Research Article

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Bioanalysis

Detection of antidrug antibodies against human therapeutic antibodies lacking Fc-effector functions by usage of soluble Fcγ receptor I

Aim: Bridging immunoassays for the detection of antidrug antibodies (ADAs) are limited to detection of bivalent molecules and are prone to interference by drug and soluble targets. Hence, alternative approaches for ADA detection are desired. **Materials & methods:** A novel ADA assay with secondary Fc detection using human soluble Fcγ receptor I (hsFcγRI) was established and compared with standard bridging assay. **Results:** Both assays showed consistent results in human and cynomolgus monkey samples. In contrast to the bridging assay, the hsFcγRI-based assay was insensitive to the presence of oligomeric targets and appeared to have better drug tolerance. **Conclusion:** The hsFcγRI-based ADA assay can serve as alternative screening assay or as orthogonal confirmation method for preclinical and clinical immunogenicity testing of IgG therapeutics lacking Fc effector functions.

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Keywords: antidrug antibody • drug interference • ELISA • FcγRI • immunogenicity • safety assessment • suppressed Fc effector functions • target interference

Uwe Wessels*[,1], Alexander Poehler[1], Miriam Moheysen-Zadeh[1], Markus Zadak[1], Roland F Staack[1], Pablo Umana[2], Julia Heinrich[1] & Kay Stubenrauch[1]

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

[2]Roche Pharma Research & Early Development, Roche Innovation Center Zurich, Wagistrasse 18, 8952 Schlieren, Switzerland

*Author for correspondence:
Tel.: +49 885 660 3814
Fax: +49 885 660 3268
uwe.wessels@roche.com

As of today, antidrug antibody (ADA) bridging immunoassays (Figure 1A) represent the state-of-the-art assay format for immunogenicity testing due to their high throughput and sensitivity [1]. However, the specificity of these assays may be challenged by the interference of dimeric soluble target molecules or matrix components (Figure 1B) [2–5]. Such interference cannot be sorted out by the drug competition test, which is typically performed as the second, confirmatory step of an ADA analysis. Thus, this analysis cannot verify that the obtained positive signals are truly generated by ADAs. Moreover, the presence of residual drug may increase the risk of false-negative results because bridging assays are unable to detect ADAs of which one biding site is occupied by an unlabeled drug molecule.

Alternative orthogonal methods of ADA detection lack these limitations and are especially desired for cases in which clinical data need detailed explanation. Indeed, approaches using an immobilized drug as capture and secondary anti-IgG detection are common for nonantibody therapeutics [6]. For therapeutic mAbs, such approaches are, however, limited to preclinical studies [7,8] since selective anti-IgG-detection of human ADAs is not possible in human samples. Yet, many mAbs bear deliberately introduced mutations in the Fc region, such as those resulting in suppressed Fc effector functions (supFcEF), and are, therefore, distinguishable from human ADAs. Thus, receptors which target the Fc region and whose binding is affected by such mutations can be used to selectively detect ADAs in the presence of a supFcEF therapeutic mAb.

The Fc gamma receptor family (FcγRs) of IgG receptors is of special interest for this approach. FcγRs have been already used to specifically detect immune complexes, taking the advantage of the low affinity binding of FcγRIII to IgG [9]. Among the identified

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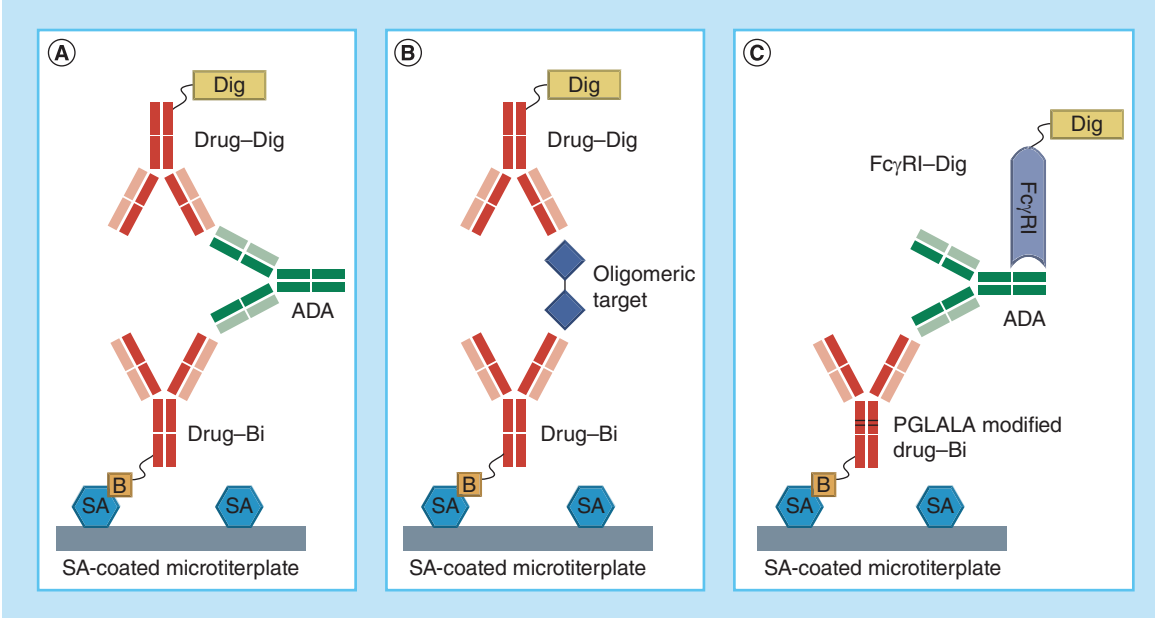

