Journal of Pharmaceutical and Biomedical Analysis 52 (2010) 249–254

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Evaluation of a generic immunoassay with drug tolerance to detect immune complexes in serum samples from cynomolgus monkeys after administration of human antibodies

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ARTICLE INFO

Article history:
Received 18 September 2009
Received in revised form
22 December 2009
Accepted 23 December 2009
Available online 4 January 2010

Keywords:
Anti-drug antibody
Immune complex
Drug interference
Cynomolgus monkey
Immunogenicity

ABSTRACT

Current state of the art bridging ELISA technologies for detection of anti-drug antibodies (ADAs) against therapeutic antibodies bear the risk of false-negative results due to interference by circulating drug. Methods to remove the drug in the sample or sample pre-treatment techniques such as acid dissociation of the immune complexes are limited, laborious and may destroy ADAs resulting again in false-negative results. The immune complex ELISA described in this publication provides a simple solution. It is designed to analyze samples from cynomolgus monkeys dosed with human antibodies; it can be used for all human antibodies since it is independent of the specific antibody and its target. The generic applicability of the ADA assay is enabled by the use of (1) a murine anti-human Fc monoclonal antibody (MAb) as capture reagent; (2) a murine anti-cynomolgus monkey IgG MAb as detection reagent; and (3) an ADA positive control conjugate consisting of cynomolgus IgG complexed with human IgG. In its basic version, the generic ADA ELISA specifically detects only immune complexes formed in vivo. Validation of the ADA assay revealed a lower limit of quantitation of 15.6 ng/mL in serum samples. Intra-assay and inter-assay precision was characterized by a coefficient of variation of less than 10% and accuracy was within 8%. Matrix effects were low as evidenced by a mean recovery of 95%. In vitro pre-incubation of the serum samples with drug makes also the free ADA in the sample amenable to measurement by the immune complex ELISA as demonstrated by analysis of ADAs from two cynomolgus monkey studies with two different antibodies. The generic and versatile nature of this ADA assay favors its use in pilot pharmacokinetic and safety studies in cynomolgus monkeys during candidate selection of antibodies. The assay can help to explain unexpected drug clearance profiles, loss of efficacy or safety events caused by immune complexes and guide further development.

1. Introduction

Monoclonal antibodies have become an increasingly important class of drugs in a variety of diseases and represent the majority of protein candidates currently in clinical development [1]. It is accepted standard practice and expected under current regulatory guidance that potential antibody formation to therapeutic biologics is being evaluated during non-clinical safety studies to describe and assess its impact on the toxicity profile [2]. Assay methodologies currently available to study the immune response against therapeutic biologics include enzyme linked immunoassays (ELISA), surface plasmon resonance (SPR), chemiluminescence and flow cytometry assays for binding antibodies and cell-based assays to assess the neutralizing potential of anti-drug antibodies (ADAs) [3]. In non-clinical studies, ADAs can affect drug exposure, thereby complicating the interpretation of the toxicity, pharmacokinetic and pharmacodynamic data [4]. In addition, immune complexes of drug and ADAs may cause toxic effects. Non-clinical safety and efficacy studies very often are conducted in cynomolgus monkeys, due to insufficient target cross-reactivity of human-specific antibodies with rodent or non-rodent laboratory animals, such as mouse, rat, rabbit and dog.

Despite the emergence of novel technologies to measure ADAs such as SPR, microtiter plate-based ELISAs are still the most widely used format for testing ADAs due to their high-throughput efficiency, relative simplicity and high sensitivity [5]. ADA ELISAs are most often designed in a bridge format which provides high selectivity and pan-species ADA detection capability, making it feasible to implement a single assay format for both non-clinical and clinical studies. In the double-antigen bridging ELISAs, the drug is immobilized on the surface of microtiter plates allowing specific binding of ADAs in the sample which in turn are recognized by the drug

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doi:10.1016/j.jpba.2009.12.029

labelled with a detection system. An apparent disadvantage of the bridging ELISA using drug for capture and detection of ADAs is the possible interference of free drug present in the sample. Free drug may compete with solid phase drug for capture of the ADA and lead to a false-negative result [6]. In addition, bridging ELISAs are incapable to detect drug–ADA complexes. Considerable efforts are underway to dissociate the drug–ADA complex to detect ADA despite the presence of bound drug or to enable detection of the complex [5].

