Novel bioanalytical method for the characterization of the immune response directed against a bispecific F(ab) fragment

Olaf Broders^{*1}, Uwe Wessels¹, Markus Zadak¹, Roland Beckmann² & Kay Stubenrauch¹

¹Roche Pharma Research & Early Development, Pharmaceutical Sciences, Roche Innovation Center Munich, Im Nonnenwald 2, 82377 Penzberg, Germany

²Roche Pharma Research & Early Development, Therapeutic Modalities, Roche Innovation Center Munich, Im Nonnenwald 2, 82377 Penzberg, Germany

*Author for correspondence: Tel.: + 49 8856 601 9809; olaf.broders@roche.com

Aim: The work was aimed at developing a bioanalytical approach to identify immunogenic parts of a bispecific F(ab) fragment and to characterize the immune response seen in a preclinical study. **Experimental:** The bioanalytical method consists of a set of domain detection assays that use germlined variants of the drug. **Results:** The method demonstrated that anti-drug antibodies (ADAs) were predominantly directed against both antigen-binding sites of the drug. **Conclusion:** The method was capable to discriminate between ADAs directed against one of the antigen-binding sites, both sites or the constant domain, allowing for an estimation of the relative binding prevalence for these subunits. The developed approach provides a practical and robust solution for exploratory characterization of ADAs against multidomain biotherapeutics.

First draft submitted: 22 March 2020; Accepted for publication: 8 April 2020; Published online: 30 April 2020

Keywords: ADA • anti-drug antibody • bispecific F(ab) • DDA • domain detection assay • immunogenicity response characterization • MDB • multi-domain biotherapeutic

Many biotherapeutics are nowadays designed to provide multiple functionalities, which enable complex mechanisms of action. This can be achieved by incorporation of several functional domains or engineered amino acid sequences [1,2]. We have recently combined both approaches to develop bispecific antibody F(ab) fragments that are designed and optimized to simultaneously recognize two different targets with high specificity and affinity. Like any other protein, these complex biotherapeutics can elicit an immune response in the recipient animal or human [3,4] and thus immunogenicity assessment becomes an essential part of their development program.

Indeed, in a preclinical nonhuman primate study with a bispecific F(ab) fragment against antigens 1 (AG1) and 2 (AG2), we found a high incidence of anti-drug antibodies (ADAs) detected by a previously published bridging assay format [5]. This finding required further characterization of the immune response as the immunogenic reaction could be caused by one of the antigen-binding sites or by the backbone of the molecule, which contained several point mutations in the constant region.

Established methods for ADA characterization are classical domain competition and domain detection assays (DCAs, DDAs) [6,7], where the biotherapeutic is enzymatically cleaved into individual domains or distinct fragments with subsequent immobilization of these fragments to capture domain-specific ADAs (DDA setup) or use as competition reagents in a combination with the labeled uncleaved drug (DCA setup) [8]. These widely used approaches are particularly valuable for large protein molecules, for example, IgG or IgG-like modalities and fusion proteins, but their utility is limited for smaller biotherapeutics, such as single-chain Fv (scFv) fragments, F(ab) fragments, or bispecific F(ab)s, because often these smaller molecules cannot be easily cleaved into subdomains or the produced subdomains are not sufficiently stable.

We therefore sought to develop a novel bioanalytical approach that would allow us to identify immunogenic parts of the bispecific F(ab) fragment and thus characterize the immune response seen in the preclinical study.