Figure 1. Principles of the antidrug antibody bridging and hsFcγRI-based assays. (A) The ADA-bridging assay relies on the predominantly bivalent nature of ADAs, which form a mixed bridging complex between immobilized drug (Drug–Bi) and labeled drug used for detection (Drug–Dig). **(B)** A soluble oligomeric target can interfere with the bridging assay by forming a bridging complex. **(C)** The alternative assay with an Fc-specific detection reagent (hsFcγRI). The principle of the assay allows its use in human samples and precludes the risk of false positive results in the presence of oligomeric drug targets.
ADA: Antidrug antibody; Bi: Biotin; Dig: Digoxigenin; SA: Streptavidin.

human FcγRs, only FcγRI (CD64) shows high affinity binding to monomeric IgG, preferentially for subclasses IgG1 and IgG3, and to a somewhat lesser extent for IgG4 [10–13]. Hence, this receptor can be used for the secondary detection of ADAs in a diagnostic assay by using the recombinantly expressed ectodomain (extracellular ligand-binding domain) of the receptor (human soluble FcγRI [hsFcγRI]) [11]. Such an assay (Figure 1C) would allow for an improved detection and characterization of ADAs formed in response to supFcEF therapeutic mAbs.

In this study, we aimed to establish a hsFcγRI-based assay as an alternative to the standard (bridging) ADA screening and confirmation assay and to evaluate its applicability for the ADA detection in preclinical and clinical samples.

Materials & methods

Chemicals, reagents & equipment

All antibodies were produced by Roche Diagnostics GmbH, Penzberg, Germany, and were stored in aliquots at -80°C until use [14]. Anti-thymic stromal lymphopoietin receptor (TSLP-R) mAb is a recombinant humanized IgG4 bearing an SPLE mutation targeting the TSLP-R [15]. Anti-X mAb is a recombinant human IgG1 mAb targeting a dimeric protein (X). Biotin (Bi)- and digoxigenin (Dig)-labeling were performed in house.

Recombinant human soluble FcγRI/CD64 was purchased from R&D Systems, MN, USA, and was labeled with Bi and Dig in house. Polyclonal sheep antibody Fab fragments against Dig coupled with horse radish peroxidase (HRP; pAb–Dig–S–Fab–HRP), phosphate-buffered saline (PBS), Tween 20 and the substrates 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid and tetramethylbenzidine were obtained from Roche Diagnostics GmbH. Low Cross buffer™ was from Candor Bioscience GmbH, Wangen, Germany. Milk powder was obtained from Merck KGaA, Darmstadt, Germany. Bovine plasma albumin was purchased from Roche Diagnostics GmbH. Pooled cynomolgus monkey and human sera for *in vitro* studies were obtained from Sera Laboratory International, Ltd, Haywords Heath, UK.

Biacore® T100 instrument and Biotin CAPture Kit, Series S were obtained from GE Healthcare Life Science, Uppsala, Sweden. Streptavidin (SA)-coated microtiter plates (MTP) were acquired from Microcoat Biotechnologie GmbH, Bernried, Germany.

Specificity of hsFcγRI binding

The specificity of hsFcγRI binding was evaluated by surface plasmon resonance using Biotin CAPture Kit, Series S and Biacore T100 instrument. Experiments were conducted according to the manufacturer's instructions. The principle of the Biotin CAPture

Kit is based on reversible formation of a streptavidin-coated surface, which is achieved by hybridization of streptavidin-oligonucleotide conjugates to complimentary oligonucleotides immobilized on the sensor chip. The dehybridization with a standard reagent followed by rehybridization regenerates the streptavidin-coated surface for each new cycle.

Experiments were conducted according to the manufacturer's instructions. Briefly, biotinylated hsFcγRI was immobilized onto a Biacore CAP-Chip by injection of hsFcγRI (10 µg/ml in HBS-P$^+$ buffer [Hepes, NaCl, Surfactant P20]) for 60 s at a flow rate 10 µl/min and temperature 25°C. Thereafter, an antibody or serum sample (10 µg/ml) was injected for 60 s at a flow rate 10 µl/min and temperature 25°C. Resonance signal was measured 80 s after the start of injection. After analysis, the chip was regenerated by removing SA from the chip surface using Biotin CAPture Kit regeneration solution (6 M guanidinium hydrochloride, 0.25 M sodium hydroxide) and the procedure was repeated for the next sample.

Bridging ADA assay

ADAs against anti-TSLP-R mAb in human serum samples were detected by a one-step ELISA. Affinity-purified anti-idiotypic polyclonal rabbit antibodies directed against the complementarity determining region of anti-TSLP-R mAb (pAb–anti-Id–anti-TSLP-R) spiked into blank matrix were used as quality control (QC) samples. Reagents and samples were incubated at room temperature with shaking at 500 rpm. Antibody preparations were made in assay buffer containing PBS and 0.5% bovine plasma albumin. PBS with 0.05% Tween 20 was used as washing buffer.

Samples were diluted 1:50 with assay buffer containing Bi- and Dig-labeled mAb anti-TSLP-R (each at 0.5 µg/ml), incubated overnight and then transferred to a SA-MTP, which was preblocked for 1 h in 10% milk powder in PBS. Thereafter, the plate was incubated for 1 h, washed three-times with 300 µl washing buffer and 100 µl polyclonal anti-Dig-S-Fab-HRP conjugate (5 mU/ml) was added for 1 h. After washing, 100 µl tetramethylbenzidine (ready to use solution) was added to each well as substrate for color reaction, which was monitored by photometrical readout at 680 nm (reference wavelength 450 nm). Measurements were performed in duplicates and mean absorbance values were calculated; a measurement was accepted if the precision of duplicates was ≤20% of coefficient of variation.

A screening cut point (CP) was evaluated according to Shankar *et al.* [16] using a floating CP and non-parametric determination. By calculating a plate specific CP (blank of pooled serum multiplied by the normalization factor), screening negative (<plate-specific CP) and screening positive (≥plate-specific CP) samples were identified. Method validation was thoroughly performed according to recommendations [1,16,17].