The present work describes an ELISA to measure ADAs in serum samples from cynomolgus monkeys exposed to human antibodies despite drug interference and to measure drug–ADA immune complexes. The ELISA uses a murine monoclonal antibody directed against the constant part (Fc) of human immunoglobulin (Ig) to capture the antibody (drug) of the drug–ADA complex, and a murine monoclonal antibody specifically directed against cynomolgus monkey IgG to detect cynomolgus monkey ADA. The antibody specific for cynomolgus monkey IgG was successfully generated in our lab despite the high sequence homology of cynomolgus immunoglobulins and human immunoglobulins of 85–98% [7].

This assay principle, thereby, provides a versatile generic format for measurement of ADAs in serum of cynomolgus monkey independent of the drug and the drug target. In its basic version, the generic ADA ELISA specifically measures immune complexes formed in vivo and, by definition, does not detect unbound ADAs. By introducing a simple pre-incubation step in which the serum sample is spiked with drug to form immune complexes, the generic ADA ELISA can be used to measure total ADA levels.

Other methods have been reported to reduce drug interference by sample pre-treatment techniques such as acid dissociation of drug–ADA complexes, or drug removal [8]. However, drug removal might also lead to removal of the drug–ADA complex. Acid dissociation to destroy the immune complexes is limited for high affinity ADAs, in addition ADA may be irreversibly denatured by acid pretreatment resulting in false-negative samples [3].

The generic ADA assay format described here is a novel and versatile method to generically detect immune complexes of human antibodies with ADAs from cynomolgus monkeys, or to analyze total ADA levels in the presence of high levels of circulating drug.

2. Experimental

2.1. Chemicals and reagents

The monoclonal antibodies used in the experiments were recombinant fully human monoclonal IgG antibodies directed against the human insulin-like growth factor-1 receptor (anti-IGF-1R) and against a human cellular surface target (MAb 2), respectively. The antibodies were produced at Roche Diagnostics GmbH, Penzberg, Germany.

The murine IgG1 monoclonal antibody R10Z8E9 was produced by Roche Diagnostics GmbH, Penzberg, Germany. This antibody is directed against a conformation epitope on the CH2 domain of all four subclasses of human Fc gamma [9]. To prepare biotinylated anti-human Fcγ-pan R10Z8E9, 10 mg/mL of the purified MAb were incubated at pH 8.5 in a molar ratio of 1:5 with D-biotinoyl-aminocaproic acid-N-hydroxysuccinimide ester dissolved in DMSO. The reaction was stopped after 60 min by addition of L-lysine and the surplus of the labeling reagent was removed by dialysis against 50 mM potassium phosphate buffer, with 150 mM NaCl, pH 7.5.

The murine monoclonal antibody against cynomolgus monkey IgG was generated from mice immunized with purified cynomolgus monkey IgG. Antibodies from hybridoma supernatants were purified by protein A chromatography. This anti-cynomolgus monkey IgG MAb (anti-cyno IgG) was digoxigenylated by incubating 2 mg/mL of the purified MAb at pH 8.1 in a molar ratio of 1:5 with digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester dissolved in DMSO. The reaction was stopped after 60 min by addition of L-lysine and the surplus of the labeling reagent was removed by dialysis against 50 mM potassium phosphate buffer, with 150 mM NaCl, pH 7.5.

Serum samples from chimpanzee, rhesus macaque, marmoset monkey and baboon were obtained by standard methods from individual zoo animals. Serum samples of 10 different individual cynomolgus monkeys were provided by Covance, Muenster, Germany. Batches of pooled cynomolgus monkey sera for use as matrix in the ELISA for calibration standards and quality control samples as well as for dilution of analyte samples were from Covance, Muenster, Germany, and from Bioreclamation Inc., Hicksville, NY, USA. Serum from dog, rat, CD-1 and NMRI mice was obtained from commercial sources (Charles River, Wilmington, MA, USA). The polyclonal antibody against human Fcγ (PAb anti-human Fcγ) was purchased from Dianova GmbH, Hamburg, Germany. The polyclonal anti-digoxigenin-horse radish peroxidase (HRP) conjugate (Fab fragments) was from Roche Diagnostics GmbH, Mannheim, Germany (cat. no. 11633716-001). The following reagents were obtained from Roche Diagnostics GmbH, Mannheim, Germany: 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate (cat. no. 11684302-001) and the wash buffer for the ELISA: phosphate-buffered saline (PBS; 50 mM K_3PO_4; 150 mM NaCl; pH 7.5)/0.05% polysorbate 20 (Tween 20) (cat. no. 11332465-001). Ready to use LowCross buffer (order no. 100500) was obtained from Candor Bioscience GmbH, Weissensberg, Germany, and was used as dilution and assay buffer in the ELISA. All chemicals were of analytical grade.