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Experimental

Chemicals, reagents & equipment

The following antibodies and reagents were provided by Roche Diagnostics GmbH, Penzberg, Germany: digoxigenin (Dig)-labeled monoclonal antibody (mAb) against cynomolgus IgG (Anti-cyno IgG), anti-Dig polyclonal sheep antibody F(ab)-fragments coupled with horseradish peroxidase (<Dig>S-F(ab)-HRP), unlabeled and Dig-labeled anti-human kappa M1.7.10 mAb (anti-human kappa IgG), Dig-labeled recombinant AG1 and AG2, phosphate-buffered saline (PBS), Tween 20, 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), bovine serum albumin (BSA). Unlabeled, biotin (Bi) and Dig-labeled human bispecific F(ab) fragment against AG1 and AG2 (<AG1/AG2>), variant F(ab) fragments with either AG1-binding (Germ/<AG2>), AG2-binding (Germ/<AG1>) or both antigen-binding sites (Germ/Germ) back-mutated to human germline were generated at Roche Diagnostics GmbH as described below. Synthetic genes encoding the F(ab) fragments were purchased from GeneArt, Regensburg, Germany. Ranibizumab was provided by Novartis, Basel, Switzerland. Antibodies, F(ab) fragments, Dig-AG1 and Dig-AG2 were stored in aliquots at -80°C until use. CaptureSelect IgG-CH1 and CaptureSelect Kappa LX resins were from GE Healthcare, Uppsala, Sweden. Individual citrate-theophylline-adenosine-dipyridamole (CTAD) plasma samples from drug-naïve female and male cynomolgus monkeys were obtained from Sera Laboratory International, Ltd., Haywards Heath, UK. Ready-to-use LowCross Buffer™ was obtained from Candor Bioscience GmbH, Wangen, Germany. Streptavidin-coated microtiter plates (SA-MTPs) were obtained from Microcoat Biotechnologie GmbH, Bernried, Germany. Uncoated Nunc Maxisorp™ MTPs were obtained from Thermo Fisher Scientific, Frankfurt, Germany.

Generation of bispecific & variant F(ab) fragments

Synthetic genes encoding the bispecific F(ab) fragment and variant F(ab) fragments Germ/<AG1>, Germ/<AG2>, and Germ/Germ were cloned into a vector for periplasmic expression in *E. coli*. To ensure isolation of only full-length, intact molecules from culture supernatants, F(ab) fragments were double affinity purified using CaptureSelect IgG-CH1 and CaptureSelect Kappa LX resins. Protein concentration of purified F(ab) fragments was determined by spectrophotometry using absorbance at 280 nm.

Study samples

Plasma samples were taken from a preclinical tolerability study in cynomolgus monkeys treated with bispecific F(ab) <AG1/AG2>. Sixteen samples from eight different animals who were tested positive in the initial ADA screening by the bridging assay were selected for further ADA characterization with the newly developed DDAs. From each animal, the Day 35 postdose sample was tested and the predose sample was used as a negative control.

Bridging ADA assay (ADA screening assay)

The initial detection of ADA-positive samples from the preclinical study was performed using the previously published bridging immunoassay [9]. In brief, study samples were diluted 1:20 with PBS/0.5% BSA and then incubated with Bi- and Dig-labeled bispecific F(ab) <AG1/AG2> fragments. The formed complexes were captured on an SA-MTP and detected with anti<Dig>S-F(ab)-HRP and ABTS. The color reaction was monitored by photometrical readout at 405 nm (reference wavelength 490 nm). Anti-human kappa IgG was used as a positive control. The floating cut point (CP) approach according to Shankar *et al.* [10], was used to differentiate between positive and negative samples. The normalization factor was determined using 40 individual plasma samples from drug-naïve female and male cynomolgus monkeys.

Domain detection assays

The DDAs were performed as a four-step solid phase ELISA. The drug molecule (bispecific F(ab) fragment <AG1/AG2>), variant F(ab) fragments (Germ/<AG1>, Germ/<AG2>, Germ/Germ), and ranibizumab were directly immobilized on a Nunc MaxiSorp™ MTP using solutions in PBS at a concentration of 3.33 µg/ml. Samples were diluted in Low Cross Buffer™ (1:100, 1:1000 or 1:10000), added to the MTP wells (100 µl) and incubated for 1 h at room temperature. After washing with PBS/0.1% Tween, the bound ADAs were detected using Dig-labeled Anti-cyno IgG (0.5 µg/ml), which specifically binds monkey IgGs but not immobilized F(ab) fragments [11]. Readout was performed with <Dig>S-F(ab)-HRP and ABTS as described for the bridging assay. All five assays were performed on a single plate to ease execution and ensure comparability and all samples were measured at three dilutions in parallel. In addition, capture control assay (CCA) 1 performed on the same plate

Figure 1. Domain detection assays. (A) Schematic representation of the developed domain detection assays (DDAs). Five different F(ab) variants were used to capture domain-specific anti-drug antibodies (ADAs): the original bispecific F(ab) fragment (<AG1/AG2>; assay 1), bispecific F(ab) with either AG1-binding (Germ/<AG2>; assay 3), AG2-binding (Germ/<AG1>; assay 2) or both antigen-binding sites (germ/germ; assay 4) back-mutated to human germline, and ranizumab, a commercial anti-VEGF F(ab) without mutations in the constant region (assay 5). The captured ADAs were recognized by Anti-cyno IgG. (B) Expected positive/negative signals for ADAs that are specific for AG1-binding site (<AG1-CDRs>), AG2-binding site (<AG2-CDRs>), or constant regions (<constant regions>). AG1: Antigen 1; AG2: Antigen 2; CDR: Complementarity-determining region; Dig: Digoxigenin; HRP: Horseradish peroxidase.

