Research paper

Detection of drug specific circulating immune complexes from in vivo cynomolgus monkey serum samples

Piotr Pieroga, Murli Krishna⁎, Aaron Yamniuk, Anil Chauhan, Binodh De Silva

Novartis Institutes for BioMedical Research, Inc., Cambridge, MA 02139, United States
Bristol-Myers Squibb Company, Princeton, NJ 08543-4000, United States
Division of Adult and Pediatric Rheumatology, St. Louis University, St. Louis, MO 63104, United States

Article info

Article history:
Received 16 September 2014
Received in revised form 13 November 2014
Accepted 13 November 2014
Available online 20 November 2014

Background: Administration of a biotherapeutic can result in the formation of anti-drug antibodies (ADAs). The resulting ADA can potentially form immune complexes (ICs) with the drug leading to altered pharmacokinetic (PK) profiles and/or adverse events. Furthermore the presence of such complexes may interfere with accurate PK assessment, and/or detection of ADA in immunogenicity assays. Here, we present two assays to detect the presence of drug–ADA immune complexes in cynomolgus monkeys.

Results: Serum samples were analyzed for IC formation in vivo. 8/8 tested animals were positive for drug specific IC. Depending on the time point tested 4/8 or 7/8 animals tested positive for ADA during drug dosing. All 8 animals were confirmed positive for ADA during the washout phase, indicating drug interference in the bridging assay. Relative amount of IC over time was determined and its correlation with PK and ADA was then assessed. Multivariate data analysis demonstrates good correlation between signals obtained from the anti-drug and FcγRIIIa based capture assays, although due to its biological characteristic FcγRIIIa based assay captured only a subset of drug specific IC. In one animal IC remained in circulation even when the drug levels decreased below detection limit.

Conclusion: Results from this study indicate the presence of IC during administration of an immunogenic biotherapeutic. Potential application of these assays includes detection of ADA in an IC during high drug levels. The results on the kinetics of IC formation during ADA response can complement the understanding of PK and ADA profiles. Moreover, the presence of IC indicates possible ADA interference in standard PK assays and potential underestimation of total drug exposure in toxicology studies. In addition this study also highlights the need to understand downstream in vivo consequences of drug–ADA IC as no animals under investigation developed adverse events.

© 2014 Elsevier B.V. All rights reserved.

Keywords:
Immunogenicity
Imunoassays
Anti-drug antibodies
Immune complex
Drug specific
Immune complexes

Abbreviations: ADA, anti-drug antibody; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSA, bovine serum albumin; BUN, blood urea nitrogen; CP, cut point; CREA, creatinine; FcγR, Fc-gamma receptor; HQC, high quality control sample; HPLC, high pressure liquid chromatography; HRP, horseradish peroxidase; IC, immune complex; IBA, ligand binding assay; IQC, low quality control sample; mAB, monoclonal antibodies; MALS, multi-angle light scattering; MRD, minimum required dilution; MSD, Meso Scale Discovery; NQC, negative quality control sample; PK, pharmacokinetic; PBS, phosphate based saline; QC, quality control; SEC, size exclusion chromatography; UV, ultra violet.

☆ This research was performed at Bristol-Myers Squibb Company, Princeton, NJ 08543-4000, United States.
⁎ Corresponding author at: Bristol-Myers Squibb Company, Mail Stop L14-07, P.O. Box 4000, Princeton, NJ 08543-4000, United States. Tel.: +1 609 252 5851. E-mail address: murli.krishna@bms.com (M. Krishna).

http://dx.doi.org/10.1016/j.jim.2014.11.007
0022-1759/© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Biotherapeutics are revolutionizing the treatment of many diseases due to multiple advantages of this class of molecules. One of the limitations with this modality of treatment is their ability to trigger an immune response. This entails the innate as well as both the cellular and humoral arms of the immune system that can result in the formation of anti-drug antibodies (ADAs) leading to altered pharmacokinetic profiles, loss of efficacy (Chirmule et al., 2012; Vugmeyster et al., 2012) and in extreme cases hypersensitivity reactions (Brennan et al., 2010).
Immunogenicity against biotherapeutics is mounted against a foreign portion of the protein (Jefferis and Lefranc, 2009; Harding et al., 2010), although immunogenicity towards recombinant human proteins may be a result of breaking self-tolerance (Eckardt and Casadevall, 2003; Schellekens and Casadevall, 2004; Torosantucci et al., 2014). Irrespective of the cause of immunogenicity an immunogenic response leads to the induction of humoral responses in the form of anti-drug antibodies. ADA found in circulation binds to the biotherapeutic resulting in the formation of immune complexes. Formation of immune complexes is the natural response of the immune system designed to neutralize and remove foreign molecules from circulation. Most downstream adverse effects of ADA generally require the formation of an IC intermediate with the drug which in turn mediates Fc mediated clearance, reduced PK, reduced efficacy, immune complex disorders, or altered potency. In extreme cases, formation of ADA may lead to severe adverse events like pure red-cell-aplasia triggered by anti-epoetin alfa antibodies cross reacting with the patient’s own erythropoietin (Eckardt and Casadevall, 2003).

