Immunogenicity testing of therapeutic antibodies in ocular fluids after intravitreal injection

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Aim: High drug concentrations in ocular fluids after intravitreal administration preclude the use of drug-sensitive immunoassays. A drug-tolerant immunoassay is therefore desirable for immunogenicity testing in ophthalmology. **Experimental:** Immune complex (IC) antidrug antibody (ADA) assays were established for two species. The assays were compared with the bridging assay in ocular and plasma samples from two preclinical studies. **Results:** The IC assays showed high drug tolerance, which enabled a reliable ADA detection in ocular fluids after intravitreal administration. The IC assays were superior to the bridging assay in the analysis of ocular fluids with high drug concentrations. **Conclusion:** The IC assay allows a reliable ADA detection in matrices with high drug concentrations, such as ocular fluids.

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Therapeutic antibodies are increasingly used in ophthalmology to treat inflammatory or angiogenic diseases [1]. Wet age-related macular degeneration is a notable example, where new therapeutics such as bevacizumab, ranibizumab and aflibercept have enabled sight-saving therapy [2]. However, the drugs require chronic intravitreal (IVT) administration with 1–2-month intervals. This high burden on patients has prompted the development of new drug candidates that would require less frequent administration thanks to slower elimination rates, increased binding affinity and higher dosing concentrations [3].

The formation of antidrug antibodies (ADAs) can affect pharmacologic properties of therapeutic antibodies. Most notably, ADAs have a major impact on pharmacokinetics, often leading to faster drug elimination and increased intersubject variability. Immunogenicity assessment therefore comprises an essential part of the development program for therapeutic antibodies, which supports the correct interpretation of pharmacokinetic, pharmacodynamic and safety data. Yet, finding an appropriate immunogenicity testing strategy may pose one of the biggest bioanalytical challenges.

ADA analysis in ophthalmology studies is usually conducted by the systemic detection of ADAs from serum or plasma samples [4] and immunogenicity has been observed after IVT administration in preclinical studies, especially in rabbit and minipig [5,6]. It is assumed that the systemic ADAs are not generated in the eye but are induced following drug clearance through the systemic circulation, for example, via anterior elimination and uveal blood flow [7], because the eye itself is an immune privileged organ with several mechanisms to inhibit immune response [8]. However, specific immune response in vitreous humor (VH) has been observed [9]. The direct ADA analysis in ocular fluids, such as VH or aqueous humor (AH), would thus support the pharmacokinetic evaluation of IVT-administered therapeutic antibodies and provide valuable insights into the eye's immune system homeostasis.

So far, ADA detection in ocular fluids has not been described. The conventional analysis with a bridging assay is hampered by high residual drug concentrations, which can persist over the whole treatment period due to high IVT doses and long drug retention time in ocular fluids (e.g., half-life for bevacizumab is 9 days) [10]. Residual

Figure 1. Principles of the bridging and immune complex assays. (A) In the bridging assay, the two antigen-binding sites of an ADA couple the immobilized drug (drug-Bi) with the digoxigenylated drug (drug-Dig) used for detection. (B) In the immune complex assay, a drug-ADA complex is captured by the immobilized anti-human IgG (anti-Human IgG-Bi) and then detected using digoxigenylated anti-animal IgG (anti-animal IgG-Dig). ADA: Antidrug antibody; Bi: Biotin; Dig: Digoxigenin; SA: Streptavidin.

unlabeled drug interferes with the conventional bridging assay by competing with the labeled drug molecules, thereby increasing the risk of false-negative results (Figure 1A). The problem is further aggravated by the low sample volume available for the analysis, with the typical AH sample volume per eye being 100 μl for human [11] and 50 μl AH for cynomolgus monkeys or minipigs. VH samples typically have larger volume; however, human VH samples can only be obtained in rare cases during surgery [12], whereas only terminal VH samples are available in animals. In addition, the VH requires pretreatment due to its high viscosity [13], which diminishes the occurrence of VH analysis in bioanalysis. Considering that the conventional ADA analysis consists of two steps (screening and confirmatory bridging assays), the available amount of ocular fluid would usually be insufficient for reliable immunogenicity testing.

