Research Article

Theme: Impact of Immunogenicity on PK/PD of Protein Therapeutics
Guest Editors: Bernd Meibohm and Naren Chirmule

Universal Immunoassay Applied During Early Development of Large Molecules to Understand Impact of Immunogenicity on Biotherapeutic Exposure

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Abstract. Immunogenicity testing during early biotherapeutic development is usually limited by resources needed for assay development, validation, and the necessity for unique product-specific controls and reagents. We describe a unique immunoassay [universal indirect species-specific assay (UNISA)] that can be applied during early phase preclinical studies to support pharmacology, pharmacokinetics (PK), and toxicology evaluation during biotherapeutic antibody candidate assessment. UNISA was evaluated across three animal species: mouse, rat, and cynomolgus monkey. For each species, a unique and specific antibody pair was generated consisting of the secondary antibody and the positive control. The secondary antibody is specific for species anti-IgG antibody while demonstrating no cross-reactivity to human antibody-based biotherapeutics. The positive control is comprised of a species-specific anti-human IgG antibody clone specific for binding to the CH2 domain of all human IgG subtypes. Applications of this platform included: (a) identifying the dose with the least immunogenicity risk; (b) characterizing the impact of immunogenicity on PK exposure profiles across multiple antibody candidates and dose regimens; and (c) characterizing the immune response specificity to the idiotype or non-idiotypic region of the biotherapeutic candidate. Due to its use of universal species-specific reagents, UNISA can overcome resource constraints and avoid extensive validation and development time to support immunogenicity testing during the early research and preclinical phase of programs. Enhanced understanding of the impact of the immunogenicity on biotherapeutic exposure and target-related immunomodulatory effects have been made possible with the use of this assay.

KEY WORDS: anti-drug antibody (ADA); immunogenicity; impact; pharmacokinetics (PK); validation.

INTRODUCTION

The early drug development process requires selection of the most suitable protein-based biotherapeutic candidates through several steps of molecule assessment. In order to identify the next target-specific biotherapeutic antibody clone that has the highest probability of commercial success, several

antibody clones are screened at this early stage. The processes used for selection of these molecules may include several critical quality attributes, such as: structure-function analyses, identification of unwanted toxicities, manufacturability, potency, immunogenicity, and evaluating efficacy and safety (1–6). Administration of biotherapeutics in animals can elicit an immune response against the drug (anti-drug antibodies; ADA) which may impact study measurements like pharmacokinetics (PK), pharmacodynamics (PD), or safety. During early biotherapeutic optimization, implementing a proactive immunogenicity assessment strategy can assist in interpretation of PK, PD, or safety findings in subsequent animal studies.

There are several outcomes of a biotherapeutic ADA response that can confound the interpretation of the PK exposure profiles (7–9). Abnormal clearance of the biotherapeutic can be attributed to either a target-mediated clearance or to an undesirable immune response to the biotherapeutic. Furthermore, abnormal clearance of the biotherapeutic in the circulation can be attributed to either clearing or sustaining antibodies that complex with the biotherapeutic and either shorten or prolong the presence of the drug, respectively. The ability to distinguish immune-mediated clearance from target-mediated uptake enables a better understanding of the exposure of the biotherapeutic. Hence, immunogenicity assessment can

Animal Use and Assurances All animal experiments were conducted in full compliance with local, national, ethical, and regulatory principles and local licensing regulations, per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care International's expectations for animal care and use/ethics committees.

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be triggered in the instance where an abnormal biotherapeutic clearance event or excessive immune response associated pathology needs to be addressed (8).

During early development of a biotherapeutic antibody, ~5–10 antibody candidates may be assessed for their immunogenic potential in clinic using sequence-based *in silico* methods (10). In preclinical studies, if the PK or PD of a dosed biotherapeutic is unexpectedly impacted, evaluating for the presence of ADA may be useful in understanding target-mediated *vs* immune-mediated clearance in the animals. Current bioanalytical methods for measuring ADA levels include assessment of binding and neutralizing antibodies (11,12). The validation of such assays requires availability of specific reagents to the biotherapeutic antibody candidate, such as polyclonal and/or monoclonal positive control antibodies, negative control sera, and conjugated biotherapeutic antibodies. Significant time prior to development of these assays is required to generate and characterize the biochemical and biophysical criteria of these critical reagents (13–17). We have developed a Universal Indirect Species-Specific Immunoassay (UNISA) to support the impact assessment of immunogenicity on associated PK, PD, or safety findings during early stage preclinical studies, while eliminating the resource-intensive factors associated with traditional assays.