2.2. Biacore assay for determination of specificity of binding

The specificity of the murine anti-cynomolgus monkey IgG MAb was evaluated by surface plasmon resonance (SPR) technology with the Biacore® 2000 instrument (Biacore, Uppsala, Sweden) using a CM5 sensor chip (BR-1000-14; Biacore, Uppsala, Sweden). Coating of an antibody to this chip was achieved by standard amine coupling. Unless otherwise stated, all incubations were performed in HBS buffer (HEPES, NaCl, pH 7.4) at 25 °C. A saturating amount of anti-cyno IgG and PAb anti-human Fcγ, respectively, was immobilized by amine coupling on different flow cells of the same CM5-chip. All animal and human sera were diluted in HBS buffer containing 1 mg/mL CM-dextran at a final concentration of 1%. Binding was analyzed by injection of the 1 in 100 diluted sera and incubation for 60 s. Dissociation was measured by washing the chip surface with HBS buffer for 180 s. Using BIAevaluation software V4.1 from Biacore, Sweden, the dissociation constant values (=K_{Diss}) were calculated with a 1:1 Langmuir fitting model. For all animal sera this calculation was based on the assumption that the IgG level was 15 mg/mL. The signal values 80 s after start of the injection of the test antibody have been chosen for the comparison of the amount of bound IgG (resonance signal units (RU) in Table 1).

2.3. Preparation of calibration standards and quality control samples

Conjugates of cynomolgus monkey IgG (Cyno IgG) with human IgG (H-IgG) were prepared for use as calibration standards (CS) and quality control (QC) samples. N-succinimidyl-3-acetylthiopropionate (SATP) was added to Cyno IgG purified from cynomolgus serum by ion exchange chromatography and gel filtration in a molar ratio of 1:5 (IgG:SATP). The mixture was incubated for 60 min at 25 °C at pH 7.1. The reaction was stopped by adding L-lysine and the surplus of SATP was removed by dialysis. In par-

Table 1
Binding signals (RU) and the corresponding dissociation constants (K_{Diss}) of sera from rodent and non-rodent animals, non-human primates and human for binding to a monoclonal anti-cynomolgus IgG and a polyclonal anti-human-Fcγ antibody in the Biacore system.

Sample (Serum)	Anti-cyno IgG MAb		Anti-human Fcγ PAb	
	RU	K_{Diss} (M)	RU	K_{Diss} (M)
Human	−26	No binding	1572	4.56×10^{-12}
Chimpanzee	−28	No binding	1918	0.37×10^{-12}
Rhesus macaque	2363	5.09×10^{-10}	1172	0.8×10^{-12}
Cynomolgus	2279	2.03×10^{-9}	1345	7.17×10^{-12}
Baboon	2039	0.94×10^{-12}	1405	6.37×10^{-11}
Marmoset	−31	No binding	532	1.41×10^{-8}
Dog	−36	No binding	564	1.15×10^{-8}
Rat	−21	No binding	48	No binding
CD1-mouse	−40	No binding	−30	No binding
NMRI mouse	−16	No binding	−23	No binding

allel, maleimidohexanoyl-N-hydroxysuccinimide ester (MHS) was added to H-IgG purified from human serum by ion exchange chromatography in a molar ratio of 1:6 (IgG:MHS). The mixture was incubated for 60 min at 25 °C at pH 7.1. The reaction was stopped by adding L-lysine, the pH was adjusted to 6.1, and the surplus of MHS was removed by dialysis.

Cyno IgG-SATP was deacetylated to Cyno IgG-SH. The deacetylated antibody was mixed with H-IgG-MH (molar ratio 1:1) to a final concentration of 7 mg/mL of total IgG. The pH was adjusted to 7.1 and the mixture was incubated at 25°C. The conjugation process was monitored with an analytical gel filtration column (TSK 3000). The conjugation was stopped by adding cysteine. After 30 min, N-methylmaleimide (NMM) was added and the pH was adjusted to 7.5. After 60 min incubation at 25 °C the conjugate was separated by gel filtration chromatography with Sephacryl S-300 to remove non-conjugated antibodies.

Stock solutions for CS and QC were prepared separately. For use as calibration standards and QC samples, the conjugates were spiked in 100% pooled cynomolgus monkey serum and stored at −80 °C until use. A single use aliquot of the CS stock solution (320 μg/mL) was thawed at the time of analysis and diluted stepwise 1:2 to yield calibrator concentrations of 200; 100; 50; 25; 12.5; 6.25; 3.13; 1.56; 0.78; 0.39; 0.20; and 0.00 ng/mL in 5% pooled cynomolgus monkey serum. The standard concentration range for routine use after validation was 80–0 ng/mL (80; 40; 20; 10; 5; 2.5; 1.25 ng/mL and blank). The four QC stock samples had concentrations of 60 ng/mL (high QC, HQC), 32 ng/mL (medium QC, MQC), 7.5 ng/mL (low CQ, LQC) and 2.5 ng/mL (lower limit QC, LLQC) in 5% pooled cynomolgus serum.

