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RESEARCH ARTICLE

Preclinical Observations of Systemic and Ocular Antidrug Antibody Response to Intravitreally Administered Drugs

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Abstract

Intravitreally administered biotherapeutics can elicit local and systemic immune responses with potentially serious clinical consequences. However, little is known about the mechanisms of ocular antidrug immune response, the incidence of ocular antidrug antibodies (ADAs), and the relationship between ocular and systemic ADA levels. Bioanalytical limitations and poor availability of ocular matrices make studies of ocular immunogenicity particularly challenging. We have recently reported a novel bioanalytical ADA assay and shown its applicability for the ADA detection in ocular matrices. In the present study, we used this assay to analyze a large set of preclinical samples from minipig and cynomolgus monkeys treated with different ocular biotherapeutics. We found a significant association between the incidence of ADAs in plasma and ocular fluids after a single intravitreal administration of the drugs. Importantly, none of the animals with ADA-negative results in plasma had detectable ADAs in ocular fluids and systemic ADA response always preceded the appearance of ocular ADAs. Overall, our results suggest the systemic origin of ocular ADAs and support the use of plasma as a surrogate matrix for the detection of ocular ADA response.

Keywords Antidrug antibody · Immunogenicity · Ophthalmology · Aqueous humor · Vitreous humor · Drug tolerance · ELISA · Immune complex assay · Safety assessment · Ocular immune response

Introduction

Unwanted immune reactions pose one of the greatest challenges for biotherapeutic drug development (1). Immune response to a biotherapeutic can reduce efficacy, alter pharmacokinetics, or lead to adverse reactions. Therefore, the assessment of immunogenicity, in particular the detection of antidrug antibodies (ADAs), forms an essential part of the development of biologic drugs (2, 3), including intravitreally (IVT) administered anti-VEGF biotherapeutics such as bevacizumab, ranibizumab, or aflibercept.

Although these ocular biotherapeutics are well-tolerated (4), cases of non-infectious intraocular inflammation have been reported in patients receiving IVT anti-VEGF treatment (5, 6). Moreover, the presence of ADAs in the systemic circulation was associated with an increased incidence of intraocular inflammation for some of these drugs (7–9). Drug-ADA immune complexes can play a role in these adverse reactions (10, 11), yet the mechanisms of ocular immunogenicity and its role in treatment-related inflammatory reactions are still poorly understood (12).

The eye is an immunoprivileged organ, where inflammatory reactions are inhibited by immune-suppressive mechanisms (13). It is therefore believed that ADAs detected in plasma after IVT drug administration (7, 8, 14) are developed in response to drug molecules leaked into the systemic circulation. These systemic ADAs can diffuse into the eye, form drug-ADA immune complexes, and thereby produce unwanted effects. Experimental evidence for this mechanism, in particular data demonstrating a relationship between systemic and ocular ADA levels, is still lacking.

Studies of ocular antidrug immune responses face considerable technical limitations. Ocular fluids are not easily accessible in clinical trials even less so in routine clinical practice. Especially vitreous humor (VH), the most important ocular matrix due to its proximity to the site of action of IVT administered drugs, can only be sampled during

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surgical procedures (15). In addition, low volume of ocular samples and usually high residual drug concentrations present a substantial bioanalytical challenge. These limitations along with the rareness of the antidrug immune response in human make the clinical assessment of immunogenicity of intraocular drugs particularly difficult.

Even though immunogenicity findings in animals are not considered to be directly translatable to human (2, 3, 16, 17), preclinical studies can provide insights into the mechanisms of ocular immunogenicity and help develop monitoring methods, which can be then applied in the clinical setting. In our previous publication, we reported a novel bioanalytical assay for reliable detection of ADAs in ocular matrices and demonstrated an association of systemic and ocular ADA responses in a limited set of samples from preclinical studies with an IVT administered biotherapeutic (18). In the present study, we used this method to analyze ADAs in a large set of samples from 7 nonclinical studies with different ocular biotherapeutics, aiming at investigating the time course and the association of systemic and ocular ADA responses after IVT administration of different biotherapeutics.