ADA-screening positive samples were tested for specificity of the ADA response by performing this assay in the presence of the unlabeled drug (10 µg/ml), which was added to the diluted samples before over-

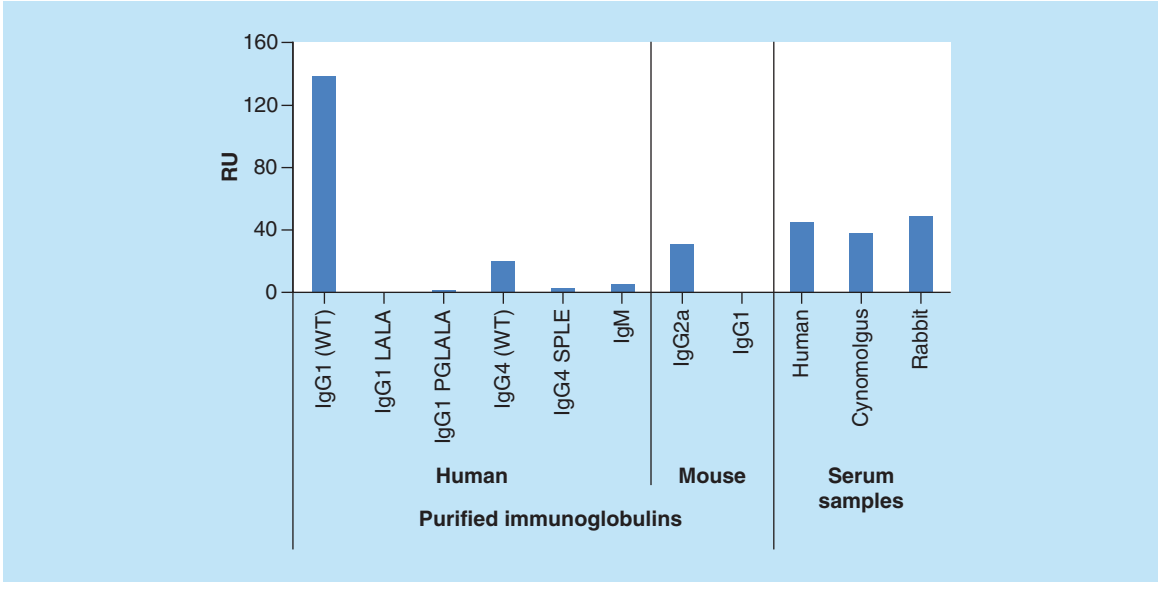

Figure 2. Specificity of hsFcγRI binding evaluated by surface plasmon resonance. The specificity was tested for purified human wild-type IgG1, wild-type IgG4, IgG subtypes with suppressed Fc effector functions (mutations PGLALA, LALA and SPLE), IgM, for purified mouse IgG2a and IgG1 as well as for human, cynomoglus and rabbit serum samples.
RU: Relative unit; WT: Wild-type.

Table 1. Validation of the bridging and hsFcγRI-based assays for detecting antidrug antibodies against anti-thymic stromal lymphopoietin receptor.

Parameter	Bridging ADA assay			hsFcγRI-based ADA assay		
Screening assay						
QC (ng/ml)	1000	200	80	3750	1800	275
Accuracy (%)	103	107	–	101	81	–
Precision (%CV)	2	1	1	4	3	5
Dilution factor	50 (2% serum)			50 (2% serum)		
Normalization factor	1.39			2.97		
Assay signal at screening cut point (mean of 6)	0.057			0.12		
Confirmation assay						
Drug assay concentration for confirmation (μg/ml)	10			200		
Confirmation cut point (%) based on 50 individuals	37			44		

ADA: Antidrug antibody; CV: Coefficient of variation.

night incubation. A confirmation cut point (CCP) was determined according to Shankar *et al.* [16] by screening of 50 blank individual serum samples in the presence of unlabeled drug. Screening positive samples showing a signal reduction higher than the CCP were considered as confirmed ADA positive samples.

The bridging assay to detect ADAs directed against anti-X mAb in cynomolgus monkey serum samples was performed as described above. A mouse anti-idiotypic mAb against the dimeric protein specificity (mAb-Id-X) spiked into blank matrix was used as QC sample. Concentrations of the Bi- and Dig-labeled drug were 1.0 μg/ml each, and 6.7 mg/ml of the unlabeled drug was used in the confirmatory analysis.

hsFcγRI-based ADA assays

A four-step solid phase ELISA as illustrated in Figure 1C was used as orthogonal ADA assay. The antibodies used as QC samples were the same as in the respective bridging assays. Reagents and samples were incubated at room temperature with shaking at 400 rpm. All antibody and sample preparations were made in Low Cross buffer. PBS with 0.05% Tween 20 was used as washing buffer. Washing was performed in three steps with 300 μl washing buffer each.

Bi-labeled anti-TSLP-R (0.5 μg/ml) was bound to a SA-MTP for 1 h and the unbound antibody was subsequently removed by washing. Thereafter, diluted (1:50) serum and QC samples were added and the plate was incubated for 1 h. After washing, Dig-labeled hs-FcγRI (0.5 μg/ml) was added, the plate was incubated for 1 h and then polyclonal anti-Dig-S-Fab-HRP conjugate (100 μl at 50 mU/ml) was added for further incubation for 1 h. 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid was used as substrate for color reaction, which was monitored by photometrical readout at 405 nm (reference wavelength 490 nm). Measurements were performed in duplicates and mean absorbance values were calculated; a measurement was accepted if the precision of duplicates was ≤20% CV. Plate-specific screening CP and assay validation parameters were determined as described for the bridging assay.

