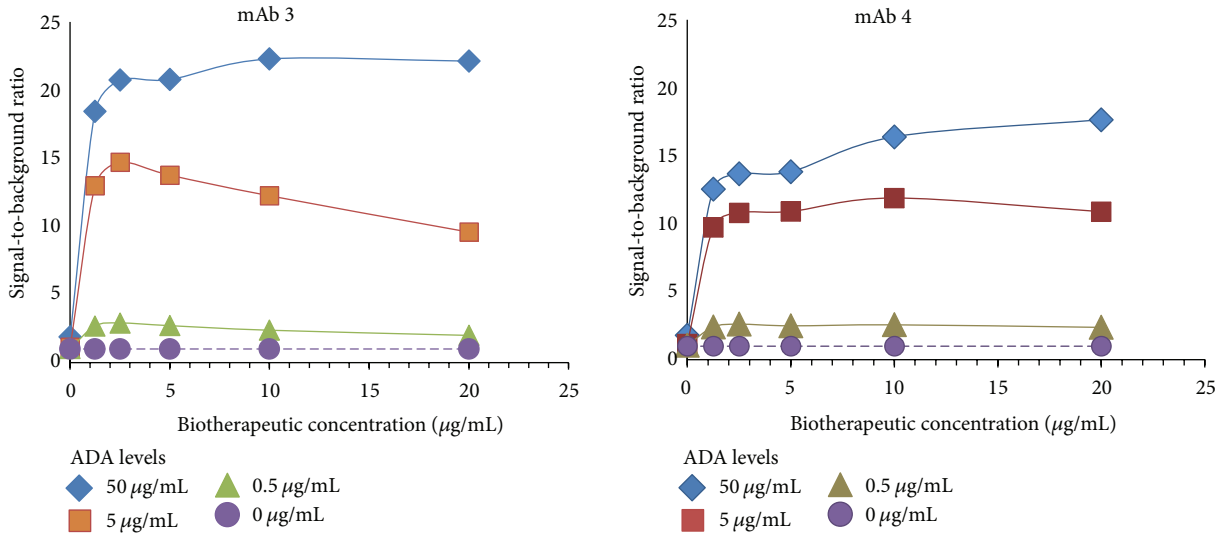

Figure 2: The same concentration of biotherapeutic can be used with different biotherapeutics to form complexes with ADA. Signal-to-background ratios obtained in a pooled cynomolgus monkey serum spiked with purified cynomolgus monkey anti-human IgG ADAs at 0 to 50 μg/mL and with 8 biotherapeutics at 0 to 20 μg/mL. The biotherapeutics consisted of 4 ADCs, 3 mAbs, and a one-arm antibody. With all the biotherapeutics, the signal-to-background ratios increased with increasing concentrations of ADA to reach a plateau at biotherapeutic concentrations between 2 and 10 μg/mL. Based on these data, a biotherapeutic concentration of 5 μg/mL in neat serum was determined to be appropriate to form complexes with ADA in the samples containing up to 50 μg/mL of ADA, the highest level tested in our study. ADAs = anti-drug antibodies; ADC = antibody-drug conjugate; mAb = monoclonal antibody.

ADAs and biotherapeutics. In all the cases, the highest signal observed was below the maximum reading absorbance limit of the microplate reader.

The amount of biotherapeutic to add to the samples was selected based on the ADA signal-to-background ratios observed for different biotherapeutics at various ADA levels. At each level of biotherapeutic, the ratios were calculated by comparing the signal in the presence of ADA with biotherapeutic to the signal with the same biotherapeutic amount in the absence of ADA.

As Figure 2 shows, in general, the signal-to-background ratios at all ADA levels with the 4 ADCs and 4 mAb-derived biotherapeutics reached the highest ratio at concentrations between 2 and 10 μg/mL with no significant changes in the ratios at higher concentrations. Based on these data, a generic biotherapeutic concentration of 5 μg/mL in neat serum was determined to be the appropriate concentration to form complexes with ADA in the samples containing up to 50 μg/mL of ADA, the highest level tested in our study.

Concentrations of biotherapeutics up to 400 μg/mL were tested in early developmental experiments. As Figure 3 depicts, the signal-to-background ratios in samples spiked with various levels of ADA decreased at levels of the biotherapeutic higher than 20 μg/mL.

Figure 4 shows that, in absence of ADA, the background of the pooled cynomolgus monkey serum spiked with 5 μg/mL of the 8 biotherapeutics above was between −21% and 31% of the unspiked serum. These differences in sample background with addition of biotherapeutic gave us a hint to expect similar differences across biotherapeutics when spiking samples from individual monkeys to evaluate biological variability in the assay. Moreover, differences in background signals across biotherapeutics would be a challenge to use the fusion positive control spiked into serum pool as a tool to determine sample positivity.

3.3. Background in Serum from Nontreated Cynomolgus Monkeys Differs with Each Biotherapeutic. Differences in background signal across molecules were observed not only in the pooled monkey serum but also in earlier assay development experiments in serum from individuals. Following industry general practices, screening cut point determination in our laboratory is determined based on the population background biological variability and normalized using a negative serum control [12]. We explored the feasibility of adopting a floating population generic cut point with an earlier version of the assay using commercial samples from 30 cynomolgus monkeys. For this approach to work, the population background should not change in the presence of the added biotherapeutic. However, different backgrounds were observed in unspiked samples and samples spiked with three mAbs. As a result, biotherapeutic-specific cut points were obtained for each mAb as summarized in Table 1. Using the cut point as determined from the population unspiked samples could result in high false positives with mAb 2 and mAb 5 but false negatives with mAb 6. Determining a biotherapeutic-specific cut point for each molecule was not a desirable characteristic for a plug-and-play assay. Thus, a different approach for estimating the cut point had to be followed.

3.4. Statistical Analysis and Determination of Individual Cut Point. Given the background and screening cut point differences across biotherapeutics, a generic approach based on

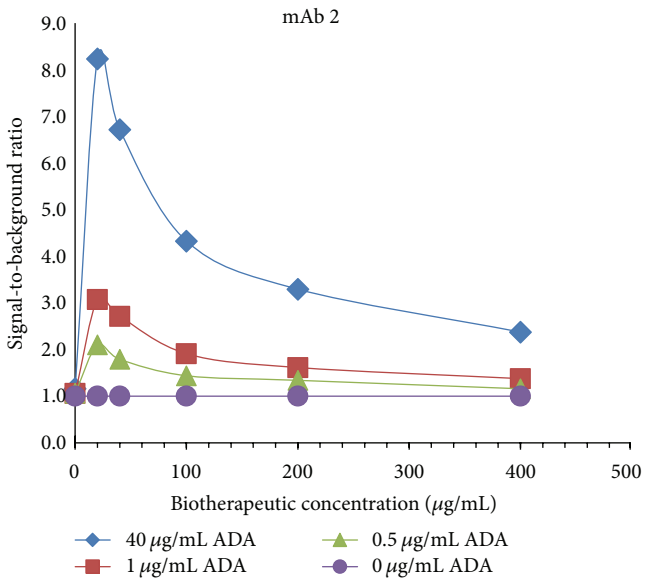

Figure 3: Early development experiments informed on biotherapeutic concentrations to test with a variety of molecules. Signal-to-background ratios obtained in a pooled cynomolgus monkey serum spiked with purified cynomolgus monkey anti-human IgG ADAs at 0, 0.5, 1, and 40 μg/mL and with mAb 2 at 0, 20, 40, 100, 200, and 400 μg/mL. The figure shows signal-to-background ratios increase with increasing concentrations of ADA. The highest signal-to-noise was observed at mAb 2 concentration of 20 μg/mL while higher levels of mAb 2 yielded decrease in the signal-to-noise values at all the ADA levels tested. Based on these data, further experiments with a variety of molecules were performed with biotherapeutic levels up to 20 μg/mL. ADAs = anti-drug antibodies; ADC = antibody-drug conjugate; mAb = monoclonal antibody.

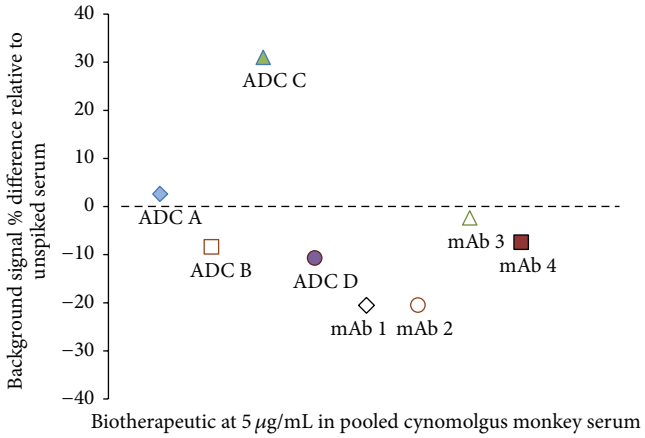

Figure 4: Background signal in pooled cynomolgus monkey serum differs across biotherapeutics in the generic assay. Eight biotherapeutics were added at 5 μg/mL to a pooled cynomolgus monkey serum and tested in the generic assay. The background signal in the presence of biotherapeutic was compared to the signal in the unspiked sample and the percent difference calculated. The background signals differed across biotherapeutics with % differences compared to the unspiked pooled monkey serum ranging between −21% and 31%. ADC = antibody-drug conjugate; mAb = monoclonal antibody.

Table 1: Difference in background responses across three biotherapeutics resulted in molecule specific screening cut points following a floating population data approach. Serum samples from 30 cynomolgus monkeys were spiked with 3 mAbs in early assay development experiments. The unspiked and mAb-spiked samples were analyzed in the generic assay format and a floating population screening cut point was evaluated. Different backgrounds were observed in the samples spiked with three mAbs compared to the unspiked corresponding samples. Therefore, molecule specific screening cut points were obtained for each mAb.

	Unspiked	mAb 2	mAb 5	mAb 6
Cut point (AU)	0.241	0.263	0.426	0.190

AU = absorbance units.

Table 2: Estimation of within monkey variability across biotherapeutics. Four types of biotherapeutics were spiked in two baseline samples taken 1 week apart from 15 cynomolgus monkeys and tested in the assay. The background data from the biotherapeutic spiked samples showed that within monkey signal variability was similar. Therefore, the overall variability obtained with these four molecules could be used to estimate a generic cut point factor.

Biotherapeutic	Type of molecule	Estimated within monkey SD
ADC C	ADC	0.095
mAb 1	One-arm mAb	0.086
mAb 2	IgG1 mAb	0.096
mAb 4	IgG4 mAb	0.087
Overall		0.091

SD = estimate of within monkey signal variability.

a floating individual cut point [12] was explored. This involved using the baseline signal from each individual animal to establish its own cut point signal.

In this experiment, two samples taken 1 week apart from 15 cynomolgus monkeys were spiked at a concentration of 5 μg/mL with 4 biotherapeutics: ADC C, mAb 1, mAb 2, and mAb 4. The background signal from each sample was determined in duplicate in the assay. A universal CPF was established based on the within monkey variability.

Figure 5 shows the normalized scores calculated as the log (sample signal/negative control signal) across all four biotherapeutics in each of the two samples obtained from 15 cynomolgus monkeys. This figure illustrates the difference in responses for each biotherapeutic spiked in the individual samples. Although background signals differed across animals and molecules, within monkey variability was independent of the molecule.

Determination of individual signal cut points depends primarily on accurate assessment of (1) within monkey variability and (2) individual mean sample OD signal. Table 2 summarizes the evaluation of the 4 biotherapeutics with differing structures but similar within monkey variability. The individual cut point signal method with any new mAb-derived biotherapeutic is based on the assumption that the generic cynomolgus monkey ADA assay will exhibit similar levels of within monkey variability with the new molecule.

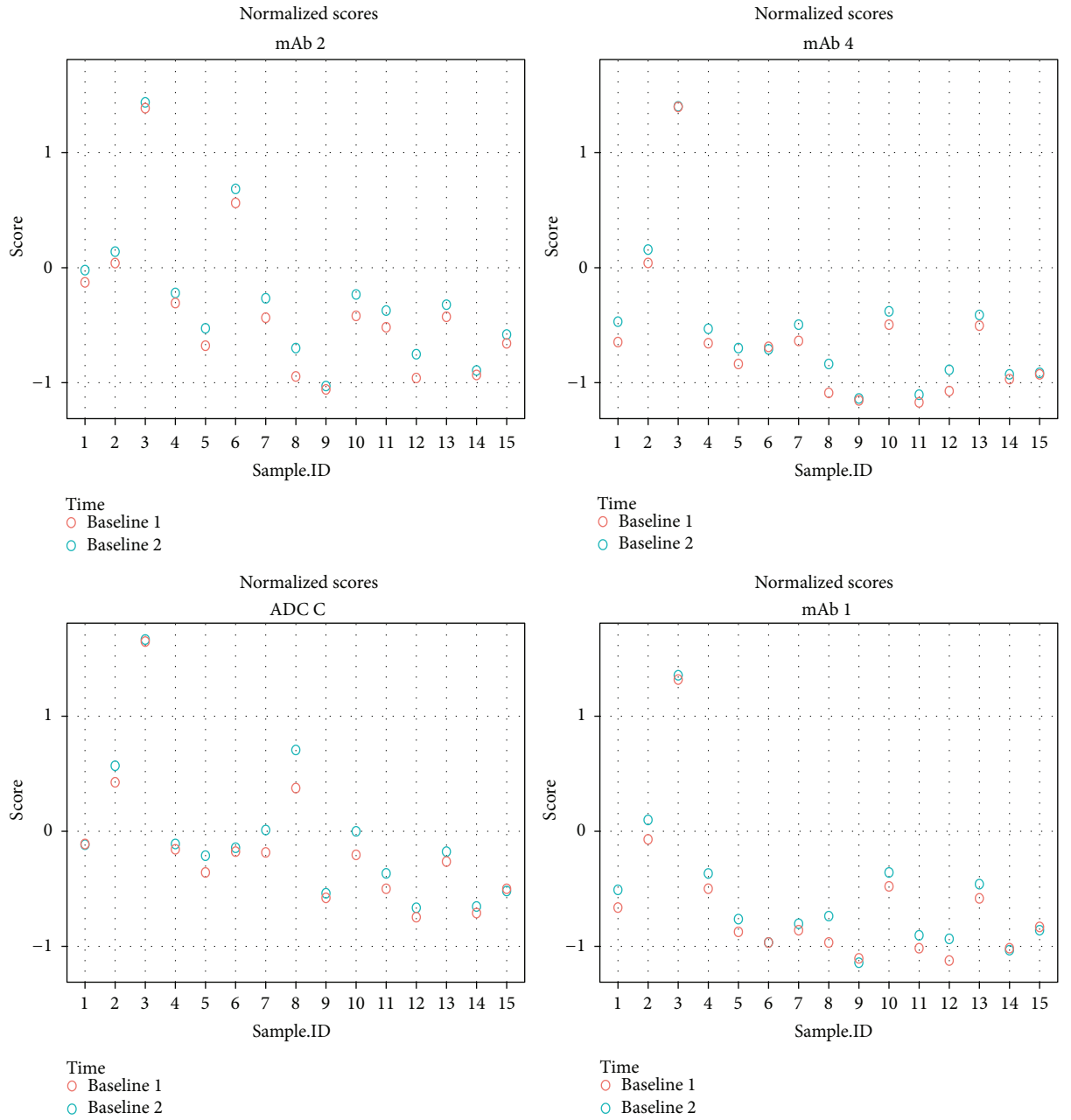

FIGURE 5: Normalized scores for background signal in samples from 15 cynomolgus monkeys across four biotherapeutics. Two samples taken 1 week apart from 15 cynomolgus monkeys were spiked at 5 μg/mL with ADC C, mAb 1, mAb 2, and mAb 4 and analyzed in the generic assay. This figure illustrates the difference in responses for each biotherapeutic spiked in the individual samples. Normalized scores were calculated for each sample as the log (sample signal/negative control signal) across all four molecules.