MATERIALS AND METHODS

Chemical and Reagent Preparation

Serum Samples

Batches of pooled normal mouse (BALB/C, C57BL/6, and CD-1 strains), cynomolgus monkey (*Macaca fascicularis*), and rat (Sprague Dawley) serum were obtained for use as assay matrix from Bioreclamation, Inc., Hicksville, NY. Test samples were obtained from Amgen-sponsored preclinical, non-GLP studies, where serum was collected according to the individual study protocols and in adherence with animal care and use/ethics committees.

General Serum Collection Process

Blood was collected in Microtainer® serum separator tubes at protocol-driven time points and maintained at room temperature following collection. Following a 30–40-min clotting period, samples were centrifuged at 2–8°C at 11,500 rpm for approximately 10 min using a calibrated Eppendorf 5417R Centrifuge System (Brinkmann Instruments, Inc., Westbury, NY). The collected serum was then transferred into a pre-labeled, cryogenic storage tube and stored at –60°C to –80°C for future analysis.

Primary Antibodies (Biotherapeutic Candidates)

The primary biotherapeutic antibodies used were either fully human monoclonal antibodies (hMab) or their rat surrogate (rMab). All primary biotherapeutic antibodies used were generated by Amgen Inc. and are proprietary materials.

Species-Specific Positive Control Antibodies—ADA Immunoassay

Affinity-purified, mouse monoclonal anti-human immunoglobulin (IgG) was produced internally at Amgen. This antibody is directed against an epitope on the CH2 domain of human IgG and can thus bind across all hMab subtype and Fc-containing peptibody-based biotherapeutics containing this region. A cynomolgus monkey IgG1 and rat IgG1 constant region (Fc) was then grafted onto the mouse variable region of this specific antibody clone. The resulting chimeric antibodies were protein A purified. The final product yielded a mouse anti-human IgG/rat IgG1 chimeric monoclonal antibody and mouse anti-human IgG/cynomolgus monkey IgG1 chimeric monoclonal antibody (Amgen Inc.). All three antibodies are specific for human IgG with no cross-reactivity to mouse, rat, or cynomolgus monkey serum. All positive control antibodies used in the ADA immunoassay were generated by Amgen Inc. and are proprietary materials.

Species-Specific Secondary Detection Antibodies—ADA Immunoassay

Goat anti-mouse IgG (Fc specific) (Sigma), rabbit anti-rat IgG (whole molecule) (Sigma) and mouse anti-cynomolgus monkey IgG (whole molecule) (Texas Labs) were the three species-specific secondary antibodies used in all experiments to detect specific mouse, rat, and cynomolgus monkey ADA, respectively. All three detectors were confirmed to have no cross-reactivity with human IgG or other non-specific IgG (e.g., rat detector does not cross-react with mouse IgG). The labeling of secondary detection antibodies with ruthenium was performed as described previously (18).

Table I highlights the qualified antibody pairs (positive control and secondary detector) and the overall key assay performance characteristics per species used to support all programs. Additional details on the UNISA method, the assay performance characteristics and related validation information can be obtained from the “[Electronic Supplementary Material](#)”.

PK Immunoassay

To measure the biotherapeutic antibody serum sample concentrations, the following method was used: ½ area black plate (Corning 3694, Corning, NY) was coated with 2 µg/ml of mouse anti-hu Fc, monoclonal antibody AA (in-house) in PBS and then incubated overnight at 4°C. The plate was then washed and blocked with I-Block™ (Applied Biosystems) overnight at 4°C. If needed, samples were diluted in pooled normal species serum. The standards, QCs (quality control samples), and samples were then diluted 1: 20 in 1X PBS +1 M NaCl+0.5% Tween 20 and 1% BSA buffer (5% serum). The plate was washed and 50 µl of diluted standards, QCs, and samples were transferred into an antibody AA coated plate and incubated for 1.5 h at room temperature. The plate was washed and then 50 µl of 100 ng/ml mouse anti-hu Fc monoclonal antibody BB-HRP conjugated (in-house) diluted in I-Block™+5% BSA was added and incubated for 1.5 h. The plate was washed, then 50 µl of Pico substrate (Thermo Fisher, Rockford, IL) was added, after which the plate was immediately analyzed with a SpectraMax 384 Plus