2.4. Immune complex ELISA assay set-up

The ELISA was evaluated in a 96-well microtiter plate (MTP) format (Fig. 1). In this generic immune complex assay, all solutions were prepared in dilution (assay) buffer. All incubation steps were performed at room temperature on a MTP shaker at 500 rpm and all wash steps were performed three times with 300 μL each of wash buffer and subsequent removal of residual fluid. Biotinylated MAb anti-human Fcγ-pan R10Z8E9 was bound to streptavidin-coated microtiter plates (SA-MTP), obtained from Roche Diagnostics GmbH, Penzberg, Germany (article no. 11645692001) at a concentration of 0.5 μg/mL and incubated for 1 h. The assay did not require specific blocking steps for reduction of unspecific binding because it used SA-MTP microtiterplates and the LowCross buffer which were specially designed to minimize unspecific binding. Furthermore, the use of purified, highly specific monoclonal capture and detection antibody reagents also contributed to reduce unspecific binding. Samples and standards were diluted with assay buffer to 5% cynomolgus monkey serum and 100 μL added to each well of the coated SA-MTP after washing and incubated for 1 h. After washing, 100 μL of the digoxigenylated anti-cyno IgG at 0.05 μg/mL were added to the cavities of the MTP and incubated for 1 h. After washing, 100 μL of the polyclonal anti-digoxigenin-HRP conjugate at 50 mU/mL were added and incubated for 1 h. The HRP of the antibody-enzyme conjugate catalyzed the colour reaction after addition of the substrate solution ABTS to the MTP. Absorption was measured by an ELISA reader at 405 nm wavelength (reference wavelength: 490 nm). The highest standard concentration had to reach an optical density (OD) value of 1.8–2.2 AU. Absorbance values of each serum sample were determined in triplicates. The obtained data was used for generating the standard calibration curve for calculating the sample concentration.

2.5. Validation of the analytical method

For validation, four independent calibration curve preparations with eleven calibrator points ranging from 200 to 0.20 ng/mL and one blank sample were measured in duplicates on one plate to define a suitable range for accurate measurement. Four independent assays were performed to take into account a potential variability by dilution of the calibrators. The lower limit of quantitation (LLOQ) was determined as the calibration point which fulfilled the criteria that its OD − 1.64 × standard deviation (SD) was higher than the blank OD + 1.64 × standard deviation (SD). The cut-point of the immune complex assay in the format for screening of samples was evaluated by analysis of 25 individual blank serum samples from cynomolgus monkeys of either sex in duplicates in two independent test runs. The cut-point was calculated using a parametric approach and defined as mean value (MV) of duplicates of all blank individual serum samples +1.645 × SD.

One vial of each QC sample was freshly thawed and diluted with assay buffer to 5% serum content to yield final concentrations of 60; 32; 7.5; and 2.5 ng/mL, to determine intra-assay precision and accuracy. Five separate vials of each QC were measured on one plate in duplicates. Results were calculated as percent recovery from target concentration (accuracy) and percent coefficient of variation (CV) (precision). Inter-assay precision and accuracy was calculated by separate analysis of QCs in nine independent assay runs. Matrix effects (variability) in individual serum samples were evaluated by spiking 10 individual neat serum samples with the CS at high concentrations (60 ng/mL) and compared to the respective unspiked (blank) and QC samples. Dilutional lin-

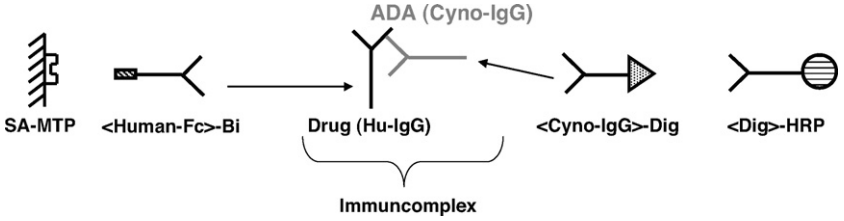

Fig. 1. Schematically illustrated assay principle of the drug–ADA immune complex ELISA.

earity was assessed by using a cynomolgus monkey study sample which was tested positive in the immune complex assay. The sample was initially diluted at the minimum of 1:10 in assay diluent, followed by increasing dilutions of 1:20, 1:30, 1:40, 1:50 and 1:100. A possible hook effect was evaluated by preparing a positive control antibody sample in cynomolgus monkey serum at 320 μg/mL which is 400-fold higher than the highest standard concentration. The 320 μg/mL concentration was serially diluted to several intermediate concentrations which were measured in the assay.