As a confirmatory step, ADA-screening positive samples were tested for specificity of the ADA response by performing this assay in the presence of the unlabeled drug (200 μg/ml), which was added to the diluted samples before incubation. Samples were defined as confirmed positive, when the signal intensities decreased below the determined CCP, which was calculated as described above.

The hsFcγRI-based screening assay to detect ADAs directed against anti-X mAb in cynomolgus monkey serum samples was performed as described above using the same concentrations of the Bi- and Dig-labeled drug. A confirmation assay was not performed by that time. However, since it has been established as an orthogonal confirmation assay, an additional confirmation seemed not to be necessary.

Determination of soluble target interference

Twelve samples of a cynomolgus serum pool were spiked with target protein X in concentrations ranging from 0.05 to 100 ng/ml. The samples were then analyzed by the bridging and hsFcγRI-based ADA assays.

Study samples

Human study samples were obtained from a single-ascending dose study with anti-TSLP-R in healthy male and female adults, which was aimed at examining safety, tolerability and pharmacokinetics. Blood samples were taken predose and at 19 time points post-dose (from 1.5 to 4500 h). After collection, the blood samples were immediately centrifuged and the obtained serum fractions were stored frozen (-70°C) until analysis. Cynomolgus monkey serum samples were obtained from a preclinical study that was aimed at evaluating the systemic tolerance and toxicokinetics of the anti-X antibody administered twice (day 1 & day 15) via intravenous injection. Blood samples were taken predose and 7 (168 h), 14 (336 h) and 16 days (384 h) after the first dosing (the last sample was taken 1 day after the second dosing). Samples were collected in serum separator tubes and allowed to clot before centrifugation; the obtained serum fractions were stored frozen until analysis.

Titration of study samples

ADA-positive anti-TSLP-R samples were initially diluted 1:25 using Low Cross buffer to reach 4% serum matrix content (titration sample 1). Subsequent serial 1:3 dilutions of this sample were performed seven-times using Low Cross buffer containing 4% human pooled serum (titration samples 2 through 8). For the bridging assay, titration samples 1 through 8 were diluted 1:2 to the final assay concentration of 2% matrix using assay buffer with the Bi- and Dig-labeled drug. For the

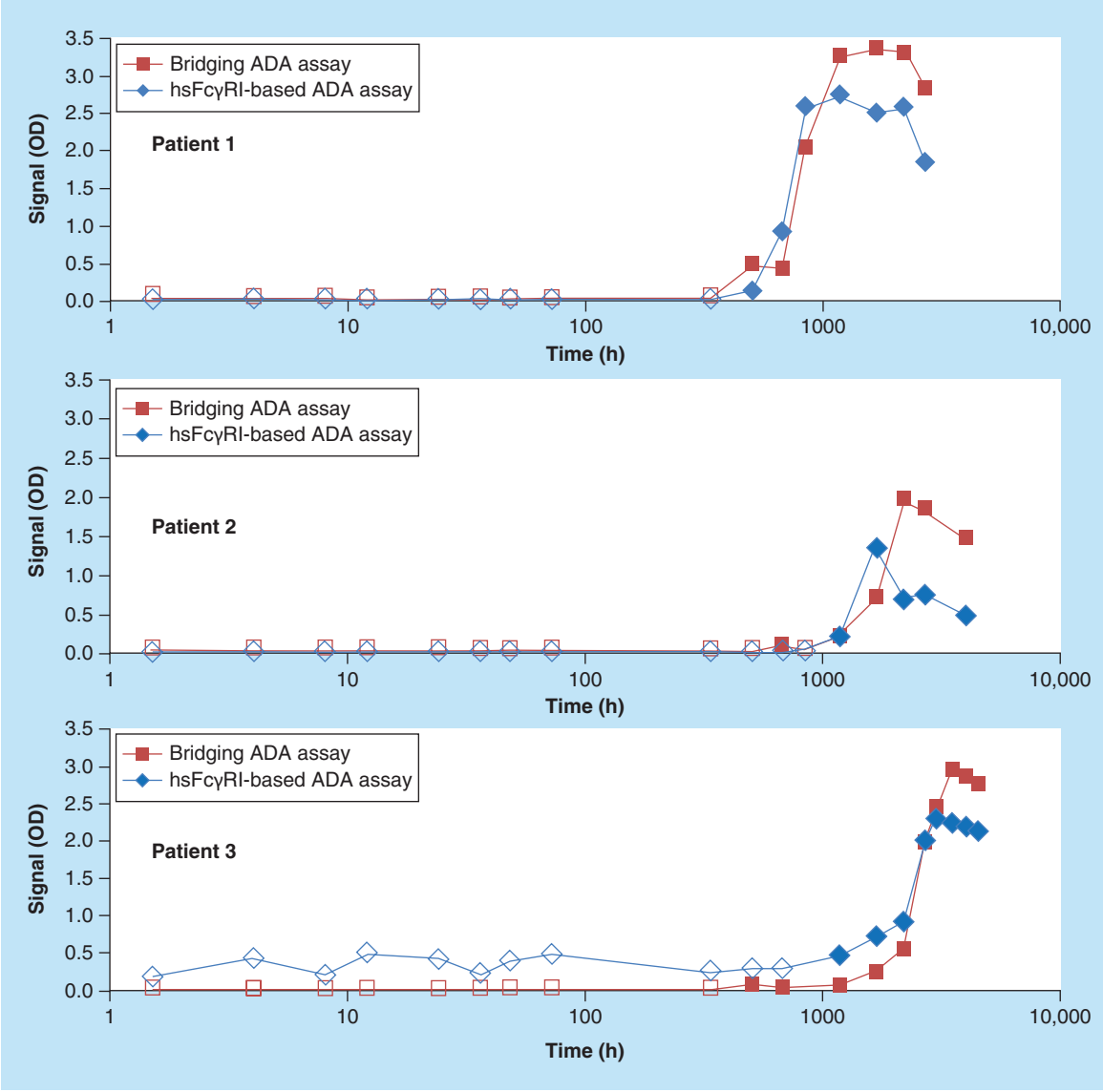

Figure 3. Time course of antidrug antibody signal measured with the bridging and FcγRI-based assays in samples of six patients treated with anti-thymic stromal lymphopoietin receptor mAb.
ADA: Antidrug antibody; OD: Optical density.

