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Detection of low-affinity anti-drug antibodies and improved drug tolerance in immunogenicity testing by Octet® biolayer interferometry

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ABSTRACT

We assessed the utility of the FortéBio Octet® system for detection of anti-drug antibodies (ADAs) against an investigational therapeutic human IgG1 monoclonal antibody (mAb), CNTO X. To understand the relative merits of this technology, key performance requirements were compared with two popularly accepted ADA detection methods, a step-wise bridging ELISA and a Meso Scale Discovery (MSD) homogeneous (single step binding) bridging ECLIA. When used to detect 13 monoclonal ADAs of varying affinities and one polyclonal ADA, all three methods demonstrated their greatest apparent sensitivity to the polyclonal sample (1, 6, and 130 ng/mL, respectively for ECLIA, ELISA, and Octet). Sensitivity to monoclonal ADAs tended to vary in accordance with their affinities, however, the sensitivity of the Octet method varied much less between ADAs. As a result, the above ranking became reversed such that Octet was the most and ELISA least sensitive for detection of low-affinity ADAs. With regard to drug tolerance, the presence of CNTO X could lead to false-negative assay results, although each method was affected to a different degree, with the Octet method tolerating up to 10 times more drug than the ECLIA method, which in turn tolerated up to 10 times more than the ELISA. Finally, the ECLIA and Octet methods were applied to the bioanalysis of cynomolgus monkey sera from a pre-clinical multiple dose study of CNTO X. Octet indicated 3 positive animals developed ADA as early as day 15 of the dosing phase while drug was present at nearly 1 mg/mL. ECLIA detected only one of these, and only in a day 57 recovery sample after drug had cleared from circulation. We conclude that the Octet is a promising platform for detection of lower affinity ADAs and is particularly suitable for ADA detection when drug persists at levels that negatively impact bridging immunoassays.

1. Introduction

The administration of therapeutic biologic drugs can induce anti-drug antibody immune responses in study subjects. These immune responses can produce a range of effects from benign and asymptomatic to altered pharmacokinetics and/or pharmacodynamics and adverse clinical sequelae [1,2]. It is therefore important to use sensitive and reliable bioanalytical methods to monitor the ADA status of study subjects during investigational treatment with biologic drug products [3,4].

Numerous assay technologies have been used for the detection of ADAs, each of which is associated with relative merits and weaknesses [5]; however, electrochemiluminescent immunoassays (ECLIA) have become very popular, and a recent survey indicates that ELISA remains the most widely used technology for this purpose, even though 90% of laboratories are also considering other technologies for potential advantages in the detection of immunogenicity [6]. To date, all ADA assay technologies are prone to false-negative results due to interference caused by drug that remains in circulation after dosing [7–9]. To make assays more "drug-tolerant" it is common practice to pre-treat samples with acid to disassociate immune complexes; however, this may inactivate some ADAs without completely eliminating drug interference. Although many techniques are available to improve the sensitivity and drug tolerance of ELISA assays [10–15], it is often easier to reach the same goals using ECLIA [16,17]. ELISA- and ECLIA-based ADA assays are often designed as bridging methods in which ADA is captured by one molecule of drug then detected when concurrently bound to a second molecule of drug. Biolayer/biomolecular interaction (BLI) paired with surface plasmon resonance (SPR) detects binding events via changes in light reflectance as proteins accumulate on a sensor, and thereby circumvent the need for a detection label, thus eliminating one binding event required of a bridging format as well as eliminating the need to conjugate the drug to a label. They are ideal for use with simple buffer systems, but are less suitable for complex matrices, such as serum, which may present clots and varied background effects between individuals. Biacore® (GE

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Healthcare, Piscataway, NJ) and ProteOn® (Bio-Rad Laboratories, Hercules, CA) are SPR-based biosensor technologies successfully used to detect clinically relevant ADAs, particularly low-affinity antibodies [18,19], however, they have not been widely adopted due to the modest throughput compared to microtiter plate methods, specialized equipment, the high price of the equipment and consumables, the extensive training required to develop expertise, and the complexity of data interpretation. The Octet® System (FortéBio, Inc., Menlo Park, CA) is another biosensor technology that employs a related BLI system (for a comprehensive review of the principles of BLI, the reader is referred to a prior publication [20] or the manufacturer's website http://www.fortebio.com). With this system, analysis occurs on disposable fiber tips in a "dip and read" manner [21–23] using 96- or 384-well microtiter plates. For determination of binding kinetics there is evidence that the Octet generates kinetic binding constants comparable to other biosensor instruments [22,24,25]. Similar comparability assessments for non-kinetic uses have not been published; however, we predicted that Octet would maintain key benefits of related biosensor technologies, while permitting simplified data analysis without microfluidics that may become clogged by serum.

We compared Octet with two of the most widely employed methods, a step-wise bridging ELISA and a MSD homogeneous bridging ECLIA, for the detection of ADAs against CNTO X, an investigational therapeutic human IgG1 monoclonal antibody (mAb) that neutralizes a soluble inflammatory human cytokine. After optimizing each method, our comparisons focused on assay sensitivity and drug tolerance, characterizing performance against sera spiked with purified ADAs and/or CNTO X. Then the two most promising methods (ECLIA and Octet) were evaluated by analyzing cynomolgus monkey sera from a pre-clinical multiple dose study of CNTO X. Although the manufacturer suggests using the Octet to detect ADA responses, to the best of our knowledge this is the first demonstration of an Octet ADA assay being applied to a pre-clinical study.

