Meeting Report

Immunogenicity of Antibody Drug Conjugates: Bioanalytical Methods and Monitoring Strategy for a Novel Therapeutic Modality

M. Benjamin Hock,^{1,5} Karen E. Thudium,^{2,5} Montserrat Carrasco-Triguero,³ and Nikolai F. Schwabe⁴

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Abstract. Immunogenicity (the development of an adaptive immune response reactive with a therapeutic) is a well-described but unwanted facet of biotherapeutic development. There are commonly applied procedures for immunogenicity risk assessment, testing strategies, and bioanalysis. With some modifications, these can be applied to new biotherapeutic modalities. For novel therapies such as antibody-drug conjugates (ADCs), the unique structural components may contribute additional complexities to both immunologic responses and bioanalytical methods. US product inserts (USPIs) for two commercially available ADCs detail the incidence of immunogenicity; however, the body of literature on immunogenicity of ADCs is limited. We recently participated in a conference session on this topic (Annual meeting of the American Association of Pharmaceutical Scientists, held November 2013 in San Antonio, TX, USA. The meeting featured the Symposium: Immunogenicity Assessment for Novel Antibody Drug Conjugates, Nonclinical to Clinical) which prompted an effort to share our perspectives on how immunogenicity risk assessment, testing strategies, and bioanalytical methods can be adapted to reflect the complexity of ADC therapeutics.

KEY WORDS: antibody-drug conjugate (ADC); antidrug antibodies (ADA); bioanalytical; immunogenicity; oncology.

INTRODUCTION

Immunogenicity is the development of an adaptive immune response to a therapeutic (1). Typically, the humoral response is assessed by assays designed to detect antidrug antibodies (ADAs; also referred to as anti-therapeutic antibodies (ATAs)). Potential effects of immunogenicity range from immune recognition of self proteins (if there are endogenous counterparts), hypersensitivity reactions (particularly types I and III), and alterations of drug exposure and activity (neutralization) to no observable clinical consequences. Accordingly, sponsor organizations are tasked with monitoring the immunogenicity of their therapeutic proteins and providing a thorough evaluation of this information to clinicians in the product label. This may include information on ADA prevalence and the impact of ADA on clinical safety and efficacy (2).

Clinicians and regulators are familiar with immunogenicity concerns of monoclonal antibody (mAb) therapeutics (3),

growth factors, and enzyme replacements. Guidance documents (4) and industry white papers (5) are excellent sources regarding strategies to evaluate immunogenicity as well as the development and validation of assays for these products. There are, however, emerging protein therapeutic modalities for which there are no current guidelines for immunogenicity evaluation (including bispecific antibodies (and variants thereof, such as dual-variable domain molecules) and BiTE® antibodies). These novel constructs have structural motifs that may carry unique immunogenicity risks: among these, the antibody-drug conjugates (ADCs) are the most advanced, with three molecules approved and two currently on the market for use in the USA.

ADCs were developed as oncology therapeutics coupling the specificity of mAbs with the cytotoxicity of a small-molecule drug (e.g., maytansinoid, calicheamicin, auristatin) (6–10). The ADC's mechanism of action hinges on the release of a cytotoxic payload after internalization by one of several processes into the target cell (11–13). ADCs are descendants of “immunotoxin” conjugate therapeutics such as denileukin diftiox (Ontak) (14), whereby a protein-targeting component is coupled to a protein toxin. Unfortunately, immunogenicity of the toxin component (proteins derived from plants or bacteria) may limit efficacy of these molecules without heroic modifications (15). Small-molecule drugs present in ADCs provide an alternative but are not without complications for immunogenicity assay development and monitoring strategies.

Evaluation of a potential humoral response against an ADC is more complex than for mAb therapeutics. While

¹Department of Clinical Immunology, Amgen Inc., One Amgen Center Dr., 30E-3-B, Thousand Oaks, California 91320, USA.

²Oncology Clinical Pharmacology, Novartis Pharmaceuticals, One Health Plaza, BLDG 337, East Hanover, New Jersey 07936, USA.