2.6. Evaluation of the drug-spiked immune complex assay variant

Serum samples of cynomolgus monkeys containing ADAs were obtained from a single-dose pharmacokinetic study of anti-IGF-1R. The therapeutic antibody was administered intravenously at a dose of 3 mg/kg and blood samples for preparation of serum were taken repeatedly over 7 weeks after administration. In vivo formed immune complexes of ADAs with anti-IGF-1R were measured in the immune complex ELISA. To measure total ADA levels with the immune complex ELISA described in Section 2.4, the samples were diluted with PBS and spiked and pre-incubated with anti-IGF-1R for 30 min before analysis. To determine the optimal drug concentration for pre-incubation, a pooled sample prepared of samples taken at time points after drug wash-out, was incubated with increasing concentrations (range: 10 ng/mL to 1 mg/mL) of anti-IGF-1R. This experiment was conducted at three different ADA concentrations obtained by dilution of the sample with PBS at ratios of 1:5000 (high titre response); 1:40,000 and 1:60,000 (both low titre responses), respectively. The results of the immune complex ELISA were compared with a state of the art bridging ELISA for measurement of ADAs in the same cynomolgus monkey serum samples [3] in which drug is used for capture and detection of the ADA.

Serum concentrations of anti-IGF-1R were measured in an ELISA format which used the target antigen (recombinant human IGF-1R) as coating and a digoxigenylated anti-human Fc MAb as detection agent. The Dig-labeled antigens were detected by an anti-Dig-HRP antibody. The HRP enzyme reacted with its substrate ABTS and absorbance was measured at 405 nm.

2.7. Application of the ADA ELISA to serum samples of cynomolgus monkeys

In a pharmacokinetic study, three adult male cynomolgus monkeys received a single i.v. dose of MAb 2 at 1 mg/kg. Blood samples were taken from the femoral vein into a test tube at 13 time points over 4 weeks after administration of the antibody and serum was prepared and stored at −80 °C. Samples were analyzed for serum concentration of the antibody as described in Section 2.6. Samples at days 13, 17, 21, and 27 were analyzed for immune complexes as described in Section 2.4. Total ADAs (immune complexed and free) were analyzed after in vitro pre-incubation of samples with 1 μg/mL of the antibody as described in Section 2.6.

3. Results and discussion

3.1. Specificity of binding of anti-cyno IgG in the Biacore assay

The species cross-reactivity of the murine monoclonal antibody against cynomolgus monkey IgG was evaluated by SPR technology with the Biacore instrument and compared with that of a polyclonal antibody against human Fcγ. Table 1 summarizes the results. The murine anti-cyno IgG MAb showed a nanomolar dissociation constant for cynomolgus monkey serum (IgG) and no measurable binding for human, chimpanzee, marmoset and the rodent and non-rodent animal species. In contrast, samples of the rhesus macaque and baboon also bound the anti-cyno IgG MAb and showed even lower dissociation constants, suggesting that the generic immune complex assay can also be used for analysis of serum samples of baboon and rhesus monkey studies. Importantly, the anti-cyno IgG did not bind to human serum, thereby confirming the specificity of the immune complex ELISA for cynomolgus monkeys dosed with human antibodies. A polyclonal anti-human Fcγ antibody was used as a positive control for binding to human IgG.

3.2. Validation of the generic immune complex ELISA

As the assay principle of the generic immune complex ELISA is independent of a target specific antigen and antibody, the calibrators and quality controls were made of generic conjugates consisting of cynomolgus monkey IgG fused to human IgG. Experiments for determination of the lower limit of quantitation (LLOQ) resulted in 0.78 ng/mL which fulfilled the definition of the LLOQ, i.e. that its OD − 1.64 × SD (=0.227) was higher than the blank OD + 1.64 × SD (=0.208) (Table 2). As the measurements were performed in 5% serum after dilution, the corresponding serum LLOQ was 15.6 ng/mL. The screening cut-point of the immune complex assay was calculated to be 41.7 ng/mL, which is within the assay range. Intra-assay and inter-assay precision and accuracy data are summarized in Table 3. Intra-assay and inter-assay precision for high, medium and low QC concentrations showed mean CV values of less than 10%. Intra-assay and inter-assay accuracy for high, medium and low QC concentrations showed mean recovery values of 93.4–107.2%. Matrix effects for the measurement of high QC concentrations (60 ng/mL) in 10 different individual cynomolgus serum samples was low: In neat sera of the 10 individuals, mean recovery was 94.5% with a range of 81.0–107.9%. All tested blank samples were below the limit of quantitation (data not shown). Dilutional linearity was evidenced by recovery of 91–105% of expected concentrations in the measurement range of the assay. There was no hook effect observed. At concentrations higher than the upper limit of quantitation the measurement signals remained constant.