2. Materials and methods

2.1. Pre-clinical cynomolgus monkey study

A non-GLP toxicity study of CNTO X was previously conducted by our company to evaluate the tolerability of the biologic and to establish a toxicokinetic profile of this mAb when administered subcutaneously (SC, 20 or 100 mg/kg) or intravenously (IV, 100 mg/kg) to cynomolgus monkeys weekly for 4 weeks. Twelve (3 per CNTO X treatment group and 3 treated with control vehicle) female cynomolgus monkeys (Mauritius-origin, Covance Research Products, Alice, TX) were used in this study. The animals were dosed on days 1, 8, 15, and 22, and measurements of CNTO X serum concentrations and detection of ADAs were performed on samples collected at several time points during the dosing phase of the study as well as a sample taken on day 57 (35 days following the last injection). CNTO X concentration determinations were performed using an ECLIA (Meso Scale Discovery, Inc.) method that employed biotin- and ruthenium-conjugated anti-idiotypic antibodies to capture and detect CNTO X. A standard curve was employed (lower limit of quantification, 0.02 μg/mL) to quantify CNTO X in the samples. ADA detection was performed using both the bridging ECLIA (Section 2.6) and the Octet method (Section 2.7) using the Octet-RED device. Individual subjects' serum concentration–time profiles were generated for comparison against ADA results.

2.2. Generation of anti-CNTO X antibodies

In accordance with applicable regulations concerning the ethical use of laboratory animals, ten Balb/c mice (12–14 weeks old) were immunized with CNTO X, a human anti-cytokine monoclonal antibody. Lymphocytes were isolated from the immunized mice and were subsequently fused to FO myeloma cells. Solid phase ELISA was used to screen hybridoma supernatants for CNTO X binding antibodies. The IgG fraction of the hybridoma culture supernatant was purified by protein G affinity chromatography. Thirteen mAbs reactive to the variable region of CNTO X were identified and further characterized.

To generate a polyclonal antibody reagent, two cynomolgus monkeys were hyperimmunized by an initial administration of a 50% emulsion of 1 mg CNTO X per kg body weight in Hunter's TiterMax (CytRx Corp., Los Angeles, CA) followed every third week by booster injections of a 50% mixture of Imject Alum (Pierce, Rockford, IL) and 0.1 mg CNTO X per kg body weight. Blood was collected from the animals after multiple rounds of boosting. Polyclonal antiserum from the animal with the greater anti-CNTO X antibody titer was purified by protein G followed by CNTO X affinity chromatography.

2.3. CNTO X conjugation

For the ELISA and Octet applications, CNTO X was labeled with biotin using EZ-Link Sulfo-NHS-LC biotinylation kits (Pierce, Rockford, IL) according to the manufacturer's instructions. The biotin:protein ratio for ELISA was 9:1 and for Octet was 3:1.

For the ECLIA application, CNTO X was conjugated with a ruthenium bipyridine-sulfo-NHS (BvTAG) according to the manufacturer's (Meso Scale Discovery, Gaithersburg, MD) instructions. The 8:1 conjugate:protein labeling ratio was optimized to provide the greatest signal-to-noise ratio between an ADA-containing sample and a negative matrix sample in the ECLIA.

2.4. Antibody affinity determination

The binding affinities of 13 ADAs (mouse monoclonal anti-CNTO X antibodies) were determined using a Biacore® 3000 optical biosensor equipped with a research-grade CM5 sensor chip (GE Healthcare, Piscataway, NJ). Amine coupling reagents, N-ethyl-N′-dimethylamino-propylcarbodiimide (EDC), N-hydroxy-succinimide (NHS) and sodium ethanolamine–HCl, pH 8.5, were obtained from GE Healthcare. Standard coupling protocols were used to tether a goat anti-mouse Fc_γ polyclonal antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) onto the biosensor surfaces [20,26]. The experiments were performed at 25 °C in PBS containing 3 mM EDTA and 0.005% P20. Anti-drug antibodies were diluted to approximately 1 μg/mL and captured on each of three immobilized anti-mouse Fc surface. Between 20 and 50 response units (RU) of each ADA were captured by the goat anti-mouse Fc_γ-specific antibody. After ADA capture, randomly ordered concentrations of CNTO X, spanning 4–500 nM, were injected in running buffer at 60 μL/min for 3 min. Dissociation was monitored for 15 min and each measurement was repeated in triplicate. Between measurements, the biosensor surfaces were regenerated with two 6-s pulses of 50 mM phosphoric acid. To increase data confidence, multiple buffer cycles were included in each of the triplicate assays [27]. Each data set was fitted globally to a 1:1 interaction model (BIA evaluation 3.1, GE Healthcare, Piscataway, NJ) to determine the kinetic parameters k_a and k_d. Apparent affinities were then calculated as a ratio (k_d/k_a) of these rate constants.

2.5. Bridging ADA ELISA

This method was developed and optimized using the hyperimmunized monkey polyclonal ADA obtained previously. Microtiter plate wells were coated with 1 μg/mL CNTO X in 0.1 M carbonate

coating buffer (pH 9.6) overnight at 4 °C. The plates were washed to remove unbound protein and the coated plate wells were then blocked with casein buffer (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). 50 μL of test or control sample (in triplicate) was added to each corresponding well on the pre-coated plate. Biotinylated CNTO X at 2 μg/mL was added to each well and the plates were incubated at 37 °C for 1 h. A 1:55,000 dilution of streptavidin-linked horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added to each well and the plates were incubated at 37 °C for an additional hour. The plates were washed and 250 μL of tetramethylbenzidine (TMB) (Sigma–Aldrich, St. Louis, MO) was added to each well, followed by incubation at 37 °C for 30 min to allow color development. The colorimetric reaction was stopped by adding 50 μL of 4 N sulfuric acid to each plate well. The optical density (OD) of the solution in each plate well was measured at 450/650 nm on a microtiter plate reader (Molecular Devices, Inc., Sunnyvale, CA). The method is referred to as a step-wise or heterogeneous bridging ELISA because binding between ADA and two drug molecules occurs over multiple steps.