Materials and Methods

Reagents

Murine monoclonal antibodies against minipig and cynomolgus monkey IgG, conjugates of minipig and cynomolgus IgG with human IgG, anti-human kappa light chain M1.7.10 antibodies and biotin (Bi) and digoxigenin (Dig)-labeled drugs and antibodies were produced by Roche Diagnostics GmbH, Penzberg, Germany. Other reagents were provided by Roche Diagnostics or obtained from commercial suppliers.

Study Samples

Study samples (plasma, aqueous humor [AH], and VH) were collected in preclinical pharmacokinetics and toxicity studies in drug naïve Göttingen minipigs and cynomolgus monkeys after a single IVT administration of a biotherapeutic drug. Six different drugs were used in these studies. The drugs were either bivalent IgG Fab fragments (50 kDa) or complete IgGs (150 kDa). The doses ranged from 1.21 µg/eye through 10 mg/eye in minipigs and from 0.15 to 10 mg/eye in cynomolgus monkeys.

Plasma and AH samples (approximately 50 µL) were taken during in-life phase (predose and up to 2904 h postdose). VH samples (approximately 200 µL) were taken at necropsy. Ocular samples were taken from the treated eye. Sodium citrate, theophylline, adenosine, and dipyridamole (CTAD) were used as an anticoagulant for plasma samples. All samples were frozen immediately after collection and thawed shortly before analysis. VH samples were centrifuged before analysis at 13,000 × g for 5 min, and the supernatant was used for the analysis.

ADA Detection

In this study, two enzyme-linked immunosorbent assay (ELISA) formats were used to detect ADAs: a conventional bridging assay for plasma samples and the novel immune complex assay for ocular samples. Only screening analysis was conducted.

In-study assay performance was controlled according to FDA recommendations (19). Assay- and drug-specific positive controls were soluble targets or anti-human kappa light chain antibody (bridging assay) and conjugates of minipig or cynomolgus monkey IgG with human IgG (immune complex assay). Quality control (QC) samples for the bridging assay were prepared in pooled normal minipig or cynomolgus monkey CTAD plasma. QC samples for the immune complex assay were prepared either in pooled animal CTAD plasma or assay buffer. Respective unspiked matrices (either pooled animal CTAD plasma or assay buffer) were used as negative controls.

Experimental procedures are briefly described below. The principles of the assay formats are shown in Fig. 1. A detailed description of the assays and experimental procedures is available in Wessels *et al*. (18).

Bridging Assay

Study and QC samples were appropriately diluted, mixed with Bi- and Dig-labeled drug, incubated, and then transferred onto a streptavidin coated microtiter plate (SA-MTP). After incubation, unbound drugs were washed out and captured drug-ADA complexes were detected using pAb-Dig-S-Fab-HRP and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) color reaction. A sample was considered screening positive if its signal was at or above the 95% upper percentile of the signals measured in 50 blank samples (cut point).

Immune Complex Assay

Bi-labeled anti-human kappa light chain antibodies were bound onto an SA-MTP. Study and QC samples were appropriately diluted, mixed with unlabeled drug, incubated, and then transferred onto the SA-MTP. After incubation, unbound drugs were washed out. Dig-labeled anti-minipig IgG or anti-cynomolgus IgG antibodies were added to the plate, and the plate was incubated again. Unbound detection antibodies were then washed out, and bound complexes were