Although several approaches can improve drug tolerance of the bridging assay format, for example, acid pretreatment [14,15], affinity capture elution assays [16] or non-ELISA technical platforms such as Meso Scale Discovery (MSD), Gyros [15], Biacore [17] or Octet [18], these approaches have not been demonstrated to be adequate for residual drug concentrations as high as 1000 $\mu\text{g}/\text{ml}$, which occur in VH after IVT administration. In addition, the pretreatments would further diminish the already low fluid sample volume. A reliable ADA analysis in ocular fluid thus requires a novel approach with high drug tolerance and low sample consumption.

We have reported several assay formats that addressed the drug tolerance problem in ADA analysis and evaluated them in preclinical studies in cynomolgus monkeys and mice [19–21]. These assay formats rely on the formation of an immune complex (IC) between an ADA and a free drug molecule (Figure 1B). The principle differs fundamentally from that of the bridging assay, requiring the presence of a free unlabeled drug, which makes them intrinsically

drug tolerant. Moreover, these IC assays do not require an additional confirmatory step because the assay principle precludes the occurrence of false positive signals, for example, by a soluble target [22]. The sensitivity of the bridging and IC assays is comparable; however, the IC assays have lower sample consumption (approximately 5 µl). Overall, these features of the IC assays make them ideal assay formats for ADA analysis in ocular fluid samples.

In this article, we present two IC assays developed for the analysis of ocular fluid samples in cynomolgus monkey and minipig preclinical studies. We evaluate their applicability for ADA analysis in ophthalmological studies and compare them with the conventional bridging assay.

Experimental

Chemicals, reagents & equipment

The murine monoclonal antibodies against cynomolgus monkey IgG and minipig IgG were generated from mice immunized with purified cynomolgus monkey or minipig IgG. Antibodies from hybridoma supernatants were purified by protein A chromatography. Conjugates of cynomolgus monkey or minipig IgG with human IgG and anti-human kappa light chain M1.7.10 were generated in house. All antibodies were produced by Roche Diagnostics GmbH, Penzberg, Germany, and were stored in aliquots at -80°C until use. Biotin (Bi)- and digoxigenin (Dig)-labeling was performed by Roche Diagnostics GmbH. Polyclonal sheep antibody Fab fragments against Dig coupled with HRP (pAb-Dig-S-Fab-HRP), phosphate-buffered saline (PBS), Tween 20, 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS; ready to use solution), bovine plasma albumin and Roche Universal buffer were obtained from Roche Diagnostics GmbH. Low Cross Buffer® was from Candor Bioscience GmbH (Wangen, Germany). Individual and pooled minipig sodium citrate, theophylline, adenosine and dipyridamol (CTAD) plasma, as well as individual and pooled cynomolgus monkey CTAD plasma for *in vitro* studies was obtained from Sera Laboratory International Ltd (Haywards Heath, Sussex, UK). Streptavidin-coated microtiter plates (SA-MTPs) were from Microcoat Biotechnologie GmbH (Bernried, Germany).

Study samples

Study samples were obtained from preclinical tolerability studies with a human IgG1 Fab fragment as the drug. Minipig samples were obtained from eight animals that received an IVT administration in both eyes at day 1 and again at day 28 at either of two different dose levels (5 or 10 mg/eye dose groups). CTAD plasma samples were taken prior dosing and 4 h and then 1, 3, 7 and 10 days after first and second dosing. One additional sample was taken terminally, 71 days after first dosing. Approximately 250 µl VH sample was taken terminally (day 35, 44 or 71) from each animal from the right eye. Although AH samples were also taken, they could not be analyzed due to insufficient sample volume.

Cynomolgus samples were obtained from eight animals that received IVT administrations in both eyes at day 1 and again at day 29 at either of two different dose levels (5 or 10 mg/eye dose groups). CTAD plasma samples were taken prior dosing and 4, 7, 24, 33, 48, 72 and 120 h after each dosing. AH of the right eye (approximately 30 µl) were taken on days 8, 35 and 43 after first dosing. VH samples (approximately 250 µl) were obtained terminally (day 43) from each animal from the right eye.

All samples were immediately frozen after acquisition and thawed prior to analysis. Cynomolgus and minipig VH samples were thawed and centrifuged prior to analysis at 13,000 ×g for 5 min. Supernatant was taken for analysis.