Current state of the art methods in bioanalysis utilizes a bridging assay where a ternary complex or “bridge” is formed between the capture reagent (e.g., biotin-labeled drug), ADA, and the detection reagent (e.g., ruthenium-labeled drug). This format has the ability to detect multivalent molecules such as IgG or IgM antibodies. However, this assay as well as most other assay formats suffer from drug interference, where biotherapeutic drug found in circulation during immunogenicity sampling prevents detection of ADA in the same sample (Hart et al., 2011). This interference is in fact caused by biotherapeutic drug being bound to ADA and several approaches e.g. acid dissociation of the sample, have been developed to minimize this interference (Butterfield et al., 2010).

The issue of drug interference has consequences for establishing clinical rate of immunogenicity which is a regulatory requirement. For example, the reported immunogenicity of adalimumab is 5%, however, true immunogenicity rate is difficult to determine and is dependent on the assay format used (van Schouwenburg et al., 2010). In fact, current data supports that a substantial number of patients develop adalimumab ADA (van Schouwenburg et al., 2013a) of which 99% are neutralizing and lead to loss of efficacy (van Schouwenburg et al., 2013b).

Moreover, anti-adalimumab antibodies were recently reported to be found in small complexes with adalimumab of sizes consistent with IgG dimer using density gradient centrifugation (van Schouwenburg et al., 2013b). Although immune complexes were present in circulation there was no high rate of hypersensitivity reactions reported clinically. According to adalimumab prescribing information hypersensitivity reaction including anaphylaxis was rare, with allergic reactions occurring in about 1% of patients. These results suggest that although formed, not all types of immune complexes will result in anaphylaxis or immune complex disease.

Therefore, when a humoral immune response is generated against a therapeutic protein and whenever the therapeutic molecule is present in circulation, the ADA and the therapeutic protein will be present both separately as well as bound together as an immune complex. In this investigation, in order to detect immunogenicity, we used the hypothesis that immune complexes are present in circulation during an immunogenic response. To investigate this hypothesis we have selected a biotherapeutic molecule lacking an Fc portion in order to take bioanalytical advantage of the Fc portion on the ADA for detection of immune complexes made of drug and ADA. In this study we clearly demonstrated that immunogenicity can be detected not only through the detection of ADA but also through the detection of drug specific immune complexes that are likely to be present in circulation at any time point at which both ADA and biotherapeutic are present, but not necessarily detectable in circulation. Furthermore, the immune complexes formed in circulation had the ability to bind to Fcγ receptors, however, the presence of biotherapeutic drug–ADA IC did not lead to adverse events in non-human primates.

2. Materials and methods

2.1. Biotherapeutic drug

Biologic molecule used in this investigation was an engineered 31 kDa monovalent recombinant human protein. Unlike most biotherapeutics this protein was not engineered on an immunoglobulin backbone and as a result lacked Fc. The preclinical lot of this material was manufactured using a bacterial expression system with standard molecular biology techniques.

2.2. Anti-drug antibody (ADA)

ADA used as a positive control in bridging assay and as ADA component of drug specific immune complexes were generated by hyper-immunizing cynomolgus monkeys with the biotherapeutic drug using standard protocols available with the vendor. Sera were collected at multiple time points and were pooled together. Polyclonal ADA was purified using Protein G sepharose affinity chromatography and was followed by affinity purification on a column with immobilized biotherapeutic drug. Affinity purified ADA was eluted using 0.1 M Sodium Citrate buffer at pH 3.0, then buffer exchanged into PBS and stored at 80 °C.

2.3. Formation of immune complexes

Biotherapeutic drug–ADA immune complexes were formed in PBS buffer by combining both components of the immune complex at twice the final concentration and allowing the binding to proceed for 1 h in a temperature controlled incubator at 24 °C, followed by an equilibration step overnight at 4 °C. Preformed immune complexes were spiked into pooled cynomolgus monkey serum (Bioreclamation) for analysis of immune complexes in matrix and analyzed at indicated quality control (QC) concentration levels. Concentrations of immune complex QC used in IC assays were always based on the final concentration of IgG (ADA) in the immune complexes.

