Adaptation of an ELISA assay for detection of IgG2a responses against therapeutic monoclonal antibodies in a mouse immunization model

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ABSTRACT
Biotherapeutic monoclonal antibodies (mAb) play important roles in clinical medicine but their potential to elicit immune responses in patients remains a major issue. In a study designed to investigate the effect of aggregation on immunogenic responses, mice were immunized with two monoclonal antibodies (mAb1 and mAb2). Serum levels of total IgG, IgG1, and IgG2a were measured by ELISA. An anti-mouse IgG2a monoclonal detection antibody cross-reacted with mAb2 but not mAb1, leading to high background when the ELISA plate was coated with mAb2. The problem was solved by use of a goat anti-mouse IgG2a polyclonal antibody that demonstrated the required specificity. IgG2a responses were similar for monomer- or aggregate-coated ELISA plates. The results demonstrate the importance of assessment of the specificity of individual reagents when measuring antibody responses against therapeutic antibodies by ELISA.

Introduction
In the last two decades biotherapeutics, and in particular monoclonal antibodies (mAb), have become increasingly important for treatment of a wide range of disorders (Geng et al. 2015). Currently, more than 250 approved biotherapeutics are available, and there are estimated to be in excess of 500 biotherapeutics at various stages of development (Shankar et al. 2007; Foltz et al. 2013). A variety of factors can influence the immunogenicity of biotherapeutics, including: product-related factors such as protein conformation or impurities, patient-related variables such as immune and genetic background, disease status, and treatment-related factors such as route and duration of exposure (Pendley et al. 2003; Tabrizi and Roskos 2007). These can impact the pharmacokinetics and efficacy of biologics; therefore, assessment of potential immunogenicity is an important element of the development and regulatory approval process (Anderson 2005; Chirmule et al. 2012; Maneiro et al. 2013; Sathish et al. 2013).

Products derived from non-human species are predicted to elicit a high incidence of antibody responses because the human immune system is not tolerated to non-human proteins. Consistent with this, there is a high incidence of Anti-Drug Antibody (ADA) development to most therapeutic proteins of bacterial origin, sometimes after a single therapeutic exposure. Aggregation is potentially an important factor which can influence immunogenicity: aggregated proteins are frequently more immunogenic than their non-aggregated counterparts (Ellis and Henney 1969; Moore and Leppert 1980; Rosenberg 2006). An experimental approach adopted in our laboratory is to use a murine intraperitoneal immunization model and humanized monoclonal antibodies or their fragments (Ratanji et al. 2017; Rane et al. 2019).

The purpose of these investigations was not to characterize the immunogenicity per se of a given biotherapeutic, but rather to understand the difference in behavior of the two mAb that are similar in structure, in the experimental settings. In the course of the experiments here, nonspecific binding and background issues were encountered in the ELISA experiments. Humanized biotherapeutic antibodies were used as ELISA substrates and the anti-murine IgG2a antibodies were used for detection. Humanized mAb were stressed to generate aggregates which were used to immunize the mice and also as ELISA substrates. One of the mAb used performed well with experimental strategy used for ex vivo IgG2a assessment; however, other mAb did not, generating high background and confounding ADA measurements. A strategy to mitigate this problem is proposed.

Material and methods
Animals
BALB/c mice (female, 8–12 week-of-age) were obtained from Envigo (Bicester, UK) for use in these experiments. Mice were housed on sterilized wood bedding with materials provided for environmental enrichment. Food (Beekay Rat and Mouse Diet #1 pellets; B&K Universal, Hull, UK) and water were available ad libitum. The housing facilities were maintained at an ambient temperature of 21 ± 2 °C with a relative humidity at 55 ± 10% and with a 12-hr light/dark cycle. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986, and approved by Home Office license.
Monoclonal antibodies

Two human IgG1 monoclonal antibodies (designated mAb1 and mAb2) were used for the current study. mAb1 has a theoretical molecular weight of ~148 kDa, and mAb2 (bi-specific antibody) has a theoretical weight of ~204 kDa. Both mAb were provided by MedImmune (Cambridge, UK).