during sample analysis served as a quasi-positive control ('surrogate positive control') to ensure the comparability of individual measurements on different plates. The CCA signal was monitored and the analysis was stopped at signal intensities of 1.8–2.2 OD_{405nm}. Cut point determination was performed with eight predose samples for each of the five DDAs; the 99th percentile was used for the CP calculation.

Capture control assays

Nunc MaxiSorp™ plates were coated with all five different molecules as described for the DDAs. Subsequently, Dig-labeled anti-human kappa (for CCA1), AG1 (for CCA2) or AG2 (for CCA3) were added at a concentration of 50 ng/ml and incubated for 1 h at room temperature. The detection was performed using <Dig> S-F(ab)-HRP and ABTS as described for the bridging assay.

Results & discussion

Assay format

We generated four variants of the bispecific F(ab) fragment using back-mutation of the respective antigen-binding sites to human germline to abolish the specificity for AG1, AG2 or both AG1/AG2. These variants were used along with ranizumab, a commercial anti-VEGF F(ab) without mutations in the constant region, which served as an additional control, to develop DDAs that can discriminate between ADAs directed against one of the specificities, both specificities, and the constant domain (CH1/Ck backbone) of the molecule (Figure 1).

Figure 2. Schematic representation of the capture control assays used to demonstrate the reproducibility of the coating and the functionality of the immobilized proteins in the domain detection assays. Digoxigenin (Dig)-labeled antigen (AG) 1, AG2, and anti-human kappa IgG were used as capture controls for the five different F(ab) variants immobilized on a microtiter plate in the DDAs (see Figure 1).

Table 1. Expected positivity/negativity in capture control assays.						
Assay	Controls	Immobilized F(ab) variants				
		F(ab) <AG1/AG2> (DDA1)	Germ/<AG1> (DDA2)	Germ/<AG2> (DDA3)	Germ/Germ (DDA4)	Ranibizumab (DDA5)
CCA1	AG1-Dig	+	+	–	–	+
CCA2	AG2-Dig	+	–	+	–	–
CCA3	Anti-hu-kappa-Dig	+	+	+	+	+

AG: Antigen; CCA: Capture control assay; DDA: Domain detection assay; Dig: Digoxigenin; Germ: Germline.

Assay validation

The availability of specific positive controls, which are required for an optimal assay validation, poses a challenge for the development of DDAs [7,9]. Positive control antibodies (specific anti-idiotypic antibodies) can be generated in mice, which is a time-consuming and laborious process involving immunization with the complete compound and a good screening concept. Alternatively, already available antibodies against individual domains can be used [8]. The latter was not possible for our approach because of the novelty of the molecule. Likewise, antibody generation does not allow a rapid assay development, especially considering that our approach is based on five different DDAs and thus it ideally requires the generation of five distinct positive controls.

Since our main goal was to demonstrate the feasibility of the developed bioanalytical method and to gain a basic understanding of the observed ADA response, we decided in favor of a rapid assay development using a combination of CCAs and an assay-specific CP determination. Although assay validation, our standard practice, was not possible without positive controls, we believe that our approach represents a practical solution, which has provided meaningful and reliable data.

Capture consistency

The robustness of the coating procedure is a prerequisite for the DDAs comparability. We have therefore established three different CCAs and used them to demonstrate the reproducibility of the coating and the functionality of the immobilized F(ab) variants (Figure 2 & Table 1). The precision values obtained in these assays reflect the reproducibility of the coating. The human anti-kappa IgG-based CCA shows the efficiency of the surface coating and the AG1- and AG2-based CCAs can confirm that the complementarity-determining regions (CDRs) of the immobilized F(ab) fragments are accessible to the antigen.

The precision data for all five individual capture proteins show little variation in the amount of the immobilized F(ab) fragments and their ability to bind antigens (Table 2), thus indicating that surface coating was robust. The data also confirmed that variants back-mutated to human germline lost their specificity for the respective target, whereas target binding by the intact specificity was not affected.

Table 2. Precision data for capture control assays.

