



An electrochemiluminescence (ECL)-based assay for the specific detection of anti-drug antibodies of the IgE isotype[☆]

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ARTICLE INFO

Article history:

Received 27 March 2013

Received in revised form 8 June 2013

Accepted 11 June 2013

Available online xxx

Keywords:

IgE

ADA

Chimeric antibody

Hypersensitivity

Immunoassay

ABSTRACT

To address a possible linkage between the occurrence of the hypersensitivity reactions and the induction of IgE anti-drug-antibodies (ADA), a drug specific IgE ADA assay was developed using electrochemiluminescence (ECL) technology. In the assay a drug-specific IgE isotype chimeric antibody was generated and used as an ADA positive control. The biotinylated drug X (an antibody) and ruthenium-labeled omalizumab (an anti-human IgE antibody) were used as capture and detection reagents, respectively.

The binding affinities of the chimeric IgE isotype positive control have been shown to be highly comparable to drug X and drug Y (drug X is the 2nd generation of drug Y), indicating that it could serve as a highly useful control to compare and contrast the relative ability of the two generations of drug to elicit IgE ADA responses. The assay cut point factor (CPF) was estimated to be 1.13. The cut point factor derived from normal human serum samples was statistically equivalent to the cut point factor determined from targeted population samples. The assay could detect less than 250 ng/mL of IgE antibodies in the presence of 300 µg/mL drug X. The assay sensitivity was <0.2 ng/mL. A minimal prozone was observed at 100 µg/mL IgE ADA, but the sample remained highly detectable. The inter-assay precision was within 12%. The assay was not susceptible to non-specific matrix effects. The performance specifications ensured that the assay was suitable for validation. The combination of the chimeric IgE positive control and the detection antibody (ruthenium-labeled omalizumab) used for the assay could potentially provide a general bioanalytical approach for other biopharmaceuticals for the detection of IgE ADA responses.

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1. Introduction

Biopharmaceuticals are used for the treatment of a wide spectrum of diseases [1,2]. The repeated administrations of biopharmaceuticals can elicit the induction of anti-drug antibodies (ADAs) [3,4], which may lead to altered pharmacokinetic (PK) and pharmacodynamic (PD) profiles, and reduced drug efficacy [3] and may cause adverse events including hypersensitivity reactions. ADAs mostly occur with IgG isotype [5]; however, IgE isotype antibodies can potentially elicit anaphylactic reactions [6].

Hypersensitivity reactions are categorized into four different types, Type I, II, III and IV, each characterized by specific biological function [7]. A Type I hypersensitivity reaction is a rapid immune response, where mast cells and basophils play a central role through release of inflammatory mediators [8]. In Type I hypersensitivity reactions, antigens interact with IgE that are bound to its receptors on the surface of mast cells [9]. It has been reported that hypersensitivity reactions correlate with IgE ADAs for several biotherapeutics, such as tocilizumab, cetuximab, natalizumab and muromonab [10–13]. Also, pre-existing IgE cross-reacting with cetuximab caused infusion reactions in some patients [14].

Evaluation of IgE ADAs is not necessary in the routine evaluations of clinical trial data. However, when subjects display hypersensitivity reactions, it is of increased importance to determine if the adverse events are related to drug-specific IgE antibodies. This approach will help to elucidate the mechanism(s) behind the adverse events [15].

In vitro test methods for drug-specific IgE antibodies have been documented. A radioimmunoassay was employed for the detection

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of specific IgE antibodies to erythromycin [16,17]. The ImmunoCap platform has been adapted for the detection of IgE antibodies specific to several biologics [14,18,19]. The AdvanSure enzyme immunoassay system is a recently developed IgE detection assay for multiple allergen screen tests [20]; however, these methods have certain limitations in terms of the assay's sensitivity, robustness, logistic operations and cost.

A drug-specific IgE assay can be used for the assessment of IgE responses in subjects participating in clinical studies, and the corresponding IgE data are used for the evaluation of a subject's safety profiles along with other clinical information. Therefore, it is essential to validate drug-specific IgE assays for clinical studies when warranted by safety and risk-benefit profiles [21]. The use of a positive control is critical in the development of a drug-specific IgE assay as it provides useful information on the performance of the assay, such as sensitivity, precision, robustness and specificity. As is commonly practiced for ADA assays, a surrogate positive IgE control is used as no control adequately reflects every possible ADA response that could be elicited in clinical trials or clinical use.

Polyclonal antibodies produced in animals or mouse anti-drug monoclonal antibodies (mAbs) are most commonly used as positive controls in ADA assays. However, none of these reagents would be suitable controls to characterize the isotype of a human ADA response. To assess human IgE ADA, the control must have an Fc region that is human IgE. Since direct production of human IgE antibodies in human IgG transgenic mice is not possible due to the lack of the proper transgene, some form of molecular engineering would be needed to produce such an antibody or a human B cell clone producing drug-specific IgE would have to be identified and immortalized. Thus, the production of a chimeric (mouse variable regions for the anti-idiotypic (id) specific drug and human IgE constant regions) was attractive as the easiest approach for generating the needed control.

This paper illustrates the generation of chimeric anti-drug-specific IgE positive control and characterization of its specificity and binding affinity. A drug-specific IgE assay using electrochemoluminescence (ECL) technology for the detection of IgE ADA in human serum was developed, characterized and qualified using this chimeric positive control. The binding affinities of the chimeric IgE positive control were highly comparable for two generations of the drug. The cross-reactive chimeric IgE positive control can be used as a positive control antibody for the comparison of the two generations of drug in terms of their potential ability to elicit IgE ADA. Utilization of omalizumab, anti-human IgE mAb as the detection reagent in the assay greatly enhances drug tolerance compared to the standard ADA assay. The combination of an IgE anti-id mAb control and omalizumab offers a novel approach to detect IgE ADA response. The assay performance warranted its suitability for validation and downstream application.