³BioAnalytical Sciences, Genentech, South San Francisco, California, USA.

⁴ProImmune Ltd., Oxford, UK.

⁵To whom correspondence should be addressed. (e-mail: mhock@amgen.com; Karen.Thudium@novartis.com)

ADAs could develop against protein components of either modality, the linker and/or the small-molecule drug may act as a hapten once conjugated to form the ADC. As such, ADA may also target the linker/small-molecule drug or the linker/small-molecule drug in a particular orientation (analogous to a haptenic group (16)) within an ADC. The immune mechanisms contributing to an anti-ADC response may also be more diverse (see “**MECHANISMS OF IMMUNOGENICITY**” below). In addition, an immune response reactive with one part of the molecule may eventually spread to other epitopes (epitope spreading). Finally, the consequences of an ADA response may differ from those of an ADC (see “**RISK ASSESSMENT**”). Due to these unique features of ADC immunogenicity, ADA domain specificity experiments (analogous to epitope mapping, where the resolution conferred by a particular bioanalytical method determines whether it is domain or epitope specific) may be included in an immunogenicity monitoring strategy.

Three ADCs have received marketing approval in the USA: Mylotarg® (gemtuzumab ozogamicin, approved in 2000 and withdrawn in 2010), Adcetris® (brentuximab vedotin, 2011), and Kadcyla® (ado-trastuzumab emtansine, 2013). The pharmacokinetics and pharmacology, toxicology, and bioanalytical strategies used for these ADCs have been reviewed (17–22). Immunogenicity support strategies (and the resulting data) for these approved ADCs form the knowledge base on what is an expanding field (Table I illustrates the examples of published ADC immunogenicity information). The 2013 annual meeting of the American Association of Pharmaceutical Scientists featured a symposium on the immunogenicity of ADCs. The intent of this manuscript is to review the most-discussed topics of that session: how unique properties of ADCs impact immunogenicity risk assessment, testing strategy, and bioanalytical methods?

RISK ASSESSMENT

Immunogenicity monitoring strategies for new biotherapeutics (or new indications) should be based on a thorough risk assessment. This consists of two parts: (1) the probability of the biotherapeutic to elicit an immunogenic response and, (2) if induced, the severity of potential consequences. The risk assessment is based on known and potential correlates of immunogenicity, *in vitro* and *in silico* analyses, and should be a “living document” that is refined by developments within the literature as well as acquisition and interpretation of product-specific preclinical and clinical data.

Some of the factors that may contribute to the development of immunogenicity against a biotherapeutic are known and have been discussed in detail elsewhere (3,23,24). Importantly, for ADCs (currently approved for oncology indications), the immunological status of patients may impact immunogenicity. This phenomenon is illustrated by the incidence of ADA against rituximab (a monoclonal antibody targeting CD20) across indications: approximately 11% of immunogenicity is observed in rheumatoid arthritis patients, while 1.1% is observed in patients with low-grade or follicular NHL (US product insert, USPI). The second example is the observation reported by Ragnhammar and colleagues that the incidence of clinically neutralizing anti-GM-CSF

antibodies in immunocompromised subjects (on intensive chemotherapy) is low (1 of 8), while neutralizing ADA (neutralizing antibodies (NABs)) developed in practically all (19 of 20) non-immunocompromised patients (25).

One hallmark consequence of a high-risk immune response is neutralization of an essential endogenous counterpart. ADAs towards ADCs are not expected to cross-react with endogenous immunoglobulin molecules and result in loss of function. Furthermore, the generally well-tolerated human immunoglobulin component, intravenous route of administration, and immunosuppressed oncology patient population are all associated with a lower immunogenicity risk. However, the limited clinical experience and the variety of potential complications caused by conjugation of the linker and small-molecule drug to a mAb require careful examination prior to inclusion of ADCs in the low-risk category.