Table I. UNISA Critical Antibody Pairs and Their Associated Key Performance Attributes for Monoclonal Antibodies (Human or Species IgG)

Test sample specifics	Mouse serum	Rat serum	Cynomolgus monkey serum
Antibody 1: Positive control (Amgen) ^a	Mouse anti-human IgG clone A	Rat anti-human IgG chimera ^b	Cyno anti-human IgG chimera ^c
Antibody 2: Secondary detector (Vendor, Catalog No.)	Goat anti-mouse IgG Fc (Sigma, M3534)	Rabbit anti-rat IgG (whole molecule) (Sigma, R3756)	Mouse anti-cyno IgG Fc (Texas Labs)
Sensitivity (ng/mL) ^a	24	64	26
Biotherapeutic tolerance (μg/mL) ^a	760	440	173
ADA to biotherapeutic ratio ^a	1:1,520	1:880	1:288
Secondary specificity	IgG1, IgG2a, IgG2b	IgG1, IgG2a, IgG2b	IgG1, IgG2, IgG3 (IgG4 weak)

Sensitivity was determined within each species by running an eight-point SS-positive control dose–response curve (7.8 to 1,000 ng/mL) in pooled SS-serum and then extrapolating the concentration corresponding to a *S/N* ratio of 1.5 on GraphPad Prism v5.04. Biotherapeutic tolerance was determined within each species by running an eight-point dose–response curve (7.8 to 1,000 μg/mL) of a fully human monoclonal antibody in pooled SS-serum containing 500 ng/mL of SS-positive control antibody. The concentration corresponding to a *S/N* of 1.5 was then interpolated using GraphPad Prism v5.04. The ADA to biotherapeutic ratio takes into account the molar equivalence of the two. For mouse and rat, the secondary detector specificity was determined by coating a bare MSD plate with the isotype/subclass and incubating with the secondary detector at a static concentration. For cynomolgus monkey, a biosensor surface was coated with the detector and the isotype/subclass was evaluated for binding reactivity. Parameters not qualified for surrogate antibody candidates. *S/N* signal to noise ratio, *ADA* anti-biotherapeutic antibody, *SS* species-specific

^a Specific for human IgG

^b Mouse anti-human IgG clone A/ Rat IgG1 Fc engineered chimeric antibody

^c Mouse anti-human IgG clone A/ Cyno IgG1 Fc engineered chimeric antibody

Luminometer (Molecular Devices, Sunnyvale, CA). The sample concentration was calculated with the Watson (v7.0.0.0.1; Thermo Fisher Scientific) data reduction package using a five-parameter logistic algorithm with 1/*Y* weighting.

RESULTS

Impact of Dose on the Immunogenic Potential of a Biotherapeutic

Case Study 1. Study and analysis details are captured in Table II. A correlation of the dose administered to the magnitude of ADA response was observed (Fig. 1a). Animals

in the mid-dose group (1 mg/kg) demonstrated an overall mean signal to noise (*S/N*) response of 38.87 by day 21. In addition, a visible impact on the PK exposure was observed for the mid-dose group, demonstrated by a diminished level of the biotherapeutic antibody clone by day 14 (Fig. 1b). In contrast, the high (25 mg/kg) and low dose (0.04 mg/kg) groups had significantly lower average *S/N* of 6.08 ($p < 0.005$) and 2.29 ($p < 0.005$), respectively. In the high-dose group, the low levels of the detected ADA decreased the detectable levels of biotherapeutic clone 1 but did not eliminate it entirely. The lower detectable ADA in the high-dose group could indicate “the dosing over or induction of tolerance” phenomenon implying that high drug levels were able to eliminate the impact of ADA. For the low-dose group, no impact of ADA on PK was observed.