2. Materials and methods

2.1. Materials

Drug X and drug Y are both humanized mAbs generated by MedImmune LLC (Gaithersburg, MD). Drug X is a second generation of drug Y. Individual serum samples from subjects similar to the target population were obtained from the pre-first dose time point of the placebo group of a historic clinical study. Pooled normal human sera were purchased from Bioreclamation (Hicksville, NY). Omalizumab (anti-human IgE mAb) is produced by Genentech (South San Francisco, CA). The chimeric anti-drug X IgE mAb was generated internally by MedImmune LLC. Meso Scale Discovery (MSD) plates and Sulfo-TAG™ Plus NHS Ester were purchased from MSD (Gaithersburg, MD). Amicon® Ultra Centrifugal Filter Units,

Ultra-50 membrane was purchased from Millipore (Billerica, MA). Protein Assay Kit was purchased from Pierce (Rockford, IL).

2.2. Preparation of reagents

2.2.1. Generation of the anti-drug X IgE antibody

The positive control reagent is a recombinant chimeric antibody consisting of the mouse heavy and light chain V-domains of an anti-id antibody against the target drug X, fused in frame to the constant domains of the human IgE and human kappa chain. The construct for expressing this recombinant IgE was made as follows. The mRNA of an anti-id hybridoma against drug X was isolated using Dynabeads mRNA Direct kit (Invitrogen, Frederick, MD). The first strand cDNA was synthesized using Invitrogen SuperScript III reverse transcriptase and random hexamer primer (Invitrogen). The mouse Ig VL (kappa) and VH were amplified by PCR using an Ig primer set (Novagen, Madison, Wisconsin). The PCR-amplified V-domains were cloned into TOPO TA vector (Invitrogen, Frederick, MD) and plasmids from 48 distinct colonies of VHs and VLs were sequenced. Except for minor point mutations, the majority of the colonies had identical VH and VL sequences. The VH and VL from representative TA clones were re-amplified by PCR. During this step, BssHII and BsiWI restriction sites were added to the 5' and 3' end, respectively, of VL. Likewise, BsrGI and Sall restriction sites were added to the 5' and 3' end of VH, respectively. The PCR products were then digested with the appropriate restriction enzymes and cloned by a 4-piece ligation into an in-house vector that coded for the constant domains of human IgE and C-kappa. This vector is a bi-cistronic vector, which expresses human Ig heavy and light chains using the CMV IE promoter in HEK293 cells. After cloning, the recombinant plasmids were sequence confirmed. Large scale plasmid prep was made using an endotoxin free plasmid Maxiprep kit from Qiagen. This maxiprep was then used for transfection of 293F cells. After six days, the conditioned media was collected by filtration. The IgE from the conditioned media was purified by drug X affinity chromatography.

2.2.2. Quality control samples

Quality control (QC) samples were prepared for evaluating different assay parameters during assay development. The high concentration quality control (HQC, 1000 ng/mL) and low concentration quality control (LQC, 10 ng/mL) samples were prepared by spiking the chimeric anti-drug X IgE positive control into pooled human serum and analyzed across multiple assays in different days.

2.2.3. Conjugation of antibody

2.2.3.1. Biotin, ruthenium conjugation of omalizumab or drug X. Drug X and omalizumab were first buffer-exchanged into phosphate-buffered saline (PBS) using Amicon® Ultra centrifuge tubes (50 kD cutoff) by centrifugation at $1500 \times g$ for 15 min. Following buffer exchange, protein concentrations were determined by A280 nm using an extinction coefficient of 1.4. Concentrations were adjusted by either concentration (using the Amicon filters) or dilution to approximately 2 mg/mL for labeling. Drug X was biotinylated using a molar ratio of 8:1 (biotin:protein). Omalizumab and drug X were conjugated to Sulfo-TAG using a 12-fold molar excess of Sulfo-TAG. The labeled proteins were separated from excess free label using the Amicon centrifugation filters as described above. The labeled proteins were washed 3–4 times by re-suspending the filter retentate in 3–4 mL of PBS containing 0.05% sodium azide and then repeating the concentration step with the Amicon filters. Protein concentrations were determined with BCA™ Protein Assay Kit and the final protein concentrations were adjusted to 1–2 mg/mL with PBS containing 0.05% sodium azide.

2.3. Methods

2.3.1. Assay procedure

The assay was formatted as a solution-phase immunoassay employing biotinylated drug X for capture and ruthenylated omalizumab for detection. The serum samples and QC samples were mixed with a label solution containing both biotinylated drug X and ruthenylated omalizumab in 96-well polypropylene plates, and incubated at room temperature (RT) overnight with shaking on an orbital shaker set at 550–650 rpm (Titermix 100, Brinkmann, Westbury, NY). The next day, streptavidin-coated MSD plates were blocked with a blocking buffer containing 3% bovine serum albumin (BSA) for 60 ± 5 min and washed 3 times with a plate wash buffer containing 0.1% Tween-20 in PBS. The test sample and label solution mixtures were then transferred to streptavidin-coated standard 2400 MSD plates. After 60 ± 5 min incubation at RT with shaking (550–650 rpm), the plates were washed 3 times and a 2X MSD “T” read buffer is added to the wells. The plates were then read by the MSD Sector Imager 6000 reader.

The mean relative light unit (RLU) of negative quality control (NQC) samples on each plate was used as the plate reference (background) sample to derive the signal to noise (background) ratio (S/B) of each test sample. A sample was designated as positive if its assay response is greater than or equal to the assay cut point factor (CPF).

2.3.2. Specificity of chimeric IgE positive control

Microtiter plates were coated with 5 µg/mL of drug X, polyclonal human IgG or a negative isotype control. Samples containing various concentrations of drug X anti-id murine IgG mAb or chimeric IgE mAb control were added into the designated wells and incubated at RT for 60 min. Anti-murine IgG Fc-conjugated with horseradish peroxidase (HRP) antibody (Santa Cruz, CA) (for detection of drug X anti-id murine IgG mAb) or omalizumab-conjugated with HRP (for detection of chimeric IgE mAb) was added, followed by substrate tetramethylbenzidine (TMB) solution. The color reaction was stopped with sodium hydroxide and optical density (OD) was measured at 450 nm.