Bridging ADA assay

ADAs against the drug (human IgG 1 Fab fragment) were detected in AH, VH and plasma samples from minipig and cynomolgus monkey using a one-step ELISA (Figure 1A). Affinity-purified anti-idiotypic monoclonal mouse antibodies directed against the complementarity-determining regions (CDRs) of the drug spiked into blank matrix were used as quality control (QC) samples. The QC concentrations were 750 ng/ml (high-concentration quality control [HQC]) and 15 ng/ml (low-concentration quality control [LQC]) for the cynomolgus and minipig assays. Reagents and samples were incubated at room temperature with shaking at 500 rpm. Roche Universal buffer was used as assay buffer; PBS containing 0.05% Tween 20 was used as washing buffer.

Samples were diluted 1:20 with assay buffer containing Bi- and Dig-labeled drug (each at 0.5 µg/ml final assay concentration), incubated for 1 h and then transferred onto an SA-MTP. The plate was incubated for 1 h and washed three-times with 300 µl washing buffer. Afterwards, 100 µl pAb-Dig-S-Fab-HRP (12.5 mU/ml) was added to each sample and the plate was incubated for 1 h. After washing, 100 µl ABTS was added to each well and

the ensuing color reaction was monitored by photometrical readout at 405 nm (reference wavelength 490 nm). Measurements were performed in duplicates and mean absorbance values were calculated; the results were accepted if the precision (% coefficient of variation) of duplicates was $\leq 20\%$.

A floating cut point (CP) was statistically evaluated using 20 male and 20 female individual blank plasma samples according to Shankar *et al.* [23]. To obtain the plate-specific CP, the signal of a pooled plasma blank sample was multiplied by the plate normalization factor. Samples with signals below the plate-specific CP were considered screening negative; samples with signals at or above the plate-specific CP were considered screening positive. Due to low sample volume, a confirmation assay was not performed.

Method qualification was conducted according to the current guidelines [23,24].

IC ADA assay

The IC assay was developed as a four-step solid phase ELISA (Figure 1B). Conjugates of cynomolgus monkey or minipig IgG with human IgG spiked into blank matrix were used as QC samples. The QC concentrations were 3000 ng/ml (HQC) and 375 ng/ml (LQC) for cynomolgus [18] and 6000 ng/ml (HQC) and 175 ng/ml (LQC) for minipig assay.

Reagents and samples were incubated at room temperature with shaking at 500 rpm. Low Cross Buffer was used as assay buffer; PBS containing 0.05% Tween 20 was used as washing buffer.

Bi-labeled anti-human kappa light chain M-1.7.10 antibodies (capture antibody [20]) were bound to an SA-MTP. Samples were diluted 1:50 with assay buffer containing drug (1 $\mu\text{g}/\text{ml}$ for cynomolgus samples, 0.15 $\mu\text{g}/\text{ml}$ for minipig samples), incubated for 30 min and then transferred onto the SA-MTP (100 μl per well). The plate was incubated for 1 h, washed and 100 μl of Dig-labeled anti-cynomolgus monkey or anti-minipig IgG was added to each well. After 1 h incubation and washing, Dig was detected using the same procedure as for the bridging assay.

The CP was evaluated as described for the bridging assay. For cynomolgus, 50 (25 for each sex) and for minipig 32 (16 for each sex) individual blank plasma samples were used in duplicates.

Drug quantification (human Fab fragment)

Drug levels were determined for AH, VH and plasma samples using a solid phase ELISA with a quantification range 0.78–50 ng/ml. Reagents and samples were incubated at room temperature with shaking at 500 rpm. Low Cross Buffer was used as assay buffer; PBS containing 0.05% Tween 20 was used as washing buffer.

Briefly, a drug-specific protein was immobilized on an SA-MTP. Samples and calibration standards diluted 1:50 in the assay buffer were added and incubated for 1 h. After washing, the bound drug was detected by adding a dioxigenylated drug-specific protein and pAb–Dig–S–Fab–HRP with ABTS used as a substrate. The color reaction was monitored at 405 nm (reference wavelength 490 nm).

Drug tolerance of minipig ADA assays

The sample was split into 16 portions, which were spiked with an increasing amount of drug (concentration range: 0.25–500,000 $\mu\text{g}/\text{ml}$). After incubation for 1 h, the spiked samples were diluted 1:50 and immediately analyzed by the bridging assay and the minipig IC assay as described above with the variation that the IC assay was performed without additional drug spiking.