3.3. Analysis of immune complexes and total ADA in study serum samples

Serial serum samples from an individual cynomolgus monkey dosed with the anti-IGF-1R antibody were analyzed for ADA–drug

Table 2
Determination of the lower limit of quantitation (LLOQ) in the generic immune complex ELISA assay for ADA conjugates (complex of cyno IgG with human IgG) in cynomolgus monkey serum (four different calibration runs with duplicate measurements each).

	Calibrators (ng/mL)											
	200	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.00
Mean OD	3.046	2.811	2.118	1.362	0.825	0.512	0.358	0.277	0.234	0.211	0.203	0.198
SD	0.0707	0.0995	0.0498	0.0385	0.0214	0.0167	0.0081	0.0060	0.0046	0.0059	0.0055	0.0058
CV (%)	2	4	2	3	3	3	2	2	2	3	3	3
OD − 1.64 × SD	2.930	2.648	2.036	1.299	0.790	0.484	0.344	0.267	0.227	0.201	0.194	0.208[a]

[a] OD + 1.64 × SD.

Table 3
Determination of intra-assay and inter-assay precision and accuracy in the generic immune complex ELISA for ADA conjugates (cyno IgG–human IgG complex) in cynomolgus monkey serum.

	ADA conjugate (ng/mL)							
	Intra-assay ($n = 5$)				Inter-assay ($n = 9$)			
	High QC	Mid QC	Low QC	LLOQ	High QC	Mid QC	Low QC	LLOQ
Spiked (expected concentration)	60	32	7.5	2.5	60	32	7.5	2.5
Mean of measured concentration	57.78	30.78	8.04	2.63	58.15	29.89	7.70	2.62
SD of measured concentration	2.10	0.70	0.20	0.25	3.30	2.00	0.50	0.29
Precision (% CV)	3.63	2.26	2.53	9.40	5.68	6.69	6.50	11.09
Accuracy (% recovery)	96.3	96.2	107.2	105.2	96.9	93.4	102.7	104.8
Accuracy range (% recovery)	90.8–100.2	93.1–98.8	104–110.7	88–116	90–104.9	86.1–103.4	94.8–111.1	79.6–118.4

complexes. The generic immune complex assay showed an increase of the assay signal at day 14, with a peak of signal intensity at day 21 and subsequent decline to pre-treatment values at day 35 (Fig. 2). This profile suggests that immune complexes in serum samples from cynomolgus monkeys dosed with anti-IGF-1R are detected as long as the antibody is present in serum, but escape detection once serum concentrations of the antibody have fallen below the detection limit. Typically, ADA formation starts between day 10 and 20 and persists for weeks or even months. When the same serum samples were pre-incubated with anti-IGF-1R to allow the in vitro formation of immune complexes, analysis of these spiked samples shows persistence of ADAs up to week 7 post-dose (Fig. 3).

The experiments to determine optimal conditions of the pre-incubation step suggested a concentration of 1 μg/mL of anti-IGF-1R for the tested serum samples (Fig. 4). Fig. 5 illustrates the relationship between the antibody level in serum and the measurement of immune complexes in the two variants of the ELISA. The immune complex ELISA without in vitro drug spiking was able to detect immune complexes formed in vivo as long as the drug was present in serum. In contrast, in vitro drug spiking led additionally to the detection of free ADAs.

The interference of drug for the analysis of ADAs by a state-of-the-art bridging ELISA without sample pre-treatment is illustrated in Fig. 6. The bridging ELISA detects ADAs once the drug level decreased to baseline values whereas the immune complex assay in combination with in vitro drug spiking detects total ADA. The generic immune complex ELISA was used to analyze serum samples from cynomolgus monkeys dosed with MAb 2. One of the three animals did not develop ADAs during the study time course. The other two cynomolgus monkeys developed ADA–drug complexes measurable by the immune complex ELISA as long as the drug was present in serum (Fig. 7). Pre-incubation of the serum samples with drug prior to analysis additionally detected ADAs at later time points when in vivo drug levels dropped to baseline levels.