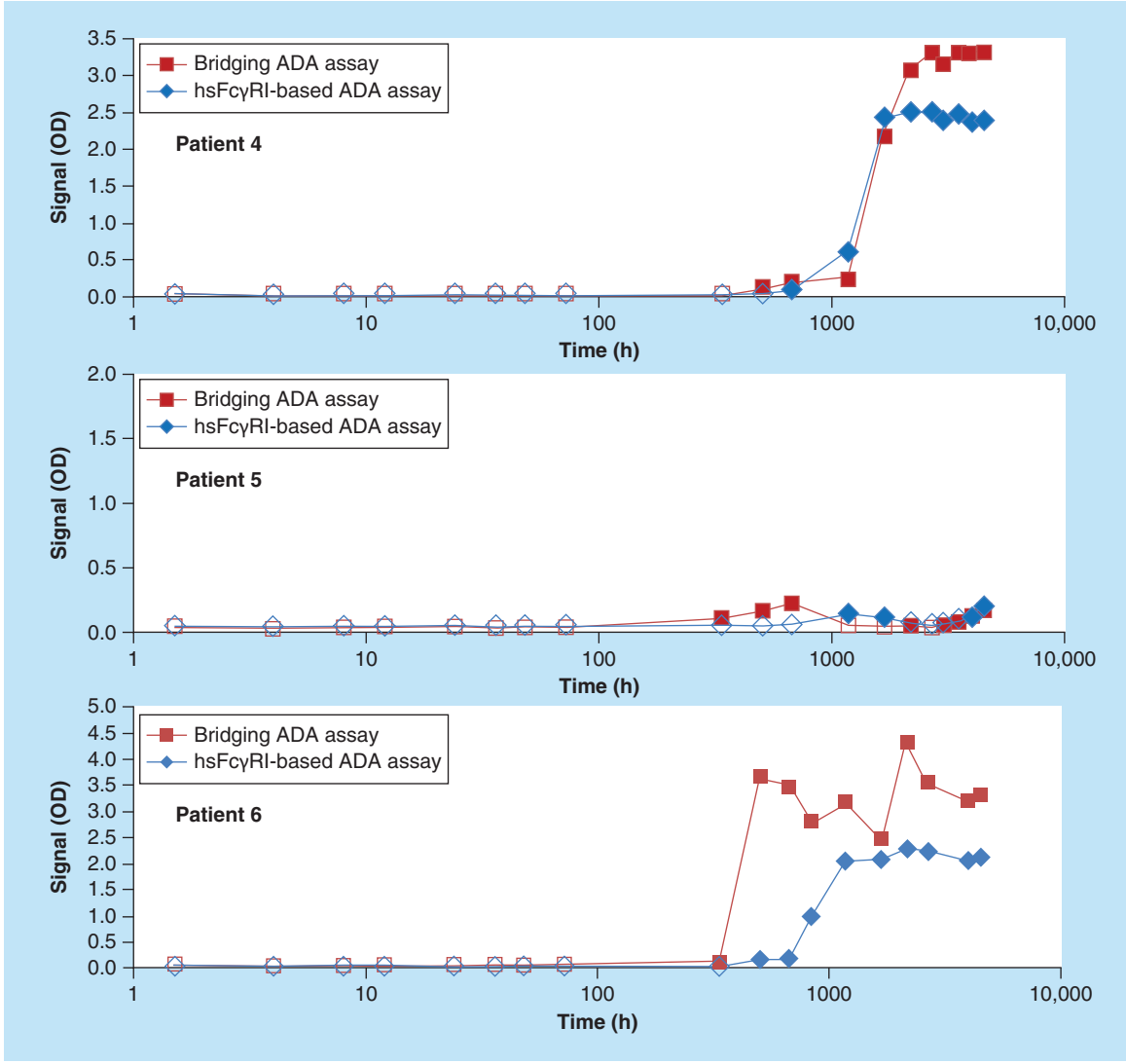

Figure 3. Time course of antidrug antibody signal measured with the bridging and FcγRI-based assays in samples of six patients treated with anti-thymic stromal lymphopoietin receptor mAb (cont.).
ADA: Antidrug antibody; OD: Optical density.

hsFcγRI-based ADA assay, titration samples 1 through 8 were diluted 1:50 to the final assay concentration of 2% matrix using Low Cross buffer. Titration and QC samples were routinely analyzed in duplicates together with four human pooled blank samples on every plate. Titers were reported as the lowest positive dilution resulting in a value above each plate-specific CP.

Results & discussion

Specificity of hsFcγRI binding

We evaluated the binding specificity of the hsFcγRI detection reagent to purified human wild-type IgG1, IgG4 and their subtypes with suppressed Fc effector functions (Figure 2). hsFcγRI had a high binding capacity to IgG1, but showed only moderate binding to IgG4, in agreement to the described binding affinities [12,13,18]. In contrast, its binding to the two engineered human IgG1 variants bearing Leu234Ala and Leu235Ala (LALA), or an additional Pro329Gly substitution (PGLALA) mutations in the Fc domain introduced to abrogate bridging to Fcγ receptors was drastically diminished. Similarly, the binding to the IgG4 variant bearing the Ser228Pro and Leu235Glu (SPLE) mutation (TSLP-R mAb) was strongly reduced. In addition, hsFcγRI showed a weak binding to IgM, substantiating its specificity to IgG. Overall, these findings demonstrate that hsFcγRI can specifically detect human IgG1 and to a lesser extent IgG4, whereas its binding to drug mAbs bearing PGLALA, SPLE and LALA modifications in the Fc-domain can be largely excluded.