Aggregate formation

Both mAb were diluted to 1 mg/ml in Dulbecco’s phosphate-buffered saline (DPBS) without Ca$^{2+}$ or Mg$^{2+}$ (Sigma, St Louis, MO). mAbI aggregates were generated using thermal stress at 60 °C for 25 min. mAb2 aggregates were generated by shaking stress at 1,500 rpm in a bench top shaker (IKA® MS 3 Digital, Oxford, UK) for 4 hr at 22 °C.

Immunizations

Mice were immunized by intraperitoneal (IP) injection on Days 0, 7, and 14 with 250 µg of mAb1 or 150 µg of mAb2 in aggregated states. All mice were then exsanguinated on Day 21. Individual serum samples and samples pooled on group basis were prepared and stored at −80 °C until analysis.

Elisa

Plastic Maxisorb® plates (Nunc, Copenhagen, Denmark) were coated with 0.1 mg/ml monomeric or aggregated mAb1 or mAb2 (prepared in PBS) and incubated overnight at 4 °C. Doubling dilutions of serum samples were then added and the plates incubated for 3 hr at 4 °C. Sera from naïve mice (NMS) were used as controls. Serum blank wells (including all other reagents) were used to calculate plate background. horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody (1:4000 dilution, AAC10P, BioRad, Killington, UK) was used for the IgG ELISAs; HRP-goat anti mouse IgG antibody (1:2000 dilution, STAR132P, BioRad) was used for the IgG2a ELISAs. Bovine serum albumin (BSA, 2% in PBS) was added as a blocking reagent and the plates were then incubated at 37 °C for 30 min. Serum dilutions were prepared in 1% BSA/PBS solution.

For the IgG2a ELISA, plate wells were coated with 0.1 mg/ml monomeric or 0.05 mg/ml thermal stress-aggregated mAb1, or 0.1 mg/ml monomeric or shaking stress-aggregated mAb2 (prepared in PBS), and then incubated overnight at 4 °C. For IgG2a detection in mice sera, various blocking reagents including 2% BSA in PBS, 5% HSA in PBS, 5% semi-skimmed milk in PBS, and 10% FCS in PBS were examined in ELISA pilot studies. On the basis of the results obtained, a 5% milk block was selected for use in all further experiments. After wells were washed with 0.05% Tween-20 in PBS, serum dilutions (prepared in 2.5% skimmed milk-PBS) were added to dedicated wells and the plates incubated for 2 hr at 4 °C. After gentle washing, wells received HRP-conjugated monoclonal rat anti mouse IgG2a antibody (clone: LO-MG2a-9, BioRad) or HRP-polyclonal goat anti mouse IgG2a antibody (BioRad), each diluted 1:1,000. For the IgG, IgG2a, and both IgG2a ELISAs, the plates were incubated for 2 hr at 4 °C. The wells were then gently washed prior to addition of HRP substrate solution.

Plates then received substrate [1.6 mg/ml o-phenylenediamine and 0.4 mg/ml urea hydrogen peroxide in 0.5 M citrate phosphate buffer (pH 5)] and were incubated for 15 min in the dark at 22 °C (RT). Reactions were then stopped by addition of 0.5 M citric acid. Absorbance in each well was then read at 450 nm using an ELx800 automated reader (BioTek Instruments, Winooski, VT), and evaluated using system-associated Gen 5 software. All data were reported out as OD450 nm values (± SEM, where appropriate) and mean antibody titers. Titers were calculated as the maximum dilution of serum at which an OD450 reading of ≥ 0.3 was recorded (i.e. three-times a reagent blank [all reagents except for serum] OD450 reading of 0.1).

Statistical analyses

Statistical analyses were performed using the Prism 7 software (GraphPad, San Diego, CA). Analysis of variance (ANOVA) was used to determine the statistical significance of differences between groups. Experiments were analyzed by non-parametric one-way or two-way ANOVA followed by a Tukey’s post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

Characterization of therapeutic mAb in pre- and post-stress

Two human IgG1 mAb, i.e. mAb1 and mAb2, were used in the current study. Both mAb were prepared at 1 mg/ml in PBS and aggregates generated by application of thermal or shaking stress. mAb2 showed no aggregation in response to thermal stress (at 45, 50 or 60 °C) but aggregates of mAb1 were generated by both methods. Sizes of the generated mAb1 and mAb2 aggregates were analyzed by Dynamic Light Scattering (DLS) (Figure 1). Both mAb monomers showed a narrow size distribution at ~10 nm, as anticipated. Application of thermal stress to mAb1 generated an aggregate population within the sub-visible size range. Aggregate sizes were much larger (~1μm) when formed by shaking stress mAb2. For aggregated mAb, DLS data shows single peak for aggregates with increased particle size, no monomer peak was observed in the same sample.