2.6. Bridging ADA ECLIA

An MSD-based ECLIA (Meso Scale Discovery, Inc., Gaithersbrug, MD) method was developed, optimized, and validated using hyperimmunized monkey polyclonal ADA obtained previously. In brief, a master mixture of 1 μg/mL biotin-labeled CNTO X and 2 μg/mL BvTag-linked CNTO X was prepared in blocker casein (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The ADA and control samples were prepared in 20% pooled NMS then combined with the master mixture at a 1:1 ratio. The samples were incubated on a shaker for 90 min at RT. Streptavidin-coated plate wells (Meso Scale Discovery, Inc., Gaithersbrug, MD) were blocked by adding 200 μL of blocker casein per well, followed by incubation on a shaker for 90 min at RT. The blocking solution was then discarded and 50 μL of the appropriate sample mixture was added in triplicate to each corresponding well on the plate. After 60 min incubation on a shaker at RT, the plates were washed and 150 μL of reaction buffer (Meso Scale Discovery) was added to each well. The ECL signal of the solution in each well was measured using a Sector® 6000 imager (Meso Scale Discovery). All test and control samples were analyzed in triplicate. The method is referred to as a homogeneous bridging ECLIA because binding between ADA and two drug molecules occurs in a single step.

2.7. Octet ADA method

Samples or buffer were dispensed into 96-well microtiter plates (Millipore, Billerica, MA) at a volume of 200 μL per well. Operating temperature was maintained at 30 °C. Streptavidin-coated biosensor tips (FortéBio, Inc., Menlo Park, CA) were pre-wetted with buffer (FortéBio) in order to establish a baseline prior to drug immobilization. Various concentrations of Biotin-CNTO X were then immobilized on the biosensor tips while agitating for 15 min at 1000 rpm. A serum matrix baseline was then established by dipping the drug-immobilized tips in 10% NMS for 13 min. Because the program was run in kinetics mode, dissociation of the anti-CNTO X antibodies occurred for 15 min in buffer agitated at 1000 rpm; however, the dissociation data were not part of the ADA detection process. Unless specified, experiments were performed on the Octet-QK device. Data were generated automatically by the Octet® User Software (version 3.1) and was subsequently analyzed from the text files using Excel 2000. The binding profile of each sample was summarized as a "nm shift" (the wavelength/spectral shift in nanometers), which represented the difference between the start and end of the 15 min sample association step. In this method, direct detection of ADA binding to drug circumvents the need for a bridging format.

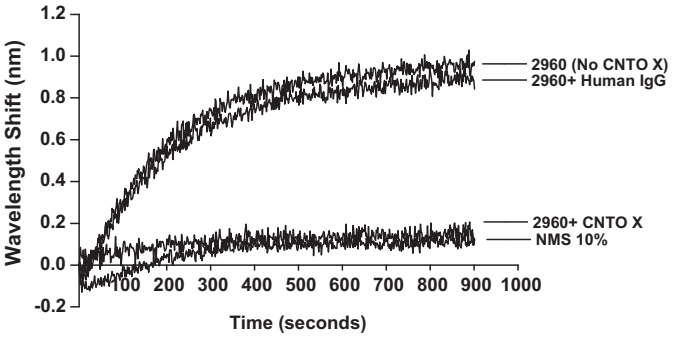

Fig. 1. Octet assay specificity. mAb 2960 (a monoclonal ADA known to bind specifically to the hypervariable region of CNTO X) was diluted to 500 ng/mL in 10% normal monkey serum (NMS), with and without 100 μg/mL of CNTO X or a monoclonal human IgG_1 isotype control and tested on the Octet-QK. Data are representative of two independent experiments.

2.8. Cut point determinations for the ELISA, ECLIA and Octet ADA assays

ADA assays discriminate between ADA negative and positive samples and calculate assay sensitivity relative to a signal cut point [7,9]. The cut point for screening samples using each of the three methods was calculated by adding the mean signal obtained from naive serum and 1.645 times the standard deviation. Consequently, initial screening would eliminate 95% of negative sera. The remaining potentially positive sera and 5% of negative sera would undergo confirmation testing that would conclusively identify the true ADA positive samples based upon specific binding to CNTO X. For screening samples using the Octet-QK device, a cut point equating to a 0.263 nm shift was calculated using data from 21 drug-naive monkey sera tested three times on separate days by one analyst (data not shown). The determination of a screening assay cut point for the ELISA was based on results from 30 drug-naive monkey sera tested by one analyst on two separate days (data not shown) and for the ECLIA was based on results from 60 drug-naive monkey sera tested by three analysts on two separate days (data not shown). The resulting cut points were 0.091 OD units for the ELISA and 143 ECL units for the ECLIA. Likewise, for the detection of ADAs using the Octet-RED device in the pre-clinical cynomolgus monkey study, a study-specific screening assay cut point of equating to a 0.484 nm shift was calculated using data (tested 3 times on separate days by one analyst) from baseline samples collected from 12 drug-naive monkeys prior to any exposure to CNTO X. To determine the specificity of the Octet method using competitive inhibition with excess CNTO X, an inhibition threshold of 56.8% was calculated and represented the 99.9th percentile of the percent inhibition values obtained from ADA negative sera.

3. Results

3.1. Octet assay specificity

It was important to verify that the Octet assay detected CNTO X-specific ADA despite the presence of high concentrations of unrelated serum proteins. To test specificity, the positive control ADA mAb 2960 (a monoclonal antibody known to bind specifically to the hypervariable region of CNTO X) was diluted to 500 ng/mL in 10% normal monkey serum (NMS), with and without 100 μg/mL of CNTO X or a monoclonal human IgG_1 isotype control. Fig. 1 shows a robust assay wavelength shift due to the presence of mAb 2960

Table 1
Sensitivity of ADA detection. Serial dilutions of anti-CNTO X antibody samples were prepared in 10% drug-naive pooled normal monkey serum and tested by the three methods. The minimum detectable concentrations of the ADA are shown for neat serum, after accounting for the dilution factor. Sensitivity numbers are the mean of two (for EIA and ECLIA) or three (Octet) experiments.