Results & discussion

Assay qualification

Both the conventional bridging assay and the minipig IC assay demonstrated comparable qualification data with acceptable intra- and interassay precision with variation coefficients below 20%. Also, both assays showed similar multiplicative normalization factors and thus comparable plate-specific CPs (Table 1). The differences in the QC concentrations reflect the differences in the assay principles and required positive control molecules. Notably, the IC assay had much lower sample consumption than the bridging assay (5 vs 12.5 μl).

Drug tolerance comparison

We investigated drug tolerance of the bridging and IC assays using a plasma sample from the minipig study, which had a high ADA signal and a residual drug level below LLOQ (0.78 ng/ml). The ADA signal in the bridging assay decreased with the increasing free drug concentration; the decrease became apparent at drug concentrations above 1 $\mu\text{g}/\text{ml}$ and the signal fell below the CP at concentrations above 100 $\mu\text{g}/\text{ml}$ (Figure 2). In contrast, the minipig IC

Table 1. Qualification data for minipig immunocomplex and bridging assays.

Parameter	Immune complex assay		Bridging assay	
	HQC	LQC	HQC	LQC
QC (assay concentration)	120 ng/ml	3.5 ng/ml	37.5 ng/ml	0.75 ng/ml
QC (matrix concentration)	6000 ng/ml	175 ng/ml	750 ng/ml	15 ng/ml
Intra-assay precision (% CV)	2	5	9	8
Inter-assay precision (%CV)	10	14	8	15
Dilution factor	50 (2% serum)		20 (5% serum)	
Sample volume for double determination	5 µl		12.5 µl	
Normalization factor	1.44 (multiplicative)		1.66 (multiplicative)	
Assay signals at screening cut point (four plates)	0.055/0.062/0.075/0.073		0.071/0.078/0.064/0.07	

HQC: High-concentration quality control; LQC: Low-concentration quality control; QC: Quality control.

Figure 2. Influence of free drug on the bridging assay and the immune complex assay. A sample with high amount of ADA was spiked with different amount of free drug and then measured using the two ADA assays. The expected range of drug concentrations after intravitreal injections are indicated.

ADA: Antidrug antibody; AH: Aqueous humor; CP: Cut point; IC: Immune complex; VH: Vitreous humor.

assay showed a positive ADA signal in the free drug concentration range from 1 to 10,000 µg/ml. At low free-drug concentrations, the amount of drug–ADA complexes was insufficient to generate a positive signal because the IC assay in this experiment was conducted without the spiking and pre-incubation with the drug (see Experimental section) and the residual drug concentration in the plasma sample was below LLOQ. This observation confirms that the signal generation in the IC assays requires the formation of an IC (Figure 1B). However, in routine analyses using the IC assay, low residual drug concentrations have no impact on ADA detection (see Table 2) because the standard IC assay procedure involves the addition of free drug corresponding to 7.5 µg/ml drug to allow for drug–ADA complex formation. The signal reduction at very high concentrations (above 1000 µg/ml) was due to limited binding capacity of the SA-MTP, with the free drug displacing the drug–ADA complexes from the bound capture antibody.

Overall, the bridging assay appears to be able to correctly detect ADA at the residual drug concentrations expected for plasma and AH samples after an IVT injection, whereas it would likely lead to false-negative results at higher

Table 2. ADA determination in minipig plasma samples using the immune complex and bridging assay.

Subject	Time point (day)	Immune complex assay	Bridging assay	Drug level (ng/ml)
		Positive/negative	Positive/negative	
201	0	Neg	Neg	BLQ
	3	Neg	Neg	77
	7	Neg	Neg	49
	10	Neg	Neg	35
	28	Pos	Pos	BLQ
	31	Pos	Pos	72
	35	Pos	Pos	88
	38	Pos	Pos	57
	71	Pos	Pos	42
202	0	Neg	Neg	6
	3	Neg	Neg	63
	7	Neg	Neg	58
	10	Pos	Neg	41
	28	Pos	Pos	17
	31	Pos	Pos	98
	35	Pos	Pos	132
	38	Pos	Pos	115
	71	Pos	Pos	4
251	0	Neg	Neg	BLQ
	3	Neg	Neg	46
	7	Neg	Neg	40
	10	Pos	Pos	22
	28	Pos	Pos	3
	35	Pos	Pos	79
	38	Pos	Pos	55
	71	Pos	Pos	BLQ
252	0	Neg	Neg	BLQ
	3	Neg	Neg	52
	7	Neg	Pos	40
	10	Pos	Pos	24
	28	Pos	Pos	3
	31	Pos	Pos	57
	35	Pos	Pos	50
	38	Pos	Pos	38
	71	Pos	Pos	26
301	0	Neg	Neg	BLQ
	3	Neg	Neg	172
	7	Neg	Neg	87
	10	Neg	Neg	57
	28	Pos	Neg	19
	31	Pos	Neg	140
	35	Pos	Neg	82
	38	Pos	Neg	72
	71	Pos	Pos	2

ADA: Antidrug antibody; BLQ: Below limit of quantification; Neg: ADA negative; Pos: ADA positive.