2.3.3. Measurement of kinetic rate and binding constants

Kinetic rate (k_{on} , k_{off}) and affinity constants for the binding of the chimeric cross-reactive (drugs X and Y) anti-id IgE mAb to immobilized drug X and drug Y were measured on a BIAcore 3000 instrument (Uppsala, Sweden). Briefly, drug X and drug Y were immobilized at low density onto the flow cell on the CM5 sensor chip using standard amino coupling chemistry as outlined by the instrument's manufacturer. The final IgG densities were 100 RUs (drug X) and 138 RUs (drug Y). A reference flow cell surface was also prepared on this sensor chip using the identical immobilization protocol, minus the protein. Serial dilutions of the chimeric IgE mAb were prepared in a buffer containing 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% P-20. Samples were injected at the flow rate of 75 µL/min. Dissociation data were collected for 15–40 min, followed by a 60 second pulse of 10 mM glycine, pH 1.5, between injections to regenerate the IgG surfaces on the chip. Several buffer injections were used along with the reference cell data to correct the raw data sets for injection artifacts and/or non-specific binding interactions commonly referred as “double-referencing” [22]. Fully corrected binding data were then globally fit to a 1:1 binding model (BIA evaluation 4.1 software, BIAcore, Inc.) that included a term to correct for mass transport-limited binding, should it be detected. This analysis determined the kinetic rate (on, off) constants from which the apparent K_D values were then calculated as $K_D = k_{off}/k_{on}$.

2.3.4. Comparison of the screening assay CPF between normal human serum (NHS) and targeted population serum samples

To evaluate the feasibility of using NHS samples as surrogate samples for the targeted population in determining the assay cut point factors and possible use for assay development and qualification, a panel of 36 NHS samples and 36 target population samples (from the pre-first dose time point of the placebo group of a historic clinical study) was tested once by a single analyst. The S/B ratio data points of tested samples in each group were generated and the statistical equivalence of the cut point factors derived from each population was evaluated. For both populations, the CPFs associated with false positive rates of 5% were determined using the parametric method on the original datasets and the non-parametric method on the log-transformed datasets, respectively. The median CPFs and the corresponding median absolute deviations were calculated along with the upper limit of a one-side 95% confidence interval of the median difference.

2.3.5. Screening assay CPF determination

For determination of the assay CPF, a panel of 51 individual NHS samples (33 male and 18 female) along with a pooled NHS sample were analyzed in 3 separate assays (one on each of three days) by a single operator.

The analyses described above produced 149 signal to noise (S/B) ratio data points for the determination of the CPF. To avoid inflation of the CPF, a box-plot outlier test was conducted on the raw data using three inter-quartile ranges (IQR) as an outlier criterion. Nine data points were identified to be outliers and removed. A normality test was performed on the reduced data set to assess the suitability of using a parametric method to determine the CPF. The test indicated that the data violated an assumption of normality ($p < 0.001$). The original data ($n = 149$) were then log-transformed. The same outlier test was repeated on the log-transformed data. As a result of this analysis, seven outliers were excluded. The remaining 142 data points were then tested for normality. The normality assumption was again violated. Subsequently, a nonparametric approach on the log-transformed data was used.

2.3.6. Sensitivity and prozone effect

The chimeric IgE ADA control was spiked at 100 µg/mL into a pooled NHS sample, and then prepared in a 1:2 serial dilution. The lowest concentration of the chimeric IgE ADA in the sample, which was still positive above the cut point factor, was reported as the estimated assay sensitivity.

2.3.7. Drug tolerance and specificity

The assessment of assay drug tolerance was performed using 1 µg/mL biotinylated drug X as capture and 2 µg/mL ruthenylated omalizumab for detection (Fig. 1a). Drug tolerance was also evaluated in the ADA assay using 1 µg/mL biotinylated drug X as capture and 2 µg/mL ruthenylated drug X for detection (Fig. 1b). Samples containing 0.25, 0.5, 1, 2, 4 and 8 µg/mL of the chimeric IgE ADA control were pre-incubated with 0, 25, 50, 100, 200 and 300 µg/mL of drug X at RT for 60 ± 5 min, and subsequently mixed with a labeled solution containing both biotinylated drug X and ruthenylated omalizumab or ruthenylated drug X. The samples were analyzed, and the results are presented as S/B ratio (drug tolerance) and % inhibition (specificity).

2.3.8. Selectivity test

Twenty individual samples of NHS (17 male, 3 female) along with the normal serum pool were tested unspiked (no chimeric IgE ADA control added) and spiked with 10 ng/mL of chimeric IgE ADA control (LQC concentration). The unspiked and spiked samples

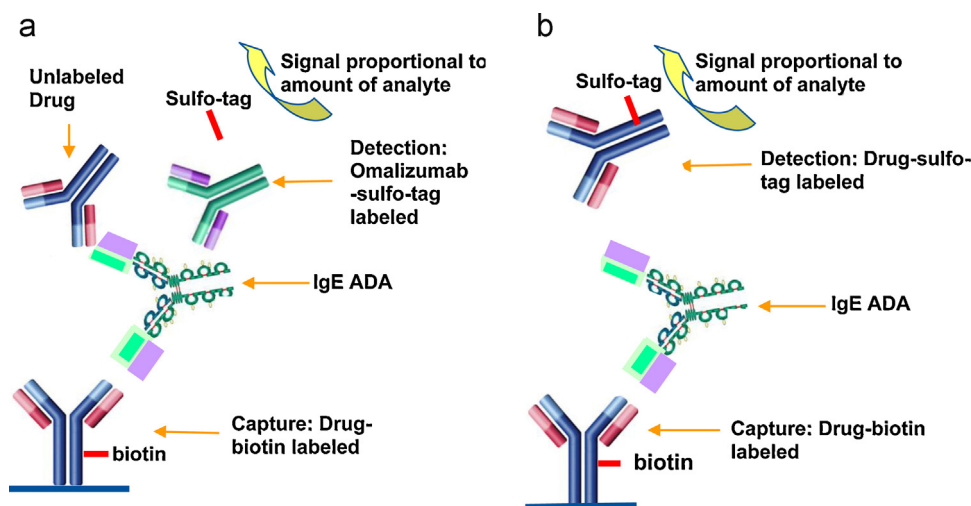


Fig. 1. Design of the biotin-drug/Sulfo-tag labeled omalizumab IgE ADA assay (a) and the biotin-drug/Sulfo-tag labeled drug (b) assay. Samples containing chimeric IgE control mAb were pre-incubated in the solution phase with a master mixture containing both the biotin-drug and Sulfo-tag-omalizumab or Sulfo-tag-drug conjugates. The complex was captured on a standard streptavidin-coated MSD plate, and a 2X MSD "T" read buffer is added to the wells. The plates are then read by the MSD Sector Imager 6000 reader.

were each analyzed in one replicate. The S/B ratio of each individual spike was calculated.