Estimates of individual signal cut points (CP_i) assume that we know the true underlying mean biotherapeutic naive sample absorbance signal. This is currently estimated using one or several observed sample absorbances prior to treatment by $\overline{x}_i$. The variability in this estimate is $SD/n^{0.5}$ where n is the number of observed sample absorbances used to estimate the mean. It is recommended to use several observations in estimating $\overline{x}_i$ in order to minimize this variability. Failure to do so can result in more false positives than expected from a given level of α. Table 3 summarizes the estimated CPF based on the false positive rate α (1%, 5%, and 10%). In the experiments presented here we have used a cut point factor targeting a false positive rate of 5% for consistency with our previous studies. However, lower positive rates are adequate based on the primary purpose of immunogenicity testing in nonclinical studies [21].

With this cut point generic approach, induced and enhanced responses are not differentiated because by definition all the baseline signals are considered negative. Additionally, for each animal the postbaseline samples have to be

Table 3: Estimation of cut point factor based on different false positive rates (α). Cut point factor (CPF) calculated at different false positive rates based on the within monkey variability observed across 4 biotherapeutics. This generic CPF can be used to establish the floating individual cut point signal according to each cynomolgus monkey baseline signal.

α	Cut point factor
0.10	1.12
0.05	1.16
0.01	1.24

α = false positive rate.

analyzed with the baseline samples to determine the cut point signal.

Although floating individual cut points are not commonly used for immunogenicity assays, this approach could be also valuable for molecule specific nonclinical and clinical assays. For example, screening assays with high baseline signal differences across individuals could result in high cut point factors when applying the widely used floating population cut point. As a consequence, the assay might not have acceptable sensitivity and drug tolerance. Using the floating individual cut point approach has the requirement of an additional sample collection, as two biotherapeutic naive samples are needed from each subject (ideally for both, assay validation and study samples) to assess individual signal variability.

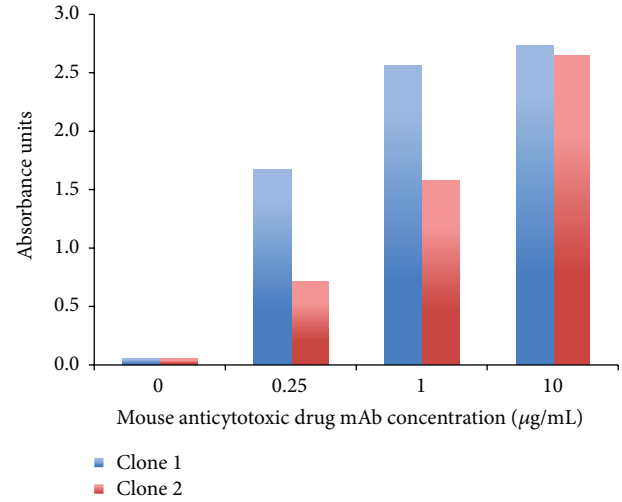

Figure 6: The generic assay detects ADAs against the linker-cytotoxic drug domain of an ADC. Two mouse anticytotoxic drug mAbs at various concentrations were spiked in pooled cynomolgus monkey serum with ADC C (5 μg/mL). The reactivity of the immune complexes was measured in a generic assay model: the ADA-ADC complexes were captured on a sheep anti-human IgG-coated plate and for detection an anti-mouse IgG-HRP antibody was used. Positive responses were obtained with the two mAb clones at all the concentrations tested. ADA = anti-drug antibody; mAb = monoclonal antibody.

3.5. A Generic Assay Format Can Detect ADAs against the Cytotoxic Drug Domain of an ADC. Immune responses to an ADC include ADAs against its different domains, such as the monoclonal antibody and the linker-cytotoxic drug. The generic assay format uses the biotherapeutic as the reagent to form complexes and it is expected that ADAs against different ADC domains will be detected. A proof of concept model assay was used to demonstrate this point. Since the affinity purified positive control used for the assay characterization binds only to the mAb domain of an ADC, it could not be used to demonstrate that ADAs against the linker-drug can be detected. A cynomolgus monkey derived anticytotoxic drug antibody was not available in our laboratory, but we had a mouse monoclonal anticytotoxic drug mAb. An assay model was needed as the mouse ADA control required changing the detection and capture reagents in the generic assay format. As a consequence, a sheep anti-human IgG was used as the capture reagent and an HRP-conjugated goat anti-mouse IgG was used for detection.

In this model assay, two mouse anticytotoxic drug mAbs (test ADA) at concentrations of 0.25, 1, and 10 μg/mL were spiked in pooled cynomolgus monkey serum with ADC C at 5 μg/mL. The ADA-ADC complexes were captured on a sheep anti-human IgG-coated plate. To detect the complexes, an anti-mouse IgG-HRP antibody was used. Figure 6 shows the positive response obtained with the two test ADA murine antibodies. These data demonstrate that ADAs against the cytotoxic drug domain of an ADC can be detected in this generic assay format.

3.6. Evaluation of Relative Sensitivity, Drug Tolerance, and Reproducibility. Immunogenicity assays should have sufficient sensitivity to detect ADAs in the presence of the biotherapeutic in circulation. It is well understood that assay sensitivity is relative to the ADA surrogate control used in the assay. It may not represent the true sensitivity of the assay in test samples. For nonclinical species, a relative sensitivity of 1000 ng/mL or better is recommended [11, 12]. Although there are no numeric recommendations regarding drug tolerance, our goal was for the assay to be able to detect ADAs in the presence of expected levels of biotherapeutic in the sample predicted from the PK.

The plug-and-play assay showed excellent relative sensitivity and drug tolerance suitable for the needs of the studies. Using the human IgG-cyno IgG fusion positive control, the assay sensitivity was 41 ng/mL. It is expected that sensitivity values with different biotherapeutics and ADA sources may vary. Sensitivity experiments were not performed with different biotherapeutics. However, the data from the experiment illustrated in Figure 2 was used to demonstrate that the assay sensitivity across biotherapeutics was better than 500 ng/mL of ADA (purified cynomolgus monkey anti-human IgG). A cut point signal was calculated for each biotherapeutic from the background signal with 5 μg/mL of biotherapeutic (baseline sample) and the CPF for a 5% false positive rate (1.16). Figure 7 shows that, with all eight biotherapeutics, the sample containing 500 ng/mL of ADA was positive with signals above their corresponding cut points. These data indicated that the plug-and-play assay

Table 4: Reproducibility of the plug-and-play assay. Assay control precision expressed as the coefficient of variation of the signal observed in 6 independent runs. The negative control is a pooled cynomolgus monkey serum. The positive control is a human IgG-cynomolgus monkey IgG fusion molecule. Interassay precision for signal values obtained with the two controls is summarised below. In addition, a ratio of the signals obtained with the positive and negative controls was calculated for assay performance evaluation.

Assay control	Number of runs	Mean measurement (units)	CV (%)
Negative control	6	0.169 (AU)	16
Positive control	6	0.302 (AU)	13
		1.80 (ratio)	11

Fusion positive control at 125 ng/mL.
AU = absorbance units; CV = coefficient of variation.
Ratio = positive control signal/negative control signal.

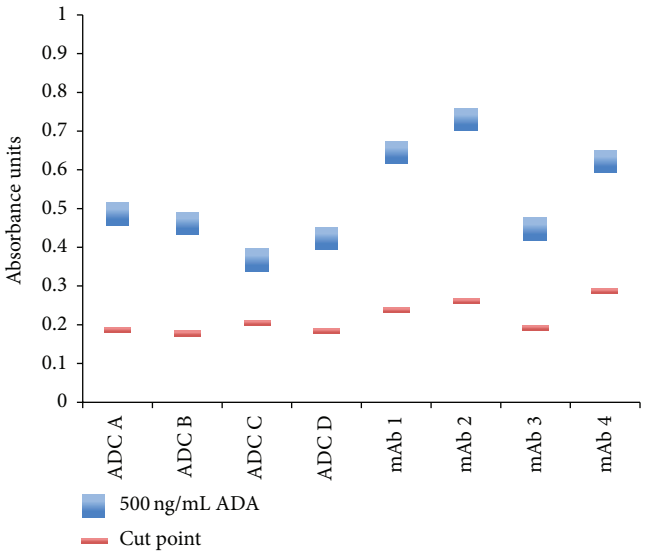

Figure 7: The plug-and-play assay showed adequate sensitivity for cynomolgus monkey studies across molecules. Pooled cynomolgus monkey serum was spiked with 5 μg/mL of eight biotherapeutics (baseline sample) or with the biotherapeutics (5 μg/mL) and 500 ng/mL of ADAs (purified cynomolgus monkey anti-human IgG). Cut points were calculated for each biotherapeutic from the signal at baseline and the generic cut point factor for a 5% false positive rate (CPF = 1.16). With all the biotherapeutics, the sample containing 500 ng/mL of ADA was positive with signals above their corresponding cut points. ADA = anti-drug antibody; ADC = antibody-drug conjugate; mAb = monoclonal antibody.

had adequate sensitivity across molecules to detect ADAs in cynomolgus monkey studies.

The drug tolerance was determined using purified cynomolgus monkey anti-human IgG positive control source spiked at 1 μg/mL in cynomolgus monkey pooled serum and varying concentration of one of the biotherapeutics (mAb 2) from 4 to 500 μg/mL. The assay was capable of detecting 1 μg/mL of the ADA positive control in the presence of up to 440 μg/mL of mAb 2.

The assay precision was evaluated by the interassay run for the signal of the negative control (unspiked pooled cynomolgus monkey serum) and the relative ADA values generated for the positive control (fusion human IgG-cyno IgG molecule). Table 4 shows that the assay is reproducible with the coefficient of variation values for the negative and positive controls below 16%.

3.7. Analysis of Samples from Studies in Cynomolgus Monkey and Comparison to Data from the Biotherapeutic-Specific ELISA. Samples from two studies in cynomolgus monkeys with ADCs A and B and their respective mAbs (A and B) were analyzed in the plug-and-play assay. The results were compared to the data obtained initially in the biotherapeutic-specific biotin-DIG ELISA. A 5% false positive rate CPF was used for the plug-and-play assay to resemble the approach followed to calculate the population cut point factor in the biotin-DIG ELISA. Immunogenicity incidence and the relative ADA levels in samples that screened positive were compared.

Table 5 summarizes the immunogenicity results obtained in the two studies with the two assays. Samples from Study 1 with both ADC A and mAb A were all negative at baseline in the biotin-DIG ELISA. In the plug-and-play assay, by definition, samples are always negative at baseline. At post-baseline time points, the plug-and-play assay detected ADAs in the two animals that had ADA positive results with the biotin-DIG ELISA. Moreover, the relative levels calculated by the ratio agreed with the titers determined in the specific biotin-DIG ELISA as Figure 8 illustrates. However, the plug-and-play assay detected more positive responses than the biotherapeutic-specific biotin-DIG ELISA. With ADC A, two more animals had postbaseline positive signals in the plug-and-play assay than in the biotin-DIG ELISA, although the signals were not very strong (Table 6). With mAb A, the plug-and-play assay detected positive responses at days 15 and 44 (ratios of 2.5 and 12.3, resp.) in one animal that was negative in the biotin-DIG ELISA. In addition, the plug-and-play assay detected ADAs earlier (at day 15) and with a stronger signal (at a ratio of 38) in the only animal dosed with mAb A that was also positive in the biotin-DIG ELISA.

In Study 2 with both ADC B and mAb B, all the baseline samples were negative in the specific biotin-DIG ELISA and all the animals had positive responses at day 43 after baseline. Likewise, in the plug-and-play assay, all the animals produced postbaseline positive signals (Table 5). Figure 8 shows general good agreement between the ADA titers determined in the specific biotin-DIG ELISA and the relative levels calculated by the ratio in the plug-and-play assay. There were two samples that deviated from the others. One postbaseline

Table 5: Summary of immunogenicity data for cynomolgus monkey Studies 1 and 2 with the biotin-DIG ELISA and the plug-and-play assay. Samples from cynomolgus monkey studies with two ADCs and their corresponding mAbs were analyzed in the plug-and-play assay. The number of animals that developed anti-drug antibodies after exposure to the above molecules and the immunogenicity incidence obtained with this assay were compared to those previously obtained in the biotherapeutic-specific biotin-DIG ELISA. In Study 1, the plug-and-play assay detected the same positive responses as the biotin-DIG ELISA plus three additional animals with ADA positive responses. In Study 2, all the monkeys had positive responses with the biotin-DIG ELISA and the plug-and-play assay.

Study	Molecule	Positive of total		Immunogenicity incidence	
		Biotin-DIG	Plug-and-play	Biotin-DIG	Plug-and-play
1	mAb A	1 of 4	2 of 4	25%	50%
1	ADC A	1 of 4	3 of 4	25%	75%
2	mAb B	3 of 3	3 of 3	100%	100%
2	ADC B	6 of 6	6 of 6	100%	100%

ADAs = anti-drug antibodies; ADC = antibody-drug conjugate; mAb = monoclonal antibody.