Table 2. ADA determination in minipig plasma samples using the immune complex and bridging assay (cont.).

Subject	Time point (day)	Immune complex assay	Bridging assay	Drug level (ng/ml)
		Positive/negative	Positive/negative	
302	0	Neg	Neg	BLQ
	3	Neg	Neg	146
	7	Neg	Neg	123
	10	Pos	Pos	98
	28	Pos	Pos	23
	31	Pos	Pos	245
	35	Pos	Pos	59
	36	Pos	Pos	40
351	0	Neg	Neg	BLQ
	3	Neg	Neg	151
	7	Neg	Neg	109
	10	Neg	Neg	77
	28	Pos	Pos	27
	31	Pos	Pos	370
	35	Pos	Pos	435
	38	Pos	Pos	394
	71	Pos	Pos	29
352	0	Neg	Neg	BLQ
	3	Neg	Neg	120
	7	Neg	Neg	122
	10	Pos	Pos	84
	28	Pos	Pos	10
	31	Pos	Pos	43
	34	Pos	Pos	BLQ

ADA: Antidrug antibody; BLQ: Below limit of quantification; Neg: ADA negative; Pos: ADA positive.

drug concentrations, which can occur in VH samples. In contrast, the IC assay demonstrated stable ADA signals over the whole range of the expected drug concentrations in ocular fluids, with only a slight signal decrease being observed at drug concentrations above 1000 µg/ml.

Applicability of bridging & IC assays for sample analysis in preclinical ophthalmological studies

Both assays showed nearly consistent results in the ADA analysis of plasma samples from the minipig study (Table 2). The ADA assay results deviated in only six of a total of 68 plasma samples, primarily at earlier time points, with no clear correlation with the residual drug concentration. All terminal plasma samples in all animals were ADA-positive in both assays. In VH analyses, the bridging assay generated clearly reduced signals as compared with those of the IC assay and it failed to detect ADA in two samples that were tested positive in the IC assay and had high residual drug concentrations (227 and 1720 µg/ml; Figure 3A). The IC assay results for VH also correlated well with those for plasma samples from the same time points (Figure 3B). Overall, the IC assay demonstrated reliable ADA detection in samples with high (VH) and low (plasma) residual drug concentrations.

Similar results were observed in the cynomolgus study (Table 3). While ADA analysis in plasma samples was nearly consistent, the bridging assay failed to detect ADAs in the majority of AH samples on day 35 and in some AH and VH samples on day 43. The false-negative results of the bridging assay (animals 60653/60605/60606/60655/60656 at day 35 and animals 60606/60656 at day 43) correlated well with the residual drug concentration in the samples. On day 35, the AH samples tested negative by the bridging assay only had higher residual drug concentrations than the two samples that were tested positive by both assays. On day 43, the lowered drug concentrations led to an increased number of positive signals in the AH samples tested by the bridging assay. Notably, the IC assay demonstrated equal results for AH and VH, suggesting that the more readily accessible AH can be used for immunogenicity assessment in ocular fluids by means of this assay.

Figure 3. Minipig immunogenicity data. Antidrug antibody detection (signals) in minipig vitreous humor (A) and matched plasma samples (B). The samples were analyzed using the immune complex and bridging assay. Sampling time points and residual drug concentrations in the samples are shown on the x-axis.

Detailed analysis of the complete dataset for cynomolgus plasma samples (data not shown) revealed positive ADA results for all eight animals in both assays. The ADA response was first detected on day 14 and it persisted until the end of the study. The majority of plasma samples (79 of a total 87) had consistent results in both assays and eight samples that showed discrepancy were all borderline positive in the IC assay and negative in the bridging assay. Two of these eight samples were taken after second dosing with higher plasma drug concentrations, the remaining six were from the animal with a weak ADA response up to day 35 (animal 60603; Table 3). Thus, the IC assay appears to be not only drug tolerant but also more sensitive, being able to detect weak ADA responses.