2.3.9. Lot comparison and storage stability of omalizumab-Sulfo-TAG

The HQC (1000 ng/mL), LQC (10 ng/mL) and NQC (0 ng/mL) were prepared in bulk and used for Sulfo-TAG-labeled omalizumab lot comparison (1st vs. the 2nd lot) and storage stability testing. Four wells of HQC and LQC, and 8 wells of NQC were analyzed for each condition on the same plate. The mean S/B ratios were compared.

3. Results

3.1. Characterization of chimeric IgE ADA positive control

The chimeric IgE ADA positive control was originally derived from a drug-specific anti-id murine IgG secreting hybridoma. As shown in Fig. 2a, the binding of the murine IgG mAb increased in a concentration-dependent manner when the IgG mAb was added to drug X-coated wells. In contrast, no binding was detected to the polyclonal human IgG coated-wells, demonstrating that the murine anti-id IgG antibody specifically bound to drug X.

A similar experiment was conducted after the chimeric IgE control was constructed into the human IgE expression vector and expressed as a recombinant protein in 293F cells. In Fig. 2b, increasing concentrations of the chimeric IgE mAb control are shown binding to drug X-coated wells, while no such pattern was observed when the IgE mAb control was interacted with two control (polyclonal human IgG and antibody isotype control) antibodies. These results showed that the chimeric IgE mAb control retained the specificity to drug X that was displayed by the original mouse IgG mAb.

3.2. Affinity of binding of IgE ADA positive control to drug X and drug Y

The kinetic rate (k_{on} , k_{off}) and equilibrium dissociation (k_D) constants for the binding of the chimeric IgE mAb to drug X and the related drug Y were determined by surface plasmon resonance technology. Individual association and dissociation rate constants were then calculated from the resulting binding curves using the BIA evaluation software available through the vendor.

From high-resolution BIAcore plots, the association rate constant (k_{on}) determined for the binding of the chimeric IgE mAb to drug X and drug Y were determined to be 5.93×10^4 /Ms and 3.63×10^4 /Ms, respectively. The same plots were also used to determine the dissociation rate constant (k_{off}) for the binding of the chimeric IgE mAb to drug X and drug Y, which measured $4.70 \times 10^{-5} s^{-1}$ and

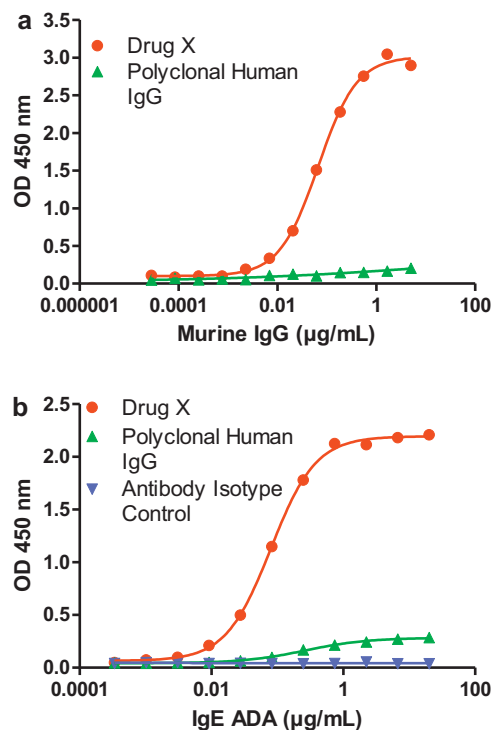


Fig. 2. (a) Specificity of anti-id murine IgG specific for drug X. The microtiter plates were coated with 5 μg/mL of drug X or polyclonal human IgG (control). The murine IgG anti-id was added at various concentrations to the wells and detected by an anti-murine Fc-HRP conjugate using TMB as a substrate. (b) Specificity of chimeric IgE control mAb specific for drug X. The microtiter plates were coated with 5 μg/mL of drug X (red), drug isotype control (blue) and polyclonal human IgG control (green). The chimeric IgE control mAb was added at various concentrations to the wells and detected by omalizumab-HRP conjugate using TMB as a substrate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

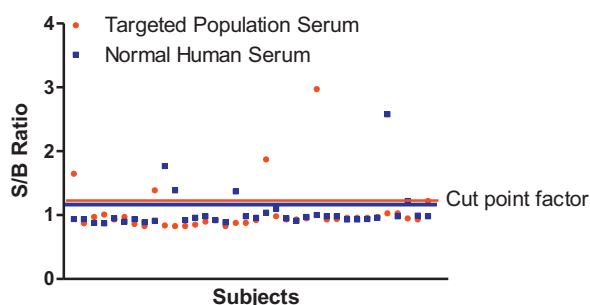


Fig. 3. Comparison of assay CPF in normal and targeted population human serum samples. A panel of 36 serum samples from healthy (normal) and targeted population serum samples were analyzed once (2 wells per sample) by a single analyst according to the assay procedure. The mean of S/B ratio for each sample is presented. Eight outliers (4 from each population) were excluded from CPF determination. The horizontal lines represent the assay cut point factor for each population.

$6.09 \times 10^{-5} \text{ s}^{-1}$, respectively. From these rate constants, the apparent dissociation binding constants (k_D) of the chimeric IgE mAb to drug X and drug Y were then calculated from the quotient of $k_{\text{off}}/k_{\text{on}}$ as 0.792 nM and 1.678 nM, respectively. Experiments were conducted in duplicate, and the results were highly reproducible. All acquired data was fit globally to calculate a single set of rate and affinity constants. The individual errors for k_{on} and k_{off} were low, and the overall fit to the data were excellent as judged by χ^2 values of <2% of Rmax, indicating the use of the 1:1 model in fitting the data was appropriate. In addition, the evaluation did not indicate binding was mass transport-limited; thus, the measured association rate constants were considered valid. Similar results were obtained with the original mouse IgG mAb. Our data suggested that the chimeric cross-reactive IgE mAb recognizes both drug X and drug Y with comparable binding parameters.