Table 6: Postbaseline ADA positive responses in cynomolgus monkey Study 1 as detected in the biotin-DIG ELISA and the plug-and-play assay. In Study 1, the biotin-DIG ELISA detected ADAs in two cynomolgus monkeys, one of them dosed with ADC A and the second one with mAb A. These two animals also had positive signals in the plug-and-play assay with good agreement between the ADA titer determined in the biotin-DIG ELISA and the relative ADA level by the ratios calculated in the plug-and-play assay (Figure 8). However, the plug-and-play assay detected ADA responses in animal 1 dosed with mAb A earlier (day 15) than the biotin-DIG ELISA. The plug-and-play assay identified positive responses in 3 additional monkeys, one dosed with mAb A and two dosed with ADC A.

Molecule	Animal	Time point	Biotin-DIG ELISA		Plug-and-play assay	
			Screening result (positive/negative)	AU or titer	Screening result (positive/negative)	AU or ratio
mAb A	1	Baseline	Negative	0.054 AU	Negative	0.067 AU
		D15	Negative	0.067 AU	Positive	Ratio of 37.9
		D44	Positive	Titer of 3.99	Positive	Ratio of 42.9
mAb A	2	Baseline	Negative	0.057 AU	Negative	0.196 AU
		D15	Negative	0.065 AU	Positive	Ratio of 2.5
		D44	Negative	0.061 AU	Positive	Ratio of 12.3
ADC A	3	Baseline	Negative	0.065 AU	Negative	0.117 AU
		D15	Negative	0.057 AU	Positive	Ratio of 1.5
		D44	Negative	0.052 AU	Positive	Ratio of 1.3
ADC A	4	Baseline	Negative	0.060 AU	Negative	0.149 AU
		D15	Negative	0.053 AU	Negative	0.119 AU
		D44	Positive	Titer of 1.79	Positive	Ratio of 18.0
ADC A	5	Baseline	Negative	0.065 AU	Negative	0.128 AU
		D15	Negative	0.052 AU	Negative	0.119 AU
		D44	Negative	0.061 AU	Positive	Ratio of 3.5

ADAs = anti-drug antibodies; ADC = antibody-drug conjugate; AU = absorbance units; D = days after dosing or after baseline; mAb = monoclonal antibody; R = ratio.
Ratio = (postbaseline AU/baseline AU).

sample from a monkey receiving mAb B had a low ADA titer in the Biotin-DIG ELISA but a high ADA ratio in the plug-and-play assay. Conversely, a monkey receiving ADC B produced a high ADA titer in the biotin-DIG ELISA and low ratio in the plug-and-play assay.

It is not uncommon to observe differences between immunogenicity data obtained from different ADA assays specially when using different formats, screening cut point, and statistical approaches for data analysis [22]. In our studies, the assay format, the approach to cut point determination, and the types of ADAs measured were all different. In the biotin-DIG ELISA, the ADAs need to be fully unbound from the biotherapeutic so they can actively react with the two biotherapeutic-conjugated reagents. Appropriate assay conditions (e.g., in-solution phase and long incubation time) favor binding of the ADAs in the sample to the conjugate reagents in the presence of the biotherapeutic in the sample. In the plug-and-play assay, only biotherapeutic-ADA complexes are detected with the ADAs bound to the biotherapeutic through one or two arms and the assay reagents recognizing the complexes. The biotin-DIG ELISA can detect ADAs with different isotypes while the plug-and-play assay can detect only ADAs of the IgG isotype. The biotin-DIG ELISA relies on biotherapeutic-conjugated reagents where the labels may potentially mask ADA epitopes and interfere with bridging of the reagents while the plug-and-play assay uses the unlabeled biotherapeutic as reagent. Antibody affinity can also play a role in the differences observed

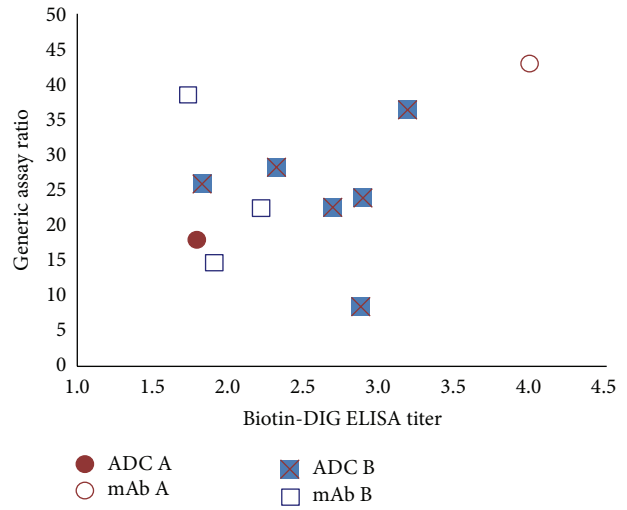

Figure 8: Good agreement between ADA titers by biotin-DIG ELISA and relative ADA ratios by the plug-and-play assay. The figure summarizes the relative levels of ADA Studies 1 (ADC A and mAb A) and 2 (ADC B and mAb B) whose samples were determined as positive by two ADA methods. The ratios calculated in the plug-and-play assay were plotted versus the titers determined in the biotherapeutic-specific biotin-DIG ELISAs. Overall, good agreement between the two procedures was observed.

between assays. As a consequence, the assays may vary in their sensitivity to detect ADAs and drug tolerance in the study samples, explaining the differences observed in the Study 1 screening data between the two assays.

4. Conclusions

Assessment of immunogenicity in banked samples from early nonclinical studies may be triggered by PK and safety data at short notice. The availability of a plug-and-play assay without the need for assay optimization and molecule specific cut points is highly valuable during drug discovery where immunogenicity may not be assessed on a routine basis.

We developed a plug-and-play assay, where the assay format and data analysis are generic. This assay is suitable for ADCs as well as other antibody-derived biotherapeutics. In addition, screening of samples and, if positive, ADA relative level determination can be performed in the same run. Sample testing from two studies in cynomolgus monkey showed that the plug-and-play assay was able to detect equal or greater number of positive samples than the biotherapeutic-specific biotin-DIG ELISA. Therefore, from this pilot study one could assume that animals with a positive ADA response would not be missed by moving to the plug-and-play assay. This approach is valuable to streamline immunogenicity assessments for molecules in the discovery phase of drug development.

Competing Interests

The authors are employees of Genentech, Inc., and own stock in F. Hoffmann-La Roche Ltd.

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References

[1] FDA Guidance for Industry, *Immunogenicity Assessment for Therapeutic Protein Products*, 2014.

[2] EMA, *Guideline on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins*, European Medicines Agency, London, UK, 2007, (EMEA/CHMP/BMWP/14327/2006).

[3] EMA, *Guideline on Immunogenicity Assessment of Monoclonal Antibodies Intended for In Vivo Clinical Use*, European Medicines Agency, London, UK, 2012.

[4] European Medicines Agency (EMA), *Guideline on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins*, Draft (EMEA/CHMP/BMWP/14327/2006 Rev1), European Medicines Agency (EMA), London, UK, 2015.

[5] M. Carrasco-Triguero, J.-H. Yi, R. Dere et al., "Immunogenicity assays for antibody-drug conjugates: case study with adotrastuzumab emtansine," *Bioanalysis*, vol. 5, no. 9, pp. 1007–1023, 2013.

[6] B. Gorovits, E. Wakshull, R. Pillutla, Y. Xu, M. S. Manning, and J. Goyal, "Recommendations for the characterization of immunogenicity response to multiple domain biotherapeutics," *Journal of Immunological Methods*, vol. 408, pp. 1–12, 2014.

[7] M. B. Hock, K. E. Thudium, M. Carrasco-Triguero, and N. F. Schwabe, "Immunogenicity of antibody drug conjugates: bioanalytical methods and monitoring strategy for a novel therapeutic modality," *The AAPS Journal*, vol. 17, no. 1, pp. 35–43, 2015.

[8] S. J. Swanson and J. Bussiere, "Immunogenicity assessment in non-clinical studies," *Current Opinion in Microbiology*, vol. 15, no. 3, pp. 337–347, 2012.

[9] ICH Harmonised Tripartite Guideline, "Preclinical safety evaluation of biotechnology-derived pharmaceuticals S6(R1)," in *Proceedings of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*, June 2011.

[10] H. Jiang, W. Xu, C. A. Titsch et al., "Innovative use of LC-MS/MS for simultaneous quantitation of neutralizing antibody, residual drug, and human immunoglobulin G in immunogenicity assay development," *Analytical Chemistry*, vol. 86, no. 5, pp. 2673–2680, 2014.

[11] A. R. Mire-Sluis, Y. C. Barrett, V. Devanarayan et al., "Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology

products," *Journal of Immunological Methods*, vol. 289, no. 1-2, pp. 1–16, 2004.

[12] G. Shankar, V. Devanarayan, L. Amaravadi et al., "Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 48, no. 5, pp. 1267–1281, 2008.

[13] K. Stubenrauch, U. Wessels, U. Essig, R. Vogel, and J. Schleypen, "Evaluation of a generic immunoassay with drug tolerance to detect immune complexes in serum samples from cynomolgus monkeys after administration of human antibodies," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 52, no. 2, pp. 249–254, 2010.

[14] A. C. Bautista, H. Salimi-Moosavi, and V. Jawa, "Universal immunoassay applied during early development of large molecules to understand impact of immunogenicity on biotherapeutic exposure," *The AAPS Journal*, vol. 14, no. 4, pp. 843–849, 2012.

[15] H. Lenz and K. G. Stubenrauch, "Detection of a therapeutic antibody in an experimental animal," European Patent 1853921B1, Hoffman-La Roche AG, Basel, Switzerland, 2011.

[16] H. Lenz and K. G. Stubenrauch, "Detection of a therapeutic antibody in an experimental animal," Unites States Patent 7,955.806 B2, Hoffman-La Roche, Basel, Switzerland, 2011.

[17] K. Stubenrauch, U. Wessels, and H. Lenz, "Evaluation of an immunoassay for human-specific quantitation of therapeutic antibodies in serum samples from non-human primates," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 49, no. 4, pp. 1003–1008, 2009.

[18] K. Peng, K. Siradze, V. Quarmby, and S. K. Fischer, "Clinical immunogenicity specificity assessments: a platform evaluation," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 54, no. 3, pp. 629–635, 2011.

[19] A. C. Bautista, L. Zhou, and V. Jawa, "Universal immunogenicity validation and assessment during early biotherapeutic development to support a green laboratory," *Bioanalysis*, vol. 5, no. 20, pp. 2495–2507, 2013.

[20] X. Wang, V. Quarmby, C. Ng et al., "Generation and characterization of a unique reagent that recognizes a panel of recombinant human monoclonal antibody therapeutics in the presence of endogenous human IgG," *mAbs*, vol. 5, no. 4, pp. 540–554, 2013.

[21] C. Kloks, C. Berger, P. Cortez et al., "A fit-for-purpose strategy for the risk-based immunogenicity testing of biotherapeutics: a European industry perspective," *Journal of Immunological Methods*, vol. 417, pp. 1–9, 2015.

[22] J. Zhang, L. Zhang, and H. Yang, "Sample size consideration for immunoassay screening cut-point determination," *Journal of Biopharmaceutical Statistics*, vol. 24, no. 3, pp. 535–545, 2014.

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Research Article

Development of Immunocapture-LC/MS Assay for Simultaneous ADA Isotyping and Semiquantitation

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Therapeutic proteins and peptides have potential to elicit immune responses resulting in anti-drug antibodies that can pose problems for both patient safety and product efficacy. During drug development immunogenicity is usually examined by risk-based approach along with specific strategies for developing "fit-for-purpose" bioanalytical approaches. Enzyme-linked immunosorbent assays and electrochemiluminescence immunoassays are the most widely used platform for ADA detection due to their high sensitivity and throughput. During the past decade, LC/MS has emerged as a promising technology for quantitation of biotherapeutics and protein biomarkers in biological matrices, mainly owing to its high specificity, selectivity, multiplexing, and wide dynamic range. In fully taking these advantages, we describe here an immunocapture-LC/MS methodology for simultaneous isotyping and semiquantitation of ADA in human plasma. Briefly, ADA and/or drug-ADA complex is captured by biotinylated drug or anti-drug Ab, immobilized on streptavidin magnetic beads, and separated from human plasma by a magnet. ADA is then released from the beads and subjected to trypsin digestion followed by LC/MS detection of specific universal peptides for each ADA isotype. The LC/MS data are analyzed using cut-point and calibration curve. The proof-of-concept of this methodology is demonstrated by detecting preexisting ADA in human plasma.

1. Introduction

Therapeutic proteins and peptides have potential to elicit immune responses [1, 2], resulting in anti-drug antibodies (ADAs) that can pose problems for both patient safety and product efficacy. Clinical consequences can range from relatively mild to serious adverse events [3–5], such as anaphylaxis, cytokine release syndrome, and cross-reactive neutralization of endogenous proteins mediating critical functions. ADA can affect drug efficacy and biodistribution and drug clearance, and complicate the interpretations of toxicity and pharmacokinetic (PK) and pharmacodynamic (PD) data [6–8]. During drug development immunogenicity is examined by risk-based approach along with specific strategies for developing "fit-for-purpose" bioanalytical approaches [9].

Characterization and analysis of ADA are a vital element of immunogenicity assessment. Enzyme-linked immunosorbent assays (ELISA) and electrochemiluminescence (ECL) immunoassays [10] are the most widely used platform for ADA detection due to their high sensitivity and throughput. Lower affinity ADA can be detected by surface plasmon resonance, biolayer interferometry, or other platforms [11]. Typically, detection of ADA is followed by assessments of the magnitude (titer) of the ADA response and the in vitro neutralizing ability of ADA, especially in late-stage clinical studies. Additional characterization of ADA such as immunoglobulin subclass or isotype determinations, domain-mapping, relative binding affinity, cross-reactivity with endogenous proteins, or complement activating ability of the ADA may be driven by product-specific, indication-specific, or risk assessment-based objectives [9, 12, 13].