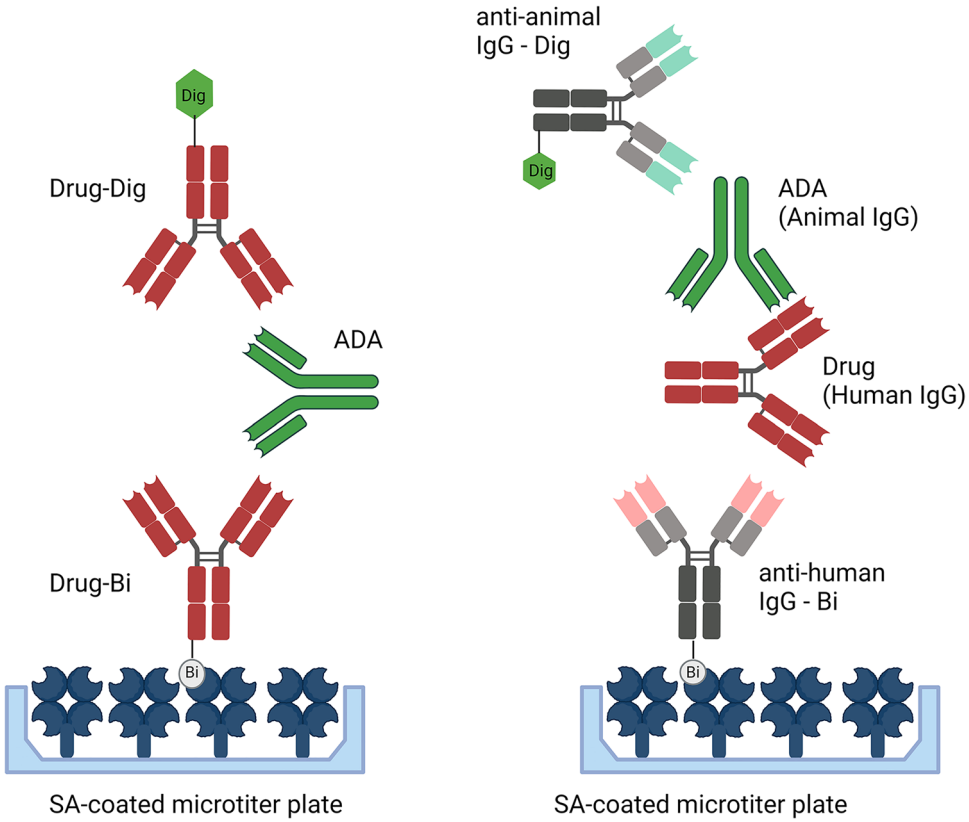


Fig. 1 Principles of the assay formats used for ADA detection. **a** In the classical bridging assay format, antidrug antibodies (ADAs) bind ("bridge") biotin (Bi)- and digoxigenin (Dig)-labeled drug molecules. The formed immune complexes are then captured onto a streptavidin (SA) coated plate and detected using anti-Dig antibodies. The assay is drug-specific because it requires labeled drug molecules but not species- or antibody isotype-specific because it does not rely on any ADA characteristics other than the ability to bivalently bind a drug. The assay cannot discriminate between ADAs and soluble targets capable of multivalent binding of a drug, such as VEGF dimers, whose presence in the sample can lead to false positive results. Since the assay requires free binding sites on an ADA molecule, it cannot detect ADAs bound to unlabeled drug. Essentially, the assay measures free ADAs, and thus it is susceptible to interference by free drug, which can decrease the amount of free ADAs by binding them into immune complexes and thus lead to a false negative result. Therefore, the assay performs poorly with samples containing high residual drug concentrations, such as vitreous humor samples. **b** In the immune complex assay format, unlabeled drug-ADA immune complexes are captured using Bi-labeled anti-human kappa light chain antibodies, which bind to human IgG-based drug molecules within the immune complexes. The captured complexes are then detected using Dig-labeled anti-animal IgG antibodies, which bind to the animal ADAs. The assay is not drug-specific, but it is species-specific because it requires anti-animal IgG antibodies to detect immune complexes. The IgG-specific detection method precludes the interference by soluble targets but also makes the assay unsuitable for the detection of early, IgM-based, immune responses. Importantly, the assay includes an incubation step with an excess of unlabeled drug, which ensures that free ADAs are bound into immune complexes detectable by the assay. Thus, in contrast to the bridging assay format, the immune complex assay can detect both free and drug-bound ADAs. This makes the assay insensitive to the presence of residual drug, allowing reliable ADA detection in ocular samples

detected and evaluated in the same way as in the bridging assay.

Statistical Analysis

Statistical analysis was performed in the statistical software "R" Version 4.2.2. Fisher's exact test was used for comparison of the ADA incidence in plasma and ocular matrices. Wilcoxon signed rank test was used for comparison of the ADA onset in plasma and AH.