There are unique potential consequences of an immune response against an ADC. For example, the ADA concentrations at which antibody-antigen immune complex formation occurs (the zone of equivalence) may be shifted for a multivalent antigen (16). Furthermore, internalization of immune complexes containing ADA and the cytotoxic drug by nontarget tissues could result in toxicity (for example, damage to cells of the monocyte phagocytic system (MPS)). Complement deposition and neutrophil response to immune complexes both have also been shown to depend on epitope density (26,27). An additional potential complication is prior exposure to a therapeutic containing the same linker or small-molecule drug. While the two ADCs present in the market utilize different small-molecule drugs, it is foreseeable that *sensitization* to a component of the ADCs would result in clinical consequences for a subsequent partially related therapeutic. Finally, the conjugation of a hapten-like linker and small-molecule drug is a unique potential immunogenicity risk itself (see “**MECHANISMS OF IMMUNOGENICITY**”).

Based on the risk assessment for each ADC, an immunogenicity assay strategy is developed for both nonclinical and clinical studies. As shown in Fig. 1, it is typically composed of a tiered assay approach with appropriate sensitivity and tolerance to the presence of the ADC in the sample (including screening, confirmatory, characterization, and possibly neutralizing assays). The immunogenicity strategy may be revised once clinical data is available based on the incidence of ADA response as well as occurrence and severity of clinical sequelae. For example, further testing such as a neutralizing assay may be needed to evaluate the impact of ADAs. On the other hand, domain specificity experiments may not be warranted for clinical studies if a relationship between ADA specificity and clinical consequences of immunogenicity is already understood.

BIOANALYTICAL STRATEGIES FOR IMMUNOGENICITY ASSESSMENT

A tiered immunogenicity strategy, often applied to mAb therapeutics, serves as a good template for ADC immunogenicity monitoring. This most often begins with the identification of *binding antibody positives*, whereby specimens are analyzed in a screening immunoassay, and putative ADA-positive specimens are confirmed in a specificity step (often

Table I. Summary of Reported ADC Immunogenicity

ADC therapeutic (manufacturer)	mAb (toxin)	Anti-ADC assay	Binding anti-ADC	ADA characterization	ADA impact	Reference
Brentuximab vedotin (Seattle Genetics)	Chimeric anti-CD30 (MMAE)	ECL, domain specificity (method NR)	47/156 (30%), transient	Epitope/mAb NAb, 36/58 (62%)	Infusion reactions	Brentuximab vedotin USPI
Trastuzumab emtansine (Genentech)	Humanized Anti-HER2 (DM1)	ECL/ELISA bridging, domain specificity (competition)	11/156 (7%), persistent 44/836 (5.3%), total 13/836 (1.6%) preexisting 28/836 (3.3%) developing	Epitope: linker/cytotoxic drug or new mAb epitopes (<i>i.e.</i> , T-DM1 epitope not shared with trastuzumab)	No observed impact on PK, safety, efficacy	Trastuzumab emtansine USPI
Gemtuzumab ozogamicin (Wyeth/Ayerst)	Humanized anti-CD33 (calicheamicin)	NR	2/182 (1.1%)	Epitope: calicheamicin/linker	Transient shortness of breath	(47)
Canzutuzumab mertansine (Glaxo)	Humanized anti-CanAg (DM1)	ELISA bridging, domain specificity (detection)	0/57 (0%)	NR	NR	(44,61)
Inotuzumab ozogamicin (Pfizer)	Humanized anti-CD22 (calicheamicin)	NR	3 ^a /118 (2.5%)	NR	NR	(62)
AVE9633 (Sanofi Aventis)	Humanized anti-CD33 (DM4)	ELISA, domain specificity (method NR)	1/54 (1.9%)	Epitope: mAb (no detectable anti-DM4)	NR	(63)
SAR3419 (Sanofi Aventis)	Humanized anti-CD19 (DM4)	ELISA, domain specificity (method NR)	1/38 (2.6%)	Epitope: mAb (no detectable anti-DM4)	None	(64)
MEDI-547 (MedImmune)	Human anti-EphA2 (mcMMAF)	ECL bridging (method NR)	0 ^b /6 (0%)	NR	NR	(65)
MLN2704 (Millennium)	Humanized anti-PSMA (DM1)	ELISA bridging, domain specificity (detection)	0/23 (0%)	Epitope: mAb (no detectable anti-DM1)	NR	(42)