Anti-CNTO X Ab	K_D (nM)	Sensitivity (ng/mL, calculated for neat serum)		
		ELISA	ECLIA	Octet-QK
Cyno Poly IgG	NA	6	1	130
mAb 7473	5.3	195	49	1000
mAb 8110	6.0	24	12	500
mAb 2825	6.2	781	391	1000
mAb 5583	6.5	195	391	1000
mAb 539	6.6	781	781	1000
mAb 8584	9.2	391	49	250
mAb 5984	11.6	391	391	1000
mAb 7942	12.2	6250	1563	5000
mAb 9698	14.0	3125	1563	1000
mAb 1773	38.9	6250	781	500
mAb 7679	50.0	6250	3125	2000
mAb 2960	65.8	12,500	6250	2000
mAb 5968	80.7	781	1563	2000

compared to the background signal of 10% NMS alone. The addition of 100 μg/mL of CNTO X reduced the wavelength shift of mAb 2960 to the level of the 10% NMS background signal (i.e., competitive inhibition of the ADA). The addition of a human IgG1 isotype control did not alter the wavelength shift of mAb 2960 alone. These findings demonstrated that the mAb 2960 signal was reduced by the specific binding of this ADA with CNTO X. Similar competitive inhibition experiments had been done previously to demonstrate the specificity of ELISA and ECLIA (data not shown).

3.2. Comparison of assay sensitivity

The sensitivities of the ELISA, ECLIA, and Octet methods were determined by assessing the detection of monoclonal and polyclonal ADAs. Serial dilutions of monkey polyclonal ADA were prepared in 10% drug-naive pooled monkey serum. Assay sensitivity was defined as the lowest concentration of ADA that produced a signal greater than the assay cut point (using the method-specific cut points described in Section 2.8) and calculated for neat serum by multiplying by 10 (the dilution factor used for sample preparation). As shown in Table 1, the ECLIA and ELISA methods, practically equivalent in sensitivity (1 and 6 ng/mL, respectively), were superior to the Octet-QK (130 ng/mL) for the detection of monkey polyclonal ADA. Additionally, a panel of 13 monoclonal ADAs spanning a range of affinities for binding CNTO X was similarly prepared in 10% drug-naive pooled monkey serum and tested by each method. The three methods were variably sensitive in detecting these antibodies (Table 1). While not directly correlated, there was a noticeable trend toward an inverse relationship between antibody binding affinity and the minimum detectable concentration. In Table 2, results from the detection of the 13 monoclonal ADAs are shown in the context of the regulatory targets for ADA sensitivity (in undiluted serum) of 250–500 ng/mL for ADA assays supporting clinical studies and 500–1000 ng/mL for pre-clinical studies [4,7]. Detection of low-affinity antibodies presented a special challenge, evidenced by the fact that most low-affinity ADAs at a concentration of 1000 ng/mL were not detected by any method; however, there are currently no specific recommendations for detection of these antibodies. Therefore, we decided to evaluate low-affinity ADA detection at an additional target concentration of 2000 ng/mL.

Monoclonal ADAs were categorized into a group of nine higher affinity (5.3–14.0 nM K_D range, Table 2A) and another group of four lower affinity (38.9–80.7 nM K_D range, Table 2B) ADAs. This grouping was empirically based on the notable gap in binding affinities between the 9th and 10th monoclonal antibodies in our possession (listed in Table 1). In our experience, monoclonal ADAs can reach much higher affinities, with 10–100 pM not uncommon in our laboratory; however, it is not clear whether such highly matured and generally more easily detected mAbs represent the affinities of typical ADA (i.e., in human subjects treated with biologic drugs). At the middle of the sensitivity targets (i.e., ≤500 ng/mL), the ELISA detected 5 of 9 and ECLIA detected 6 of 9 of high-affinity monoclonal antibodies and were clearly superior to the Octet method, which detected only 2 of the 9 ADAs (Table 2A). All three methods detected more high-affinity antibodies when ADA concentrations were increased to 1000 ng/mL; however, the level of improvement

Table 2
Comparison of ADA detection by ELISA, ECLIA, and Octet-QK in context of the current regulatory expectations of assay sensitivity. The detection in each method of nine high-affinity (K_D 5.3–14.0 nM, A) and four low-affinity (K_D 38.9–80.7 nM, B) CNTO X-specific monoclonal ADA was enumerated per the indicated range of sensitivity. The denominator represents the total number of antibodies tested whereas the numerator indicates the number that were detectable at that concentration.

Method	Sensitivity target		
	≤250 (ng/mL)	≤500 (ng/mL)	≤1000 (ng/mL)
A			
ELISA	3/9	5/9	7/9
ECLIA	3/9	6/9	7/9
Octet-QK	1/9	2/9	8/9

Method	Sensitivity target			
	≤250 (ng/mL)	≤500 (ng/mL)	≤1000 (ng/mL)	≤2000 (ng/mL)
B				
ELISA	0/4	0/4	1/4	1/4
ECLIA	0/4	0/4	1/4	2/4
Octet-QK	0/4	1/4	1/4	4/4

Table 3
Comparison of ADA detection in the presence of interfering drug. The detection of the indicated concentrations of two high-affinity (6.5 and 9.2 nM) and two low-affinity (50.0 and 65.8 nM) CNTO X-specific monoclonal ADA was tested in the presence of varying amounts of CNTO X. CNTO X tolerance in each assay is shown in terms of concentration as well as molar ratio to the amount of ADA applied in the experiment.