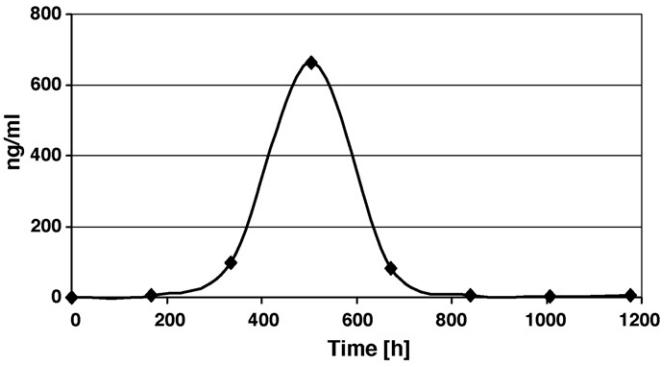

Fig. 2. Time course of in vivo drug–ADA immune complex formation in cynomolgus monkey serum after administration of anti-IGF-1R analyzed by the immune complex ELISA.

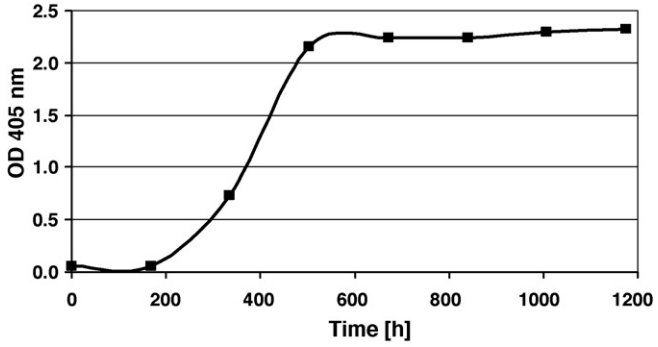

Fig. 3. Time course of in vivo ADA formation in cynomolgus monkey serum after administration of anti-IGF-1R analyzed by the immune complex ELISA after in vitro drug spiking.

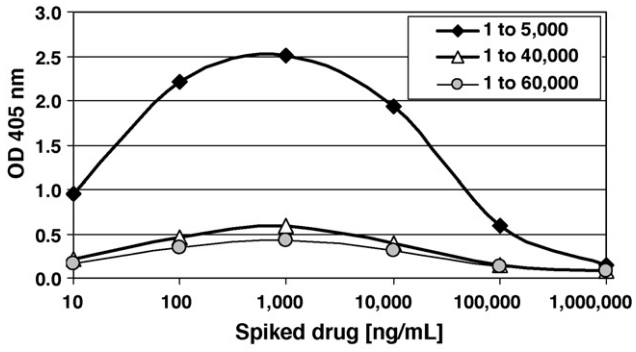

Fig. 4. Determination of the optimal drug concentration for in vitro drug spiking for the immune complex ELISA by dilution of a study serum sample by 1:5000, 1:40,000 and 1:60,000.

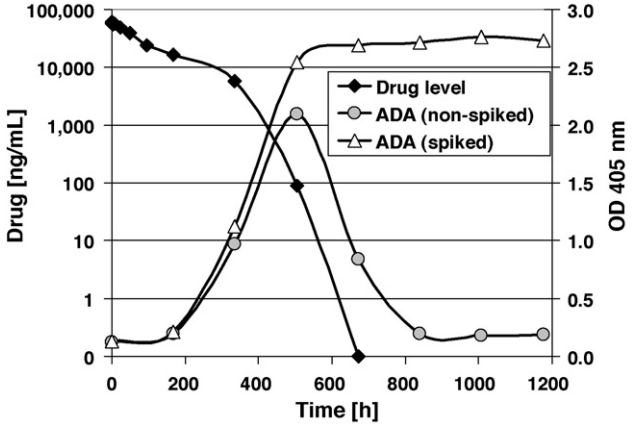

Fig. 5. Relationship between the serum concentration–time profiles of anti-IGF-1R, in vivo immune complexes and in vitro formed immune complexes of samples after a single i.v. dose of 3 mg/kg of anti-IGF-1R in a cynomolgus monkey.

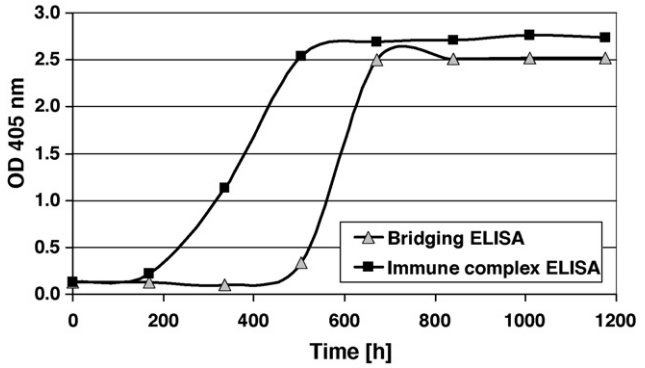

Fig. 6. Comparison of the time course of total ADA levels in one individual cynomolgus monkey after a single i.v. dose of 3 mg/kg analyzed in the immune complex ELISA with in vitro drug spiking and a state-of-the art bridging ELISA (drug used for capture and detection).