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ADC antibody-drug conjugate, mAb monoclonal antibody, NAb neutralizing antibody, NR not reported, USPI US product insert, ECL electrochemiluminescence, ELISA enzyme-linked immunosorbent assay

^a In the presence of rituximab; may include pre-dose

^b One subject was pre-dose positive, but negative at the end of study

Fig. 1. Immunogenicity assay strategy. Depicted are the potential bioanalytical components of an immunogenicity testing scheme. Screening and confirmatory assays in this case are shown to identify and confirm ADA reactive with the ADC therapeutic. A variety of assay strategies are available to facilitate characterization of both domain specificity and neutralizing activity. Implementation of any of these components should be driven by a robust risk assessment. Reprinted with permission from (66)

competitive binding with the biotherapeutic). Binding antibody-positive specimens can be tested for neutralizing activity in a variety of platforms (including cell-based or ligand binding assays (28)). Inclusion of several tiers of testing works to minimize false positives. As we will discuss, additional tiers of characterization may be implemented to support ADCs (Fig. 1).

While recommendations for ADA assay validation are covered by several excellent manuscripts (5) and guidance documents (1,4), two topics deserve special attention: cut-point determination and confirmation of binding antibody-positive specimens. The assay threshold that serves to distinguish positive from negative specimens, known as the assay cut-point (ACP), is determined statistically through a screen of drug-naïve donor specimens (preferably from patients with the same indication as the study population). An often overlooked issue, however, is the treatment of *biologic* outliers resulting from the ACP experiments. Both biologic and analytical outliers can be identified within the ACP data set (29). Analytical outliers are those whose replicates throughout the experiment are aberrant (across days or among analysts for example, perhaps suggesting that assay errors are to blame), whereas biologic outliers are those that consistently show an atypical response (e.g., a small number of individuals are identified that consistently have high levels of reactivity in the assay). The provenance of this signal is often unknown but should be investigated. Our experience has been that varieties of cross-reactivity may

increase as modalities become more complicated (such as inclusion of a small-molecule drug in a mAb). The decision to include biologic outliers within the ACP data set has implications for both assay parameters and study support. Exclusion of these individuals will result in a lower cut-point, thereby increasing the apparent sensitivity of the assay. However, since these varieties of specimen may be encountered during study support, the other consequence is likely to be an increased number of *pre-dose positive* specimens. Thoughtful ADA immunoassay validation requires that one should investigate and be aware of the consequences of biologic outliers in the ACP calculation.

Confirmation of binding antibody-positive specimens is frequently accomplished through a competitive binding or competitive inhibition assay, whereby addition of unlabeled therapeutic ablates specific ADA-derived signal. A confirmatory cut-point (CCP) is determined in a manner analogous to the ACP experiments described above, resulting in a threshold ($X\%$ inhibition) above which demonstrates a specific ADA-derived signal and below which results in an ADA-negative specimen. An important investigation is to examine the relationship between signals resulting from the screening and confirmatory assays. The assumption that the two assays are behaving in an orthogonal manner and that the confirmation truly results in the removal of false positives from the screening assay should be empirically determined (30). Importantly, one bioanalytical approach for domain characterization of ADA (see “ADA DOMAIN SPECIFICITY

STRATEGY”) relies on competitive inhibition. Understanding the analytical noise among competitive inhibition assays with various ADC components is essential if results of those assays will be used in concert to define the specificity of an ADA.