Assessment of anti-mAb IgG and IgG1 responses in immunized mice sera

The aim of the study was to investigate effects of aggregation of two mAb on immuno-genic responses. Animals were immunized with 250 µl of 1mg/mL of mAb1 and 150 µl of 1 mg/ml of mAb2 (monomeric or aggregated) in PBS. Following immunization of the mice with aggregated mAb1 or mAb2, the vigor of the IgG response and the isotype distribution of the serum antibody response were analyzed by ELISA using monomeric or aggregate mAb substrates to coat the plate. Pooled serum samples from aggregate-immunized mice were analyzed and comparisons made with serum from naïve animals. Immunization with both mAb in aggregated form provoked vigorous IgG antibody responses compared with IgG levels in naïve mice (Figure 2(A)). To further characterize the ADA, IgG1 and IgG2a were measured in a similar manner, but using appropriate detection antibodies for subclasses. There was little difference in IgG or IgG1 antibody profiles for either mAb depending on whether monomeric or aggregated protein substrate was immobilized on the plate, although somewhat higher IgG1 responses were observed for monomer mAb1 over that of aggregated substrate (Figure 2(B)).

IgG2a ELISA assays were performed using similar experimental set-ups and anti-IgG2a detection antibodies. This resulted in very low background (serum blank wells were < 0.3 OD450 nm) and very low levels of binding of negative control (naïve) mouse
serum samples. However, for mAb2 monomer and aggregate substrate coated plates (Figure 3(A), left panel), the readings were similar in immunized and naïve mice serum samples, did not titrate out upon dilution of serum and serum blank wells also displayed high readings, indicating nonspecific binding of anti-mouse IgG2a secondary antibody to the mAb2 substrate (both monomeric and aggregated forms). The use of a number of different blocking reagents including bovine serum albumin (BSA) in PBS (2%, w/v), skimmed milk (5%, w/v), human serum albumin (10%, w/v), and various combinations thereof, were assessed to reduce background signals and increase the specific signal from immunized mice sera (Supplementary Figures 1(A,B)). None of these blocking agents were effective at providing a specific signal with reduced background.

In an additional attempt to reduce the high background, analyses were repeated using an alternative detection antibody, a

Figure 1. Characterization of mAb1 and mAb2 aggregates by DLS. mAb1 and mAb2 were diluted to 1 mg/ml in PBS. In both panels, the dashed line represents monomer (10 nm) for each mAb and the solid line represents thermal (~90 nm) and shaking (~1 μm)-stressed aggregated material from mAb1 and mAb2, respectively.

Figure 2. IgG and IgG1 responses induced by immunization with aggregated mAb1 or mAb2. (A) Total IgG and (B) IgG1 antibody in serum from immunized mice assessed using ELISA assay. Doubling dilutions (in 1% BSA/PBS) of serum samples from mAb1 aggregate-immunized animals, mAb2 aggregate-immunized animals, or naïve negative controls were analyzed against both monomeric and aggregate forms of immunizing protein as substrate (vs monomer and vs aggregated protein). Data are representative of pooled serum for immunizations with aggregated mAb1 (n = 5), and mAb2 (n = 5).
horseradish peroxidase (HRP)-conjugated polyclonal goat anti-
mouse IgG2a antibody. Somewhat surprisingly, despite being a
polyclonal rather than a monoclonal antibody, use of this reagent
markedly improved the signal to noise ratio, with reduced back-
ground for the analyses of the mAb 2 anti-sera (Figure 3(B)).
Serum blank values were \(< 0.3 \text{ OD}_{450} \text{ nm}, there was no signal
above background for naïve mouse serum samples and a specific
signal was detected for the serum samples from mAb2-immu-
nized mice with the expected reciprocal dilution profile. The
polyclonal detection antibody was also suitable for the analysis of
the serum from mAb1-immunized mice (Figure 3(B)) with a vig-
orous signal detected regardless of whether the coating substrate
was monomeric or aggregated protein. Comparison of blocking
agents (Supplementary Figure 1(A)) for the polyclonal detection
agent in mAb2-immunized mice serum ELISAs indicated that 5%
milk protein was the most appropriate block in terms of mini-
mizing background and maximizing signal.