Controls	Capture				
	F(ab)<AG1/AG2>	Germ/<AG1>	Germ/<AG2>	Germ/Germ	Ranibizumab
AG1-Dig					
Intra-assay precision (%CV)	1	1	— [†]	— [†]	1
Inter-assay precision (%CV)	5	10	— [†]	— [†]	4
AG2-Dig					
Intra-assay precision (%CV)	2	— [†]	2	— [†]	— [†]
Inter-assay precision (% CV)	3	— [†]	9	— [†]	— [†]
Anti-human-kappa-Dig					
Intra-assay precision (%CV)	1	3	2	1	2
Inter-assay precision (%CV)	2	2	2	2	4

[†] Construct without binding competency to the respective capture control.
AG: Antigen; CV: Coefficient of variation; Dig: Digoxigenin; Germ: Germline.

Table 3. Cut point determination for domain detection assays.

Parameter	Immobilized F(ab) variants				
	F(ab)<AG1/AG2> (DDA1)	Germ/<AG1> (DDA2)	Germ/<AG2> (DDA3)	Germ/germ (DDA4)	Ranibizumab (DDA5)
MRD	100	100	100	100	100
Sample volume (for double determination)	3 µl	3 µl	3 µl	3 µl	3 µl
Normalization factor	1.48	1.40	2.00	1.66	1.34
Assay signals at screening cut point	0.117	0.206	0.159	0.104	0.049

Study-specific CPs were calculated by measuring the corresponding predose samples (n = 8).
DDA: Domain detection assay; MRD: Minimum required dilution.

Cut point determination for DDAs

Study-specific CPs were calculated for all five DDAs using values obtained for the corresponding predose samples (Table 3). The signals of the predose samples were well comparable between the DDAs (Figure 3), which further supported the chosen approach to CP determination. Of note, our calculations are based on data from eight animals, whereas the current recommendations suggest using at least 50 baseline samples for CP calculation [10,12]. Such a high sample number is not feasible for many preclinical studies, including the one used in this work. However, given the exploratory nature of the established DDAs, we believe that the chosen approach is sufficiently reliable for the purpose of the assays.

Sensitivity

The three different CCAs were performed with 50 ng/ml of the detection agent (AG1, AG2, or human anti-kappa IgG) and the signals reached in these assays were ten- to 20-times above the corresponding CPs of the five DDAs (data not shown). Therefore, we estimate the sensitivity of the DDAs to be above 50 ng/ml.

Drug tolerance of DDAs

The samples used in this study were from late time points (day 35 postdose) and therefore they had low levels of the residual drug (data not shown). We thus do not expect that the residual drug has had any impact on the obtained results. Theoretically, drug tolerance of the developed DDAs should be at least as good as that of a standard bridging assay because the F(ab) fragments are immobilized at a very high concentration in the DDAs, in other words, they will normally outcompete the residual drug in the samples. If necessary, the drug tolerance of the DDAs can be improved in a similar way as for bridging ADA assays, for example, by pretreatment with acid to dissolve existing drug–ADA complexes [13].

Figure 3. Domain detection assay results for anti-drug antibodies from the preclinical study with the bispecific F(ab) fragment. Day-35 post-dose optical density (OD) signals and respective predose signals are shown. AG1: Antigen 1; AG2: Antigen 2; Germ: Germline.

Sample analysis with DDAs

Sample analysis with the developed DDAs revealed four distinct patterns of ADA responses (Figure 3 & Supplementary Table 1): a strong response equally to both CDRs and a moderate response to the backbone (animals one and two), a moderate or strong response equally to both CDRs and no response to the backbone (animals three and four), a strong response equally to both CDRs and a weak response to the backbone (animals five and six) and a weak and unequal response to the CDRs and no response to the backbone (animals seven and eight). Since all predose samples were ADA-negative in all DDAs, we conclude that observed immunogenicity response in the postdose samples was related to the treatment. This was also a good indicator of the specificity of the method.

Different sample dilutions were used when comparing DDAs results because the linear range of the chromogenic ABTS substrate is limited and samples need to be appropriately diluted depending on the strength of the immune response for clearly distinguishable signal intensities. A fluorogenic substrate, such as 3-p-hydroxyphenylpropionic acid (HPPA), can potentially help mitigate this problem [14].