In addition to the preceding points on ADA assay validation, there are unique concerns for bioanalytical reagents that support an ADC immunogenicity assay (these add to existing discussions of bioanalytical critical reagent characterization, qualification, and storage (31,32)). As with any ligand binding assay, the biophysical properties (including charge, hydrophobicity, and solubility) and conformation of critical target reagents have profound effects on assay characteristics. Labeling and choice of assay buffers have been proven relevant for the use of mAbs as reagents in routine ADA immunoassays and are more critical now that the protein therapeutic in question has already been through one round (or more) of conjugation to a small-molecule drug (see (33,34) for examples of the consequences of conjugation on mAb biophysical properties). For example, if both conjugation and labeling chemistry utilize amine groups of lysine residues, the net positive charge of the doubly labeled ADC reagent may be reduced. The solubility of these reagents is more likely to differ from the parent mAb when the additional “label” is a hydrophobic biotin molecule. Furthermore, several tiers of modification may alter the immunologic reactivity of critical reagents. Sites for conjugation within the complementarity-determining regions (CDRs) may be more likely to be modified, and the ability of critical reagents to detect ADA that target these regions (likely to be NABs) could be compromised by high levels of labeling. Analytical techniques that probe the biophysical parameters of the therapeutic and variants thereof (size-exclusion chromatography to detect aggregation and capillary isoelectric focusing for charge) should be applied to identify issues early in assay development (35), allowing for optimization of labeling and assay buffers. Likewise, an SPR qualification step can demonstrate that immunologic reactivity of critical ADA reagents is similar to the parent molecule (within the scope of whatever surrogate controls are available).

Finally, to improve the drug tolerance of ADA assays, acid pretreatment is often utilized in both colorimetric ELISA and MSD screening and specificity ADA immunoassay methods (36). Complete understanding of the linker chemistry is essential here, as ADCs may incorporate acid-sensitive linkers. If acid is used for pretreatment, the sample must be completely neutralized prior to incubation with labeled ADCs to maintain the integrity of critical reagents.

ADA DOMAIN SPECIFICITY STRATEGY

For ADCs, domain specificity characterization may be a useful addition to the immunogenicity monitoring strategy (24,37). A variety of forms of the ADC may be generated *in vivo*. For example, variants derived from ADCs, based on maleimide adducts have been described (38–40). Reactivity of ADA for all three structural components of the ADC (mAb, linker, and small-molecule drug) can be determined. Importantly, screening and confirmatory assays for detection of binding ADAs should utilize the entire ADC molecule, thus allowing ADA with specificity for any component of the

intact ADC to be identified. The decision to include additional domain specificity assays (and the inclusion of any particular components (linker or linker/small-molecule drug for example)) should be based on a thorough risk assessment. Given the need to generate novel reagents, often specificity is determined against the antibody *vs.* linker/cytotoxic drug.

Two approaches for determination of domain specificity are available. In a competition strategy, samples are incubated with separate ADC domain(s) in the confirmatory portion of the screening assay (41). Ablation of specific signal during competition with unlabeled ADC components (mAb or cytotoxic drug) indicates domain specificity. An alternative strategy is a direct detection method, whereby specific ADC components are used for capturing and/or detecting ADAs directed against a certain domain (42–44). Use of a single structural component to determine specificity may be inconclusive: a negative result infers but does not test specificity to domains that were not included (41).

Both competition and direct detection strategies will provide informative data regarding domain specificity, but there are pitfalls associated with either approach. For example, the competition strategy is capable of identifying “haptenic group” reactivity (*i.e.*, the linker/small-molecule drug in a particular mAb context), which is only apparent by differences in signal strength among the various detection assays. In contrast, an advantage of the detection approach is its sensitivity for minor components of a polyclonal antibody response. Hoofring *et al.* demonstrate that with surrogate controls, detection of a *minor* anti-linker/toxin or anti-mAb ADA (in the presence of 300-fold or greater molar excess of ADA with specificity for the alternative domain) was only possible with a direct detection strategy (43). Whether antibodies that represent a minor variant of the anti-ADC response are of clinical significance remains undetermined. If meaningful minor anti-ADC responses occur in a large subset of patients that seroconvert to ADA positive (in a screening format assay), then associations between ADA status and clinical consequences may still be identified. However, if the same minor anti-ADC responses are infrequent, then a valuable clinical insight (and its corresponding bioanalytical marker) may be overlooked.