Recommendations for ADA assay development, method validation, and testing strategies have been published by the Ligand-Binding Assay Bioanalytical Focus Group (LBABFG) of American Association of Pharmaceutical Scientists (AAPS) [10, 12, 14–16]. Additionally, scientific publications on risk-based approaches to immunogenicity assessments [9, 12, 17–19] and regulatory documents from the US Food and Drug Administration (FDA) and the European Medicine Agency (EMA) are also available [20–23]. Together,

these documents provide ample guidance for the application of appropriate ADA detection methods in clinical studies.

Since the late 1990s, liquid chromatography coupled to mass spectrometry (LC/MS) has been a dominant tool for sensitive, accurate, and rapid analysis of small-molecule drugs, metabolites, and biomarkers [24]. In recent years, LC/MS has emerged as a promising platform for quantitation of biotherapeutics and protein biomarkers in biological matrices [25–27]. The vast majority of LC/MS-based protein quantifications are performed at peptide levels, mainly due to consideration of assay sensitivity [28]. A typical procedure for LC/MS-based quantification includes enzyme digestion and quantification of the target proteins based on selected signature peptides derived from the target [29, 30].

Recently, Furlong et al. developed a universal peptide method to quantitate human antibody Fc region-containing therapeutic protein candidates in nonclinical species [31]. Surrogate tryptic peptide VVSVLTVLHQDWLNGK for IgG1, IgG3, and IgG4 and VVSVLTVVHQDWLNGK for IgG2 were identified in the Fc region of human immunoglobulins (IgG), respectively. The method was shown to be capable of supporting bioanalysis of a diversity of human Fc region-containing therapeutic protein candidates in plasma samples of all commonly used animal species, thus eliminating the need to develop unique peptide methods for each individual therapeutic candidate. With a similar approach, Dongen et al. achieved a higher sensitivity of 4 ng/mL for a monoclonal Ab drug, infliximab, using a different universal peptide (SLSLSPGK) from the C-terminal of Fc [32]. Li et al. used a stable isotope labeled common mAb as internal standard for quantitation of therapeutic mAb in preclinical samples [33]. Not only was the common whole Ab internal standard able to correct for variations from the beginning of sample processing to ionization in the mass spectrometer but also it allowed rapid method development with flexible choice of a suitable surrogate peptide for new applications, such as different species or different mAb. Stable isotope labeled human monoclonal Ab incorporating [$^{13}C_6$,$^{15}N_4$]-arginine and [$^{13}C_6$,$^{15}N_2$]-lysine is now commercially available and can be used for the universal peptide methods.

LC/MS has also been reported to assess ADA in the presence of excess protein therapeutic in support of clinical programs addressing the safety and tolerability of human growth hormone analogues [34]. This methodology overcame drug tolerance issues, which are often associated with traditional ADA detection [35–37], by completely saturating available ADA binding sites with the addition of excess therapeutic. Drug-ADA complexes were then isolated using protein G immobilized on magnetic beads, followed by elution and digestion. Resultant peptide from the target therapeutic proteins was quantified by LC coupled with matrix-assisted laser desorption MS and the results were correlated to the binding capacity of total ADA.

In another application, LC/MS was used to evaluate neutralizing Ab (NAb) assay by simultaneously quantitating residual mAb-drug, endogenous IgG, and NAb-positive control in BEAD eluates [38]. In the study, the low levels of the residual drug and human IgG in the BEAD eluates indicate that the BEAD efficiently removed the high concentration drug and serum components from the serum samples. Meanwhile, the NAb-positive control recovery (~42%) in the BEAD provided an acceptable detection limit for the cell-based assay. This novel application of LC/MS to immunogenicity assay development demonstrates the advantages of LC/MS in selectivity and multiplexing, which provides direct and fast measurements of multiple components.

We describe here an immunocapture-LC/MS-based approach for simultaneous ADA isotyping and semiquantitation in human plasma. Biotinylated drug or anti-drug Ab is used to capture ADAs or drug-ADA complexes in plasma, respectively. The resulting ADA-drug or ADA-drug-Ab complexes are then immobilized on streptavidin magnetic beads and separated from matrix by a magnet. After washing, ADA is released from the beads and subjected to trypsin digestion followed by LC/MS detection of specific universal peptides for each ADA isotype.

2. Materials and Methods

Protein Z (containing no human Fc) was a proprietary experimental biotherapeutic of Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT) and produced in-house. The mouse anti-Protein Z monoclonal Ab (mAb) was supplied in-house. Human IgG1, IgG2, IgG3, IgG4, and IgM as well as bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO), human IgE was from MP Biomedicals (Solon, OH), human IgA1 was from Abcam (Cambridge, MA), and human IgA2 was from EMD Millipore (Billerica, MA). Internal standard peptides with stable labeled C-terminal [$^{13}C_6$,$^{15}N_4$]Arg or [$^{13}C_6$,$^{15}N_2$]Lys were synthesized at GenScript (Piscataway, NJ). Streptavidin magnetic beads (1 μm dia.), TPCK trypsin, and EZ-Link Sulfo-NHS-LC Biotinylation kits were obtained from Thermo Scientific (Rockford, IL). RapiGest SF was purchased from Waters (Milford, MA). All other lab chemicals, reagents, and buffer solutions were obtained from Sigma Aldrich, Thermo Scientific or Invitrogen (Grand Island, NY). Human preexisting ADA (PEA) positive and negative plasma were obtained in-house.

2.1. Biotinylation. Biotinylation of Protein Z and the mouse anti-Protein Z mAb was performed using an EZ-Link Sulfo-NHS-LC Biotinylation kit. A 1 mg/mL solution of the drug or mAb was combined with a 10-fold molar excess of biotin and allowed to react at room temperature for 60 minutes. Excess biotin was removed using desalting columns provided in the kit. A HABA assay was used to determine the amount of biotin incorporation in the sample after desalting. Typical biotin incorporation was approximately 2 biotins per drug and 4–7 biotins per mouse mAb. The biotinylated drug and biotinylated mouse mAb solutions were diluted to 0.1 mg/mL in water and stored at −80°C prior to use.

2.2. Immunocapture with Biotinylated Drug. Streptavidin magnetic beads were prepared freshly for each assay batch. The magnetic beads (10 mg/mL) were transferred to a polypropylene tube and placed on a magnetic stand to remove supernatant and collect the beads. The beads were then

washed with 10x volume of Tris buffered saline containing 0.1% Tween-20 (TBS-T) and resuspended in 2x volume of TBS-T to yield a final working bead concentration of 5 mg/mL.

An aliquot of 95 μL of human plasma sample and 5 μL of 5.85 M acetic acid were pipetted into a 96 deep-well polypropylene plate. The plate was incubated with gentle mixing for 1 hr at room temperature. After adding 75 μL aliquot of 0.1 mg/mL biotinylated Protein Z and 40 μL of Trizma base (1.5 M Tris, pH 10) to each sample, the plate was incubated at room temperature for 1.5 hr. A 40 μL aliquot of freshly prepared 5 mg/mL magnetic beads and 475 μL of TBS-T binding buffer were added to each sample and the plate was gently mixed for 1 hr at room temperature. The beads were then separated, washed three times with 300 μL of TBS-T and once with 300 μL of water, and eluted with 150 μL of 0.1 M glycine (pH 2.0) on a Kingfisher Flex bead handler (Thermo Scientific, San Jose, CA). The eluent was immediately neutralized with 45 μL of 1 M Tris-HCl (pH 8.0) followed by the addition of 10 μL of 0.1% BSA.

2.3. Immunocapture with Mouse mAb. An aliquot of 144 μL of human plasma sample and 6 μL of 5 mg/mL Protein Z aqueous solution were pipetted into a 96-deep-well polypropylene plate. The plate was incubated at 37°C with gentle mixing for 1 hr and then stored at −80°C overnight. A 100 μL aliquot of 0.1 mg/mL biotinylated mouse mAb was added to each sample and the plate was then incubated at room temperature for 2 hrs. A 100 μL aliquot of freshly prepared 5 mg/mL magnetic beads and 475 μL of TBS-T binding buffer were added to each sample and the plate was gently mixed for 1 hr at room temperature. The beads were separated, washed three times with 300 μL of TBS-T and once with 300 μL of water, and then eluted with 150 μL of 0.1 M glycine (pH 2.0) on a Kingfisher Flex bead handler. The eluent was immediately neutralized with 45 μL of 1 M Tris-HCl (pH 8.0) followed by addition of 10 μL of 0.1% BSA.

2.4. Matrix Calibration Standards. Commercial stock solutions of the different immunoglobulins (Igs) ranged from 1 to 4.18 mg/mL. A series of 50–10,000 ng/mL spiking calibration standards were prepared in 0.1% BSA using the stock solutions and stored at −80°C prior to use. Pooled human blank plasma was processed using either immunocapture procedure. Matrix calibration standards were prepared by adding 10 μL of the spiking calibration standards to the magnetic bead eluent of the pooled human blank plasma.

2.5. Trypsin Digestion. To the immunocapture eluent, matrix calibration standards, or neat Ig PBS solution, 5 μL of 0.1% RapiGest in 100 mM ammonium bicarbonate was added and the plate was gently mixed for 5 minutes. Five μL of a stable labeled internal standard solution of the universal peptides (0.2 μg/mL) and 5 μL of 50 mM TCEP in 100 mM ammonium bicarbonate were added and followed by incubation at room temperature for 20 min. After adding 5 μL of 50 mM iodoacetamide in 100 mM ammonium bicarbonate, the plate was gently shaken for 20 min while protected from light. A 5 μL aliquot of solution containing trypsin (0.2 mg/mL) and calcium chloride (0.2 M), prepared immediately before use, was added to each sample and the plate was incubated at 37°C overnight with gentle mixing. Digestion was quenched by adding 5 μL of 20% TFA. The samples were mixed for 40 min at 37°C and then centrifuged at 4400 rpm for 10 min prior to LC/MS analysis.

2.6. LC/MS Analysis. Eksigent Ekspert MicroLC 200 coupled with AB Sciex 6500 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) was used. Chromatographic separation was performed using ACQUITY UPLC Peptide BEH C18 column (1 mm × 50 mm, 1.7 μm, 300 Å) operated at 60°C. Mobile phases consisted of (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile running at a flow rate of 60 μL/min. For information-dependent acquisition (IDA), the LC gradient was 5% to 50% B over 18 minutes. For ADA isotyping and semiquantitation, the LC gradient was 12% to 17% B over 2.8 minutes and then to 47% B over 6.2 minutes.

The mass spectrometer was operated in in positive electrospray ionization mode. Key instrument parameters were as follows: +5000 V electrospray voltage, 65 nebulizer gas units, 30 axillary gas units, 375°C ion source temperature, 10 collision gas units, and unit resolution on both Q1 and Q3. For identifying unique peptides for each ADA isotype with IDA, up to 4 multiple-reaction-monitoring (MRM) pairs were used for screening followed by enhanced product ion scan. For ADA isotyping and semiquantitation, 13 MRM pairs of universal peptides were used along their respective internal standards.

3. Results and Discussion

Despite its many advantages and potential, the use of LC/MS for protein quantitation is not as straightforward as for small-molecules and oftentimes demands comprehensive method development. Plasma and serum are very complex matrices that contain several hundreds of thousands of proteins and protein isoforms in a wide concentration range [39]. Upon digestion these are all cleaved into multiple peptides, from which just one or a few have to be quantified. When no protein or peptide purification is employed, LC/MS sensitivity is significantly compromised due to matrix interference from the peptide-rich digest. For high sensitivity LC/MS applications, immunopurification is the most effective way to improve assay sensitivity and robustness. Immunopurification can be done either at the protein-level prior to digestion [40, 41] or at the peptide level after digestion [42]. Immunopurification of peptides requires anti-peptide Ab for each peptide of interest. Unlike proteins, small peptides are usually less or even not immunogenic, which presents significant challenges for anti-peptide Ab production. Moreover, ADA and/or drug-ADA complex has to be pulled down prior to digestion and peptide pull-down. In consideration of these factors, we employed protein-level immunopurification for sample preparation.

Antibodies are secreted by plasma cells and come in different isotypes with genetic variations or differences in the

constant regions of the heavy and light chains. In humans, there are five heavy chain isotypes (α: IgA; δ: IgD; γ: IgG; ε: IgE; and μ: IgM) and two light chain isotypes (κ and λ). In addition, IgG has 4 subclasses (IgG1, IgG2, IgG3, and IgG4) and IgA has 2 subclasses (IgA1 and IgA2). Relative Ab abundance (% total Igs) in human varies significantly among isotypes/subclasses: IgG1 (65%), IgG2 (25%), IgG3 (5%), IgG4 (5%), IgA1, IgA2 (13% IgA1 + IgA2), IgE (<0.003%), IgM (8%), and IgD (<1%). Abs can present as soluble and/or membrane-bound on the surface of B cells and only the soluble Abs can be found in plasma or serum [43]. All isotypes can be found in normal serum. Among them, IgG1–IgG4 are the most abundant antibody isotypes found in the circulation and provide the majority of antibody-based immunity against invading pathogens [44]. Although IgE is the least abundant isotype, it plays an essential role in type I hypersensitivity and allergic conditions [45, 46], such as anaphylactic reactions to certain drugs. IgA is an antibody that plays a critical role in mucosal immunity and is found in small amounts in blood. IgD makes up about 1% of proteins in the plasma membranes of mature B-lymphocytes but only represents about 0.25% of Igs in serum and is thus excluded in the immunocapture-LC/MS assay.

3.1. Selection of Unique Peptide. ADA isotyping and semiquantitation were based on the surrogate peptides of ADAs instead of whole molecules, mainly in consideration of sensitivity [28–30]. The semiquantitative measurement relied on the existence of a stoichiometric (quantitative) relationship between ADA and its surrogate peptide. By far the most critical element of this approach was to identify proper peptide(s) that were unique to each antibody isotype/subclass. Three main factors were considered in selecting the unique peptides. First of all, the surrogate peptide(s) to each isotype/subclass must come from the constant region. Although ADAs of the same isotype come in many different forms in terms of amino acid sequence in their variable regions, they all have the same constant region. Secondly, the surrogate peptides should be unique to each ADA isotype/subclass and should not be formed in any other isotypes. Thirdly, the surrogate peptides should not contain the amino acids of ADA allotype polymorphic residues [47]. Peptides that met the three criteria represented a certain ADA isotype regardless of differences in ADA variable regions, thus allowing ADA isotyping and semiquantitation by LC/MS.