2.4. SEC–HPLC–MALS

Immune complexes formed in vitro were characterized using Agilent 1100 series with HPLC Shodex Protein KW 803 column (8 mm × 300 mm). Samples were injected using the Agilent auto sampler and run at 0.5 mL/min with 200 mM...
K$_2$HPO$_4$, 150 mM NaCl (pH 6.8 with HCl), with 0.02% Sodium Azide buffer. Three online detectors were utilized: Agilent diode array UV/vis spectrophotometer, Wyatt Technologies mini-Dawn three angle laser light scattering detector and Wyatt Optilab DSP interferometer refractometer. Data were collected and analyzed using Astra (Wyatt) and Chemstation (Agilent) software.

2.5. Bridging assay

Study samples and positive control QCs were diluted 20 fold (MRD20) in 1% BSA in PBS with 0.05% Tween 20 (1% PTB) containing experimentally optimized concentrations of ruthenium labeled drug and biotin labeled drug for 1 h. Following bridge formation, samples were incubated for 30 min on a pre-blocked MSD streptavidin coated Gold 96 well plate and read using 4x MSD Read Buffer T with Surfactant on a MSD Sector Imager 2400 Model 1250 (Meso Scale Discovery, Gaithersburg, MD). Unless otherwise stated all incubation steps were carried out shaking at 400 rpm in temperature controlled incubator at 24 °C and all washes were repeated 4 times.

2.6. Immune complex assays

Taking advantage of the unique structure of our biotherapeutic molecule and the absence of any cross reactive epitopes with an IgG molecule we proceeded with the classical approach of capturing the drug and detecting the IgG bound to the drug as a means of detecting immune complex. In order to elicit the presence of these complexes believed to be large enough to have potential in vivo FcyR mediated effects we chose to develop a second assay format utilizing FcγRIIIa capture that would capture a subset of drug specific immune complexes capable of binding to the low affinity FcγRIIIa.

2.7. Anti-drug capture assay

A monoclonal mouse anti-drug antibody was generated in house and was labeled with biotin using EZ-Link NHS-LC-Biotin according to the manufacturer’s recommendations (Thermo Scientific Rockford, IL) for use as a capture reagent. Streptavidin 96-well black plate (Greiner Bio-one, Monroe, NC) was coated with this monoclonal anti-drug capture antibody at 1.2 μg/mL for 1 h. The plates were then blocked with 5% BSA in PBS. LowCross buffer (Candor Bioscience GmbH) was used as a dilution buffer for samples and detection antibody to decrease non-specific binding of serum IgG. According to the manufacturer’s literature (Candor Bioscience GmbH) LowCross buffer contains a proprietary formulation capable of reducing interference, non-specific binding and matrix effect. It is possible that the formulation may contain non-physiological concentrations of salts or higher levels of detergents which might have the potential to alter the size or composition of immune complexes. LowCross buffer was used in assay format B with anti-IgG + IgM detection due to its ability to decrease assay background and increase assay sensitivity to detect immune complexes irrespective of their size as compared to other buffers tested. Although it is possible that the equilibrium of IC size was potentially affected by LowCross buffer, we were still successful in detecting immune complexes using additional assays. As an example, LowCross buffer was not used in assay where presumably larger immune complexes bind to FcγRIII (format C).

Samples and QCs were diluted 50 fold (MRD50) in LowCross buffer and were incubated on the plate for 30 min and washed 4 times with PBS containing 0.05% Tween 20. Detection antibody was used at 20 ng/mL and was purchased from Jackson ImmunoResearch (affinity purified HRP labeled anti-human IgG + IgM (H + L), cat # 309-035-107). Cross reactivity of this reagent to cyno IgG was determined experimentally (data not shown). Following 1 h incubation with the detection reagent and washes as described earlier the HRP activity was detected using luminol based SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific Rockford, IL). All washes were repeated total of 4 times using ELx405 Select CW plate washer (Biotek, Winooski, VT). Luminescence signal was recorded using SpectraMax M5e (Molecular Devices Sunnyvale, CA).

2.8. FcγRIIIa capture assay

Streptavidin 96-well black plate (Greiner Bio-one, Monroe, NC) was coated with an anti-polystyhistidine monoclonal antibody labeled with biotin at 1 μg/mL (R&D Systems, cat # BAM050) for 1 h and blocked with 5% PTB. This step was followed by a wash and capture of recombinant cynomolgus monkey FcγRIIIa bearing 6HIS residues (Sino Biological, Inc. cat # 90013-C08H) at 1 μg/mL for 1 h. QCs and study samples were diluted 50 fold in 1% PTB followed by an overnight capture. Detection of captured drug specific complex was conducted using 400 ng/mL of monoclonal mouse anti-drug antibody custom labeled with HRP (Innova Biosciences Lightning-Link HRP Conjugation Kit, cat # 701-0000). All washes were conducted 5 times. HRP activity was detected using luminol based SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific Rockford, IL). Luminescence signal was recorded using SpectraMax M5e (Molecular Devices Sunnyvale, CA).