3.3. Comparison of normal adult and target population serum samples toward determination of CPF

For determination of the assay CPF, test samples used in assay development should be as similar as possible to the actual study samples. The sample volumes in the clinical studies for assessment of immunogenicity were not sufficient to support cut point factor determination during pre-study assay validation. To overcome this hurdle, normal human samples and study population samples were evaluated for equivalence. Samples from NHS ($n = 36$) and from targeted population similar to that in the actual clinical study ($n = 36$) were tested. Data from the two populations were demonstrated to be statistically equivalent (CPF of 1.029 for NHS, and CPF of 1.031 for targeted population) (Fig. 3); therefore, NHS samples were considered suitable for use during assay development, pre-study assay validation and subsequent sample analysis.

3.4. Screening assay CPF

Given the limitation of the target population serum sample supply, the cut point factor was determined with 51 individual NHS samples (33 male and 18 female) as surrogate. The S/B ratio of each sample was calculated, and the CPF was established. The CPF was determined to be 1.13 (Table 1) for a false positive rate of 5%.

3.5. Assay sensitivity and prozone effect

To estimate the assay sensitivity, the chimeric IgE ADA control was spiked into a pooled NHS sample at a concentration of 100 $\mu\text{g}/\text{mL}$ and 1:2 serial dilutions were performed. The lowest concentration of the chimeric IgE ADA in the sample that measured positive (above the 1.13 cut point factor) was the estimated assay

Table 1
Screening assay cut point factor.

Sample no.	Day 1 (S/B)	Day 2 (S/B)	Day 3 (S/B)
1	1.01	1.04	1.09
2	1.62*	1.16	1.24
3	1.03	1.03	1.09
4	1.18	1.13	1.28
5	1.03	1.07	0.98
6	1.04	1.04	1.09
7	0.97	1.00	1.14
8	0.92	1.08	0.88
9	0.96	1.01	1.11
10	0.99	1.06	0.96
11	14.41*	1.08	0.98
12	1.03	1.08	0.99
13	1.08	1.02	1.06
14	1.00	1.10	0.99
15	1.01	0.97	1.03
16	0.98	0.99	1.01
17	1.00	1.00	1.03
18	1.02	1.11	0.96
19	0.99	1.06	0.98
20	1.04	1.09	0.98
21	0.96	1.03	0.95
22	0.95	0.99	1.05
23	0.97	1.03	0.95
24	0.98	1.03	1.00
25	0.95	1.04	1.04
26	0.89	0.99	1.02
27	13.41*	1.05	0.98
28	0.99	1.04	1.03
29	1.00	1.01	1.02
30	0.92	0.97	1.02
31	1.02	1.00	1.03
32	1.24	1.11	1.02
33	0.98	1.07	0.95
34	1.02	1.03	1.00
35	0.98	1.02	0.95
36	1.01	1.04	1.04
37	0.87	1.01	1.02
38	0.91	0.96	1.00
39	0.98	0.99	0.98
40	1.06	1.01	1.01
41	0.92	1.01	0.99
42	1.02	1.03	1.03
43	0.98	0.95	1.07
44	1.00	1.00	1.01
45	1.07	1.02	1.03
46	3.36*	2.47*	3.18*
47	1.04	0.97	1.01
48	ND	1.29	1.53*
49	ND	1.06	1.04
50	ND	1.06	1.08
51	ND	1.10	1.05
Mean S/B	1.02		
Std. Dev. S/B	0.066		
Cut point factor	1.13		

A panel of 51 individual normal human serum samples was analyzed three times by one analyst according to the assay procedure. Each value represents the S/B ratio (mean RLU sample/mean RLU NHS pool) of each well (2 wells per sample). S/B ratio = signal to noise ratio. ND = not determined. Std. Dev. = standard deviation. RLU = relative light unit. Outliers, noted with an asterisk (*), were excluded for cut point factor analysis.

sensitivity. As shown in Fig. 4, the assay sensitivity was estimated to be 0.2 ng/mL of the chimeric IgE ADA. A minimal prozone effect was observed at a concentration of 100 $\mu\text{g}/\text{mL}$ chimeric IgE ADA, but the sample remained highly positive in the assay.

3.6. Drug tolerance

High levels of free drug in the blood can potentially interfere with the detection of drug-specific antibody. Therefore, the drug tolerance of the IgE ADA assay was of concern given the low concentration of total IgE in circulation. During assay development,

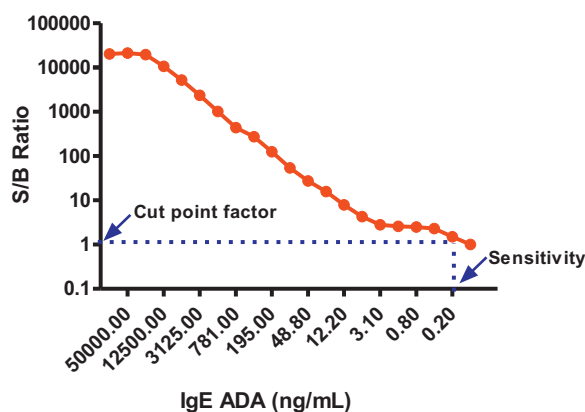


Fig. 4. Sensitivity of IgE ADA assay. The chimeric IgE control mAb was spiked into a pooled NHS sample at a concentration of 100 $\mu\text{g/mL}$ and then a 1:2 serial dilution was prepared twice. Each dilution was measured in duplicate (2 wells) to produce the result. Each data point represents the mean of two measurements (4 wells). The lowest concentration of the chimeric IgE control mAb in the sample which was above cut point factor 1.13 was 0.2 ng/mL.

we evaluated if omalizumab as detection antibody offered superiority in drug tolerance compared to using drug for detection, although we recognized that the assay format is not specific for IgE antibody. Pooled serum was spiked with chimeric IgE ADA ranging from 250 ng/mL to 2000 ng/mL and was tested in each of the assay formats in the presence of various drug levels. The results presented in Fig. 5a (omalizumab detection) showed that 250 ng/mL of the chimeric IgE control was easily detectable (>16 S/B ratio) in the presence of 300 $\mu\text{g/mL}$ of drug X. As the S/B ratio (16.26)