Table 3. ADA determination in cynomolgus monkey samples using the immune complex and bridging assay.

Dosing group	Animal	Immune complex assay			Bridging assay			Drug concentration (µg/ml)		
		AH	VH	Plasma	AH	VH	Plasma	AH	VH	Plasma
Day 8										
5 mg/eye	60603	Neg	NS	Neg	Neg	NS	Neg	77	NS	0.2
	60604	Neg	–	Neg	Neg	–	Neg	88	–	0.3
	60653	Neg	–	Neg	Neg	–	Neg	66	–	0.6
	60654	Neg	–	Neg	Neg	–	Neg	49	–	0.8
10 mg/eye	60605	Neg	–	Neg	Neg	–	Neg	194	–	0.2
	60606	Neg	–	Neg	Neg	–	Neg	262	–	0.4
	60655	Neg	–	Neg	Neg	–	Neg	230	–	0.5
	60656	Neg	–	Neg	Neg	–	Neg	217	–	0.8
Day 35 R										
5 mg/eye	60603	Neg	NS	Pos	Neg	NS	Neg	67	NS	0.5
	60604	Pos	–	Pos	Pos	–	Pos	63	–	BLQ
	60653	Pos	–	Pos	Neg	–	Pos	83	–	0.3
	60654	Pos	–	Pos	Pos	–	Pos	57	–	1.7
10 mg/eye	60605	Pos	–	Pos	Neg	–	Pos	211	–	NS
	60606	Pos	–	Pos	Neg	–	Pos	187	–	0.8
	60655	Pos	–	Pos	Neg	–	Pos	199	–	NS
	60656	Pos	–	Pos	Neg	–	Pos	239	–	1.8
Day 43 R										
5 mg/eye	60603	Neg	Neg	Pos	Neg	Neg	Pos	7	28	0.1
	60604	Pos	Pos	NS	Pos	Neg	NS	213	624	NS
	60653	Pos	Pos	Pos	Pos	Pos	Pos	2	47	BLQ
	60654	Pos	Pos	Pos	Pos	Pos	Pos	2	36	0.2
10 mg/eye	60605	Pos	Pos	Pos	Pos	Pos	Pos	5	56	blq
	60606	Pos	Pos	Pos	Neg	Pos	Pos	25	125	NS
	60655	Pos	Pos	Pos	Pos	Pos	Pos	8	63	BLQ
	60656	Pos	Pos	Pos	Neg	Neg	Pos	22	90	0.2
AH: Aqueous humor; BLQ: Below limit of quantification; Neg: ADA negative; NS: No sample available; Pos: ADA positive; VH: Vitreous humor.										

AH: Aqueous humor; BLQ: Below limit of quantification; Neg: ADA negative; NS: No sample available; Pos: ADA positive; VH: Vitreous humor.

Overall discussion

Drug tolerance comparison demonstrated the superiority of the IC assay over the bridging assay when analyzing samples with high drug concentrations. In particular, the IC assay showed a reliable ADA detection over the entire range of the expected residual drug concentrations in ophthalmological samples. The reliability and transferability of the IC assay was further confirmed in real study samples from two preclinical studies in two different species.

The results of plasma sample analysis showed only minor differences between the assays, proving that the IC assay performs comparable to the bridging ADA assay under 'normal' conditions. Because of the IVT administration of the drug, the residual drug concentration in the plasma samples was below 1 $\mu\text{g/ml}$, in other words, below the concentration at which poor drug tolerance of the bridging assay became apparent in the drug tolerance experiment. Consequently, the bridging assay was not affected by the residual drug and showed a reliable ADA detection. In contrast, high residual drug concentration in the VH and AH samples (e.g., up to 1720 $\mu\text{g/ml}$ in VH) prevented a reliable ADA detection using the bridging assay, while the IC assay demonstrated consistent results for ocular fluid samples with very high residual drug concentrations.