Among the Ig isotypes, only IgG1–IgG3 and IgA2 have different allotypes [47]. At present, 26 human allotypes are known [47, 48]: 6 for IgG1 (G1m1, G1m2, G1m3, G1m17, G1m27, and G1m28), 1 for IgG2 (G2m23), 13 for IgG3 (G3m5, G3m6, G3m10, G3m11, G3m13, G3m14, G3m15, G3m16, G3m21, G3m24, G3m26, G3m27, and G3m28), and 3 for IgA2 (A2m1, A2m2, and A2m3). In addition, 3 allotypes were found for κ light chain (Km1, Km2, and Km3) [48]. Except for G1m3 and G1m17 located on the CH1, all heavy chain allotypes are localized on the Fc region, either on CH2 or on CH3. IgG1 heavy gamma chain allotypes differ in amino acid (AA) residues at 214, 356, 358, and 431. The heavy chains of G2m23 allotype differ in AA residues 214 and 282. IgG3 has the most allotypes, and they differ at AA residues at 291, 292, 379, 384, 397, 409, 419, 422, 435, and 436. For IgA2, A2m1, A2m2, and A2m3 differ at AA residues at 115 and 124. The unique surrogate peptide(s) for each isotype/subclass should be present in all of its allotypes. Since many isotypes share the same light chains (κ and λ), light chains were excluded from the peptide selection.

Additional criteria that are commonly used in selecting a surrogate peptide include the following [49]: (a) avoid peptides containing methionine which are prone to oxidation; (b) avoid peptides containing asparagine-glycine or aspartic acid-glycine, which are prone to deamidation; (c) avoid peptides containing N-terminal glutamine or glutamic acid, which are prone to N-terminal cyclization; (d) avoid peptide sequences containing arginine-arginine (RR) and lysine-lysine (KK), which yield inconsistent tryptic digestion; (e) keep away from peptide sequences containing arginine-proline (RP) and lysine-proline (KP) which are difficult to break down by digestion; (f) keep peptide length between 5 and 15 amino acids to minimize the number of ionization charge states, achieve efficient MS/MS fragmentation and high sensitivity, and obtain desirable chromatographic retention; and (g) avoid peptide sequences containing glycosylation sites.

To identify candidates for unique peptides, 15 μg/mL of individual Ig PBS buffer solution was digested and the resulting digest was assayed by LC/MS using IDA. The IDA setup consisted of MRM survey scan for the formation of tryptic peptides based on in silico prediction, followed by enhanced product ion scans to confirm peptide identity. Only peptides that met all the criteria set forth as discussed above were included in the survey scan. Peptide separation was achieved with a 20 min ultrahigh performance liquid chromatography (UPLC) gradient program. The top 1–3 most sensitive peptides for each ADA isotype/subclass from the neat solution digestion were selected as unique peptide candidates and carried on for further method development. Of these peptides, either doubly or triply charged parent ions were the most abundant. Upon collision activation in MS/MS, the multiple charged parent ions fragmented into single charged b-ions and y-ions and the most sensitive y-ions were selected.

After suitable surrogate peptide candidates were identified, immunocapture was performed, and sensitivity and matrix interference were assessed for each MRM pair. MRMs with the best sensitivity, specificity, and selectivity were selected. Two different immunocapture approaches were used for immunopurification. One was to capture ADAs with biotinylated Protein Z, while the other was to capture drug-ADA complexes with a biotinylated mouse mAb. Each has its own advantages and shortfalls and is discussed below in detail. The workflow of the immunocapture-LC/MS assay is depictured in Figure 1.

3.2. Drug as Capture Reagent. Using drug as capture reagent is straightforward, similar to traditional drug-bridging ECL assays [50]. After spiking to human plasma samples, biotinylated Protein Z bound to ADA to form (biotinylated)drug-ADA complexes. Upon adding streptavidin magnetic beads, the (biotinylated)drug-ADA complexes were immobilized on

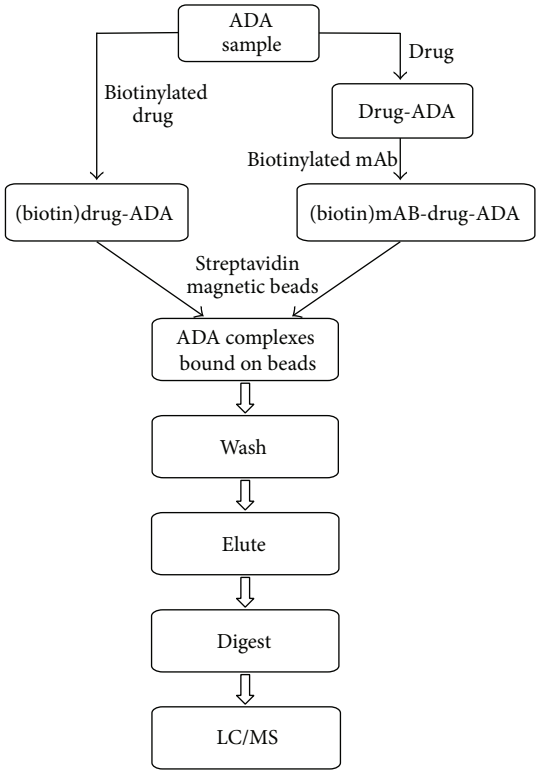

Figure 1: Immunocapture-LC/MS workflow chart.

the beads and then separated from matrix using a magnet. After washing, ADAs were eluted from the beads and subjected to trypsin digestion followed by LC/MS analysis. The basis of this approach was that biotinylated Protein Z was able to capture ADAs of different isotypes/subclasses present in the samples and the ADAs can be detected by LC/MS as long as the (biotinylated)drug-ADA complexes survived the washing steps. No special reagents were required.

The primary goal of the immunocapture was to isolate ADA from the plasma matrix. The efficiency of the immunopurification should be assessed using human ADA positive controls. Because there were no human ADA standards available, commercial human Igs were used as surrogates. Although different in their variable regions, the surrogate human Igs have the same constant regions as human ADAs and thus produce the unique peptides of human ADA isotype/subclass upon trypsin digestion which can then be detected and quantitated by LC/MS. Unlike human ADA, however, the surrogate Igs would not bind to biotinylated Protein Z to form (biotinylated)drug-Ig complexes. Therefore, instead of directly spiked into blank human plasma, the surrogate Igs were spiked into the magnetic beads eluent of blank human plasma after the immunocapture steps. The spiked and unspiked magnetic bead eluent were then digested and assayed by LC/MS. Only the MRM pairs that had the best sensitivity with minimal matrix interferences were chosen for immunocapture-LC/MS assay. Table 1 lists the final MRM pairs of each unique peptide. The MRM pairs were all y-ions with m/z greater than respective parent m/z. This was not surprising as the y-ions contained a basic amino acid (either arginine or lysine) at the N-terminal and thus showed better response in positive MS than b-ions. In addition, interference was reduced significantly when m/z values of fragment ions were greater than the doubly or triply charged parent peptide m/z values.

Table 1: List of unique peptides and MRMs for ADA isotopes/subclasses used in the immunocapture-LC/MS assay.

Isotype/subclass	Unique peptide sequence	MRM pairs
	Quantitation peptide	
IgG1	GPSVFPLAPSSK	593.83 → 699.40
IgG2	GLPAPIEK	412.75 → 654.38
IgG3	WYVDGVEVHNAK	708.85 → 698.48
IgG4	GLPSSIEK	415.73 → 660.36
IgE	AEWEQK	395.69 → 590.29
IgM	GQPLSPEK	428.23 → 670.38
IgA1, IgA2	YLTWASR	448.73 → 620.32
	Confirmation peptide	
IgG1, IgG3	ALPAPIEK	419.76 → 654.38
IgG1, IgG3, IgG4	VVSVLTVLHQDWLNGK	603.34 → 1110.57
IgE	LEVTR	309.18 → 375.24
IgM	VSVFVPPR	450.77 → 615.36
IgA1, IgA2	VAAEDWK	409.71 → 648.30
IgA1	TFTC[CAM]TAAYPESK	688.31 → 765.38

The final MRM pairs were grouped into two types: quantitation and confirmation. The quantitation MRMs were the most sensitive and used for isotyping and quantitation. The confirmation MRMs were less sensitive and results from the confirmation peptides are expected to be similar to those from quantitation peptides. In case there is a large discrepancy between the quantitation and confirmation peptide results, investigation on the assay may be needed. The AA sequence length was 6–12 for the quantitation peptides and 5–16 for the confirmation peptide. The quantitation peptides for IgG1, IgG2, IgG3, and IgG4 were GPSVFPLAPSSK, GLPAPIEK, WYVDGVEVHNAK, and GLPSSIEK, respectively. In addition, peptides ALPAPIEK and VVSVLTVLHQDWLNGK were also sensitive and found in IgG1/IgG3 and IgG1/IgG3/IgG4, respectively. These 2 peptides were not unique to any one single Ig isotype/subclass and were included as confirmation peptides. Likewise, quantitation peptides AEWEQK and GQPLSPEK and confirmation peptides LEVTR and VSVFVPPR were identified for IgE and IgM, respectively.

Both IgA1 and IgA2 shared the same peptide, YLTWASR, which was much more sensitive than any other unique peptides and thus used for quantitating the total of IgA1 and IgA2. In addition, peptide TFTC[CAM]TAAYPESK was unique to IgA1. However, TFTC[CAM]TAAYPESK was much less sensitive (2500 ng/mL limit of detection human plasma after immunocapture) and thus not very useful for low level ADA detection. For both IgA1 and IgA2, VAAEDWK was also used as a confirmation peptide.

After the final MRMs were selected, LC was optimized and total runtime was shortened from 20 min to 8 min. All

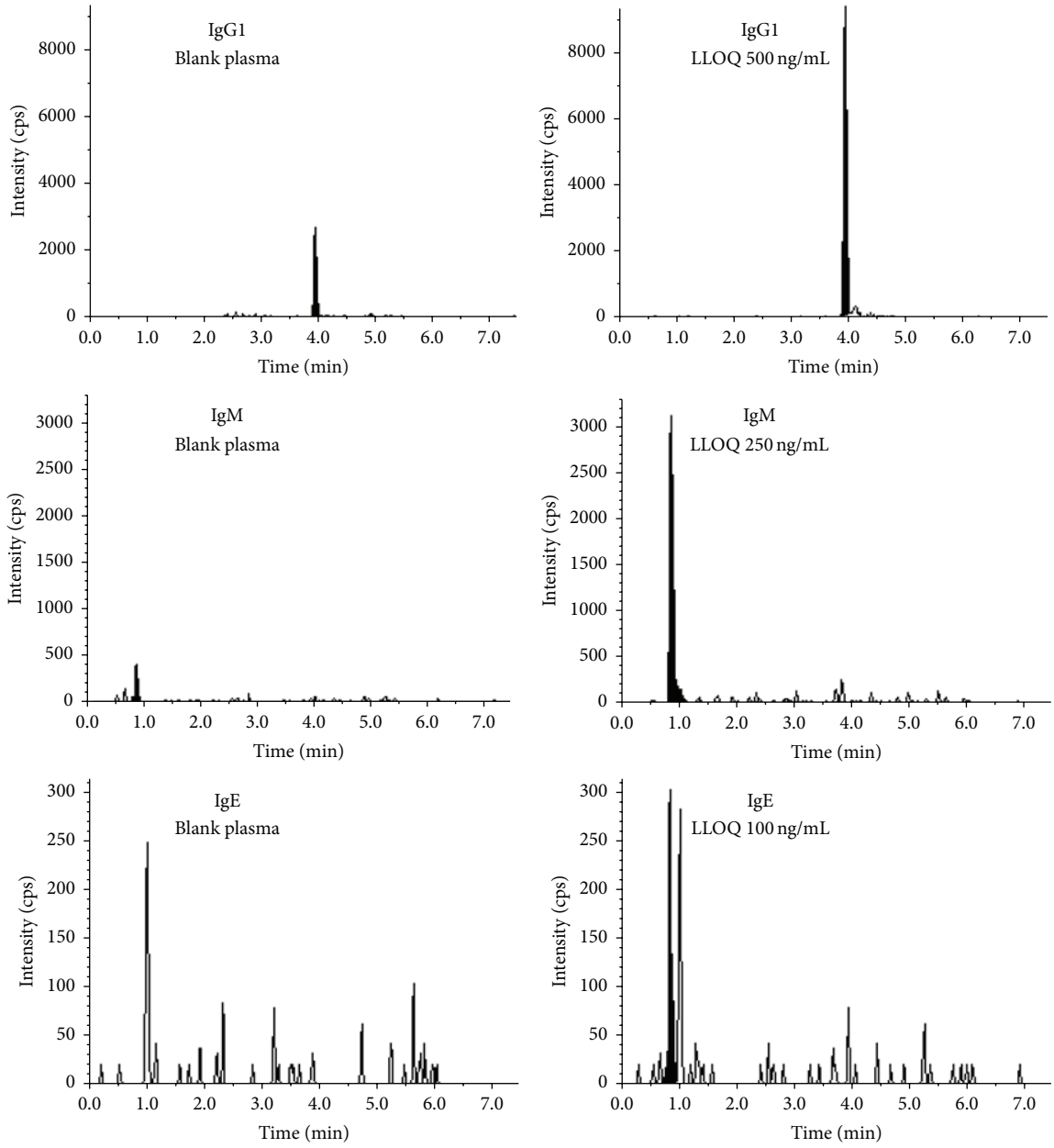

Figure 2: LC/MS chromatograms of unique peptides of IgG1 (top), IgM (middle), and IgE (bottom) from blank human plasma (left) and LLOQ samples (right) after immunocapture when using drug as ADA capture reagent.

the selected peptide came between 0.8 and 4 min in the 8 min run. Representative LC/MS chromatograms of IgG1, IgE, and IgM from the pooled ADA-free blank human plasma and low limit of quantitation standards (see discussions later) are shown in Figure 2.

The LC/MS assay was tested with blank human plasma with and without preexisting ADA (PEA) for Protein Z. The blank plasma was obtained from 20 in-house healthy donors and had been screened for PEA with a drug-bridging ECL assay. Based on the ECL assay, plasma from 9 donors was PEA negative whereas it was PEA positive from the remaining 11 donors.

The 20 plasma samples were processed using the immunocapture procedures with biotinylated Protein Z as the capture reagent. Along with the 20 plasma samples, a set of calibration standards were prepared in beads eluent from the pooled PEA negative blank plasma. After digestion, the plasma samples and the calibration standards were assayed using the LC/MS ADA method. The results were evaluated using either a cut-point or a calibration curve.