Analysis of the serum antibody titers revealed that there were
no false positive IgG, IgG1 or IgG2a antibody readouts in the
negative control samples, regardless of whether aggregated or
monomeric protein was used as the substrate. Relatively high
expression levels of mAb IgG, IgG1 antibodies were found in
sera isolated from monomer or aggregate immunized mice, with
virtually identical titration curves irrespective of whether immu-
nization was with the monomer or the aggregated form, or
whether the substrate was monomeric or aggregated. In contrast,
only immunization with the aggregated form of mAb resulted in
a high level of IgG2a antibody production. This was robust and
reproducible finding observed in independently repeated exper-
iments. Thus, in each experiment, equivalent IgG and IgG1
antibody titers were recorded following immunization with either
forms of the mAb, and regardless of the substrate used in the
ELISA. A significantly higher titer IgG2a antibody response was
recorded in sera from mAb1 aggregate compared with mAb1
monomer immunized mice (Rane et al., 2019). But results were
inconclusive for IgG2a for mAb2 immunization experiments. This
paper gives the details on how this issue was resolved.

A summary of the results and IgG2a proportion of total IgG
generated in response to aggregated mAb1 or mAb2 immuni-
zations (vs monomer or vs aggregate protein substrate) is
shown in Figure 4. For mAb1 ELISAs using monomeric and
aggregate substrates, serum titers could be determined using
both the anti-IgG2a antibodies (polyclonal as well as monoclo-
nal detection antibodies) and are shown as proportion of total
IgG (Figure 4(A,B)). Pie charts illustrate the proportion of
IgG2a within total IgG for individual mice per group. Using
monomer and aggregate substrates for ELISA experiments dem-
strated subtle differences in individual mice responses; how-
ever, that did not have a profound effect in overall
experimental outcome, and are demonstrated in a pie chart for
individual mice (Figure 4(C)). Naïve mice sera were used for
comparison and demonstrated very low IgG2a titers compared
to mAb1 and mAb2 immunization groups. Summary of serum
titers obtained in mAb1- and mAb2-immunized groups using
both the anti-IgG2a detection antibodies and both monomeric
and aggregate protein substrates is shown in Figure 4(D).
Serum titers for IgG2a were generated using both the detection
antibodies for mAb1-immunized mice whereas for mAb2-immu-
nized mice sera titers could be generated with polyclonal detec-
tion antibody only in ELISA experiments.

Figure 3. IgG2a responses induced by immunization with aggregated mAb1 or mAb2. Serum samples were analyzed for IgG2a, against both forms of immunizing pro-
teins as substrate (vs monomeric and vs aggregated protein) by ELISA. (A) HRP-rat anti-mouse IgG2a mAb was used for detection of serum IgG2a. (B) Serum IgG2a
responses detected using polyclonal anti-mouse IgG2a secondary antibody are shown. Naïve negative control serum samples were analyzed in parallel. Data are repre-
sentative of two independent experiments using pooled serum for mAb1 (n = 5), and mAb2 (n = 5) and pooled serum for naïve control (n = 3).
Choice of monomeric or aggregated protein substrate used in the ELISA experiment did not have any significant effects on the results obtained. No significant difference between mAb1 monomeric substrate with polyclonal or monoclonal detection antibody as well as aggregate substrate with polyclonal or monoclonal detection antibody was observed. With mAb2, no significant difference was observed between polyclonal detection on monomeric or aggregate substrate, though high background issues were encountered with monoclonal detection antibody.

Discussion
With the advent of novel analytical technologies, there are significant improvements in the ability to analyze and characterize...
biotherapeutics. Immunoassays provide cost-effective, high-throughput methods and could therefore represent an effective strategy for evaluation of potential immunogenicity. Humanized animal models are now being established to study these biological effects (Jiskoot et al. 2016). ELISA provides an effective method to monitor the emergence of antibody responses with time and treatment (Li et al. 2001; Tabrizi and Roskos 2007; Geng et al. 2015). In the current investigation, a BALB/c murine model was used and human mAb (at monomeric and aggregated states) were assessed to optimize the IgG and isotype ELISA assays. These observations were consistent with those reported previously (Ratanji et al. 2017; Rane et al. 2019).