2.9. Assay cut point determination

Cut point was determined using methods and calculations as previously described (Shankar et al., 2008) using normal serum from at least 30 cynomolgus monkeys. Cut point factor corresponding to 5% false positive rate was used for all three assays. The same serum lots were used in all three assays.

2.10. PK sandwich ELISA

Measurement of drug level in study samples was conducted using standard colorimetric sandwich assay format. A commercially obtained biotinylated anti-drug goat polyclonal antibody at 1 μg/mL was used to coat the Greiner streptavidin plate for 90 min. Standards, analytical QCs and study samples were diluted 10 fold (MRD10) in PTB and incubated on the plate for 90 min. Captured analyte was detected with custom made purified rabbit anti-drug antibody at 1 μg/mL. The detection reagent bound to a distinct portion of the drug away from the capture reagent binding site. Secondary detection was conducted using donkey anti-rabbit IgG HRP (Jackson ImmunoResearch, cat # 711-035-152) at 1/50,000 dilution. Each step was followed by 5 washes. TMB peroxidase substrate was added to detect HRP enzymatic activity. Following color development the reaction
was stopped using 1 M phosphoric acid. Results were read at OD 450 nm with 620 nm reference wavelength using the Tecan Genios plate reader.

2.11. Animal study design

This animal study was reviewed and approved by the Drug Safety Evaluation group and the Veterinary sciences group at Bristol-Myers Squibb. In this investigative study the biotherapeutic drug was administered at 2.5 mg/kg through subcutaneous injection. Cynomolgus monkeys (Macaca fascicularis) were injected with drug or with vehicle control twice weekly for one month, followed by a one month recovery period. Study samples from eight animals that had been collected for drug exposure were randomly chosen for analysis of free ADA, the presence of drug specific immune complexes, and for determination of the kinetics of their formation. The study samples were matched with pre-dose samples drawn from the same animals prior to biotherapeutic administration.

2.12. Graphical and statistical software

JMP 8.0 (SAS, Cary, NC) was used for design of experiments (DOE), multivariate data analysis and for analysis of distribution of signal from normal cynomolgus sera for cut point calculations. Graphs were constructed using SoftMax Pro v5.4.1 (Molecular Devices Sunnyvale, CA) and GraphPad PRISM v 5.01 (San Diego, CA).

3. Results

3.1. Immune complex formation and characterization

To prepare and characterize surrogate positive IC controls for the immune complex assay, HPLC coupled to UV and MALS detectors was used. Preparations of biotherapeutic drug (eluting ~20 min) as well as free ADA (eluting ~17 min) were mostly monomeric with aggregation <3% for all un-complexed samples and consistent with expected masses (Fig. 1). Following combination of free drug and free ADA, drug–ADA IC was formed as indicated by appearance of additional peaks eluting at 14.5 and 12 min. Size of ICs formed with fixed concentration of a polyclonal antibody (Table 1) varied depending on molar ratio of biotherapeutic drug to the ADA as indicated by a difference in the heights of 14.5 and 12 min peaks (Fig. 1). SEC-HPLC analysis of immune complexes is limited to IC of sizes physically small enough to pass through SEC column beads. To prevent physical clogging of analytical SEC column all IC samples were filtered using 0.2 μm spin filter. Therefore, it is possible any IC larger than 0.2 μm if formed, would be filtered out and not analyzed in this experimental setup. Due to these limitations of SEC, additional methods may be utilized in the future to further characterize the size of IC. Examples of additional methods include: analytical ultra-centrifugation coupled to fluorescence detector or Resonant Mass Measurement (RMM) analysis of sub-visible particle ranging from 300 nm to 5 μm.

Immune complexes formed at excess of free ADA (0.25:1 molar ratio of drug–ADA), resulted in formation of largest immune complexes (~1.4 MDa). Instead, IC formed at excess of free drug (molar ratio of 4:1 drug–ADA) resulted in formation of highest proportion of smallest complexes (~510 kDa). Therefore, immune complexes composed of a polyclonal ADA and monovalent biotherapeutic can form IC of various sizes which is partially driven by the relative concentration of drug and ADA. Based on these findings IC formed at 0.5:1 molar ratio was chosen for formation of immune complex QCs of diverse sizes used in the ligand binding assays for detection of immune complexes (Fig. 2).