Table II. Case Study Design

Case study	Study species	<i>N</i>	Biotherapeutic: group/dose regimen	PK time points (nominal day post-dosing)	ADA time points (nominal day post-dosing)
1	Mice (strain: BALB/C)	30 (10/group)	rMab IgG1: three groups: clone 1 dosed at 0.04, 1, or 20 mg/kg, single, IV	Prestudy, 3, 7, 14, 21	14, 21
2	Mice (strain: C57BL/6)	12 (3/group)	hMab IgG2: four groups: clones 2.1, 2.2, 2.3, or 2.4 dosed at 5 mg/kg, Single, IV	Prestudy, 0.003, 0.2, 0.4, 0.8, 0.16, 1, 2, 3, 4, 7, 14, 21, 28	14, 21, 28
3	Cynomolgus monkey	30 (6/group)	hMab IgG2: five groups: clones 3.1, 3.2, 3.3, 3.4, or 3.5 dosed at 4 mg/kg, single, IV	Prestudy, 0.02, 2, 4, 8, 10, 14, 21, 28, 35, 42, 49	Prestudy, 14, 21, 28
4	Cynomolgus monkey	6	hMab IgG2: one group: clone 3.6 dosed at 5 mg/kg, weekly, IV	N/A	Prestudy, 7, 14, 28, 41

rMab rat monoclonal antibody, *hMab* human monoclonal antibody, *PK* pharmacokinetic, *ADA* anti-biotherapeutic antibody, *D* day, *S/N* signal to noise. * $P < 0.0005$, significantly different

Fig. 1. Case study 1: Impact of biotherapeutic dose; dose-dependent immune-mediated clearance observed. **a** Impact of dose on average ADA level at day 21 where the mid-dose group (1 mg/kg) demonstrated a more robust immune response than the low- and high-dose groups. **b** Antibody-mediated clearance of the circulating biotherapeutic serum concentrations demonstrated by day 14 for the mid-dose group. Note: For all case studies, standard deviation *error bars* reflect the biological variability observed in the animal population and not necessarily the analytical variability, magnitude of the ADA response is proportional to the *S/N* level, and study details can be found in Table II

Distinguishing Multiple Biotherapeutic Antibody Clones

Case Study 2. Study and analysis details are captured in Table II. A significant and robust average ADA response in all animals was associated with clone 2.1 compared to clone 2.3 ($p < 0.005$) and clone 2.4 ($p < 0.005$) by day 28 (Fig. 2a). Clone 2.1 was associated with a mean *S/N* response of 12.44 and 34.31 by days 21 and 28, respectively, and demonstrated antibody-mediated clearance in the PK exposure profile (Fig. 2b). ADA response for clone 2.2 was lower in magnitude than clone 2.1, and observed in only three of the nine animals. No impact on the PK exposure was evident for this clone. In contrast, clones 2.3 and 2.4 were ADA negative in all samples tested. The PK exposure profile for clone 2.4 was atypical, suggesting potential target-mediated clearance and not immune-mediated clearance.

Case Study 3. Study and analysis details are captured in Table II. A robust immune response (greater than a *S/N* of 10) was observed with antibody clones 3.1, 3.2, 3.3, and 3.5 in all animals tested ($N=6$ animals per clone; Fig. 3a) and visible impact on the biotherapeutic serum concentration was observed (Fig. 3b). The clone 3.2 even though had a relatively lower magnitude of ADA (*S/N* of 16.91 when compared to other clones), but was still a significant response above the assay cut point and impacted the PK. For clone 3.3, early onset of a robust

immune response in all six animals was detectable with an average *S/N* of 52.48, 274.65, and 456.49 at days 14, 21, and 28 respectively. Antibody-mediated clearance on the PK exposure profile for this clone was demonstrated by day 14, where the area under the curve (AUC) was 9,770 $\mu\text{g h/mL}$ as compared to clone 3.4 with an AUC of 12,700 $\mu\text{g h/mL}$. Unlike the other clones assessed, clone 3.4 triggered a slower onset of an immune response with a lower incidence (2/6 animals by day 28 were positive for ADA) and magnitude. However, clone 3.4 demonstrated the least impact on the average PK exposure profile.