Next, we tested whether hsFcγRI can also bind to nonhuman IgGs (Figure 2). Both cynomolgus monkey

and rabbit serum samples yielded signals which were comparable to those for human serum and similar binding was also observed for mouse IgG2a but not for mouse IgG1. These results suggested that the new assay can be applied to ADA detection in cynomolgus monkey and probably also in rabbit and mouse. Moreover, the results also supported the suitability of rabbit pAb and mouse IgG2a as QCs for the hsFcγRI-based analysis of human or cynomolgus samples.

Assay validation parameters for bridging & hsFcγRI-based ADA assays

The bridging and hsFcγRI-based ADA assays were thoroughly validated for human (Table 1) and cynomolgus samples (data not shown). The accuracy and precision of QCs were comparable for both assays and were within the accepted deviation range of 20%. Due to higher unspecific binding in blank serum samples, the normalization factor for CP calculation was higher for the hsFcγRI-based assay, thus leading to higher CPs in this assay. The amount of drug needed for the confirmation of positive signals was 20-times higher in the hsFcγRI-based assay as compared with the bridging assay (Table 1), indicating a better drug tolerance of the hsFcγRI-based assay. In addition, the assays differed in substrates used for the color reaction with little difference in the sensitivity ranges (Table 1).

Comparison of bridging & hsFcγRI-based ADA assays in human samples

When used in real study samples derived from a clinical study with the anti-TSLP-R mAb, the bridging

Table 2. Antidrug antibody determination in human study samples derived from anti-thymic stromal lymphopoietin receptor treated patients using bridging and hsFcγRI-based assay.

Time (h)	Patient 1		Patient 2		Patient 3	
	Bridging ± (titer)	hsFcγRI ± (titer)	Bridging ± (titer)	hsFcγRI ± (titer)	Bridging ± (titer)	hsFcγRI ± (titer)
72	-	-	-	-	-	-
336	-	-	-	-	-	-
504	+ (50)	+ (50)	-	-	+ (50)	-
672	+ (50)	+ (150)	+ (50)	-	+ (50)	-
1176	+ (450)	+ (4050)	+ (50)	+ (50)	+ (150)	+ (50)
1680	+ (450)	+ (1350)	+ (50)	+ (150)	+ (450)	+ (150)
2184	+ (1350)	+ (1350)	+ (450)	+ (150)	+ (450)	+ (150)
2688	+ (*)	+ (*)	+ (450)	+ (150)	+ (1350)	+ (1350)
3000	†	†	+ (450)	+ (150)	+ (1350)	+ (1350)
4000	+ (*)	+ (*)	+ (*)	+ (*)	+ (*)	+ (*)
4500	+ (*)	+ (*)	+ (*)	+ (*)	+ (*)	+ (*)
Time (h)	**Patient 4**		**Patient 5**		**Patient 6**	
	Bridging ± (titer)	hsFcγRI ± (titer)	Bridging ± (titer)	hsFcγRI ± (titer)	Bridging ± (titer)	hsFcγRI ± (titer)
72	-	-	-	-	–	-
336	-	-	-	-	+ (50)	-
504	-	-	+ (50)	-	+ (12150)	+ (50)
672	+ (50)	-	+ (50)	-	+ (1350)	+ (50)
1176	+ (50)	+ (150)	-	+ (50)	+ (450)	+ (450)
1680	+ (450)	+ (1350)	-	+ (50)	+ (450)	+ (1350)
2184	+ (4050)	+ (1350)	-	-	+ (1350)	+ (1350)
2688	+ (4050)	+ (1350)	-	-	+ (*)	+ (*)
3000	+ (4050)	+ (1350)	-	-	-	-
4000	†	+ (*)	+ (*)	+ (*)	+ (*)	+ (*)
4500	†	+ (*)	+ (*)	+ (*)	+ (*)	+ (*)

Time points earlier than 72 h were consistently ADA negative in both assays.
†No data available, not determined.
-: ADA negative; + ADA positive; ADA: Antidrug antibody.

Table 3. Assay comparison for qualitative evaluation of study samples derived from mAb anti X-treated cynomolgus monkeys.

Time (h)	C3405		C3407		C3416		C3406		C3413		C3419		C3420	
	hsFcγRI	Bridge	hsFcγRI	Bridge	hsFcγRI	Bridge	hsFcγRI	Bridge	hsFcγRI	Bridge	hsFcγRI	Bridge	hsFcγRI	Bridge
0	-	-	-	-	-	-	†	†	†	†	†	†	†	†
168	-	-	-	-	-	-	-	-	-	-	-	-	-	-
336	QNS	+	+	+	+	+	+	+	+	+	+	+	+	+
384	+	-	+	+	+	+	+	-	+	-	+	+	†	†

†Not determined.
QNS: Quantity not sufficient for analysis.

and hsFcγRI-based ADA assays yielded similar time courses of signal intensities for 19 examined time points (Figure 3). In particular, both assays detected the start of ADA formation at similar time points (between 504 and 672 h postdose) in five of a total of six patients and in four patients the assays showed no difference in the timeline of the steep signal increase. In one patient, only moderate signal intensities were measured by both assays, thus reflecting rather low ADA concentrations in the patient's samples during the study, whereas in another patient the bridging assay detected the steep signal increase markedly earlier than did the hsFcγRI-based assay.