3.2. Immune complex assay performance

The lack of an Fc portion in our drug enabled us to design two assays to detect drug–ADA IC. Specifically, it ensured that in assay format B there would be no cross reactivity of the detector reagent to the captured therapeutic while in assay format C there would be no detection of aggregated drug alone binding to the capture reagent. In an attempt to quantify IC we have generated standard curves with immune complexes formed at several molar ratios. Concentration of the biotherapeutic drug present in an IC, and potentially the efficiency of IC formation, resulted in distinct signal responses at multiple concentrations of IC QCs (Fig. 3). Given that there are two variables, drug and ADA concentration, and both can vary during a time course of a study it may not be possible to reliably quantify the concentrations of immune complexes. Additional level of complexity is due to variability in stoichiometry of ADA binding to the drug, since the binding is not 1:1 and since multiple size species were formed with this polyclonal preparation. In the light of these factors further quantification of immune complexes was not carried out. Immune complexes formed at molar drug–ADA ratio of 0.5:1 consistently resulted in highest signal, at most QC levels, in ligand binding assay format B.

Based on the signal response data obtained from the freeze thaw experiments, drug–ADA immune complexes were found to be stable in cynomolgus serum for at least 6 freeze thaw cycles (Fig. 4A). Loss of signal due to freeze thaw cycles was under 20% for HQC and under 25% for LQC. The assay to measure immune complexes in serum had high precision with under 20% for HQC and under 25% for LQC. The assay to detect the presence of drug on a bridging assay and on drug–ADA IC resulted in distinct signal responses at multiple concentrations of IC QCs (Fig. 1). The assay was determined to be suitable for analysis of study samples for the presence of immune complexes.

Next we took steps to characterize the effect of excess of free drug on a bridging assay and on drug–ADA IC assay. Standard immunogenicity bridging assay was negatively affected by interference from unlabeled drug (Fig. 5). In the presence of 5 μg/mL of unlabeled drug the sensitivity of the bridging assay decreased to 500 ng/mL of ADA QC from sensitivity of below 50 ng/mL with 0 ng/mL of unlabeled drug. When unlabeled drug was present at 100 μg/mL sensitivity of the bridging assay was above the tested concentration of 2 μg/mL of QC. Contrary to bridging assay, the assay detecting immune complexes tolerated excess unlabeled drug at levels of the highest tested concentration of 100 μg/mL. Although the assay detecting immune complexes has a sensitivity of 500 ng/mL the assay was not affected by the presence of excess unlabeled drug. Therefore the assay detecting immune complexes can be used to detect the presence of drug–ADA IC during an ADA immunogenic response under high levels of systemic drug in circulation. Since the assay was dependent on association kinetics of the complex at its varying concentrations and lacked a truly representative calibrator it may be classified as quasi-quantitative.
3.3. Detection of immunogenicity and immune complex formation in study samples

Seven out of eight tested animals screened and confirmed positive for ADA using ADA bridging assay (Table 2). One animal which was negative at this time was screened and confirmed positive during drug washout period. However, assessment of immunogenicity using this method required separate sample collection when circulating drug levels were reduced. When standard PK samples were analyzed for the presence of ADA using bridging assay, signal below cut point was detected in some animals, indicating drug interference. When the same samples were analyzed for the presence of drug specific immune complexes using IC assay format B, unique patterns of formation of immune complexes were identified in all tested animals (Fig. 6).

Response pattern varied between individual animals and it was determined that in some animals circulating immune complexes could be detected before free ADA. In some cases immune complexes remained detectable in circulation even when drug levels fell below limit of quantitation, indicating ADA interference in standard PK assay. Therefore, an assay detecting drug specific immune complexes provides information on an immunogenic response manifested by formation of immune complexes and complements data from PK and ADA.

Table 1

<table>
<thead>
<tr>
<th>Biotherapeutic drug/ADA molar ratio</th>
<th>Concentration drug/ADA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:1</td>
<td>251 μg:300 μg</td>
</tr>
<tr>
<td>2:1</td>
<td>126 μg:300 μg</td>
</tr>
<tr>
<td>1:1</td>
<td>63 μg:300 μg</td>
</tr>
<tr>
<td>0.5:1</td>
<td>31 μg:300 μg</td>
</tr>
<tr>
<td>0.25:1</td>
<td>16 μg:300 μg</td>
</tr>
</tbody>
</table>
assays. When drug was cleared from circulation (or became undetectable due to ADA interference), levels of immune complexes declined as well. At the same time points when immune complex levels were low or undetectable the signal obtained from free ADA bridging assay (format A) reached highest RLU values. This data indicates the presence of equilibrium between circulating drug, free ADA, and ADA in an immune complex with the biotherapeutic drug during an immunogenic response. Therefore results obtained from both assay can supplement each other for interpretation of immunogenicity through ADA responses. Moreover, this data opens the potential for investigating the effect of formation of drug–ADA immune complexes on the PK profiles or ADA mediated clearance of administered biotherapeutic.