ADA#	K_D (nM)	ADA concentration (μg/mL)	Concentration of CNTO X tolerated					
			ELISA		ECLIA		Octet-QK	
			μg/mL	Molar excess[a]	μg/mL	Molar excess[a]	μg/mL	Molar excess[a]
mAb 5583	6.5	0.1	1	10	1	10	10	100
mAb 8584	9.2	0.1	1	10	10	100	10	100
mAb 7679	50.0	0.5	0.1	0.2	1	2	10	20
mAb 2960	65.8	1.25	0.01	0.008	10	8	100	80

[a] Molar excess relative to ADA concentration.

was not consistent between methods. Compared to the 500 ng/mL level, detection improved by a mere 17% for ECLIA (1 additional antibody detected) and 39% for ELISA (2 additional antibodies detected), whereas detectability improved 300% (6 additional antibodies detected) using the Octet method. Although the sensitivity of all methods was relatively inferior for the detection of low-affinity antibodies, only the Octet method detected such antibodies (1 of 4) at the 500 ng/mL limit. All three methods detected 1 out of 4 low-affinity antibodies at the 1000 ng/mL limit. At the highest sensitivity target of ≤2000 ng/mL, all of the low-affinity mAbs were detected by Octet, whereas ECLIA and ELISA detected just 2 out of 4 and 1 out of 4, respectively. These observations indicated that within the recommended sensitivity range [4,7] for clinical ADA methods (i.e., ≤500 ng/mL), the ELISA and ECLIA offered better high-affinity ADA detection capability compared to the Octet; however, within the less stringent sensitivity range (≤1000 ng/mL) recommended for pre-clinical ADA methods [7], the Octet was superior. For the detection of low-affinity ADAs, the Octet offered a significant advantage.

3.3. Comparison of drug tolerance

The main source of interference in an ADA assay is usually the drug itself and this interference is a particularly severe problem for bridging assay formats, leading to lower assay sensitivity in the presence of drug. Thus, it is a recommended practice to determine a "drug tolerance limit" that can be described as the maximum concentration of drug that does not inhibit detection of a low level of an ADA positive control in the assay [9]. Therefore, having demonstrated that there were distinct differences in ADA detection sensitivity between the three methods, the next objective was to examine whether such differences also existed relative to drug tolerance. To test this concept, two high-affinity (6.5 and 9.2 nM) and two low-affinity (50.0 and 65.8 nM) monoclonal ADAs were incubated at RT for 30 min in 10% serum containing serial dilutions of CNTO X. The samples were then tested using the ELISA, ECLIA, and Octet methods. As an additional assessment of drug tolerance, the molar excess of drug tolerated by each method was also determined (Table 3). That the antibodies categorized as high- versus low-affinity in our experiments had less than a log-fold difference in binding affinities could have been a constraint in demonstrating drug tolerance disparities between the methods. Nonetheless, the results adequately showed that the drug tolerance of the three methods was in the order: Octet > ECLIA > ELISA.

3.4. Detection of ADA in samples from a pre-clinical study

Having experimentally established the suitability of the Octet for ADA detection in the presence of drug, the ability of this method to detect ADAs in test samples from a pre-clinical non-GLP toxicity study was compared to that of the ECLIA. Cynomolgus monkeys were administered 4 weekly injections of vehicle control ($N = 3$, not shown) or different dose levels of CNTO X ($N = 9$). ADA assessments were performed on samples collected at six time points spanning the study duration. Serum CNTO X concentrations were analyzed separately and individual subjects' serum concentration–time profiles were generated for comparison with ADA results. Samples from 6 of 9 CNTO X-treated monkeys had no detectable ADA by Octet-RED or ECLIA (not shown). The remaining three CNTO X-treated monkeys had ADA positive samples detectable by the Octet screening assay and one of those animals had detectable ADA by ECLIA (Table 4). In the animal (Monkey #7) identified as ADA positive by both methods, the ECLIA detected ADA in the end-of-study sample (day 57) when CNTO X was no longer detectable in the serum, whereas the Octet identified ADA much earlier (day 15) in the study, soon after administration of the 2nd CNTO X injection and in the presence of a high serum level (537.09 μg/mL) of CNTO X. Similarly, the Octet identified early ADA at day 15 in Monkey #10, and day 22 in Monkey #11, also in the presence of very high levels (951.89 and 731.33 μg/mL, respectively) of CNTO X. The serum concentration–time profiles (not shown) of Monkey #7 and Monkey #11, but not Monkey #10, showed enhanced drug clearance that was consistent with treatment-induced formation of ADA. The profile in monkey #10 was similar to that of the ADA negative CNTO X-treated monkeys possibly because the relatively lower level of ADA in this animal may have been insufficient to create a noticeable impact on drug clearance.

In order to confirm that the putative positive samples detected by Octet-RED were CNTO X-specific ADAs, competitive inhibition tests were performed by exogenously adding excess CNTO X or an isotype matched human IgG1 antibody control. ADA negative samples from CNTO X-treated and vehicle-treated monkeys were also tested similarly as additional controls. As shown in Table 5, all the samples identified as putatively positive in the screening assay were inhibited to an extent greater than the specificity assay threshold level of 56.8% (see Section 2.8) by CNTO X, but not by the isotype matched control. Furthermore, the additional ADA negative control samples were not inhibited beyond the specificity assay threshold by either CNTO X or the isotype matched control. These results unequivocally confirmed that the positive samples identified by the Octet-RED contained CNTO X-specific ADAs.

4. Discussion

Administration of biopharmaceutical proteins, such as CNTO X, may induce an ADA immune response, potentially causing a reduction in drug efficacy and/or eliciting other adverse effects. It is well known that the initial immune response is typically comprised of low-affinity, low-concentration IgM. Although rarely observed, low-affinity ADAs with drug-neutralizing activity were reported in a clinical study of panitumumab which found ELISA to be more sensitive for the detection of high-affinity mAbs, and the (Biacore) SPR method better able to detect low-affinity ADAs [8]. Therefore, development of ADA detection methods that measure low-affinity

Table 4
Comparison of ECLIA and Octet-RED screening assays for detection of ADA in a pre-clinical study of repeated dose CNTO X. Cynomolgus monkeys were administered CNTO X on days 1, 8, 15, and 22. Blood was collected prior to dosing and on days 4 and 56. Data were normalized to the assay cut point (0.484 nm for Octet and 143 units for ECLIA) to enable comparative interpretation; a value <1 represents a negative result and a value ≥1 represents a potentially ADA positive result that qualifies the sample for confirmation of reactivity in the subsequent competitive inhibition method. Confirmed results (from the experiment shown in Table 5) are indicated within the boxes.