Indeed, a detailed inspection of the ADA response revealed a moderate delay in the detection of ADAs by the hsFcγRI-based assay, with the bridging assay demonstrating ADA formation one to two time points earlier in most patients (Table 2). The delay can be explained by the inability of the hsFcγRI-based assay to detect IgM (Figure 2), which is usually the first antibody class formed during an immune response [19]. An exploratory IgM evaluation (data not shown) corroborated this interpretation, especially for Patient 6, whose early sample (504 h) revealed an exceptionally high titer as measured by the bridging assay. However, in later time points, both assays had comparable sensitivity, as demonstrated by the titer determination (Table 2); the observed interpatient variability may have resulted from different sample compositions, including differences in ADA affinity and Ig isotypes.

Comparison of bridging & hsFcγRI-based ADA assays in cynomolgus monkey samples

The high sequence homology of human and cynomolgus monkey Fc regions [20] and the results of the binding experiments using cynomolgus monkey serum (Figure 2) justified the transfer of the hsFcγRI-based ADA assay to the cynomolgus monkey model, which is frequently used for preclinical studies. To test the feasibility of the hsFcγRI-based assay in this model, we used serum samples derived from a preclinical study with the anti-X mAb and compared the assay results with the data that had been obtained by a bridging assay during that study.

Both assays showed comparable results for up to four measured time points, with predose and early (168 h) samples being tested negative and samples at 336 h

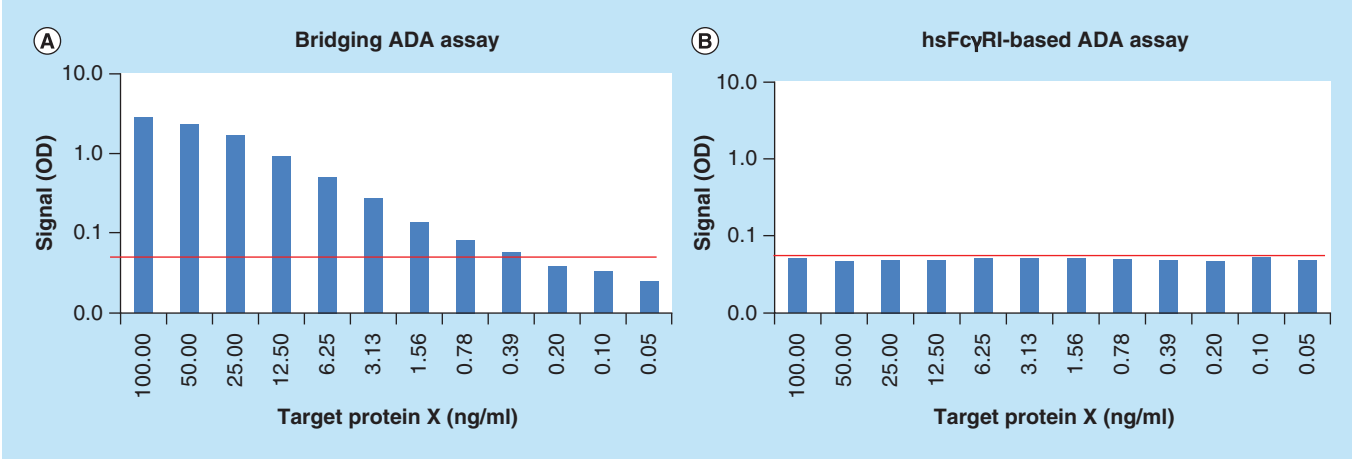

Figure 4. Interference of oligomeric soluble target with the bridging and FcγRI-based assays. The assays were performed in 12 samples of cynomolgus serum pool spiked with increasing concentrations of protein X. Red lines indicate assay-specific cutoff.
ADA: Antidrug antibody; OD: Optical density.

Table 4. Summary of assay comparison.

Assay characteristics	hsFcγRI-based ADA assay	Standard bridging ADA assay
IgG class detected	IgG1 (IgG3, IgG4)	IgG1, IgG2, IgG3 (IgG4)
IgM detected	No	Yes
Affinity of detected ADAs	High-to-low affinity	High-to-medium affinity
Applicability to therapeutic mAbs	FcR effector function deficient mAb	No restrictions
Applicability to species	Human, cynomolgus monkey†	No restriction
Drug tolerance	High	Moderate
Oligomeric target	Detected negative	Detected false positive
Positive control	FcγRI-reactive IgG, e.g., anti-idiotypic polyclonal rabbit or monoclonal mouse antibodies	Any anit-idiotypic bivalent polyclonal rabbit or anit-idiotypic bivalent monoclonal mouse antibody

†Using the species-specific FcγRI the assay principle is applicable to any other species.
ADA: Antidrug antibody.

being consistently detected positive (Table 3). However, the bridging assay failed to detect ADA positive samples at 384 h in three of six animals, whereas the hsFcγRI-based assay yielded positive results in all cases. The observed discrepancy is most likely due to better drug tolerance of the hsFcγRI-based assay, as the 384 h samples were taken 24 h after the second dosing and thus had the highest concentration of unlabeled drug of all tested time points (data not shown). Differences in drug tolerance are discussed below ('Overall assay comparison' section).

Thus, these experiments indicate that the hsFcγRI-based assay can be potentially used as an alternative ADA screening assay and show that the hsFcγRI-based ADA assay is also applicable for ADA determination in cynomolgus monkey samples.

Enhanced target tolerance of the hsFcγRI-based ADA assay

In case of mAb anti X, the presence of the soluble target protein X as dimers or oligomers in samples can complicate ADA interpretation. These structures can form a bridge between the two labeled drug mAbs in a bridging assay, thereby generating signals that are indistinguishable from those of ADA (Figure 1B) and that cannot be ruled out by a confirmatory analysis done by the presence of drug excess [4,5,21]. By contrast, the principle of the hsFcγRI-based assay precludes such interference, as this assay does not rely on a labeled drug for detection (Figure 1C).