### 3.4. Detection of immune complexes capable of binding to FcγR

In order to mimic biological interactions of IC in vivo with FcγR bearing cells we have developed a plate based method for evaluating the potential of circulating immune complexes to interact with immobilized FcγRIIIa. Immune complexes pre-formed at molar ratio drug–ADA of 0.5:1 were efficiently captured by FcγRIIIa based assay (Fig. 7). These results correlate with the presence of IgG in large or intermediate immune complexes with the target drug as previously confirmed by SEC-HPLC (Fig. 1). As expected, capture of free IgG ADA by FcγRIIIa was minimal as this lot of IgG ADA was previously analyzed by SEC HPLC and less than 3% of this ADA IgG was aggregated (panel A). Taken together this data support current literature...

---

### Table: Assay Formats

<table>
<thead>
<tr>
<th>Format</th>
<th>Analyte</th>
<th>Isotype</th>
<th>Drug Tolerance</th>
<th>Sensitivity</th>
<th>CP Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (ADA)</td>
<td>Free ADA</td>
<td>All bridging isotypes</td>
<td>5 μg/mL at 500ng/mL PC</td>
<td>&lt;50 ng/mL</td>
<td>1.50</td>
</tr>
<tr>
<td>B (IC)</td>
<td>IC of all sizes</td>
<td>IgG, IgM</td>
<td>&gt;100μg/mL</td>
<td>&lt;500 ng/mL</td>
<td>1.55</td>
</tr>
<tr>
<td>C (IC)</td>
<td>IC with &gt;2 ADA in a complex</td>
<td>IgG1,3 &gt;IgG2,4</td>
<td>&gt;100μg/mL</td>
<td>&lt;500 ng/mL</td>
<td>1.35</td>
</tr>
</tbody>
</table>

---

### Fig. 2. Summary of ligand binding assay formats. Standard bridging assay was used as a gold standard method to detect the rate of immunogenicity in study samples (format A). Additionally, 2 ligand binding assays were designed to capture and detect immune complexes composed of biotherapeutic drug and ADA (drug–ADA IC) in cynomolgus monkey serum samples, following administration of a biotherapeutic. Anti-drug capture of drug specific immune complexes has the advantage of detecting all sizes of IgG and IgM bearing immune complexes present in circulation (format B). Assay for detection of drug–ADA IC capable of binding to immobilized FcγRIIIa was developed to detect IC with FcγRIIIa binding characteristics (format C). Detailed experimental conditions for each assay are described in the Materials and methods section.

---

**Fig. 3.** Detection of immune complexes with varying molar ratios of drug-to-ADA. In order to determine whether drug–ADA immune complexes can be quantified, curves at multiple levels of ADA in an immune complex were generated at varying molar ratios of drug–ADA. Preparations of preformed immune complexes, which were analyzed in Fig. 1, were serially diluted from 300 μg/mL starting concentration of ADA in an immune complex to the indicated concentration of ADA in an immune complex. Diluted QCs were analyzed using anti-drug capture ligand based assay (format B). Data representative of one of two independent experiments was graphed as a mean ± SD of replicate wells (N = 2). ADA alone or biotherapeutic drug alone was not detected in anti-drug capture (format B) ligand based assay.
that FcγRIIIa bind immune complexes composed of multiple Fcγ chains originating from ADA present in the complex (Luo et al., 2009). The FcγRIIIa had good performance with %CV <25% for HQC and LQC. Moreover, the FcγRIIIa capture assay was tolerant to systemic drug up to the tested level of 100 μg/mL of spiked drug. Therefore, the FcγRIIIa was used to test study samples for the presence of immune complexes in circulation during an immunogenic response.

Immune complexes capable of binding to FcγRIIIa were detected in most animals using FcγRIIIa based capture assay. Data from a representative animal is shown in Fig. 8A. Visual correlation between results obtained from both assay formats was confirmed using multivariate data analysis (Fig. 8B). Multivariate data analysis demonstrated good correlation (R = 0.9441) between signals obtained from the anti-drug (IC, format B) and FcγRIIIa (FcR, format C) based capture assays. The positive correlation between the two assays was confirmed using Spearman’s nonparametric ρ test (ρ < 0.0001). Therefore, anti-drug capture (IC) and FcγRIIIa capture assays can both be used to detect IC in serum and at least a fraction of immune complexes capable of binding to FcγRIIIa remains in circulation. The lowest correlation was observed between the bridging ADA assay (format A) and both IC assays (format B or C), indicating that kinetic results obtained from the immune complex assays provide distinctive information from the results obtained from the bridging ADA assay. This data further supports that the IC assay could be used as an orthogonal method for confirming ADA responses when drug is present in circulation.