Monkey#	Dose group	Study day	CNTO X concentration (μg/mL)	ADA detection by ECLIA		ADA detection by Octet-RED	
				Value[a]	Result	Value[a]	Result
#7	100 mg/kg, SC	1[b]	<0.02[c]	0.83	Neg	0.70	Neg
		4	799.03	0.61	Neg	0.73	Neg
		8[b]	552.33	0.57	Neg	0.51	Neg
		15[b]	537.09	0.59	Neg	1.80	Pos
		22[b]	257.92	0.50	Neg	2.70	Pos
		57	<0.02[c]	1.70	Pos	5.90	Pos
#10	100 mg/kg, IV	1[b]	<0.02[c]	0.28	Neg	0.46	Neg
		4	1083.73	0.59	Neg	0.38	Neg
		8[b]	738.62	0.59	Neg	0.44	Neg
		15[b]	951.89	0.58	Neg	1.60	Pos
		22[b]	941.08	0.40	Neg	1.90	Pos
		57	82.5	0.30	Neg	1.80	Pos
#11	100 mg/kg, IV	1[b]	<0.02[c]	0.92	Neg	0.38	Neg
		4	965.86	0.62	Neg	0.36	Neg
		8[b]	547.90	0.60	Neg	0.31	Neg
		15[b]	615.99	0.60	Neg	0.60	Neg
		22[b]	731.33	0.50	Neg	1.40	Pos
		57	8.02	0.50	Neg	2.40	Pos

[a] Normalized value, determined by the ratio of an assay result to the respective assay cut point.
[b] Animals were dosed on the indicated days, after a sample was taken for ADA assessment.
[c] Lower limit of quantification of the serum CNTO X concentration assay.

ADAs during clinical studies may provide relevant insight into the potential consequences of immunogenicity. Although ELISA and ECLIA are commonly used to assess immunogenicity [6,7,16] these assays often provide limited detection of low-affinity ADAs and detection is further complicated by the presence of circulating drug. Furthermore, assay comparisons may be misleading because the apparent assay sensitivity and drug tolerance are highly dependent upon the ADAs selected, such that most, but not all assays show high ADA concentrations produce better apparent drug tolerance and high-affinity ADAs produce better apparent sensitivity values [9]. We have tried to avoid such flaws.

This study was conducted in order to evaluate the potential of the Octet to detect ADAs relative to the most commonly used ELISA and ECLIA platforms and to subsequently measure ADAs in a pre-clinical toxicokinetic and tolerability study of CNTO X mAb. Under idealized conditions (using high-affinity ADAs absent contaminating drug), the Octet method was less sensitive for detecting ADAs than ELISA and ECLIA and performed only marginally within the acceptable range for nonclinical ADA detection [4,7]; however, this particular experiment employed the Octet-QK device, which we were later able to replace with the Octet-RED device that is intended to enhance signal-to-noise ratio and actually enhanced sensitivity to our polyclonal ADA 10-fold in a separate experiment (not shown).

We used 13 monoclonal ADAs to assess the impact of ADA affinity on assay sensitivity across three platforms. The ELISA and ECLIA were less sensitive than the Octet-QK for the detection of low-affinity ADAs. It is interesting that the more sensitive ECLIA and ELISA assays (based on better detection of the polyclonal ADA and most high-affinity monoclonal ADAs) failed to detect lower affinity monoclonal ADAs that were detected by the otherwise less sensitive Octet assay, albeit at our proposed 2000 ng/mL sensitivity target, which exceeds current recommendations [7]. While data from a single evaluation of one assay per technology cannot universally represent the detection capabilities of these three platforms, it raises the question of whether the contemporary industry practice and regulatory expectation of assessing sensitivity using polyclonal ADA derived from hyperimmunized animals or high-affinity mAb

Table 5
Confirmation of ADA in CNTO X-treated Cynomolgus monkey samples identified as putative positive by Octet-RED. CNTO X-specific reactivity was assessed by competitive inhibition using excess CNTO X added exogenously or an isotype matched human IgG1 antibody control. ADA negative samples from CNTO X-treated and vehicle-treated monkeys were also tested similarly as additional controls. Results are the mean of two (day 15) or three (days 22 and 57) experiments. Values exceeding the previously established specificity cut point (56.8%) confirm positive specific binding.

Monkey#	Dose group	Study day	% Inhibition of ADA signal (mean ± SD) by	
			CNTO X	Isotype matched human IgG
#1	Vehicle	22	4.5 ± 5.7	3.8 ± 4.2
#12	CNTO X 100 mg/kg, IV	57	28.1 ± 32.5	10.7 ± 26.3
#7	CNTO X 100 mg/kg, SC	15	65.4 ± 2.3	ND[a]
		22	62.9 ± 3.3	3.6 ± 2.1
		57	80.9 ± 2.2	12.8 ± 0.9
#10	CNTO X 100 mg/kg, IV	15	67.7 ± 0.2	ND[a]
		22	76.6 ± 2.2	6 ± 3.1
		57	86.1 ± 1.9	2.4 ± 0.9
#11	CNTO X 100 mg/kg, IV	22	79.6 ± 2.5	7.6 ± 4.4
		57	96.2 ± 1.9	1.9 ± 5.5

[a] Not done.