To evaluate the target tolerance of the hsFcγRI-based assay, we compared the two ADA assays in cynomolgus serum pool samples spiked with increasing concentrations of protein X. Indeed, significant and concentration-dependent target interference was observed for the bridging assay starting from the protein X concentration of 0.39 ng/ml, whereas for the hsFcγRI-based assay no positive signal was observed for all tested concentrations (Figure 4). These results clearly demonstrate that the hsFcγRI-based assay provides high robustness of ADA detection in the presence of soluble targets and thus can be of benefit when used as a confirmation assay in combination with the standard bridging assay.

It has to be stated that another theoretical target interference needs to be considered: both assay formats can be interfered by soluble monomeric target due to the competition of target and ADA to binding to the immobilized drug. In case of very high target concentrations, this could then lead to reduced or even false-negative ADA signals. Oligomeric target would have the same effect for the FcγRI-based assay but would lead to enhanced or false-positive results in the bridging assay. In practice, this may be rare case since target concentrations are typically low and plate capacities of bound drug will probably be much higher as ADA and target levels.

Overall assay comparison

Both bridging and hsFcγRI-based assays have identical ADA-capture and signal generation principles and only differ in their secondary detection reagent (Figure 1A & C). Due to its drug-independent detection, the hsFcγRI-based assay requires only one free binding site at an ADA, thus lacking several limitations of the bridging assay. It is able to detect nonbridging-competent ADAs as well as bivalent ADAs in which one binding site is occupied by an unlabeled drug molecule, the latter leading to better drug tolerance of the assay. In addition, this assay is presumably able to detect bivalent ADAs of lower affinity since ADAs may bind bivalently to the capturing mAb resulting in avidity effects. Finally, the principle of the assay makes it extremely tolerant to the presence of oligomeric

targets as compared with the standard bridging assay (Figure 4).

The drug-independent detection also facilitates the development of the hsFcγRI-based assay for a new drug, limiting the experimental efforts to solid-phase coupling of the new drug mAb and possibly an optimization of the used reagent concentrations. Moreover, as shown in this work, a generic detection reagent allows for the application of the same assay in both preclinical (cynomolgus) and clinical studies, eliminating the need for an assay switch from animal to human, which is, for instance, required in case generic immune complex ELISAs are used [7,8]. Using FcγRI of different species would also allow adaption to other species. The commonly used rabbit pAbs or mouse mAbs are generally feasible as QCs for assay validation, however, small species-specific differences in secondary detection are visible for the FcγRI-based assay due to varying FcγRI affinity to QCs, and not relevant for the bridging assay.

The major disadvantage of the hsFcγRI-based assay as compared with the bridging assay is its inability to detect a broader range of antibody classes and subclasses, especially IgM (Figure 2). However, the IgG-specificity of the hsFcγRI-based assay may be of benefit if this assay is used as an orthogonal method. By comparing the results of both assays, it would be possible to identify non-IgG related signals such as IgM. Another potential limitation of the hsFcγRI-based ADA assay could be unspecific binding of IgG to the MTP surface resulting in relatively high normalization factors for the determination of the CP. This unspecific background can, however, be reduced and compensated by buffer optimization and by sample dilution, which is possible due to a good sensitivity of the assay; for example, we achieved good assay performance with 2% serum content in the sample while having a very low sample consumption of about 5 µl per sample (Table 1).

Conclusion

In this work, we present a novel hsFcγRI-based assay for ADA detection. We compared it to the standard bridging assay using both human and nonhuman study samples (summarized in Table 4). The hsFcγRI-based assay enabled a reliable detection of ADA formed in response to IgG therapeutic mAbs with suppressed Fc effector functions, showing robust sensitivity and greatly enhanced oligomeric target tolerance. In fact, the detection of bridging and nonbridging-competent IgG ADAs without the interference from oligomeric targets represents the major advantage of the novel assay. The assay appears to also have a better drug tolerance as compared with the standard bridging assay and can be easily adapted to a new drug or other species. Overall, both assays showed similar and consistent results.

Future perspective

In our view, the advantages of the reported hsFcγRI-based ADA assay justify its use as an alternative assay for immunogenicity testing of therapeutic mAbs with suppressed Fc effector functions. The assay may be applied in a combination with the bridging assay, for example, as an orthogonal step for specificity confirmation or as a screening assay if drug interference is an issue. In addition, this assay can provide additional information on the nature of the induced Ig, contributing to appropriate interpretation of ADA responses. Since the hsFcγRI-based ADA assay provides more reliable information about the nature of immune response, we expect that this assay would become a crucial tool for immunogenicity assessment of biotherapeutics, being especially valuable for projects in which high concentration of soluble targets is an issue. We also consider FcγRI-based detection as a useful tool for the development and setup of bioanalytical assays and thus envision its application for a variety of bioanalytical questions, particularly in projects related to supFcEF antibodies.

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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.

Executive summary

- A novel hsFcγRI-based assay for antidrug antibody detection was established and compared with the standard bridging assay in both human and nonhuman samples.
- The hsFcγRI-based assay enabled a reliable detection of antidrug antibody formed in response to IgG therapeutic mAbs with suppressed Fc effector functions. Both assays showed similar and consistent results in human and nonhuman samples; however, the hsFcγRI-based assay was unable to detect early IgM-based immune response.
- In contrast to the bridging assay, the hsFcγRI-based assay was insensitive to the presence of oligomeric targets and also appeared to have better drug tolerance.
- The hsFcγRI-based assay will become a crucial tool for immunogenicity assessment of biotherapeutics, especially in projects in which high concentration of soluble targets or drug interference is an issue. Overall, FcγRI-based detection can serve as useful tool for the development and setup of bioanalytical assays and can be applied to a variety of bioanalytical questions.

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