Results

Dataset

We analyzed 953 samples collected from 89 animals in 7 nonclinical studies (3 minipig and 4 cynomolgus monkey studies; Table I). Plasma and AH samples were collected in all studies, whereas VH samples were not available in 2 cynomolgus monkey studies. All samples were evaluable, and none of the samples were excluded from the dataset. All predose samples were tested ADA-negative. Since all

Table I Overview of the Dataset

	Studies				Animals			Samples		
	Plasma	AH	VH		Plasma	AH	VH[a]	Plasma	AH	VH[a]
Minipig	3	3	3	Total, *n*	32	24	32	206	28	32
				ADA+, *n* (%)	15 (47%)	3 (13%)	9 (28%)	54 (26%)	3 (11%)	9 (28%)
Cynomolgus monkey	4	4	2	Total, *n*	57	57	20	410	257	20
				ADA+, *n* (%)	45 (79%)	19 (33%)	10 (50%)	211 (51%)	30 (12%)	10 (50%)
Overall	7	7	5	Total, *n*	89	81	52	616	285	52
				ADA+, *n* (%)	60 (67%)	22 (27%)	19 (37%)	265 (43%)	33 (12%)	19 (37%)

ADA+, number of samples tested positive for antidrug antibodies (ADA) or number of animals with at least one positive sample; *AH*, aqueous humor; *VH*, vitreous humor

None of the samples were unevaluable or ADA-inconclusive

[a]Equal number of animals and samples (one VH sample per animal)

plasma samples had low residual drug concentration (data not shown) and ocular samples were measured with a drug tolerant assay (Fig. 1b), assay signals below the cut point were unlikely caused by drug interference and therefore all negative ADA results were considered ADA-conclusive. The bridging assay used for plasma and the immune complex assay used for ocular fluids have shown similar performance and sensitivity (18), allowing a direct comparison of ADA incidence in plasma and ocular samples analyzed in this study. Overall, our dataset provided a median 12 (range 6–27) data points per animal (time points and matrices).

ADA Incidence in Different Matrices

ADAs were detected in plasma and ocular fluids of animals treated with IVT administered biotherapeutics (Table I and Fig. 2a). All animals with ADA-negative plasma samples also had ADA-negative ocular samples. However, the presence of ADAs in plasma was only partially reflected in ocular fluids, with ADA-positive VH and AH samples seen in, respectively, 58% and 40% of animals tested positive for systemic ADAs. Overall, our data indicated a significant association between the ADA status in plasma and ocular fluids ($p < 0.0001$, Fisher's exact test, Fig. 2a).

ADA status in AH was also significantly associated with that in VH ($p < 0.0001$, Fisher's exact test, Fig. 2a). In contrast to plasma, ADA-negative results in AH were not fully predictive of ADA-negativity in VH, with three animals showing discordant results. In one of these animals, the ADA signal in VH was low and consequently a lower signal in the AH sample (as expected for AH, see below) could have led to a negative test result in AH. In two other animals, the delayed onset of ocular ADAs (see next section) could account for the observed discrepancy, as the last AH samples were taken approximately 200 h before VH sampling. The reasons for another discrepancy seen in three animals with ADA-positive AH samples and ADA-negative VH samples remain unclear. Low sample volume did not allow for sample re-analysis to exclude analytical errors, whereas re-sampling was technically impossible because VH samples were taken at necropsy.

The strength of ADA signal was comparable in ADA-positive plasma and VH samples and was markedly lower in AH samples (Figs. 2b and 3a), which can explain a lower proportion of ADA-positive AH samples in the dataset as compared with plasma and VH (Table I). ADA-positive AH samples were almost exclusively seen in animals with strong ADA signals in plasma (Fig. 2c).

Time Course of the ADA Response

We consistently observed a delayed ocular ADA response as compared with the systemic response (Fig. 3a and b). ADAs in plasma were detected as early as 168 h postdose, and the number of ADA-positive plasma samples markedly increased after 240 h postdose (Fig. 3c and Table II). In contrast, ADA-positive AH samples were first observed 336 h postdose, with most ADA-positive AH samples appearing after 840 h postdose (Fig. 3c and Table II). Notably, in all animals, ADA positivity was detected in plasma samples first. ADA-positivity persisted in most (95%) animals in plasma and in all animals in AH until the last sample, although a decrease in ADA signal in some late AH samples (Fig. 3a) may indicate declining ADA levels in AH.