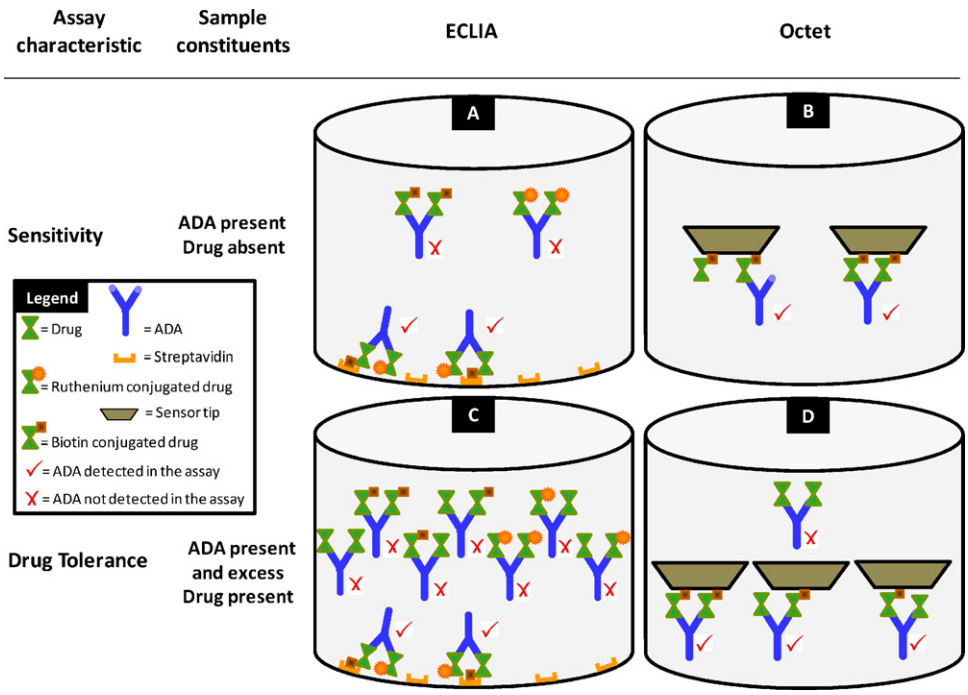

Fig. 2. Conditions affecting assay sensitivity and drug tolerance. A simple model depicting possible molecular interactions between ADA, drug, and the test reagents in the bridging ECLIA and Octet platforms.

is in fact the ideal approach for the selection and development of ADA detection methods, especially when one or more low-affinity monoclonal ADAs can be obtained.

ADA detection assay sensitivity can also be affected by interference, mainly from the drug itself [9]. Although challenging, it is important to reduce drug interference because the prolonged half-life of pharmaceutical antibodies and some fusion proteins, and the fact that they are typically administrated at relatively higher doses, renders ADA assays especially prone to false-negative results unless drug administration is followed by a substantial washout period to allow for adequate drug clearance prior to ADA assessment. Our experiments with CNTO X and high- or low-affinity ADAs demonstrated that the Octet was the most drug-tolerant of the three methods. In our experience, the ECLIA format presented here has generally offered better drug tolerance than the classical ELISA bridging format, but the current study demonstrated that the Octet presented a log-fold better drug tolerance advantage over the ECLIA. As expected in the pre-clinical cynomolgus monkey study, the ECLIA failed to detect ADAs until CNTO X was cleared from circulation. Although drug tolerance values derived from our panel of monoclonal ADAs may not be representative of all polyclonal study sera, the observed 1–10 μg/mL drug tolerance range of the ECLIA (Table 3) was far below the circulating concentrations of CNTO X found in Monkeys 7, 10, and 11, except for the day 57 samples of Monkeys 11 (8.02 μg/mL) and 7 (no detectable drug). This knowledge might explain why the ADAs escaped detection by ECLIA, except for the day 57 sample from Monkey 7. In contrast, the Octet detected ADAs in early sera obtained during the dosing phase of study in the presence of very high concentrations of drug that far exceeded the 10–100 μg/mL drug tolerance range determined under experimental conditions with monoclonal antibodies (Table 3). Given our findings on the superior sensitivity of the Octet for detecting low-affinity antibodies, one might surmise that the Octet would be able to detect such antibodies, which are generally understood to occur earlier in an immune response. As the antibodies undergo affinity maturation later, coupled with a reduction in serum concentration of the drug, they may be more amenable for detection by an ECLIA or ELISA. This was in fact what was observed, as the Octet allowed for detection of ADAs in animals as early as day 15 of the dosing phase of study and within a week after the 2nd dose. In contrast, the ECLIA could identify ADA in only one sample after a prolonged drug washout period of 35 days. Thus switching from the ECLIA to the Octet method could have allowed us to eliminate the washout period of this pre-clinical animal study, resulting in a significant savings.

Our pre-study experiments convincingly demonstrated that the Octet was not as sensitive for the detection of high-affinity ADAs, and, when testing ideal samples (i.e., those without interfering drug), it did not meet regulatory targets for sensitivity at an equivalent frequency to that of the ECLIA or ELISA. On the other hand, when testing non-ideal samples that contained drug, Octet was successful at identifying three animals that had developed ADAs, whereas the ECLIA identified only one animal from a late follow-up sample that had no detectable drug present. It could be inferred that the two additional monkeys identified by the Octet method had developed very low-affinity ADAs that were elusive to ECLIA-based detection. The poor sensitivity of the ECLIA (or ELISA) to low-affinity ADA might be explained as an inherent caveat to the bridging formats and wash steps employed by these techniques. The former lessens sensitivity by requiring two concurrent binding events for capture and detection, while the latter may remove weakly binding ADA, especially those with rapid dissociation rates [16]. In contrast, Octet does not involve pre-read washing and detects binding between ADA and drug-coated sensors in one label-free step, eliminating the need for a bridging format. The presence of both drug and low-affinity ADA may be found in non-ideal test samples, and so this apparent paradox of better detection by the less sensitive technology is probably due to a combination of superior drug tolerance and low-affinity ADA detection by the Octet platform.