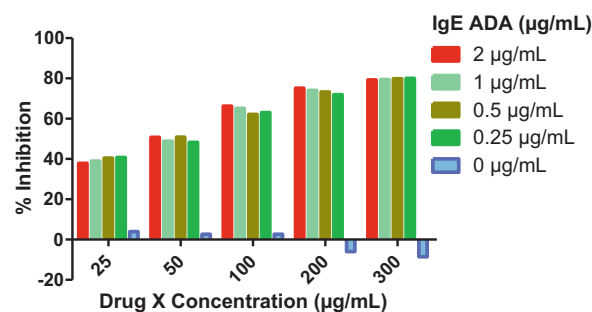


Fig. 6. Specificity of IgE ADA assay. Increasing concentrations of drug X inhibit the signal output in the experiment described in Fig. 5a. However, the chimeric IgE ADA remains detectable even in concentrations of drug X as great as 300 $\mu\text{g/mL}$.

was much higher than the 1.13 cut point factor, it was clear that much less chimeric IgE ADA could be detected in the presence of 300 $\mu\text{g/mL}$ of drug X. The assay format commonly used for ADA assays in which drug labeled with biotin and ruthenium serves as capture and detection antibodies, respectively, is shown in Fig. 1b. The data revealed that in the presence of 300 $\mu\text{g/mL}$ drug X, this spiked sample (250 ng/mL of chimeric IgE ADA) was undetectable. In the presence of 100 $\mu\text{g/mL}$ drug X, a response of 1.25 (S/B ratio) was achieved, which was just above the assay cut point factor (1.13) (Fig. 5b). This result indicates that the assay with omalizumab as the detection antibody exhibits greater drug tolerance and sensitivity than the assay format used in the screening assay for ADA to drug X.

3.7. Specificity

Specificity of the chimeric IgE ADA method was demonstrated in two ways. First, the chimeric IgE ADA control has been shown to be highly specific to drug X (Fig. 2b). Second, the data generated from experiments for determination of drug tolerance showed that 25 $\mu\text{g/mL}$ of drug X displayed approximately 40% inhibition in all chimeric IgE ADA spiked samples (Fig. 6). This level of inhibition increased with increasing concentrations of drug X although the chimeric IgE ADA remained detectable (above cut point factor) as described above. Thus, the method was demonstrated to be specific for measurement of the drug-specific IgE antibodies.

3.8. Selectivity

Selectivity of the chimeric IgE ADA detection was assessed using 20 individual (17 male, 3 female) serum samples from healthy donors. The chimeric IgE control mAb, at a concentration of 10 ng/mL (LQC concentration), was spiked into each individual serum sample along with a normal serum pooled sample, and the S/B ratios were calculated. The S/B ratio of each individual spike was above the cut point factor 1.13 (Fig. 7). The inter-sample variability was 16% and 18 out of 20 samples were within 25% compared to the serum pool. These results indicated that the assay was not susceptible to non-specific matrix effects.

3.9. Assay precision and QC sample performance

Precision across assays was evaluated by testing multiple replicates of QC samples in several runs performed over several days. The combined QC sample performance is shown in Table 2. The results for HQC and LQC samples are presented as S/B ratios, whereas the results for NQC samples are presented as RLU. The % CV of results obtained for each QC sample was calculated. Inter-assay precision was less than or equal to 11.5% across control levels. The established ranges (mean $\pm$ 2 standard deviation) were 318–508

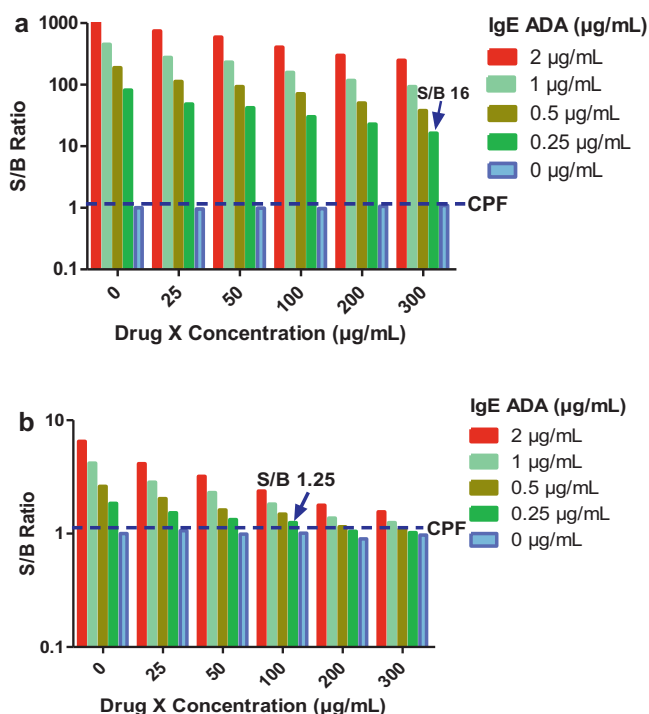


Fig. 5. Demonstration of drug tolerance in two assay formats. Samples containing the chimeric IgE control mAb from 0 to 2000 ng/mL were pre-incubated with varying concentrations of drug X from 0 to 300 $\mu\text{g/mL}$ at RT for 1 h, and simultaneously analyzed in two assays; (a) Sulfo-tag-omalizumab detection; (b) Sulfo-tag-drug X detection. The results are shown as S/B ratios. The dotted line represents the cut point factor. The arrows indicate the relative drug tolerance of a sample containing 250 ng/mL IgE control mAb. The chimeric IgE ADA at 250 ng/mL was detectable in the presence of 300 $\mu\text{g/mL}$ drug (a) and 100 $\mu\text{g/mL}$ drug (b) with 16 and 1.25 S/B ratios, respectively.

Table 2

Assay precision and QC sample performance.

	HQC (1000 ng/mL) (S/B ratio)	LQC (10 ng/mL) (S/B ratio)	NQC (RLU)
Run #1	394	2.93	80.5
Run #2	466	3.72	78.5
Run #3	435	3.3	74.8
Run #4	357	3.05	79.4
Mean	413	3.25	78.3
Std. Dev.	47.6	0.35	2.49
% CV	11.5	10.6	3.18
Accepted ranges (± 2 Std. Dev.)	318–508	2.56–3.94	73.3–83.3

HQC, LQC and NQC samples were prepared by spiking 1000, 10 and 0 ng/mL of the chimeric IgE control mAb into pooled NHS, respectively. QC samples were analyzed by one analyst on four plates over different days. The results for HQC and LQC samples are presented as S/B ratio, and the results for NQC samples are presented as RLU.