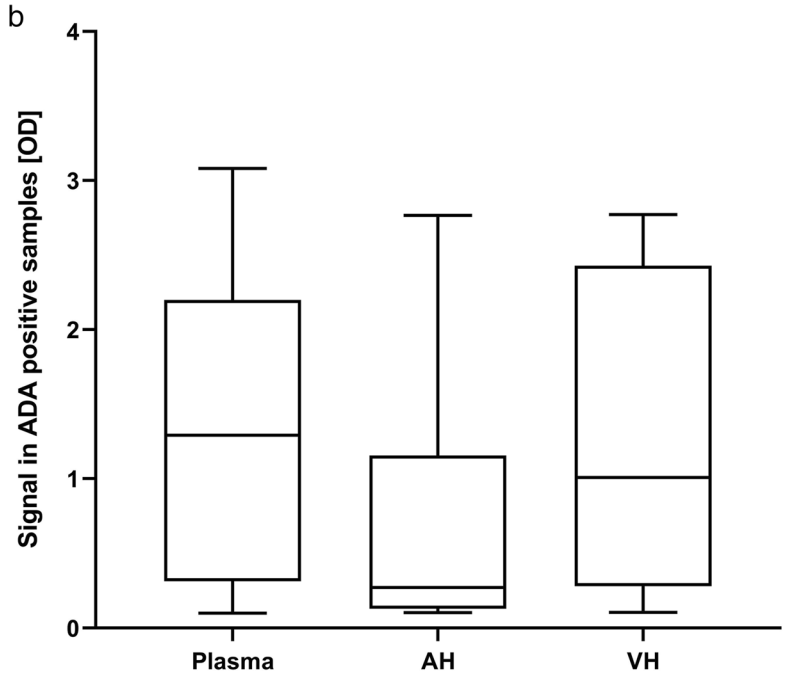
The analysis of VH samples provided limited information about the timing of the ADA response in VH because these samples were collected only at necropsy. The earliest ADA-positive VH sample was taken at 408 h postdose, indicating that ADAs can appear in VH as early as 408 h after the first dose. The proportion of animals with ADA-positive VH was higher at later necropsy time points (64% of animals with systemic ADAs at 840 h postdose or later *versus* 37% at earlier time points), which may indirectly suggest a delayed ADA appearance in VH.