We speculate on the features of biosensor-based methods that allow for relatively greater resistance to the drug interference problem that is inherent to bridging immunoassay techniques. Fig. 2 illustrates the immunochemical interactions that conceivably occur between ADA, drug, and conjugated drug reagents within assay samples that can affect the sensitivity and drug tolerance of the bridging (ECLIA or ELISA) and Octet biosensor methods.

Notwithstanding other potential cross-reactivities or interferents in a test sample, the assay sensitivity assessment scenario involves the detection of ADA in the absence of drug within the sample, whereas the drug tolerance scenario involves the detection of ADA in the presence of excess drug. The bridging assay format, exemplified by our ECLIA method, requires two concurrent binding events and the molecular complex thus detected is the molecule of ADA bound to both biotinylated drug as well as ruthenylated drug. This complex may represent just two of four possible molecular species that might occur in the sample, the remaining two of which are not conducive to detection (Fig. 2A). For illustration simplicity, one of each type of molecular complex is shown; obviously, differences in the concentrations of drug or conjugated forms (reagents) in the bridging assay reaction can produce different proportions of these molecular species and affect ADA detection differentially. In contrast, the Octet, a label-free platform that detects bound mass directly, captures ADA with biotinylated drug without the need for a detection reagent. Theoretically, the opportunity for two biotinylated drug molecules to bind the ADA (Fig. 2B) should allow Octet to take advantage of avidity in order to enhance the ability to capture and retain low-affinity ADA on the surface of the biosensor. Despite this apparent advantage, the fact that the Octet produced lower sensitivity results compared to ELISA and ECLIA might be explained by signal detection technology/electronics differences between these platforms, and certainly the enzyme-based signal amplification in the case of ELISA. A more complicated assay condition involves the additional presence of drug in the sample (a "non-ideal" sample), which affects the sensitivity of the assay depending on the drug concentration (drug tolerance of the assay). In this scenario, the formation of the bridge in the ECLIA method still requires concurrent binding of ADA to both biotinylated drug and ruthenylated drug for detection, but this molecular complex represents just two of nine entities that might occur in the sample, with the remaining seven escaping detection (Fig. 2C). In contrast, both free ADA and ADA bound to drug on one arm are detectable by the Octet. The only molecular complex that escapes detection is the one in which both ADA arms are bound to circulating drug, i.e., the form of drug that is not exogenously added as an assay reagent (Fig. 2D). Hence, the presence of drug in a sample can drastically lower the ADA detection capability of a bridge method such as ECLIA or ELISA, while the Octet is inherently less susceptible to this interference.

A common tactic to improve the drug tolerance of bridging methods is to pre-treat samples with acid, which can dissociate some drug–ADA complexes. Then, neutralization with a high pH buffer containing the desired reagent forms of the drug (conjugates) leads to improved ADA bridging and ultimately improved drug tolerance [10,11,13,15]. The pros and cons of acid pre-treatment, however, require careful elucidation on a case-by-case basis during method development. Despite this process, not all ADAs in the sample will form a detectable bridge and, due to the heterogeneous nature (different affinities for binding the drug, and potentially varying liabilities to acid treatment) of ADA immunoglobulins in a study population, it may be impossible to ensure that some ADAs were not adversely affected by the acid treatment. In an alternative approach, some labs have employed Biacore to measure ADAs [22] and reported that this platform could detect low-affinity ADAs in the presence of higher molar ratios of drug [8]. Likewise, we have demonstrated that the Octet platform has better drug tolerance than ELISA and ECLIA without a need to pre-treat the test samples with acid.

An assessment of assay precision of the Octet-QK and -RED based methods resulted in estimates of 12.9% intra-run and 11.8% inter-run variability (not shown), meeting the contemporary bioanalytical expectation of 20% or less imprecision.

An important concern for us was that use of Octet for larger scale bioanalytical applications would be significantly more expensive than ECLIA and ELISA, with much of the cost-per-sample attributed to the high price of the disposable sensors. Thus, if sensor regeneration were possible for the Octet, it would provide a more cost effective approach. In order for regeneration to be successful, one would expect immobilized drug to be stable and retain binding activity over multiple regeneration cycles and that previously captured ADA would be completely dissociated during regeneration. After testing numerous conditions in the Octet method described here, we found that each ADA measurement could be followed by alternately dipping sensor tips in 10 mM pH 2 Glycine for 30 s then 10% NMS for 30 s and repeating for a total of three cycles before testing the next ADA sample (30 °C and 1000 rpm agitation were maintained). Using regenerated sensors to make 9 repeated measurements of mono- and polyclonal ADA samples the results remained between 91% and 115% of the value obtained by a pristine sensor regardless of the type, affinity, or order of the ADAs measured (data not shown). Thus, each sensor could be regenerated 8 times and used to make 9 consecutive measurements. We could not determine the number of regenerations required to cause sensor failure because our instrument automatically discarded tips after completing a 96-well plate. The ultimate number of regeneration cycles possible may be resolved by others in the near future through the use of an Octet 384-well system or one of the newer models that allows for sensor retention across multiple runs. Nonetheless, we could reduce the cost of sensors by almost 90% by regenerating sensors nine time, thereby lowering the reagent cost-per-sample to less than our ECLIA and nearly equivalent to our ELISA.

5. Conclusion

Compared to ELISA- and ECLIA-based bridging immunoassays, the Octet method was a less sensitive means of detecting high-affinity ADAs, but facilitated detection of low-affinity ADAs and was more resistant to interference from circulating drug. Although the instrument itself was expensive, we showed that the cost of disposables per test is competitive when appropriate regeneration conditions are used. Use of the Octet may provide additional savings by allowing one to shorten the duration of drug trials that otherwise include an extended follow-up period for the purpose of reducing the drug's interference upon ADA detection. Thus, we concluded that this platform represents a promising tool for immunogenicity assessments, particularly of lower affinity ADAs, and is suitable for the analysis of samples in which the presence of drug can cause negative interference in bridging immunoassays.

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