3.4. Consequences of immune complex formation

We have detected immune complexes in all animals tested and in a fraction of the animals tested immune complexes capable of binding to Fcγ receptor were detected. Although the presence of immune complexes was confirmed, there were no abnormal treatment-related findings in serum chemistry biomarkers tested. All results were within acceptable ranges for each analyte tested. Results of representative biomarkers for kidney and liver functions, such as: aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CREA) and blood urea nitrogen (BUN) are shown in Fig. 9. Although animals were not analyzed for deposition of immune complexes, there were no abnormal gross pathological changes in these animals at the end of the study. Therefore, it appears that at least some level of these drug–ADA immune complexes can be tolerated by study animals during the investigated time period.

4. Discussion

The observations of ADA interference in PK assays as well as drug interference in the immunogenicity assay led us to investigate the hypothesis of the presence of biotherapeutic drug–ADA complexes in circulation. In this study we present the data on the identification of immune complexes found in non-human primates (NHPs) during an immunogenic response to a biotherapeutic protein. Because the biological activity of ADA is to bind to the antigen, this being a biotherapeutic in this study, it is not unexpected that both the free and bound species of ADA exist in the same sample.

Immune complexes used as QCs in this study to monitor the performance of the assays were not cross-linked; they were allowed to bind in the spiked samples in order to closely mimic immune complexes found in circulation. In instances when the binding affinity of the complexes is not high it might be necessary to create chemically cross-linked complexes. Authors of previous publication on development of drug tolerant
immunogenicity assay created chemically cross-linked IgG and ADA composed of one molecule of each component (Stubenrauch et al., 2010). However, the authors took into consideration formation of only single size species of IC. According to our HPLC analysis of IC formed ex vivo, this assumption may be inaccurate and it may be impossible to accurately quantify the amount of IC due to a polyclonal nature of an immune response and formation of multiple species of IC. Our findings suggest that polyclonal immune complexes formed ex vivo efficiently trap the biotherapeutic antigen and that the rate of immune complex dissociation is low. This observation was specific to the analytes used for this study and may not reflect a general behavior of other ADAs. This interaction and the concentration of both free and complexed species will be driven by binding equilibrium. Although we have not conducted equilibrium dissociation studies, it appears that immune complexes formed in this study are stable in solution and that dilution to final QC concentration or freeze/thaw cycles did not result in significant dissociation of complexes. This observation is consistent with the hypothesis that multiple polyclonal antibodies form a stable immune lattice with the antigen, thus efficiently sequestering the biotherapeutic. Polyclonal binding can be beneficial when an immune system responds to invading bacteria or viruses, however, when a polyclonal response occurs against a biotherapeutic it may result in an unwanted neutralization and clearance leading to loss of efficacy. The presence of these highly stable complexes also poses a problem in bioanalytical approaches to measure either ADA or biotherapeutic present during an immunogenic response. However, IC assay presented in this study was able to detect IC during high drug levels in all animals tested.

Humoral immune response driven by antibody mediated formation of immune complexes is a normal physiological process of clearing non-self antigens. Currently there are a number of assays to measure total immune complexes,
however, each of these assays uses some unique characteristic of immune complexes such as complement opsonization for quantification and their clinical significance is not well established (Van Hoeyveld and Bossuyt, 2000). Concentration of immune complexes in circulation of normal human individual ranges between 2.3 μg/mL (Senbagavalli et al., 2011) and 5 μg Eq/mL (Quidel, MicroVue, CIC-RAJI EIA, Cat # A002) depending on the method used. Therefore, IC may be present in circulation of normal individuals without causing disease, indicating steady state clearance of foreign or abnormal antigens. We have attempted to measure total biotherapeutic drug–ADA immune complexes in NHP with multiple commercially available human IC kits; however, none of these generic assays were able to recognize cynomolgus immune complexes formed in this study. These results can potentially be due to the fact that the biotherapeutic used in this study was a non-Fc bearing entity, and the only Fc found in these complexes was of cynomolgus IgG ADA origin, making the commercial kits unable to recognize NHP IC with sufficient sensitivity.

Clearance of immune complexes is mediated by the reticulocyte and the phagocytic system (Schifferli et al., 1988; Halma et al., 1992). Recently, it was shown that infliximab IC is trapped in the spleen and liver of patients who developed ADA and the IC clearance correlated with clearance of infliximab from circulation in non-responders (van der Laken et al., 2007). Despite the presence of this IC in two subjects under investigation only one subject developed infusion reaction. These findings suggest that certain types or certain levels of immune complexes can be tolerated. Immune complex pathology may occur when the levels of IC overwhelm the complement (reticulocyte) or FcγR (phagocytic) mediated clearance or phagocytosis (Heyen et al., 2014). To investigate the potential biological function of IC we have developed a second assay to detect the presence of drug