As noted previously for screening and confirmatory ADA techniques, there are unique bioanalytical requirements for domain characterization assays. These approaches require generation of specialized control reagents. Surrogate positive controls generated through adjuvant-assisted immunization campaigns may contain immunoreactivity against all the necessary components of the molecule. Unfortunately, the nature and frequency of biotransformation events may differ between nonclinical model species and what occurs in the clinic. In order to account for the basic possibilities, mAbs specific for the linker, small-molecule drug, or both will prove to be beneficial for assay development. Two technical points are relevant for direct detection assays: first, an unrelated carrier protein such as bovine serum albumin (BSA) allows detection of ADA with reactivity specific for the linker and small-molecule drug (and distinguished from reactivity towards haptenic groups). Second, assay buffers should include excess carrier protein to inhibit the detection of nonspecific antibodies against the carrier.

PRECLINICAL MONITORING STRATEGIES

Regulatory agencies provide guidance for preclinical immunogenicity assessment. Briefly, immunogenicity assessment should be implemented when there is evidence of altered PD activity, unexpected changes in exposure in the absence of a PD marker, or immune-mediated reactions (45). As a consequence, ICH S6 R1 recommends collection of ADA specimens, such that an analysis of ADA can be implemented to facilitate interpretation of a preclinical study.

Preclinical ADA results for ADCs have been reported in several studies to date. While the incidence of immunogenicity in a preclinical model is not predictive of clinical immunogenicity, general principles of ADC immunogenicity (*e.g.*, role of linker/small-molecule drug) and study interpretation may both benefit from this support. Carrasco-Triguero and colleagues report that an ADA titer greater than 4 (log scale) resulted in decreased exposure for a model trastuzumab ADC in cynomolgus monkey (41). In contrast, an ADA incidence of 4.2% (eight out of 190) was reported in ado-trastuzumab emtansine (T-DM1)-dosed cynomolgus monkeys across eight preclinical studies, with no impact on pharmacokinetics (PK) (41). These results (among others (20)) for trastuzumab confirm that the particular linker and small-molecule drug may contribute unique immunogenicity properties. Hoofring *et al.* also demonstrate that ADA in a rat preclinical study can impact PK (43). This study also used a direct detection domain specificity approach to show that most individual animals developed immunoreactivity to both the mAb and the linker/small-molecule drug component of the ADC.

CLINICAL IMMUNOGENICITY FOR ADCS

There is limited clinical data available describing the incidence and impact of immunogenicity for ADCs. We have compiled examples in Table I. For products currently on the market, the ADA incidence ranges from 5.3% for ado-trastuzumab emtansine (41) to approximately 37% for brentuximab vedotin (46). For gemtuzumab ozogamicin, a total of 182 patients were evaluated for immunogenicity, of these, only two tested positive (both developed antibodies to the calicheamicin/linker portion) (47). Immunogenicity has not been reported in pediatric patients treated with gemtuzumab ozogamicin (48). In each of these cases, an analysis of safety-by-antibody status was conducted. The incidence of infusion reactions was greater in patients with anti-brentuximab vedotin ADA, transient shortness of breath was associated with anti-gemtuzumab ozogamicin ADA (in a single patient), and no clinical sequelae were associated with the development of ADA reactive with ado-trastuzumab emtansine.

MECHANISMS OF IMMUNOGENICITY

Immunogenicity is the result of factors intrinsic to the biotherapeutic as well as extrinsic ones (introduced in the “**RISK ASSESSMENT**” section above). A variety of molecular and immunologic techniques can be applied to determine whether and how conjugation of a small-molecule drug to a mAb impacts immunogenicity. The use of chemical haptens

as a tool to probe the cellular and molecular basis for an antibody response was pioneered in 1903 by Obermayer and Pick (with nitrated serum proteins), and the foundation for modern immunology was established through elegant experiments with these tools [reviewed in (49)]. It has recently been argued, however, that a T cell-dependent anti-hapten antibody response may have different requirements for innate immune signals. Building on mouse studies showing anti-DNP antibody responses in the absence of innate immune signaling modules (50), Palm and Medzhitov demonstrate that several model-haptenated proteins are uniquely immunogenic and do not require TLR signalling (51). The immunogenicity demonstrated in these murine models may be enhanced by the foreign nature of the protein component (OVA, HSA, or KLH) or the use of adjuvants (and is not in keeping with the immunogenicity determined to date for ADCs). However, these works do suggest that immune mechanisms behind an anti-hapten response may be unique, and allow for potential immunogenicity that differs between an ADC and an unconjugated mAb.