Most animals showed equal ADA responses against both CDRs and in all animals the ADA response to both CDRs was positive. Thus, our data do not indicate that one of the two CDR regions of the bispecific F(ab) fragment was the predominant driver of the observed immune response. The assay setup provides information about polyclonal immune responses and therefore we are not able to make statements about the exact epitope of the ADA. However, the developed DDAs addressed our primary question, namely to identify immunogenic parts of the bispecific F(ab) fragment.

In this cynomolgus monkey study, another point that we tried to address with the developed DDAs was whether the constant backbone of the engineered bispecific F(ab) bears an increased risk of immunogenicity due to its slightly different scaffold compared with the wild-type F(ab) fragment. Although four of eight tested animals had antibodies against the constant regions, in none of these animals the signal was significantly higher for the Germ/Germ variant as compared with ranibizumab, an ocular therapeutic known for its low immunogenicity [15]. Thus, the DDAs did not provide evidence of an immune response against a neoepitope on the Germ/Germ variant that does not exist on ranibizumab.

Conclusion

We have developed bioanalytical assays to identify predominant immunogenic parts of a bispecific F(ab) fragment. The assays are based on a combination of molecular engineering and an efficient and easy-to-establish DDA setup, providing a practical and robust solution for exploratory characterization of ADA against smaller biotherapeutics with good data quality. Using these assays, we found different immunogenicity response patterns elicited by the bispecific F(ab) fragment. ADA response was largely targeted at the CDRs with little difference between the two antigen-binding sites, whereas the constant parts of the drug molecule were not more immunogenic than ranibizumab.

Future perspective

Immunogenicity monitoring using bridging ADA screening assays can detect the overall immunogenicity, but it cannot provide insights into potential immunogenic hotspots [4]. However, depending on the mechanism of action of a drug, immunogenicity assessment may need to be extended beyond immunogenicity monitoring to closely characterize the epitopes or domains recognized by ADAs.

The presented approach can help identify immunogenic domains of complex biotherapeutics. In contrast to classical bioanalytical ADA characterization, which often relies on enzymatic cleavage of the drug molecule before analysis [4,8], this approach does not require generation of subfragments and thus it will be especially advantageous for smaller protein biotherapeutics such as F(ab)s or scFv.

Although immunogenicity response pattern in a nonhuman species is generally not predictive of that in human, the new approach can still be relevant as a preclinical proof of concept during the development of the new modalities. From the discovery perspective, early evaluation of an intrinsic immunological risk and understanding of immunogenic properties will greatly contribute to developability assessment. The presented assays, which use germlined variants of clinical lead candidates, are robust yet easy-to-establish and thus they will be particularly suitable for such early exploratory evaluations.

Furthermore, generation of specific positive controls can enable optimal assay validation and thus the adaptation of the method to nearly all antibody-based therapeutics. Such an adapted method can add value to clinical immunogenicity assessment and characterization of multidomain biotherapeutics.

Executive summary

Background

- We developed an easy-to-establish bioanalytical method to identify predominant immunogenic parts of a bispecific F(ab) fragment.

Experimental

- We combined a molecular engineering approach with an easy-to-establish ELISA setup to develop a set of domain detection assays.

Results & discussion

- Using these assays, we were able to characterize immunogenic response to a bispecific F(ab) fragment in a preclinical cynomolgus monkey study.
- We were able to distinguish different immunogenicity response patterns and we found that anti-drug antibodies were predominantly directed against both antigen-binding sites of the drug.

Conclusion

- The assays were capable to discriminate between anti-drug antibodies directed against one of the antigen-binding sites, both sites, and the constant domain, allowing for an estimation of the relative binding prevalence for these molecular subunits.
- This novel approach can be a valuable and easy-to-establish tool for preclinical immunogenicity assessment of multidomain proteins, especially smaller protein biotherapeutics.
- The method can be further adapted to larger antibody therapeutics and derivatives and it can also be used to complement clinical immunogenicity assessment and characterization.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/bio-2020-0064

Acknowledgements

The authors thank all colleagues involved from Large Molecule Bioanalytical R&D, Large Molecule Research, Pharmaceutical Sciences and Diagnostics R&D for generation of assay tools, inspiring discussions and review. In addition, we thank Florian Neff and Rubén Álvarez Sánchez for critical review and Alexander Nürnberg (Roche Basel) for the diligent preparation of the manuscript.

Financial & competing interests disclosure

All authors are employees of Roche Diagnostics GmbH. Some authors are owners of Roche bonus shares and profit certificates. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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