---

Fig. 6. Immune complex detection by anti-drug capture in study samples. In this investigative study the biotherapeutic drug was subcutaneously administered twice weekly at 2.5 mg/kg in cynomolgus monkeys. Biotherapeutic injection times are indicated by an arrow (↑) below the graph. Eight animals were randomly chosen for analysis of ADA and drug specific immune complexes. Pharmacokinetic (PK) profiles (red filled triangles, ▲) are graphed as concentration on the left y-axis, whereas results from ADA bridging assay (format A, black filled diamonds, ⬤) and the IC anti-drug capture (format B, green filled circles, ●) assay are graphed on the right y-axis and presented as RLU. Kinetics results from two representative animals are shown. Asterisk (*) indicates that the sample was above plate cut point for the presence of ADA in the bridging assay format.
specific IC in circulation capable of binding to plate immobilized FcγR. Our results indicate that a fraction of drug specific IC present in circulation can bind to FcγR (Smith and Clatworthy, 2010).

Autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus (SLE), where immune complexes are part of the pathology lead to deposition of immune complexes in various physiological locations and are shown to trigger inflammation. In fact only certain types of immune complexes isolated from SLE patients trigger an inflammatory response and IC formed purely from protein does not trigger activation of dendritic cells (Means et al., 2005). Some reports suggest that IC can result in maturation of mouse DC, however, such findings have not been proven in human DC (reviewed in (Platzer et al., 2014)). These publications support the possibility of differences not only of IC composition but also differences in DC subset and host species having an effect on maturation.

In some cases IC deposited in glomeruli triggers activation of complement and Fcγ receptor signaling which can result in IC glomerulonephritis (Kurts et al., 2013). To our knowledge with the exception of replacement protein therapeutics that carry foreign sequences to which the host has not been tolerized, there are no reports of autoimmune like consequences of biotherapeutic drug–ADA IC such as glomerulonephritis or proteinuria in patients receiving the typical antibody based biologic therapeutics indicating that immune complexes formed in circulation are efficiently cleared by the spleen and liver. Although, not directly monitored in this study there were no reports of immune complex disease or adverse events observed in animals under investigation as
supported by normal serum chemistry reports in all animals. These findings support the hypothesis that certain level of immune complexes can be tolerated without immediate side effects like anaphylaxis or effect on kidney function.

Although we have taken first steps to characterize immune complexes in vitro and confirmed immune complex presence in circulation during an immunogenic response, significant amount of work remains to be completed. Immune complexes

Fig. 8. Study sample analysis and correlation between assay formats. All PK samples from eight animals in the study were re-analyzed for the presence of drug–ADA IC using FcγRIIIA assay (format C). The results from a representative animal over length of the study are plotted on the left y-axis for the FcγRIII assay (blue filled square, □). Data from the IC anti-drug capture assay (format B) obtained from the same sample was overlaid on the same graph and plotted on the right y-axis (green filled triangle, ▼) (panel A). Multiparametric data analysis of the results obtained from: PK, bridging (format A), IC (format B) and FcγR (format C) for all eight animals, was conducted using JMP 8.0 multivariate data analysis function (panel B). Study samples analyzed after an immunogenic response of Day 29 were used in this correlation analysis and correlation was confirmed using Spearman’s nonparametric ρ test.
formed in vivo have diverse forms in size, clonality and IgG subtype distribution and so can be the resulting consequences. Pleiotropic nature of immune complexes can be driven by the immunoglobulin subtype, isotype, clonality and affinity which results in formation of immune complexes of specific size, charge and stability. All of these physical characteristic of immune complexes result in differential ability to bind to FcγR, fix complement, and trigger adverse events. The mere presence of immune complexes may not be indicative of induction of adverse events the same way as the presence of ADA doesn’t necessarily translate into adverse events. Despite the presence of immune complexes in cynomolgus monkey serum samples none of these animals had any evidence of Type III mediated hypersensitivity related adverse effects. However, the presence of immune complexes with specific characteristic may potentially be a risk factor. Based on findings by our group and others, it is very likely that high proportion if not all of the subjects developing ADA will have some type of immune complex present without onset of adverse events. The question remains what biophysical properties make these immune complexes pathogenic. Therefore, further investigations to identify the qualitative characteristics and quantitative requirements that distinguish clinically impactful IC from clinically benign IC are warranted. Future research into characterization of immune complexes may lead to identification of biomarkers differentiating benign and harmful immune complexes.

**Acknowledgments**

The authors of this publication would like to recognize Suk Kwok and Surendran Rajendran for sharing the pharmacokinetic data. Alexander Kozhich and Dharmesh D. Desai for insightful discussions and expert ideas on assay development and formation of drug specific immune complexes. Steven Piccoli assisted in reviewing this manuscript and in scientific discussions.

**References**