Table 3

Omalizumab-Sulfo-TAG: lot comparison and storage stability.

QCs	Baseline ^a (2nd lot)				–60 °C to –80 °C for 2.5 month (1st lot)				2–8 °C for 2.5 month (1st lot)			
	S/B	Mean	Std. Dev.	%CV	S/B	Mean	Std. Dev.	%CV	S/B	Mean	Std. Dev.	%CV
HQC	373	362	16.1	4.00	382	356	29.0	8.00	430	389	47.0	12.0
	378				381				429			
	347				330				352			
	349				332				344			
LQC	3.89	3.70	0.16	4.00	4.02	3.92	0.43	11.0	4.38	3.93	0.44	11.0
	3.78				4.48				4.24			
	3.52				3.63				3.63			
	3.62				3.54				3.49			
NQC	1.01	1.00	0.03	3.00	1.04	1.00	0.04	4.00	1.00	1.00	0.04	4.00
	0.98				1.00				1.03			
	1.02				1.04				1.05			
	0.99				1.00				1.05			
	1.01				0.94				0.96			
	0.94				0.95				0.95			
	1.03				0.99				0.98			
	1.02				1.03				0.98			

HQC (1000 ng/mL), LQC (10 ng/mL) and NQC (0 ng/mL) samples were prepared in bulk, and subsequently aliquotted. HQC and LQC samples (4 wells each level), and NQC samples (8 wells) were tested for each assigned condition. The S/B ratio at each level of QCs was calculated.

^a Freshly thawed reagents were considered as baseline.

(S/B ratio), 2.56–3.94 (S/B ratio), and 73.3–83.3 (RLU) for HQC, LQC, and NQC samples, respectively.

3.10. Lot comparison of omalizumab-Sulfo-TAG

To assess variability between different preparations of omalizumab-Sulfo-TAG, two lots were tested. Two QC levels (HQC [1000 ng/mL] and LQC [10 ng/mL], 4 wells each level) and NQC (0 ng/mL, 8 wells) were tested. The S/B ratio at each level of QC samples was comparable between the two lots of omalizumab-Sulfo-TAG as shown in Table 3. The results indicate that there were minimal differences seen in the S/B ratios generated with the two lots of omalizumab-Sulfo-TAG.

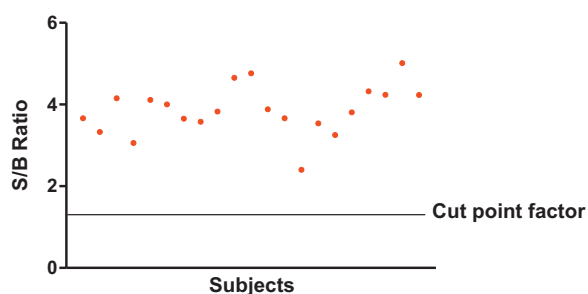


Fig. 7. Selectivity in normal human serum. Serum from 20 (17 male and 3 female) individuals along with the normal serum pool was tested by spiking 10 ng/mL of the chimeric IgE control mAb. Each spiked sample was measured in duplicate (2 wells) to produce the result. Each data point represents the S/B ratio for one individual serum sample.

3.11. Omalizumab-Sulfo-TAG storage stability at 2–8 °C and at –60 °C to –80 °C

For long-term use, omalizumab-Sulfo-TAG was stored at –60 °C to –80 °C. During validation or sample analysis, this reagent may be stored at 2–8 °C on certain days. To test the stability of omalizumab-Sulfo-TAG at 2–8 °C after removing it from –60 °C to –80 °C freezer, two sets of replicates (2 wells per replicate), each of HQC (1000 ng/mL) and LQC (10 ng/mL) (4 wells total) and 4 sets of replicates NQC (0 ng/mL, 8 wells total) were tested with freshly thawed reagents, while another set was tested with reagents stored at 2° to 8 °C for 2.5 months. The S/B ratio of all samples for freshly thawed and tested reagents were comparable (Table 3). These results indicated that thawed omalizumab-Sulfo-TAG could be stored at 2–8 °C for 2.5 months without compromising assay performance.

The first lot of omalizumab-Sulfo-TAG was kept at –60 to –80 °C for 2.5 months. A second lot of labeled omalizumab was prepared and freshly stored, in aliquots at –60 to –80 °C. The comparability of the performance of the two lots (2.5 months old first lot and freshly thawed aliquot from the second lot) demonstrated that the two lots were comparable, indicating that the labeled omalizumab was stable when stored at –60 to –80 °C for 2.5 months (Table 3) and that inter-lot reproducibility was good.

3.12. Sample testing in IgE isotype assay for one clinical study

The tiered approach has been widely applied for ADA sample testing. After screening (tier 1) and confirmatory (tier 2) assays, only positive samples are characterized in an IgE isotyping assay when needed. In the course of drug X clinical development, samples

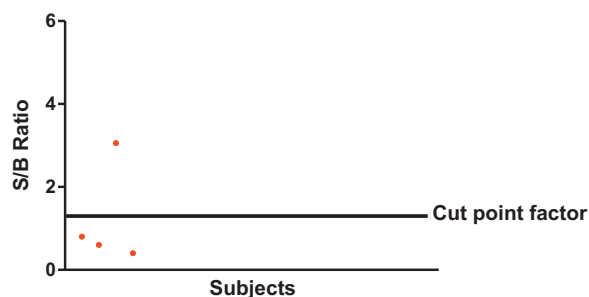


Fig. 8. Anti-drug X IgE isotype antibody levels in human serum samples from a clinical study. The four serum samples confirmed positive for anti-drug X antibodies, were subsequently tested for anti-drug X IgE antibody. Only one sample was found to be low titer positive. Each data point represents the S/B ratio for one individual serum sample.

from one clinical study that measured positive for anti-drug X antibodies were analyzed for anti-drug X IgE isotype antibodies using the validated ECL IgE isotype assay. For that clinical study, 735 samples were tested for anti-drug X antibodies. Of the samples tested, 4 samples confirmed positive for anti-drug X antibodies. Subsequently, these four samples were measured for anti-drug X IgE isotype antibodies. Only one sample was shown to have low titer for IgE antibodies against drug X (Fig. 8; 1:30, the minimal required dilution in the IgE titration assay).