The two cynomolgus monkey studies show that the immune complex ELISA described in this publication can be used as a generic assay. It is possible to detect ADA–drug immune complexes in vivo. Pre-incubation of samples with drug allows detection of total ADA levels. The assay might also find up-side use for determination of the specificity of ADAs. A drug-unspecific immune response could be assumed if pre-incubation of the sample with non-related antibodies directed against other targets also lead to a positive signal. On the contrary, a negative signal would indicate a drug-specific immune reaction against the complementarity determining regions (CDR) of the antibody. The generic applicability of the assay is based on the use of human and cynomolgus monkey specific MAbs as capture and detection reagents, respectively. An ADA standard conjugate consisting of cynomolgus monkey IgG fused to human IgG was used as a positive control to demonstrate assay validation parameters.

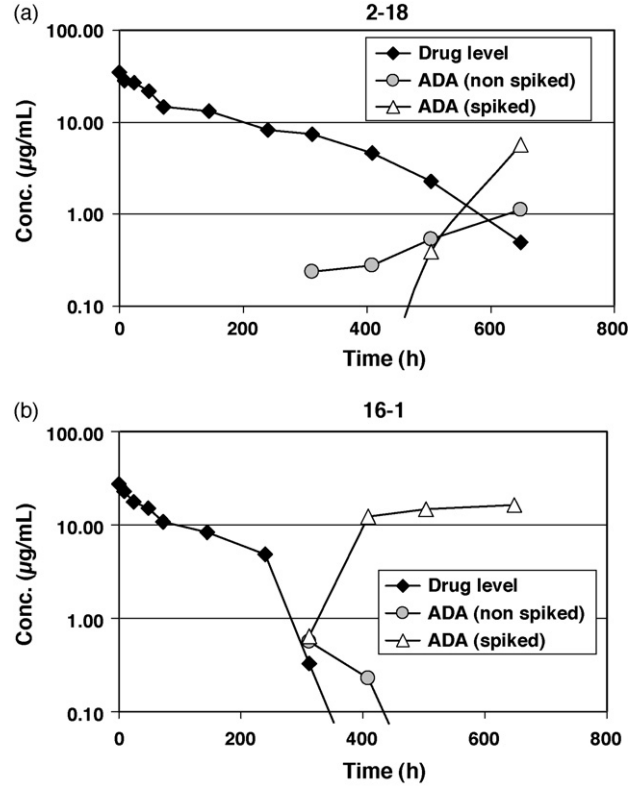

Fig. 7. Relationship between serum concentration-time profiles of MAb 2, in vivo immune complexes and immune complexes after in vitro drug spiking in cynomolgus monkey 2-18 (a) and 16-1 (b) after a single i.v. dose of 1 mg/kg of MAb 2.

The advantage of using a generic assay allows availability in early drug discovery and development of therapeutic antibodies. By definition it is developed for the cynomolgus monkey which is the most common species for pilot pharmacokinetic and safety studies of therapeutic antibodies. The basic version of the immune complex assay may help to explain unexpectedly occurring short half-life values or safety events of a drug candidate by specifically demonstrating the presence of immune complexes.

4. Conclusions

Current state-of-the-art bridging ELISA technologies for detection of anti-drug antibodies (ADAs) against therapeutic monoclonal antibodies are prone to false-negative results due to interference by circulating drug. Methods to remove the drug from the sample or sample pre-treatment techniques such as acid dissociation of the immune complexes are laborious and bear disadvantages such as denaturation of ADAs. The immune complex ELISA characterized in the present work provides a simple solution for serum samples from cynomolgus monkeys dosed with human therapeutic antibodies. It can be used generically due to its independency of the specific human antibody and its target. This is achieved by the use of (1) a murine anti-human Fc MAb as capture reagent; (2) a murine anti-cynomolgus monkey IgG MAb as detection reagent; and (3) an ADA standard conjugate as positive control consisting of cynomolgus monkey IgG fused to human IgG to mimic an immune complex. In its basic version, the generic ADA ELISA specifically detects ADA–drug immune complexes formed in vivo. In vitro pre-incubation of the serum samples with drug allows detection of total ADAs. The generic and versatile nature of this ADA assays favors its use during early pilot pharmacokinetic and safety studies in cynomolgus monkeys during candidate selection of therapeutic antibodies where it helps to explain unexpected clearance profiles, loss of efficacy or safety events caused by immune complexes.

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