3.2.1. Cut-Point with Drug as Capture Reagent. The LC/MS responses (analyte/IS peak area ratio) of each ADA isotype/subclass from the 9 PEA negative and the 11 PEA

Table 2: LC/MS peak area ratio response and cut-points of ADA isotopes/subclasses in PEA negative human plasma with drug as ADA capture reagent.

Plasma lot #	IgG1	IgG2	IgG3	IG4	IgE	IgM	IgA1 + IgA2
1	0.0277	0.0099	—	0.0107	—	0.1130	0.0041
2	0.0107	0.0008	—	0.0019	—	—	0.0059
3	0.0263	—	—	0.0012	0.0150	0.0084	0.0078
4	0.0093	0.0023	—	0.0053	—	0.0072	0.0057
5	0.0249	0.0244	0.0210	0.0099	0.0110	0.0474	0.0136
6	0.0060	0.0044	0.0110	0.0035	—	0.0326	—
7	0.0129	—	—	0.0037	0.0160	0.0159	0.0062
8	0.0637	0.0205	0.0570	0.0353	0.0140	0.0066	0.0174
9	0.0127	0.0008	—	0.0111	—	0.0035	0.0075
Mean	0.0216	0.0070	0.0099	0.0092	0.0061	0.0261	0.0086
SD	0.0177	0.0093	0.0192	0.0105	0.0074	0.0361	0.0043
Cut-point (95%)	0.0507	0.0224	0.0416	0.0265	0.0183	0.0854	0.0156

—: no LC/MS response was detected.

Table 3: LC/MS peak area ratio responses and calculated ADA isotope/subclass levels in PEA positive human plasma with drug as ADA capture reagent (numbers in bold italic are above respective cut-points).

Lot #	IgG1	IgG1 conc. (μg/mL)	IgG2	IgG3	IG4	IgE	IgM	IgA1 + IgA2
1	***0.0874***	—	**0.0033**	***0.0476***	0.0096	—	0.0499	***0.0441***
2	***0.0838***	—	***0.0452***	***0.0762***	0.0070	—	0.0421	***0.0422***
3	***0.4200***	0.660	0.0061	0.0263	0.0088	—	0.0111	***0.0847***
4	***0.1970***	—	0.0047	—	0.0020	0.0113	0.0068	***0.0497***
5	0.0239	—	0.0044	0.0119	0.0020	—	0.0007	***0.0480***
6	***0.0827***	—	0.0141	0.0205	0.0192	0.0054	0.0316	***0.0771***
7	0.0188	—	0.0041	—	—	—	0.0127	***0.0239***
8	0.0150	—	0.0109	0.0315	0.0017	0.0119	0.0027	***0.0712***
9	***0.0788***	—	0.0009	—	0.0009	0.0150	0.0099	***0.0727***
10	***0.0641***	—	0.0076	0.0192	—	0.0155	0.0314	***0.0424***
11	***0.4330***	0.680	0.0041	0.0303	0.0031	—	***0.1010***	***0.0652***
Cut point (95%)	0.0507	—	0.0224	0.0416	0.0265	0.0183	0.0854	0.0160

—: below the limit of quantitation for IgG1 concentration or no peak was detected for other isotypes/subclasses.

positive human plasma samples are listed in Tables 2 and 3, respectively. Similar to traditional drug-bridging assays [10], the cut-point was set at 95% to allow a rate of 5% false positives and determined with the 9 lots of PEA negative human blank plasma. The LC/MS peak area ratios of ADA IgG1 isotope ranged from 0.0060 to 0.0637, with a mean of 0.0216 and a standard deviation (SD) of 0.0177 (Table 2). Using the standard calculation formulation for 95% cut-point, mean + 1.645 × SD, the calculated cut-point value was 0.0507 for IgG1. Compared to the cut-point, 8 of 11 PEA positive samples were also ADA (IgG1) positive by the immunocapture-LC/MS assay (Table 3). The ADA (IgG1) response in the remaining 3 samples (lots 5, 7, and 8) ranged from 0.0150 to 0.0239, which was below the cut-point.

Likewise, cut-points were calculated for all other ADA isotypes/subclasses (Table 2). Based on the cut-points, there were one ADA positive plasma lot each for IgG2 and IgM and 2 for IgG3 while none for IgG4 and IgE by the immunocapture-LC/MS assay. In contrast, all these 11 samples were ADA positive for IgA1 and/or IgA2. As discussed below, except for IgG1, the LC/MS responses of all other isotypes/subclasses were below limit of quantitation (BLQ) and, therefore, their contributions to the overall ADA amounts were negligible.

3.2.2. Calibration Curve with Drug as Capture Reagent. ADA levels in the 11 PEA positive samples were semiquantitatively determined using a calibration curve. A series of matrix calibration standards ranged from 0.05 to 10 μg/mL were prepared in the magnetic bead eluent of pooled blank human plasma by spiking neat Ig isotype standard solutions. The matrix calibration standards were then digested and assayed by LC/MS. The calibration curve for each Ig isotype/subclass was constructed using LC/MS peak area ratios of peptide versus respective stable isotope labeled IS. Linear regression with $1/x^2$ weighting was used. The ADA concentration in the plasma samples was back-calculated using the calibration curve.

Table 4: Calibration curve ranges and regression coefficients (r) of ADA isotype/subclasses using drug or mouse mAb as ADA capture reagent.

Isotype/subclass	Unique peptide	Calibration curve parameters			
		Drug capture		mAb capture	
		Range (μg/mL)	r	Range (μg/mL)	r
IgG1	GPSVFPLAPSSK	0.5–10	0.9919	0.5–10	0.9940
IgG2	GLPAPIEK	0.25–10	0.9939	0.1–10	0.9964
IgG3	WYVDGVEVHNAK	0.25–10	0.9858	0.25–10	0.9952
IgG4	GLPSSIEK	0.1–10	0.9919	0.25–10	0.9909
IgE	AEWEQK	0.1–10	0.9947	0.1–10	0.9976
IgM	GQPLSPEK	0.25–10	0.9988	0.25–10	0.9909
IgA1 + IgA2	YLTWASR	0.1–10	0.9974	0.1–10	0.9966

During the immunocapture process, the (biotinylated)drug-ADA complexes were separated from the plasma by a magnet. However, as endogenous plasma Igs were at much higher levels [44], a small amount still remained on the beads even after the washing. The endogenous Igs surviving the washing step were carried on in subsequent elution and digestion and eventually detected as background peaks in the LC/MS assay. The endogenous interference peaks of IgG1 and IgM and IgE were clearly seen in the chromatograms of the blank plasma sample (Figure 2). The endogenous interference peak intensity seemed to follow the order of Ig abundance in human plasma [44]. IgG1 had the highest interference peak while IgE had the lowest. Similar to traditional LC/MS assay, the low limit of quantitation (LLOQ) of the calibration curve was defined such that LC/MS response at LLOQ was equal to or greater than 4x matrix background response. As shown in Figure 2, the LLOQ peaks were much higher than the corresponding matrix interference peaks and the peak areas were accurately measured. The LLOQ determined for each isotype/subclass ranged from 0.1 to 0.5 μg/mL. A lower LLOQ corresponded to a higher assay sensitivity. Among the ADA isotypes/subclasses, IgG4, IgE, and [IgA1 + IgA2] had the highest assay sensitivity with LLOQ of 0.1 μg/mL.

The higher limit of quantitation (HLOQ) of the calibration curve range depended on the beads and capture reagent capacity. However, as the calibration standards were prepared after immunocapture, the beads and capture reagent capacity could not be readily assessed. Based on our experiences with immunocapture using similar experimental settings, the HLOQ was arbitrarily set at 10 μg/mL for all isotypes/subclasses, which should be well within the capacity of the assay.

The calibration linear range was defined from LLOQ to HLOQ. The curve linear regression correlation coefficients (r) were all >0.9910 except for peptide WYVDGVEVHNAK (IgG3, which was 0.9858). The calibration linear ranges and correlation coefficients (r) are listed in Table 4. Representative calibration curve of IgG1 in human plasma eluent after immunocapture is shown in Figure 3.

Using the calibration curve, only 2 out of the 11 PEA positive samples had ADA levels (for IgG1 only) above the LLOQ 0.5 μg/mL. Lots 3 and 11 had ADA IgG1 level of 0.660 and 0.680 μg/mL, respectively. The ADA IgG1 levels in the remaining 9 samples were BLQ. It is expected that ADA IgG1 levels in some of the 9 samples could be quantitated if the LC/MS assay sensitivity was further improved. Obviously, in order to increase the assay LC/MS specificity, one has to further eliminate endogenous Igs to minimize the background response. This effort is currently ongoing in our lab. In all 11 samples, the levels of ADAs of other Ig isotypes/subclasses were BLQ. This was consistent with the fact that IgG1 is the most dominant antibody in human plasma [44].

The biggest caveat of the semiquantitation approach was that the calibration standards did not go through the

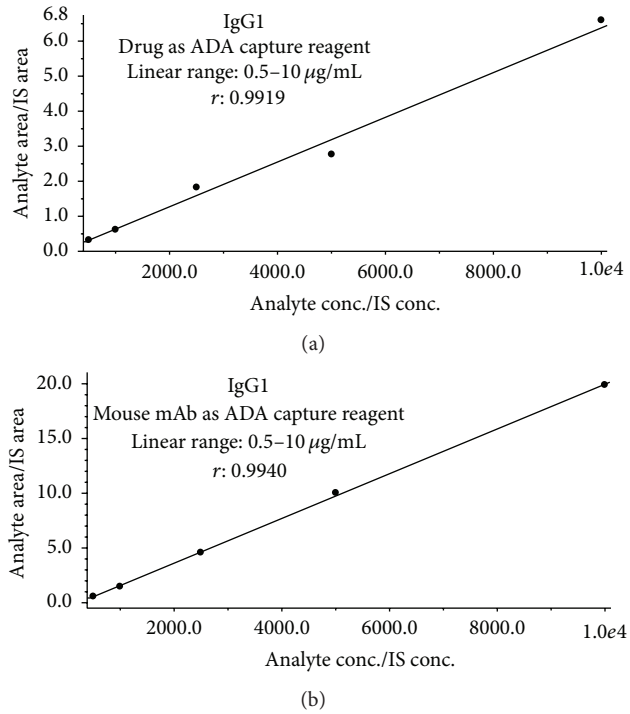

Figure 3: Calibration curves of IgG1 in human plasma eluent after immunocapture when using either drug (a) or mouse mAb as ADA capture reagent (b).

immunocapture process whereas the study samples did. The calibration thus did not take immunocapture recovery into account. The recovery could be estimated using well characterized polyclonal human ADA positive controls, which were not available for Protein Z. Based on our experiences with immunocapture in similar experimental setting and those reported in literature [38], immunocapture recovery varied from project to project but usually falls within a 30–50% range. If this also held true for Protein Z, the measured ADA levels in the PEA plasma would be around 30–50% of actual concentrations.

Different from ECL assays, the ADA levels measured by the immunocapture-LC/MS represent absolute amounts. This allows one to compare ADA isotype levels between samples, studies, and different biotherapeutics. Database of such information could be gradually built and provide valuable insight to better understand immunogenicity and immunology of biotherapeutics.

As with traditional ECL drug-bridging assays, the immunocapture-LC/MS method could be hampered by drug tolerance issues when drug is present [51]. As a consequence, the assay sensitivity can be severely compromised. This limitation may be overcome by using acid dissociation to break up the drug-ADA complex and release ADA [37]. Biotinylated drug is then added to the samples so that the biotinylated drug competes with the existing drug in forming (biotinylated)drug-ADA complexes. If the amount of the biotinylated drug is much more than that of existing drug which is usually determined with a PK assay, the drug interference is greatly reduced and assay sensitivity is improved. In ECL drug-bridging assays, one binding domain of ADA has to bind to biotinylated drug while the other binds to sulfotagged drug in order to form (biotinylated)drug-ADA-(sulfotagged)drug complex and be detected. In the immunocapture-LC/MS assay, on the other hand, only one arm of ADA needs to bind to biotinylated drug and the other can still bind to the unlabeled drug. Therefore, drug interference is expected to be less in immunocapture-LC/MS assay platform.

It should be noted that if drug contains the human Ig Fc region, it may also bind to the beads via nonspecific binding just like endogenous proteins and could contribute to interference in the LC/MS assay. On the other hand, biotinylated drug that binds to streptavidin beads will not be eluted out under the elution conditions due to very strong biotin-streptavidin interaction [52]. The binding between streptavidin and biotin has long been regarded as the strongest, noncovalent, biological interaction known, with a dissociation constant K_D in the order of 4×10^{-14} M [52]. The bond forms very rapidly and is stable in wide ranges of pH and temperature [53, 54].

It was evident that the results from the immunocapture-LC/MS assay confirmed PEA positive results in most of these samples and were in good agreement with the drug-bridging ECL assay.

3.3. Anti-Drug Ab as Capture Reagent. The second immunocapture approach was using a mouse anti-Protein Z mAb to capture ADA in human plasma. In this approach, all ADAs had to be first completely converted to drug-ADA complexes by adding excessive Protein Z to the samples [34]. Biotinylated mouse mAb was then added to capture the Protein Z-ADA complexes along with free Protein Z. In the presence of mAb, the drug-ADA complexes and free drug were converted to drug-ADA-mAb complexes and drug-mAb complexes, respectively. After adding streptavidin magnetic beads, the complexes were immobilized on the beads and were subsequently separated from plasma using a magnet. ADA was then eluted from the beads, digested, and assayed by LC/MS following the same procedures with drug as the capture reagent described previously.