Clones 3.3 and 3.4 were further analyzed by individual animals as they represented the shortest and longest biotherapeutic exposure profiles. Results demonstrate the inverse relationship between strength of the immune response and biotherapeutic exposure. At day 14, all animals demonstrated a robust ADA immune response (10 to 100 *S/N*) with consequent impact on the PK. In contrast, only one animal dosed with clone 3.4 showed an ADA response greater than 10 and visible impact on PK (Fig. 4). By day 21, all animals dosed with clone 3.3 demonstrated an increased magnitude of ADA (100 to 1,000 *S/N*) and a loss of exposure to the biotherapeutic. However, for clone 3.4, a gradual increase in ADA magnitude was observed in two animals, coinciding with diminished exposure. By day 28, a persistent ADA response was observed for all animals dosed with clone 3.3 and in 3 animals dosed with clone 3.4 (*S/N* above 10) with visible impact on PK. Overall clone 3.3

Fig. 2. Case study 2: antibody clone-specific ADA impact on biotherapeutic serum concentrations for target 2. **a** Clone 2.1 has significantly higher average *S/N* response as compared to clones 2.3 and 2.4 by day 28. **b** Antibody-mediated clearance demonstrated on the average PK exposure profiles for clone 2.1. See additional “Note” in Fig. 1

Fig. 3. Case study 3: Rank ordering antibody clone specific ADA impact on PK profiles for therapeutic target 3. **a** Day 28 ADA analysis shows that dosing with clone 3.3 induces the strongest immune response. **b** Average PK exposure profiles confirm antibody-mediated clearance for clone 3.3. Interestingly, abnormal clearance was observed in clone 3.2 which had the lowest average ADA S/N response, while the average exposure was not impacted for clone 3.4, where two of the six animals demonstrated ADA greater than 100 S/N by day 28. See additional “Note” in Fig. 1

demonstrated a high incidence, early onset of a robust immune response which lead to the loss of biotherapeutic exposure by day 21. In contrast, clone 3.4 elicited a lower ADA incidence rate with a slower onset. Low levels of measurable ADA (10 S/N and below) could not be associated with any direct impact on biotherapeutic serum levels.

Mapping Specificity of the Immune Response

Case Study 3 (Continued). Study and analysis details are captured in Table II. The specificity analysis involved depletion with a relevant or irrelevant antibody hence confirming the ADA response as idiotype (ID) specific for all clones except clone 3.5, where the ADA reactivity was predominantly directed against the non-ID region of the antibody (Fig. 5a). It was notable that by day 49, the impact on exposure (measured by the AUC) was not as profound for the non-ID-specific ADA (clone 3.5, AUC of 13,200 $\mu\text{g h/mL}$) as compared to the ID-specific ADA (clone 3.3, AUC of 9,790 $\mu\text{g h/mL}$).

Case Study 4. Study and analysis details are captured in Table II. Of the six animals analyzed, 33% were positive by day 14, 66% by day 21, and 100% by day 28 for ADA in the UNISA (Fig. 5b). None of the animals demonstrated non-ID-

specific antibodies, demonstrating high specificity of the immune response to the ID region of clone 3.6.

DISCUSSION

Several attributes are taken into consideration during the selection process of a biotherapeutic candidate during early biotherapeutic development. These may include *in vitro* potency, target binding, safety, functional assessment, and selectivity, assessed primarily in studies conducted in animal models (2–4,19). Utilizing the UNISA, immunogenicity testing can be performed *ad hoc* if the study PK, PD, or toxicology findings warrant such analyses. The authors would like to note that preclinical immunogenicity does not necessarily reflect the outcome in the clinic (20,21). However, having the necessary information on the immunogenic potential of a molecule during early development can help explain abnormal biotherapeutic exposure profiles in preclinical species by distinguishing target vs ADA-mediated clearance and providing information on sustaining vs clearing antibodies. This strategy supports a quality by design approach to biotherapeutic development, whereby understanding the risk and associated impact of immunogenicity across antibody clones early on, enables proactive informed decisions related to program progression.

Fig. 4. Case study 3: Inverse correlation of ADA to PK response observed. Clone 3.3 and 3.4 represent the shortest and longest biotherapeutic exposure profiles respectively in individual animals across 3 time points. An early onset of the immune response and the overall magnitude (S/N of above 10) directly correlate to a diminished PK response as compared to low (S/N of 10 and less) or ADA negative animals. Observe that, early onset of the immune response and the overall magnitude directly correlate to a diminished PK response as compared to low (ADA response less than 10) or negative ADA animals. Dashed line represents a response of 10 or below. See additional “Note” in Fig. 1