a

Plasma	Vitreous humor ***	
	ADA+	ADA−
ADA+	19	14
ADA−	0	19

Plasma	Aqueous humor***	
	ADA+	ADA−
ADA+	22	33
ADA−	0	26

Aqueous humor	Vitreous humor***	
	ADA+	ADA−
ADA+	12	3
ADA−	3	26



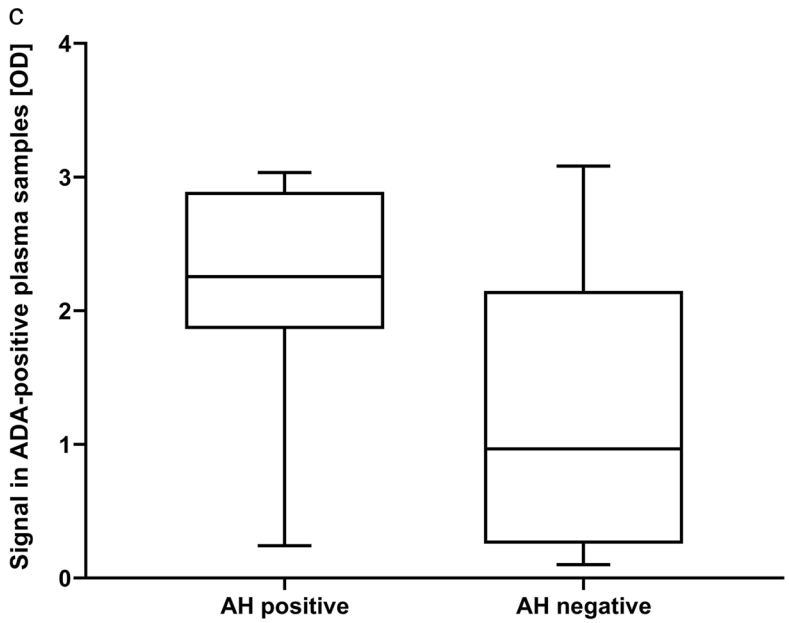


Fig. 2 ADA incidence and signal strength in plasma, aqueous, and vitreous humor. **a** Frequencies of ADA incidence in plasma and ocular fluids. The cross-tabulations show the number of animals; an animal was counted as ADA-positive (ADA +) for a given matrix if at least one sample of the matrix was tested ADA-positive. ***$p < 0.0001$, Fisher's exact test. **b** ADA signal strength in ADA-positive plasma, AH and VH samples ($n = 256$ for plasma, 33 for AH and 19 for VH). **c** ADA signal strength in time-matched ADA-positive plasma samples from animals with ADA-positive AH samples (AH positive, $n = 33$) and with no ADA-positive AH samples (AH negative, $n = 223$). ADA, antidrug antibody; AH, aqueous humor; OD, optical density (arbitrary units); VH, vitreous humor. Whiskers indicate the minimum and maximum values