The merit using anti-drug Ab as the capture reagent lies on that drug no longer interferes with the assay. This offers a huge advantage when drug levels in the study samples are high enough such that drug tolerance becomes a concern in other types of assays. The most important element of this approach is that the capture Ab should not compete with ADA for the drug; that is, the two should not share the same binding domain on the drug. To confirm this for the mouse mAb, the immunocapture recovery of Protein Z from ADA positive samples was assessed. Protein Z was spiked at 5 ng/mL to the pooled PEA negative and the 11 positive human plasma samples and its concentration was determined using an immunocapture-LC/MS PK assay. The PK assay was developed in our lab to support clinical studies. In the PK assay, Protein Z was captured using the mouse mAb, and the resulting drug-mAb complex was then immobilized on magnetic beads, separated from plasma, eluted out from beads, digested, and analyzed by LC/MS. A unique peptide from Protein Z was monitored by LC/MS and used to quantitate Protein Z. The immunocapture recovery was determined by comparing Protein Z concentrations in the PEA positive human plasma with the pooled PEA negative plasma. No difference in Protein Z concentration was observed between the PEA positive and PEA negative samples (data not shown), and Protein Z recovery was more than 82% with averaging 97%. It was evident that the mouse mAb was indeed able to capture Protein Z regardless of whether it is in ADA-Protein Z complexes or free form. However, one has to be cautious as human ADAs come in many different forms and some may bind to the same domain on the drug as the capture Ab. Therefore, it is recommended to run this test using ADA positive samples from the study.

Similar to the first approach using drug as capture reagent, the 11 PEA positive and 9 PEA negative blank human plasma samples were used to evaluate immunocapture using the mouse mAb as the capture reagent. Besides using a different capture reagent, the only difference between the two approaches was that in the second approach there was an additional step to convert ADA to ADA-drug complexes. To ensure a complete conversion, 6 μL of 5 mg/mL Protein Z buffer solution was added to 144 μL of human plasma. The amount of Protein Z added was overwhelmingly more than the PEA level (≤680 ng/mL) estimated by the first approach. The same amount of Protein Z was also added to the pooled PEA blank plasma used for preparation of calibration standards.

Table 5: LC/MS peak area ratio response and cut-points of ADA isotopes/subclasses in PEA negative human plasma with mouse mAb as ADA capture reagent.

Lot #	IgG1	IgG2	IgG3	IG4	IgE	IgM	IgA1 + IgA2
1	0.0495	0.0107	—	0.1110	—	0.0422	0.0224
2	0.0340	0.0054	—	0.0357	0.0152	0.0702	0.0223
3	0.0400	0.0035	—	0.0548	—	0.0277	0.0538
4	0.0243	0.0084	—	0.0259	—	0.0358	0.0264
5	0.0265	0.0108	—	0.0677	—	0.0593	0.0237
6	0.0210	0.0511	—	0.0868	—	0.0341	0.0688
7	0.0815	0.0384	—	0.0009	—	0.0622	0.0087
8	0.0231	0.0294	—	0.0866	—	0.0029	0.0274
9	0.0253	0.0027	—	0.0719	—	0.0015	0.0094
Mean	0.0361	0.0178	—	0.0601	0.0017	0.0373	0.0292
SD	0.0194	0.0175	—	0.0345	0.0051	0.0245	0.0197
Cut-point (95%)	0.0680	0.0466	0.0000	0.1168	0.0100	0.0775	0.0617

—: no LC/MS response was detected.

Table 6: LC/MS peak area ratio responses and calculated ADA isotope/subclass levels in PEA positive human plasma with mouse Ab as ADA capture reagent (numbers in bold italic are above respective cut-points). Plasma samples were spiked with addition of excessive drug (+drug) or without (−drug) addition of excessive drug.

Lot #	IgG1		IgG2		IgG3		IgG4		IgE		IgM		IgA	
	−drug	+drug	−drug	+drug	−drug	+drug	−drug	+drug	−drug	+drug	−drug	+drug	−drug	+drug
1	0.0369	***0.1860***	0.0077	0.0106	—	—	0.0668	0.0823	—	—	0.0159	0.0094	0.0194	0.0411
2	0.0145	0.0563	0.0072	0.0066	—	—	0.0584	0.0458	—	—	0.0316	0.0340	0.0033	0.0113
3	0.0354	***0.2330***	0.0074	—	—	—	0.0634	0.0594	—	—	0.0268	0.0098	0.0405	0.0483
4	0.0212	***0.0715***	0.0240	0.0314	—	—	0.0484	0.0369	—	—	0.0045	0.0190	***0.1380***	***0.2300***
5	0.0350	0.0381	—	0.0059	—	—	0.0182	0.0150	—	—	0.0053	0.0118	0.0325	***0.0692***
6	***0.0731***	***0.1800***	0.0122	0.0237	—	—	0.0325	0.0544	—	—	0.0575	0.0310	***0.1810***	***0.1900***
7	0.0231	0.0343	***0.0469***	***0.0739***	—	—	0.0685	0.0768	—	—	0.0234	0.0321	0.0339	0.0476
8	0.0463	0.0474	0.0086	0.0045	—	***0.0915***	0.0572	0.0528	—	—	0.0089	0.0394	0.0142	0.0329
9	0.0396	***0.1160***	0.0011	0.0026	—	—	0.0661	0.0404	—	—	0.0094	0.0125	0.0437	***0.0681***
10	0.0188	***0.1200***	0.0020	0.0039	—	—	0.0151	0.0225	—	—	0.0275	0.0329	0.0029	0.0208
11	***0.0974***	***0.5690***	0.0094	0.0075	—	—	0.0294	0.0305	—	—	***0.2570***	***0.2850***	***0.1040***	***0.1050***
Cut-point (95%)	0.0680		0.0466		0.0000		0.1168		0.0100		0.0775		0.0617	

—: no LC/MS response was detected.

3.3.1. Cut-Point with mAb as Capture Reagent. Tables 5 and 6 provide the LC/MS peak area ratio response of ADA in the PEA negative and positive samples, respectively. The LC/MS chromatograms of IgG1, IgM, and IgE unique peptides from the blank human plasma and LLOQ samples are shown in Figure 4. The LC/MS response for ADA (IgG1) from the 9 PEA negative samples ranged from 0.0210 to 0.0815, with a mean of 0.0361 and SD of 0.0194. The calculated cut-point at 95% was 0.0680 for IgG1. Using the cut-point, 7 of the 11 PEA positive samples were also ADA positive with the immunocapture-LC/MS assay. These 7 plasma lots were also ADA positive in the first approach using drug as capture reagent. Plasma lot 2 was ADA positive in the first approach but negative in the second approach. In both approaches, LC/MS response of plasma lot 2 was close to the respective cut-point, so it was not surprising to see the discrepancy between the two approaches.

Calculated cut-points for all other ADA isotypes/subclasses are listed in Table 5. Based on cut-points, lot 7 was ADA positive for IgG2, lot 8 was ADA positive for IgG3, lot 11 was ADA positive for IgM, and lots 4, 5, 6, 9, and 11 were ADA positive for IgA1 + IgA2. No positive lot was found for IgG4 or IgE. However, lot 7 for IgG2, lot 11 for IgM, and lots 6 and 11 for IgA1 + A2 were considered false positive due to the presence of interference as discussed below.

The mechanism of ADA capture using the mAb was more complicated than using drug. The ADA must be bound to the drug first, and the resulting drug-ADA complexes had to be bound to the mouse mAb and survive the immunocapture procedure in order to be detected by LC/MS. Endogenous components such as Igs that cross-reacted with the mAb could also interfere with the assay and give false positive results. Although this potential interference was already accounted for in the cut-point determination, it was

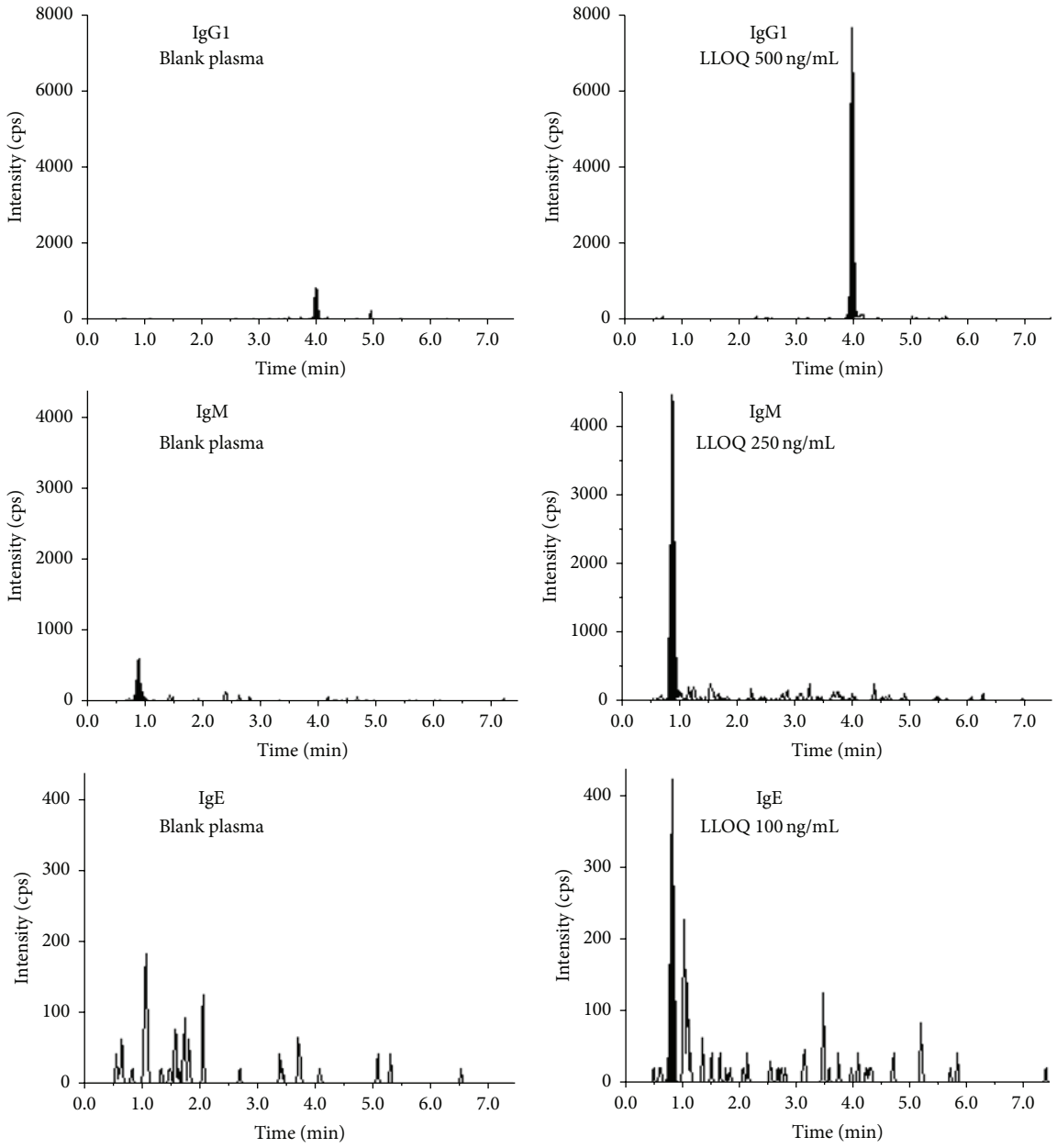

Figure 4: LC/MS chromatograms of unique peptides of IgG1 (top), IgM (middle), and IgE (bottom) from blank human plasma (left) and LLOQ samples (right) after immunocapture when using mouse mAb for ADA capture.

further assessed for the PEA positive plasma samples without the addition of excessive Protein Z. The "−drug" plasma samples were spiked with the mAb and then processed with the immunocapture procedure followed by LC/MS analysis. The results are provided in Table 6. For IgG1, the LC/MS responses from the "−drug" samples of lots 6 (0.0731) and 11 (0.0974) were above the cut-point of 0.0680, suggesting possible interference. However, both responses were slightly above the cut-points and much less than those (0.1800 and 0.5690) from their respective "+drug" samples. Therefore, lots 6 and 11 were still considered ADA positive despite the presence of small interference. Likewise, Lot 4 was considered positive for IgA1 + A2, as the above cut-point "−drug" response (0.1380) was much less than "+drug" responses (0.2300). The remaining "−drug" positive samples, lot 7 for IgG2, lot 11 for IgM, and lots 6 and 11 for IgA1 + A2 gave similar responses as their respective "+drug" samples and thus were considered false positive. Overall, seven of the eleven PEA positive plasma samples were positive for IgG1, one was positive for IgG3, and three were positive for IgA1 + IgA2 using the mAb as ADA capture reagent.

3.3.2. Calibration Curve with mAb as Capture Reagent. Calibration curves were established for each ADA isotype in the same way as in the first approach. The calibration linear ranges and curve regression correlation coefficients

are provided in Table 4. Calibration curve of IgG1 is shown in Figure 3. HLOQ was also set at 10 μg/mL for all isotypes/subclasses. The correlation coefficient (r) was >0.98 in all cases. The LLOQ, calibration ranges, and r were all similar to those from the first approach using drug as capture reagent.

Using the calibration curve, ADA (IgG1) level in lot 3 and lot 11 plasma was determined to be 0.570 and 1.25 μg/mL, respectively. These two plasma samples were also the only ones with ADA (IgG1) level above LLOQ in the first immunocapture approach. The ADA (IgG1) level from the first approach was 13.6% and −45.6%, respectively, compared with the second approach. Given the two totally different immunocapture approaches and the limited sample size, the two sets of semiquantitative data were considered in good agreement with each other.

Besides IgG1, ADA levels for other ADA isotypes/classes were all BLQ in these 11 PEA positive samples. This was consistent with the first approach.

It should be noted that the anti-drug Ab capture approach may not be used if the biotherapeutic proteins contain constant human Fc regions. Unlike using drug as capture reagent, anti-drug Ab captures both free drug and drug-ADA complexes and during the ADA elution step drug is also eluted out from magnetic beads and thus interferes with LC/MS detection. For instance, Humira (adalimumab), a TNF inhibiting anti-inflammatory drug and the first fully human monoclonal antibody drug approved by the FDA, is an IgG1 made by phage display technology with amino acid sequences only from the human germline, making it indistinguishable in structure and function from natural human IgG1 [55]. Based on in silicon digestion prediction, Humira would yield the universal peptides of human IgG1, GPSVFPLAPSSK, and thus interfere with the universal peptide ADA assay. In this case, unique peptide(s) from the drug instead of the ADA peptides might be monitored by LC/MS and the results can be qualitatively correlated to ADA, as Neubert et al. [34] reported. Another option is to use the first immunocapture approach with biotinylated drug as the ADA capture reagent.