Using the IC assay, we were able to demonstrate for the first time the presence of ADA in ocular fluids such as AH or VH after IVT administration in preclinical ophthalmology studies. Moreover, the IC assay enabled us to assess the correlation of the systemic ADA response with the presence of ADA in the eye by simultaneous analysis of plasma and ocular fluid samples. In all animals, the presence of ADA in the eye was accompanied by the systemic ADA response, indicating that the immune response to the drug was generated systemically first and then the systemically generated ADA were transported into the eye. This finding supports the concept of the eye as an immune privileged area with several mechanisms to inhibit immune responses [8]. However, our

current understanding of the mechanism and pathway of the immune response in the eye is far from being fully understood, as IgG antibodies were detected in VH [9] and the innate immune system of the eye can be activated by IVT administration of a drug [25].

A good correlation between the AH and VH samples also indicates that AH, the preferred sampling matrix due to easy (within seconds) [26] and safe access [24], would be predictive for the ADA presence in the eye. This is in line with the previous findings demonstrating a good correlation of protein composition between AH and VH, for example, for cytokine concentrations [27] and for drug elimination profiles after IVT administration of bevacizumab [28].

Overall, the systemic ADA assessment using the bridging assay performed alongside ophthalmic investigations and safety observations seems to be an appropriate strategy to assess immunogenicity after IVT administrations. If an additional analysis of the ADA response in AH or VH samples is required, for example, for the interpretation of pharmacokinetic data, we suggest using the IC assay to generate the most reliable data.

Conclusion

In ophthalmological studies, systemic ADA detection using the bridging assay seems to be sufficient for the immunogenicity assessment. The IC assay can, however, be used to address specific questions requiring ADA detection in ocular fluids, such as possible ADA-related toxicological findings or lack of efficacy due to aberrant pharmacokinetic profile in the eye. The good correlation of the ADA data between AH and VH can be considered for the design of future preclinical ophthalmology studies, in particular in the light of the 3R principle because termination of a study animal to obtain a VH sample may not be necessary. Additionally, AH analysis would enable a continuous monitoring of the ADA response in the eye.

Overall, the IC assay allows for a reliable ADA detection in matrices with high residual drug concentrations, such as ocular fluid samples after IVT injections, in which the bridging assay leads to false-negative results due to its poor drug tolerance. Low sample consumption (5 µl) of the IC assay provides an additional benefit for the analysis. Thus, the IC assay would be particularly suitable for studies with rare and low sample volumes and high residual drug concentrations.

Future perspective

The bridging assay format will likely remain the method of choice for immunogenicity assessment in ophthalmological studies in the short term because of its widespread use, which ensures data comparability, and its ability to detect the early IgM-based response. However, regulatory authorities are encouraging sponsors to explore approaches for an improved drug-tolerance, including the use of alternative assay formats [29]. Therefore, we believe that the IC assay will be increasingly used to address specific questions, especially those requiring analysis of samples with high residual drug concentrations. The IC assay has been so far developed and used for the analysis of cynomolgus monkey, minipig and mouse samples but the assay principle allows its ease extension to other nonhuman species. However, the development of an IC ADA assay for human samples is challenging and thus will remain one of the aims of future bioanalytical research.

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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

- The bridging and the immune complex (IC) assays demonstrated comparable qualification data. The IC assay had much lower sample consumption than the bridging assay (5 vs 12.5 µl).
- The IC antidrug antibody (ADA) assay was superior to the bridging assay in samples with high residual drug concentrations in an *in vitro* experiment, showing a reliable ADA detection over the entire range of the expected residual drug concentrations in ocular fluids.
- Using the minipig-specific IC assay, we were able for the first time to demonstrate a reliable ADA detection in vitreous humor (VH) after an intravitreal (IVT) injection.
- Transferability of the assay was demonstrated by the analysis of aqueous humor (AH), VH and plasma samples in a cynomolgus monkey study.
- Both assays showed similar results for the plasma sample analysis. However, the bridging assay generated inconsistent results for AH and VH samples with high residual drug concentrations.
- After IVT injection, systemic ADAs were detected in plasma earlier than in ocular fluids. Not in every case the systemic ADA response was accompanied by the presence of ADA in ocular fluids.
- The good correlation between the ADA detection in AH and VH samples justifies AH as a predictive matrix for the ADA detection.
- The IC assay allows for a reliable ADA detection in matrices with high residual drug concentrations, such as ocular fluid samples after IVT injections, in which the state-of-the-art bridging assay leads to false-negative results due to its poor drug tolerance. The IC assay would be particularly suitable for studies with rare and low sample volumes and high residual drug concentrations.

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