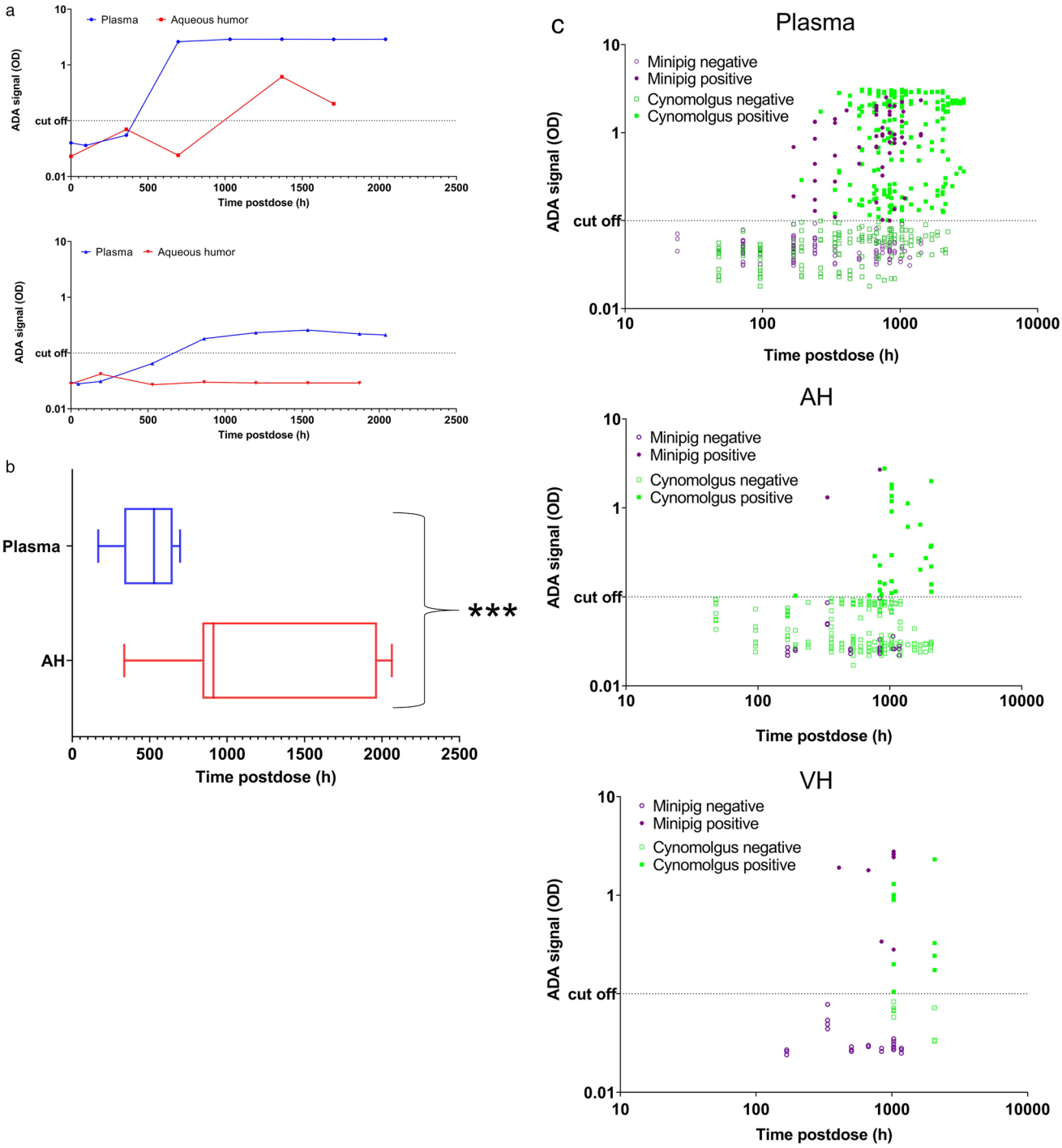


Fig. 3 ADA kinetics in plasma, aqueous, and vitreous humor. **a** Exemplary time course of ADA signal strength in plasma and AH in animals with systemic ADA response and ADA-positive AH (upper panel) and ADA-negative AH (lower panel). **b** ADA onset in plasma and AH ($n = 22$). Whiskers indicate the minimum and maximum values. ***$p < 0.0001$, Wilcoxon signed rank test. **c** ADA signal strength in plasma, AH, and VH samples at different time points. ADA, antidrug antibody; AH, aqueous humor; OD, optical density (arbitrary units); VH, vitreous humor

Table II ADA Onset in Plasma and Aqueous Humor

	Median onset time (range) postdose, h
Plasma (all animals, n=89)	564 (168–1032)
Plasma (animals with ADA+ AH, n=22)	528 (168–696)
AH (n=22)	912 (336–2064)

Discussion

In the present study, we conducted ADA analysis in a large set of plasma and ocular samples from minipigs and cynomolgus monkeys treated with different intraocular biotherapeutics. Our analysis revealed a significant association between ADA incidence in plasma and ocular fluids in full agreement with our previous preliminary observations (18). In particular, none of the animals had detectable ocular ADAs in the absence of detectable ADAs in plasma. ADAs appeared in plasma at median 22 days postdose, about 16 days earlier than in AH. Taken together, our data showed that ocular ADAs appear only in animals with preceding systemic ADA response, thus suggesting the systemic origin of ocular ADAs.

Only about a half of the animals with positive test results in plasma had detectable ADAs in ocular fluids. Given that ocular ADAs seem to originate from the systemic circulation, it is likely that this discordance in the test results was due to low levels of ocular ADAs caused by the anatomical and physiological barriers to the diffusion of macromolecules into in the eye (the blood-aqueous and the blood-retinal barriers). Thus, ocular ADA levels may not reach the detection limit, especially when systemic ADA levels are low. This effect was indeed apparent in AH, which has low concentration of proteins due to both the ocular barriers and its high turnover rate (20). Consequently, ADA incidence in AH was the lowest among the three matrices, and ADA-negative results in AH did not fully exclude ADA-positivity in VH. The disruption of the blood-ocular barrier seen in the target indications of IVT biotherapeutics (e.g., due to inflammatory neo-vascularization) can facilitate the diffusion of macromolecules into the eye and potentially result in a better concordance between the presence of systemic and ocular ADAs in patients. However, leaky vessels do not appear to be a prerequisite for the appearance of ocular ADAs, as ocular ADAs were detected in our study in healthy animals with an intact blood-ocular barrier.

The observed kinetics of ADA response to IVT administered drugs appears to be in line with the systemic origin of ocular ADAs. Slow permeation of systemic ADAs through the ocular barriers can explain the observed time lag between the appearance of ADAs in plasma and AH. Although the immune complex assay used for ocular matrices cannot detect the IgM isotype, which appears at early stages of an immune response, this limitation unlikely impacts the results observed in our study because IgM were not detected or detected at very low levels in AH of animals and humans (21–23). However, IgM was detected in the eyes of animals and humans with inflammatory conditions (24, 25) and in patients treated with IVT administered drugs (26), indicating that the onset and composition of ocular ADAs may differ in healthy and patient populations.