4. Conclusions

We demonstrated for the first time that immunocapture-LC/MS can be used for simultaneous ADA isotyping and semiquantitation in human plasma. Either biotinylated drug or biotinylated anti-drug Ab could be used as the immunocapture reagent, each with its own merits and shortfalls. Biotinylated drug can readily capture ADA but drug interference could be an issue if drug levels in the samples are high. On the other hand, immunocapture using an anti-drug Ab eliminates drug interference, providing that the Ab is able to capture drug-ADA complex in addition to free drug. With this method, unique peptides from each ADA isotype/subclass were identified and monitored by LC/MS. ADA isotyping was performed by the detection of isotype-unique peptides. Absolute ADA amount was determined semiquantitatively using surrogate calibration standards. Similar to traditional drug-bridging ELISA assay, cut-points at 95% were established. The assay was used for screening, isotyping, and semiquantitating preexisting ADAs in human plasma. It could be also used as a confirmatory assay. Endogenous Ig interferences need to be reduced in order to improve the assay sensitivity and specificity, and human positive ADA controls will be needed for more accurate ADA quantitation.

Owing to LC/MS's advantages such as high specificity, selectivity and reproducibility, wide dynamic range, and multiplexing capability, it is expected that, with further improvements, immunocapture-LC/MS will become an invaluable tool in immunogenicity assessment. It can be easily implemented in bioanalytical lab settings for routine ADA isotyping and semiquantitation. As ADA levels measured by immunocapture-LC/MS represent absolute amounts, one can compare ADA isotype levels between samples, studies, and different biotherapeutics, providing that consistency in positive controls is achieved to determine recovery. Database of such information could be gradually built and provide valuable insight to better understand immunogenicity and immunology of biotherapeutics.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] H. Schellekens, "Bioequivalence and the immunogenicity of biopharmaceuticals," *Nature Reviews Drug Discovery*, vol. 1, no. 6, pp. 457–462, 2002.

[2] C. A. Schnabel, S. E. Fineberg, and D. D. Kim, "Immunogenicity of xenopeptide hormone therapies," *Peptides*, vol. 27, no. 7, pp. 1902–1910, 2006.

[3] K. Kuus-Reichel, L. S. Grauer, L. M. Karavodin, C. Knott, M. Krusemeier, and N. E. Kay, "Will immunogenicity limit the use, efficacy, and future development of therapeutic monoclonal antibodies?" *Clinical and Diagnostic Laboratory Immunology*, vol. 1, no. 4, pp. 365–372, 1994.

[4] E. Koren, L. A. Zuckerman, and A. R. Mire-Sluis, "Immune responses to therapeutic proteins in humans—clinical significance, assessment and prediction," *Current Pharmaceutical Biotechnology*, vol. 3, no. 4, pp. 349–360, 2002.

[5] H. Schellekens and N. Casadevall, "Immunogenicity of recombinant human proteins: causes and consequences," *Journal of Neurology*, vol. 251, supplement 2, pp. II4–II9, 2004.

[6] S. Tangri, B. R. Mothé, J. Eisenbraun et al., "Rationally engineered therapeutic proteins with reduced immunogenicity," *Journal of Immunology*, vol. 174, no. 6, pp. 3187–3196, 2005.

[7] J. M. Sailstad, L. Amaravadi, A. Clements-Egan et al., "A white paper—consensus and recommendations of a global harmonization team on assessing the impact of immunogenicity on pharmacokinetic measurements," *The AAPS Journal*, vol. 16, no. 3, pp. 488–498, 2014.

[8] G. Shankar, S. Arkin, L. Cocea et al., "Assessment and reporting of the clinical immunogenicity of therapeutic proteins and peptides—harmonized terminology and tactical recommendations," *The AAPS Journal*, vol. 16, no. 4, pp. 658–673, 2014.

[9] G. Shankar, C. Pendley, and K. E. Stein, "A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs," *Nature Biotechnology*, vol. 25, no. 5, pp. 555–561, 2007.

[10] A. R. Mire-Sluis, Y. C. Barrett, V. Devanarayan et al., "Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products," *Journal of Immunological Methods*, vol. 289, no. 1-2, pp. 1–16, 2004.

[11] J. A. Lofgren, S. Dhandapani, J. J. Pennucci et al., "Comparing ELISA and surface plasmon resonance for assessing clinical immunogenicity of panitumumab," *Journal of Immunology*, vol. 178, no. 11, pp. 7467–7472, 2007.

[12] E. Koren, H. W. Smith, E. Shores et al., "Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products," *Journal of Immunological Methods*, vol. 333, no. 1-2, pp. 1–9, 2008.

[13] I. C. Büttel, P. Chamberlain, Y. Chowers et al., "Taking immunogenicity assessment of therapeutic proteins to the next level," *Biologicals*, vol. 39, no. 2, pp. 100–109, 2011.

[14] G. Shankar, V. Devanarayan, L. Amaravadi et al., "Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 48, no. 5, pp. 1267–1281, 2008.

[15] S. Gupta, S. R. Indelicato, V. Jethwa et al., "Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics," *Journal of Immunological Methods*, vol. 321, no. 1-2, pp. 1–18, 2007.

[16] S. Gupta, V. Devanarayan, D. Finco et al., "Recommendations for the validation of cell-based assays used for the detection of neutralizing antibody immune responses elicited against biological therapeutics," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 55, no. 5, pp. 878–888, 2011.

[17] A. S. Rosenberg and A. Worobec, "A risk-based approach to immunogenicity concerns of therapeutic protein products. Part 1. Considering consequences of the immune response to a protein," *BioPharm International*, vol. 17, no. 11, pp. 22–26, 2004.

[18] A. S. Rosenberg and A. S. Worobec, "A risk-based approach to immunogenicity concerns of therapeutic protein products, part 2: considering host-specific and product-specific factors impacting immunogenicity," *BioPharm International*, vol. 17, no. 12, pp. 34–42, 2004.

[19] A. S. Rosenberg and A. Worobec, "A risk-based approach to immunogenicity concerns of therapeutic protein products—part 3—effects of manufacturing changes in immunogenicity and the utility of animal immunogenicity studies," *BioPharm International*, vol. 18, pp. 32–36, 2005.

[20] European Medicines Agency, "Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins," April 2008, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003946.pdf.

[21] U.S. Food and Drug Administration, "Guidance for industry assay development for immunogenicity testing of therapeutic proteins," Draft Guidance, U.S. Food and Drug Administration, 2009, http://www.fda.gov/downloads/Drugs/.../Guidances/UCM192750.pdf.

[22] US Food and Drug Administration, "Guidance for industry: immunogenicity assessment for therapeutic protein products," August 2014, http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm338856.pdf.

[23] European Medicines Agency, "Guideline on immunogenicity assessment of monoclonal antibodies intended for in vivo clinical use," May 2012, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/06/WC500128688.pdf.

[24] H. Trufelli, P. Palma, G. Famiglini, and A. Cappiello, "An overview of matrix effects in liquid chromatography-mass spectrometry," *Mass Spectrometry Reviews*, vol. 30, no. 3, pp. 491–509, 2011.

[25] O. Heudi, S. Barteau, D. Zimmer et al., "Towards absolute quantification of therapeutic monoclonal antibody in serum by LC-MS/MS using isotope-labeled antibody standard and protein cleavage isotope dilution mass spectrometry," *Analytical Chemistry*, vol. 80, no. 11, pp. 4200–4207, 2008.

[26] S. Pan, R. Aebersold, R. Chen et al., "Mass spectrometry based targeted protein quantification: methods and applications," *Journal of Proteome Research*, vol. 8, no. 2, pp. 787–797, 2009.

[27] B. An, M. Zhang, and J. Qu, "Toward sensitive and accurate analysis of antibody biotherapeutics by liquid chromatography coupled with mass spectrometry," *Drug Metabolism & Disposition*, vol. 42, no. 11, pp. 1858–1866, 2014.

[28] M. Blackburn, "Advances in the quantitation of therapeutic insulin analogues by LC-MS/MS," *Bioanalysis*, vol. 5, no. 23, pp. 2933–2946, 2013.

[29] V. Lange, P. Picotti, B. Domon, and R. Aebersold, "Selected reaction monitoring for quantitative proteomics: a tutorial," *Molecular Systems Biology*, vol. 4, article 222, 2008.

[30] D. C. Liebler and L. J. Zimmerman, "Targeted quantitation of proteins by mass spectrometry," *Biochemistry*, vol. 52, no. 22, pp. 3797–3806, 2013.

[31] M. T. Furlong, Z. Ouyang, S. Wu et al., "A universal surrogate peptide to enable LC-MS/MS bioanalysis of a diversity of human monoclonal antibody and human Fc-fusion protein drug candidates in pre-clinical animal studies," *Biomedical Chromatography*, vol. 26, no. 8, pp. 1024–1032, 2012.

[32] W. D. Dongen, F. van Holthoon, R. Bas, and A. Kleinnijnhuis, "Low ng/mL bioanalysis of human monoclonal antibody therapeutics using UPLC-MS," in *Proceedings of the 62nd ASMS Conference on Mass Spectrometry and Allied Topics*, Baltimore, Md, USA, June 2014.

[33] H. Li, R. Ortiz, L. Tran et al., "General LC-MS/MS method approach to quantify therapeutic monoclonal antibodies using a common whole antibody internal standard with application to preclinical studies," *Analytical Chemistry*, vol. 84, no. 3, pp. 1267–1273, 2012.

[34] H. Neubert, C. Grace, K. Rumpel, and I. James, "Assessing immunogenicity in the presence of excess protein therapeutic using immunoprecipitation and quantitative mass spectrometry," *Analytical Chemistry*, vol. 80, no. 18, pp. 6907–6914, 2008.

[35] H. W. Smith, A. Butterfield, and D. Sun, "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," *Regulatory Toxicology and Pharmacology*, vol. 49, no. 3, pp. 230–237, 2007.

[36] J. S. Bourdage, C. A. Cook, D. L. Farrington, J. S. Chain, and R. J. Konrad, "An Affinity Capture Elution (ACE) assay for detection

of anti-drug antibody to monoclonal antibody therapeutics in the presence of high levels of drug," *Journal of Immunological Methods*, vol. 327, no. 1-2, pp. 10–17, 2007.

[37] A. Patton, M. C. Mullenix, S. J. Swanson, and E. Koren, "An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen," *Journal of Immunological Methods*, vol. 304, no. 1-2, pp. 189–195, 2005.

[38] H. Jiang, W. Xu, C. A. Titsch et al., "Innovative use of LC-MS/MS for simultaneous quantitation of neutralizing antibody, residual drug, and human immunoglobulin G in immunogenicity assay development," *Analytical Chemistry*, vol. 86, no. 5, pp. 2673–2680, 2014.

[39] G. S. Omenn, R. Menon, M. Adamski et al., "The human plasma and serum proteome," in *Proteomics of Human Body Fluids*, vol. 3, pp. 195–224, Humana Press, 2007.

[40] M. Dubois, F. Fenaille, G. Clement et al., "Immunopurification and mass spectrometric quantification of the active form of a chimeric therapeutic antibody in human serum," *Analytical Chemistry*, vol. 80, no. 5, pp. 1737–1745, 2008.

[41] M. F. Ocaña and H. Neubert, "An immunoaffinity liquid chromatography-tandem mass spectrometry assay for the quantitation of matrix metalloproteinase 9 in mouse serum," *Analytical Biochemistry*, vol. 399, no. 2, pp. 202–210, 2010.

[42] A. N. Hoofnagle, J. O. Becker, M. H. Wener, and J. W. Heinecke, "Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry," *Clinical Chemistry*, vol. 54, no. 11, pp. 1796–1804, 2008.

[43] L. Borghesi and C. Milcarek, "From B cell to plasma cell: regulation of V(D)J recombination and antibody secretion," *Immunologic Research*, vol. 36, no. 1–3, pp. 27–32, 2006.

[44] G. B. Pier, J. B. Lyczak, and L. M. Wetzler, *Immunology, Infection, and Immunity*, ASM Press, 2004.

[45] H. J. Gould, B. J. Sutton, A. J. Beavil et al., "The biology of IgE and the basis of allergic disease," *Annual Review of Immunology*, vol. 21, pp. 579–628, 2003.

[46] W. E. Winter, N. S. Hardt, and S. Fuhrman, "Immunoglobulin E: importance in parasitic infections and hypersensitivity responses," *Archives of Pathology and Laboratory Medicine*, vol. 124, no. 9, pp. 1382–1385, 2000.

[47] M.-P. Lefranc and G. Lefranc, "Human Gm, Km, and Am allotypes and their molecular characterization: a remarkable demonstration of polymorphism," *Methods in Molecular Biology*, vol. 882, pp. 635–680, 2012.

[48] R. Jefferis and M.-P. Lefranc, "Human immunoglobulin allotypes—possible implications for immunogenicity," *mAbs*, vol. 1, no. 4, pp. 332–338, 2009.

[49] S. T. Wu, Z. Ouyang, T. V. Olah, and M. Jemal, "A strategy for liquid chromatography/tandem mass spectrometry based quantitation of pegylated protein drugs in plasma using plasma protein precipitation with water-miscible organic solvents and subsequent trypsin digestion to generate surrogate peptides for detection," *Rapid Communications in Mass Spectrometry*, vol. 25, no. 2, pp. 281–290, 2011.

[50] H. Yang, J. Zhang, B. Yu, and W. Zhao, "ADA assay development and validation," in *Statistical Methods for Immunogenicity Assessment*, chapter 2, pp. 23–48, CRC Press, Boca Raton, Fla, USA, 2006.

[51] Y.-M. C. Wang, L. Fang, L. Zhou, J. Wang, and H.-Y. Ahn, "A survey of applications of biological products for drug interference of immunogenicity assays," *Pharmaceutical Research*, vol. 29, no. 12, pp. 3384–3392, 2012.

[52] N. M. Green, "Avidin and streptavidin," in *Methods in Enzymology*, vol. 184, pp. 51–67, Academic Press, New York, NY, USA, 1990.

[53] D. Savage, G. Mattson, S. Desai, G. Nielander, S. Morgensen, and E. Conklin, *Avidin-Biotin Chemistry: A Handbook*, Pierce Chemical Company, Rockford, Ill, USA, 1992.

[54] X. Tong and L. M. Smith, "Solid-phase method for the purification of DNA sequencing reactions," *Analytical Chemistry*, vol. 64, no. 22, pp. 2672–2677, 1992.

[55] J. Salfeld, Z. Kaymakcalan, D. Tracey, A. Roberts, and R. Kamen, "Generation of fully human anti-TNF antibody D2E7," *Arthritis & Rheumatology*, vol. 41, supplement 9, article S57, 1998.