In order for any protein (including ADCs) to stimulate a T cell-dependent antibody response, the protein needs to be taken up by antigen-presenting cells and cleaved by proteolytic enzymes in the cell into peptide fragments. These peptides can then be loaded onto MHC class II molecules, which are restricted in the repertoire of peptides they can bind. Finally, MHC-peptide complexes potentially can be recognized by T cells. The analysis of T cell antigenicity therefore includes the following: (i) the potential of peptide sequences to bind to MHC, (ii) whether such sequences are actually processed and presented in antigen-presenting cells, and (iii) whether the presented MHC-peptide complexes have the actual ability to stimulate T cells to potentially provide T cell help for antibody response to the protein.

For ADCs specifically, the question arises: how does introducing the linker and small-molecule drug into the antibody affect T cell recognition of the resulting ADC? Linear amino acid sequences that carry linkers and small-molecule drugs could become novel T cell epitopes. Such events will be very dependent on the size and type of the linker/small-molecule drug and its degradation products. This means that sequences in unconjugated form may not be recognized by T cells but could become recognizable when conjugated, because the haptenated peptide is now directly recognized by relevant T cell clones. This phenomenon has been demonstrated in the case of site-specific modifications of murine TNF- α or EGF with unnatural amino acids (*p*-nitrophenylalanine, sulfotyrosine, or 3-nitrotyrosine) (52), where CD4 T cells specific for mutant residues mediate induction of a polyclonal IgG response reactive with the otherwise “self” protein. In addition, conjugation of a mAb to the linker and small-molecule drug could alter antigen presentation.

Peptides available for MHC binding are generated by proteolytic cleavage of their source proteins. Such cleavage is dependent upon enzyme recognition of sequences adjacent to the cleavage site. Therefore, if a sequence is altered materially through the introduction of a linker/small-molecule drug, the presentation of sequences that are in fact upstream and downstream of the conjugation site can be affected. As a result, self-protein sequences (including human

antibody germ line sequences) have the potential to be recognized as foreign, *as a consequence of occurring in a conjugated protein entity alone*. Observations related to this possibility have been made in murine models, where modifications in protein conformational stability (53) or susceptibility to lysosomal proteolysis (54) have been shown to contribute to immunogenicity. Another example is the conjugation of a hen egg lysozyme (HEL) to phosphorylcholine. In HEL-transgenic B6 mice, immunization with PC-HEL (but not HEL) results in a robust antibody response that depends on CD4 T cells. The quantity and variety of HEL-derived peptides that are presented by antigen-presenting cells (APCs) is enhanced by conjugation to phosphorylcholine (55). Human antigen presentation assays have also been used to demonstrate that the repertoire of presented peptides can be altered by protein modifications (results from ProImmune Limited, N. Schwabe, personal communication). Typically, the ADC or unconjugated antibody is co-cultured with APCs in culture, such as monocyte-derived dendritic cells. Once full maturation of the dendritic cells is induced after protein loading, cells are lysed and MHC-peptide complexes are recovered in an MHC-specific immunoprecipitation step. Peptides are then separated from MHC molecules and sequenced in high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS).

In summary, there is a body of immunology research that relies on haptenated proteins as model antigens. This work suggests that the linker and small-molecule drug components of an ADC have the potential to form novel T or B cell epitopes. Recent efforts suggest that the immune response against haptenated proteins may differ from unconjugated ones (reflected in different requirements for co-signalling). Some of those differences may be due to the biophysical alterations that accompany conjugation of linker and small-molecule drug to a mAb, resulting in altered antigen presentation. As a consequence, both the antigenicity and immunogenicity of any conjugated protein is influenced by site of conjugation and the chemical moiety itself (as demonstrated by the results discussed previously in the “[PRECLINICAL MONITORING STRATEGIES](#)” section (20,41)). Our expectation is that these evolving topics will be considered during a risk assessment that accompanies the development of a clinical immunogenicity strategy for each ADC.