Fig. 5. Case studies 3 and 4: Application of UNISA to screen and differentiate idiotypic (ID) *versus* non-ID-specific immune response. **a** All animals dosed with clone 3.5 elicited a robust non-ID-specific immune response confirmed by depletion with irrelevant biotherapeutic (or Fc). By day 28, all animals dosed with clones 3.1, 3.2, and 3.3 were positive for the presence of ID-specific ADA. Interestingly, two out of the six animals dosed with clone 3.4 elicited an immune response mapped to both anti-ID and non-ID specificity, representing a maturation of the immune response. **b** All animals dosed with clone 3.6 demonstrated a robust anti-ID-specific immune response by day 42. Non-ID-specific antibodies were not detected. UNISA (universal indirect species-specific assay). See additional “Note” in Fig. 1

The advantages of the UNISA include (1) the use of a generic species-specific detector that binds to any antibody of that individual species and eliminates the need for biotherapeutic specific detection reagents for each candidate, (2) the specific detection of antibodies directed to any region of the biotherapeutic, and (3) ease of assay performance and maintenance.

We have shown that immunogenicity testing utilizing the UNISA format can (1) provide understanding of the biotherapeutic dose that will have minimal immunogenicity and impact on exposure, (2) help characterize the impact of immunogenicity on PK exposure profiles across multiple

doses and antibody clones, and (3) map the immune response specificity to the ID or non-ID region of the biotherapeutic candidate. As evident from case study 1, a single dose at 1 mg/kg of the biotherapeutic was highly immunogenic; hence during design of future studies, a higher dose could be evaluated to dose over the immune response. Case studies 3 and 4 demonstrate that UNISA could map the specificity of the immune response to the ID region of the biotherapeutic, in the absence of a neutralizing bioassay, and differentiate reactivity between the multiple antibody candidate clones. We acknowledge that this immunogenicity profile is not indicative of what may happen in the clinic, but such

Table III. Assessment of Impact of ADA Across Modalities and Biotherapeutic Targets

Biotherapeutic antibody target	Modality	UNISA application
Target 1—case study 1	Rat surrogate monoclonal antibody (Mab) IgG1	Monitor an immunomodulatory molecule and evaluate impact of dose on the immunogenic potential of the biotherapeutic
Target 2—case study 2	Fully human Mab IgG2	Distinguish antibody <i>vs</i> target-mediated clearance to understand abnormal profiles in early PK studies
Target 3—case studies 3 to 4	Fully human Mab IgG1	Assess impact of immunogenicity on PK in support of lead biotherapeutic antibody candidate ranking
Target 4	Fully human Mab IgG1	Monitor an immunomodulatory molecule associated with immune complex-mediated reactions
Target 5	Fully human Mab IgG1	Distinguish antibody <i>vs</i> target-mediated clearance to understand abnormal profiles in early PK studies
Target 6	Fully human Mab IgG1	Evaluate impact of dosing regimen on the immunogenic potential of the biotherapeutic for an intravenous toxicology study (weekly <i>vs</i> twice weekly dosing)

information earlier on can help make informed decisions along the biotherapeutic development process, such as eliminate molecules with ID reactivity by either re-engineering or selecting a back up that has less or no reactivity (1,2).

Overall, the case studies described here (as well as additional programs summarized in Table III) have shown that ADA assessment across early lead antibody biotherapeutic candidates helped understand changes in PK. In addition, with minimum sample volume requirement, UNISA characterized the ADA response specificity through a competitive confirmation step. Thus, the minimal resources required for this assay, can allow the UNISA to become a screening tool and an integral part of the biotherapeutic candidate selection process early on and hence support the quality by design paradigm (22).

CONCLUSION

Biotherapeutics, when dosed in animals, have the potential to elicit an immune response with possible impact on exposure and pharmacodynamic end points. We have developed a universal immunoassay or UNISA that can support immunogenicity assessment in an *ad hoc* mode. The UNISA is a quick and easy platform to assess immunogenicity with limited resource requirements, has the ability to screen multiple antibody candidates within the same test, and map the specificity of the ADA response for its ID or non-ID reactivity. This straightforward approach and implementation of universal critical reagents makes UNISA desirable over traditional bridging or biotherapeutic specific ADA assays that are too resource intensive to be feasible during early stages of molecule development. Utilizing the UNISA to monitor the immune response can help delineate the impact on PK exposure due to antibody *versus* target-mediated clearance and subsequent effects on PD and toxicity. The case studies explored here were driven by requests from the research team leads in response to unexpected PK exposure profiles and are not routinely performed.

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