4. Discussion

Biopharmaceuticals carry potential risks of adverse events [23,24] such as hypersensitivity reactions. Different mechanisms have been proposed that may contribute to drug-related hypersensitivity reactions, including IgE-mediated [25] and non-IgE-mediated reactions [26]. Since these reactions can in turn pose a potential hurdle for drug development, there is a compelling need to properly characterize the drug related hypersensitivity reactions to understand the risks.

The assay development for drug-specific IgE antibody can be technically challenging, mainly due to the following reasons [21]. First, an IgE ADA positive control is difficult to obtain, and it is often unavailable when the drug is at the early clinical development stage. Second, the assay requires high sensitivity due to low IgE levels in the circulation. Finally, high drug levels can interfere with the assay depending on the time of sample collection relative to dosing.

For bioanalytical assays, appropriate controls are important to include in each run to track variability of the assay [27], sample stability and acceptance/rejection criteria of runs. It is widely accepted that a surrogate positive control for the ADA assay is very useful for characterizing the specificity of the assay and monitoring the systematic performance of the assay, such as precision and robustness. Affinity purified polyclonal antibodies generated in animals are generally the preferred surrogate control for the monitoring ADA assay performance. However, the generation of a polyclonal anti-drug X of the IgE isotype is not easily accomplishable. One possible approach might be to try induce anti-drug X IgE antibodies in a human IgE transgenic mouse line. However, the likelihood of success, as well as, the ability to generate a large and reproducible supply of the critical control reagent is uncertain. In order to overcome the technical challenges for IgE ADA assay development, we generated a chimeric, drug-specific IgE positive control. Recombinant DNA technology was successfully employed through the grafting of the variable regions of a mouse drug-specific mAb into a human IgE expression vector to create a chimeric mAb. The resultant chimeric IgE control mAb specifically bound to drug X (Fig. 2b). The comparable binding affinities of the chimeric IgE control mAb

to drug X ($K_D = 0.792$ nM) and drug Y ($K_D = 1.678$ nM) demonstrated that the newly generated mAb was an appropriate positive control for the evaluation of possible IgE responses against two generations of drug. A serum pool was used as a standard curve (with IgE antibody calibrated relative to the World Health Organization International standard) to measure IgE antibody to purified allergens [19]. Since our chimeric IgE control mAb is generated through recombinant DNA technology, it displays some advantages, such as lot-to-lot consistency and speed of generation of an appropriate control provided a drug-specific mAb is available.

Circulating drug levels could potentially interfere with the detection of drug-specific antibodies, and this is particularly challenging for the detection drug-specific IgE since the level of IgE antibodies in blood is very low compared with other isotypes of immunoglobulin [19]. Different approaches have been taken into account to overcome drug interference with the assays, such as sample pre-treatment with acid to disassociate the drug and ADA complex [28]. The approach we took was to employ ruthenylated omalizumab as a detection antibody in conjunction with biotinylated drug capture. The assay format (biotinylated drug/ruthenylated drug) is often used for ADA assays, in which both arms of ADA bind to labeled drugs and form a bridge. Omalizumab is a mAb that specifically binds to Fc portion of IgE, thus (depending on the amount of free drug) leaving one arm of IgE antibody available for binding with the unlabeled drug (Fig. 1a). Our drug tolerance data has shown that increasing concentrations of drug X interfered with the ability to detect chimeric IgE ADA in a dose-dependent manner (Fig. 5a). Furthermore, chimeric IgE ADA at 250 ng/mL (S/B ratio 16) can be detected in the presence of 300 μ g/mL of drug X in the assay with omalizumab as the detection antibody (Figs. 1a and 5a). It is worth noting that 250 ng/mL of chimeric IgE ADA generated an S/B ratio 16, which was significantly above assay cut point factor (1.13). The high drug tolerance of the assay was confirmed during validation, in which chimeric IgE ADA levels as low as 125 ng/mL were still detectable in the presence of drug X at all levels, up to 300 μ g/mL (data not shown). Our data clearly demonstrated that omalizumab as the detection antibody offers greater drug tolerance as compared to a standard ADA assay (Figs. 1b and 5b) despite the fact that the ADA assay is not specific for an IgE ADA.

It should be noted; however, that 125 ng/mL of IgE antibody is still a high amount of antibody of that isotype. Thus, in working with IgE ADA detection assays, it will be important to determine concentrations of drug where much lower levels of IgE ADA can be detected [29,30]. Similar to other ADA assays, the best sensitivity will be achieved in the absence of drug. In contrast to the long half-life of IgG antibodies, IgE antibodies have been reported to have shorter half-life [30]. Thus, the balance of sensitive detection with drug tolerance will be particularly important to consider in developing and using assays for detecting of IgE ADA. Our assay achieved sensitivity as low as 200 pg/mL (Fig. 4) in the absence of drug X.

The assay with omalizumab as the detection antibody displays excellent drug tolerance, specificity, high sensitivity, and the labeled omalizumab was also stable for 2.5 months at 2–8 °C and –60 °C to –80 °C, respectively (Table 3). Moreover, the two lots of labeled omalizumab showed comparable performance. All these data together support that omalizumab is an acceptable reagent for the detection of drug-specific IgE.

Like a standard ADA assay, the assay cut point factor for the IgE ADA assay was determined in a similar way to that established for the screening assay [31,32]. The CPF for our assay determined in NHS samples, was 1.13. The equivalent CPFs from the two populations allowed us to use NHS as a surrogate for serum samples from the targeted population during assay development, pre-study assay validation and subsequent sample analysis.

Interference of matrix on our assay performance was tested using serum samples from a healthy population. The results showed that there was no matrix interference. In addition, assay precision for HQC, LQC and NQC levels was determined to be less than 12%. The robustness of this method was evaluated during internal validation by 2 analysts performing the assays with multiple instruments over multiple days. The assay subsequently performed well in a variety of environments with successful validation at multiple laboratories and had sustained performance through use in clinical studies (data not shown).

In conclusion, we have developed, qualified and validated an IgE ADA assay for the detection of anti-drug X IgE ADA in human serum. The described technique for the generation of a chimeric anti-drug specific IgE mAb positive control and utilization of omalizumab as a detection reagent in the assay illustrates a general approach that can be used for other biopharmaceuticals.

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