DISCUSSION

Administration of any biotherapeutic can result in an immune response reactive with the therapeutic. An immunogenicity strategy is developed based on a risk assessment. Both sample collections and *in vitro* assays (possibly including screening, confirmatory, and characterization) are designed to inform clinicians of the likelihood and consequences of an ADA response. This immunogenicity approach has been developed with recombinant protein therapeutics (enzymes, cytokines, Fc fusions, and mAbs) and is being applied to other modalities (peptides (some modified ex: lipidated): lixisenatide, peginesatide, teduglutide, liraglutide, and oligos: mipomersen sodium). We have sought to highlight how the unique structural components of ADCs complicate immunogenicity assessment.

The cellular and molecular mechanisms by which immunogenicity occurs may differ for ADCs from monoclonal antibody therapeutics. In addition, there are outstanding questions with regard to the clinical consequences of immunogenicity for ADCs. Among those are whether there are unique consequences of ADA-ADC immune complexes (as discussed in the “[RISK ASSESSMENT](#)” section). This is an issue that will require a careful analysis of immunogenicity data and clinical safety outcomes.

The role of previous exposure (and potential for cross-reactivity among neutralizing antibodies) has been investigated for IFN beta-1a and beta-1b (56,57). The related possibility for ADCs is sensitization, whereby prior exposure to mAb, linker, or small-molecule drug could result in altered immunogenicity or consequences thereof. Sensitization would require that mAb, linker, or small-molecule drug components are antigenic (recognized by either an immunoglobulin molecule (B cell) or a T cell receptor (T cell)) themselves. This may occur for portions of the mAb, and immunoreactivity towards the mAb may facilitate epitope spreading to other components of the ADC. In the case of the linker or small-molecule drug, we presume that the risk of antibody cross-reactivity only would apply for identical or highly similar chemical entities (the basis for this presumption lies in experiments demonstrating antibody specificity among haptens of alternative stereoisomers, described in (49)). One example of serious consequences resulting from sensitization is the hypersensitivity observed on dosing with cetuximab. In this case, hypersensitivity responses were associated with preexisting IgE antibodies against a carbohydrate epitope (α -gal) present on the cetuximab heavy chain (58). Whether sensitization is a bona fide concern for ADCs can be determined through immunogenicity monitoring and an analysis of safety that includes both ADA results and prior exposure to related biotherapeutics.

The ADCs that have been approved to date are all in the oncology therapeutic area. However, it may prove that ADCs are a promising and safe platform for other indications such as inflammatory diseases (59) and antibacterial therapy (60). The unique facets of immunogenicity support for ADCs will remain in non-oncology indications, but the risk assessment will have to be modified to reflect the population-specific likelihood (including immune status) and consequences of immunogenicity.

Finally, several domain specificity formats are available (competition or direct detection). Each offers unique advantages (as discussed in “[ADA DOMAIN SPECIFICITY STRATEGY](#)”), and diverse approaches have been followed to assess and describe immunogenicity data for different ADC molecules (see Table I). A recent white paper suggests harmonized definitions and terminology to describe product immunogenicity (2). A similar effort may eventually come about with regard to immunogenicity assessment for ADCs. At present, there are no clinical consequences that have been shown to correlate with ADC immunogenicity domain specificity data. Our hope is that a body of data will become available (through publication of immunogenicity analyses for ADCs) to allow a data-driven discussion of whether the potential safety consequences of an anti-ADC response merit general implementation of these additional assays or whether they should be limited to extraordinary circumstances (such as the sensitization example above).

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Conflict of Interest K.T. is employed by Novartis Pharmaceuticals and declares no competing financial interests. M.B.H. is employed at Amgen and declares no competing financial interests. M.C.T. is employed by Genentech and declares no competing financial interests. N.F.S. is the CEO of ProImmune and declares no competing financial interests.

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