When experiments were repeated using same experimental set up for mAb2-immunized group, nonspecific cross-reactivity was observed in IgG2a ELISAs - with higher background readings in test wells as well as in negative controls. This was later resolved using different a detection antibody system. The ADA responses reflect impact on both the T-dependent and T-independent compartments of the immune system (Larocca et al. 1989; Amalfitano et al. 2001; Bertolotto et al. 2003), and underscores the need to characterize the immunogenic responses to biotherapeutic mAb individually. The current investigation highlighted the importance of optimization and evaluation of detection system used for individual biotherapeutic mAb before concluding negative observations, in this case absence of Th1-polarized immunogenicity in response to mAb2 immunizations.

Protein aggregation can be described as the self-association of monomers in their native or partially unfolded forms (Chi et al. 2003; Roberts 2007), and is a common phenomenon observed in biopharmaceutical preparations. There is evidence that aggregates can stimulate an anti-drug immune response which may impair drug efficacy. However, the mechanisms through which immunogenicity is enhanced or conferred on proteins are only poorly understood. Aggregates that may be present in protein products can range from dimers to subvisible or visible particles and can be formed during different stages of production, transport or delivery to the patient, in response to diverse stresses (Chi et al. 2003; Mahler et al. 2009). Aggregates formed in biotherapeutic monoclonal antibodies under the influence of various stresses have been characterized by various techniques on the basis of their sizes, ranging from nm to micron dimensions (Fifis et al. 2004; Morefield et al. 2005; Filipe et al. 2010; Joubert et al. 2011). The mAb aggregates employed in this study fall within this range and can therefore be regarded as typical, at least in terms of size, compared with those studied previously.

The link between aggregation and enhanced immunogenicity is well established in mouse models (including transgenic animals). For example, aggregate percentage and the extent of denaturation of interferon (IFN)β-1a have been shown to influence the ability of aggregates to break tolerance in transgenic mice (van Beers et al. 2010). Aggregates range in size and dimensions in the 0.1–10 μm range have been identified as being the most immunogenic (Cromwell et al. 2006). Characterization of the aggregated mAb using Raster Image Correlation Spectroscopy (RICS) has been reported previously (Rane et al. 2019). Percentages of aggregates to determine the level of aggregation that increases immunogenicity may not be relevant in the context of this study. Characteristics of aggregates which may contribute to immunogenic potential include the formation of neo-epitopes, multiple valency, post-translational modifications, concentration, and size (Braun et al. 1997; Ryff and Schellekens 2002; Schellekens 2002). The murine model used here helps us to understand that humanized mAb, though with similar backbone and structure, may behave differently under similar experimental conditions assessing mAb immunogenicity. Thus, it is important that each mAb is studied on a case-by-case basis.

In the current investigation, it was noted that the ELISA detection system used plays a crucial role in the experimental outcome and conclusions derived. The epitopes identified by the commercially-available detection antibodies have the potential for cross-reactivity with the ELISA substrate due to the exposed epitopes; the blocking reagents used also play important roles in preventing nonspecific binding of antigen and antibodies to microtiter plates as described previously (Chart et al. 1998). Assays utilized to assess and characterize immunogenicity should be designed to be sensitive and specific for the intended purpose. Appropriate experimental design, reagents used, and timepoints should be determined along with recommendations for an assay procedure, when needed, to detect meaningful antibody responses at pre-clinical and clinical stages in the development of ELISA assays using therapeutic mAb.

Conclusions

The present study confirmed that the binding epitope of anti-mouse secondary/detection antibodies used in the experiments play a critical role in study design as well as experimental outcome. It also proved beneficial in the current study to optimize and assess different blocking reagents used, commercially-available and commonly-used secondary antibodies that highlighted the differences in IgG2a responses. It was also observed (with comparison to a previously-published study) that although both mAb have IgG1 subclass, subtle structural differences in the mAb can give rise to neo-epitopes inducing altered experimental outcome. This may play a critical role in characterization of ADA responses. Hence it is important to assess and optimize individual biotherapeutic monoclonal antibody proteins in the experimental systems.

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Author contributions

SSR and RJD conceived the idea; SSR performed the experiments and wrote the manuscript; JPD, RJD and IK reviewed the manuscript critically.

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