Plasma and AH samples in our study were taken over a short period of time after a single IVT drug administration, and only one terminal VH sample was available for each animal. Therefore, the duration of ADA responses could not be reliably assessed based on the data in our study; the observed decrease of ADA signal in the late AH samples should be interpreted cautiously in the absence of titration data. Moreover, the kinetics of ocular ADA response after a single dose can differ from that after multiple doses because high drug concentration in the eye expected after chronic administration can lead to an accumulation of ADAs in ocular fluids in form of drug-ADA complexes. Thus, we expect a higher concordance between ADA-positive plasma and ocular samples in studies with chronic drug treatment and longer sampling period.

The data available in the study only allowed an assessment based on signal levels, which provide limited comparability regarding the magnitude of the immune response—a fundamental problem of ADA assays (27). This can be partially compensated by sample titration, which allows a quasi-quantitative measure of the magnitude of the ADA response, yet ocular samples cannot be obtained in large amount required for such approach. Therefore, the comparisons based on ADA signal levels ought to be interpreted as exploratory owing to the lack of titer data, use of two different assays, and some measurements being close to the upper limit of the assay signal range.

Immunogenicity in animals is per se not predictive of the incidence of clinical immunogenicity owing to immunologic incompatibility of preclinical species with human or humanized biotherapeutics (1, 3). However, given the anatomical and physiological similarity between minipig, monkey and human eyes (28, 29), it is likely that ocular ADA response in humans has the same underlying mechanism as in these animals, namely, the formation of systemic ADAs first and their subsequent entry into VH. The possible contribution of ocular ADAs to intraocular inflammation could not be analyzed in this study due to the lack of histopathology data. It is worth noting that other factors, such as drug impurities and protein aggregates, can play a role in intraocular inflammation after IVT biologic drug administration (10).

Our study has provided so far the most comprehensive analysis of ocular ADAs in preclinical species. More studies are certainly needed to further elucidate the mechanisms of ocular antidrug immune response and their clinical

relevance. Isotyping and neutralizing assays can be used to characterize ADA response and predict potential clinical sequalae, such as loss of efficacy without apparent adverse reactions caused by neutralizing non-compliment binding antibodies. Moreover, such in-depth analysis may provide another line of evidence for the systemic origin of ocular ADAs by showing that ocular and systemic ADAs have the same characteristics. To our knowledge, no studies with conventional isotyping or neutralizing assays have been reported for ocular matrices likely because the analysis of ocular samples poses a substantial bioanalytical challenge. The novel immune complex assay format can be potentially adapted to selectively detect ADA isotypes (30, 31), enabling detailed analysis of ocular ADA response.

Overall, our data support the use of plasma as a surrogate matrix for the detection of ocular ADA response. Apart from better concordance with the test results in VH, plasma offers other advantages over AH in the clinical setting. These include much better availability, which enables frequent monitoring and extensive bioanalysis, such as re-analysis and titration, and the early onset of ADAs in plasma, which allows timely implementation of mitigation measures before ADAs appear in the eye. Moreover, spared AH samples can be used for exploratory investigations, such as biomarker or target measurements. The key question yet to be answered is however not the detection of ADAs per se but whether we can determine a threshold for the magnitude of the systemic ADA response above which the achieved ocular ADA levels would lead to clinical consequences.

Conclusions

Our data showed a significant association between ADA incidence in plasma and ocular fluids after a single IVT administration of different biotherapeutics in preclinical studies with minipigs and cynomolgus monkeys. ADAs appeared about 16 days earlier in plasma than in AH. Overall, our study provided evidence for the systemic origin of ocular ADAs and demonstrated that plasma can serve as a suitable surrogate matrix for the detection of ocular ADA response. Particularly, negative ADA results in plasma appear to exclude the presence of ADAs in ocular fluids.

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Author Contribution UW analyzed data, designed figures and tables, wrote, and revised the manuscript. MZ and AMW collected data. TS aided in the analysis of the data. KS conceived the idea for the study and led discussions. All authors discussed the presentation and interpretation of the data.

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Declarations

Research Involving Animals 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.

Conflict of Interest The authors are employees of Roche Diagnostics GmbH, Penzberg, Germany.

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