Journal of Immunological Methods xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

A simple and cost-effective assay for measuring anti-drug antibody in human patients treated with Adalimumab

Maohua Li[a,1], Hongzhen Li[b,1], Kai Gao[c], Muyang Wang[a], Wenqi An[d], Yaran Zhu[a], Li Ding[a], Lan Wang[c], Jingliang Gu[b], Conglin Zuo[b], Le Sun[a,*]

[a] AbMax Biotechnology Co., Ltd, Beijing, China
[b] Joinn Laborateries, Beijing, China
[c] National Institutes for Food and Drug Control, Beijing, China
[d] Hualan Genetic Engineering Co., Ltd, Xingxiang, Henan, China

ARTICLE INFO

Keywords:
Anti-tumor necrosis factor mAb (Adalimumab)
Anti-drug antibody (ADA)
Bridging ELISA assay
Anti-Adalimumab monkey pAb

ABSTRACT

It has been reported that 90% of the anti-drug antibody (ADA) to Adalimumab in human patients bound to the TNF-binding area, resulted in the annual loss of responses to Adalimumab up to 24%. It is of urgency to develop a cost-effective and easy-to-use ADA diagnostic kit for diagnosis of potential drug-resistance in patients treated with Adalimumab in clinic hospitals to avoid the tremendous economic and human costs to patients and health-care providers. In this study, we reported the generations of mouse monoclonal and monkey polyclonal antibodies against Adalimumab as assay standards and positive quality controls respectively. A Bridging ELISA assay was successfully developed with a limit of detection (LOD) between 22–80 ng/ml. The preliminary validation of assay was carried out first with 50 normal human sera, further validated by screening the ADA in 192 serum samples from monkeys treated with or without Adalimumab. Our data showed that the Bridging ELISA kit is very sensitive, highly specific and ready for study in human clinic trials.

1. Introduction

Human anti-tumor necrosis factor (TNF) monoclonal antibody (Adalimumab) is very effective in treatment of Rheumatoid Arthritis, Psoriasis and many other inflammatory human diseases (Weinblatt et al., 2003). However, it has been reported that the annual loss of response to Adalimumab in some diseases was estimated up to 24% (Billioud et al., 2011) and the anti-drug antibody (ADA) was considered as the cause of treatment failures (Ben-Horin et al., 2011; Bartelds et al., 2010; Garces et al., 2012; Van Schouwenburg et al., 2014). Recently, it has been confirmed that 90% of the ADA developed in patients treated with Adalimumab bound to the TNF-binding site of Adalimumab (vanSchie et al., 2015), further proven that ADA is the main cause of drug resistance. Therefore, the appearance of detectable ADA in patients treated with Adalimumab could be an early indication of drug-resistance prior to significant progression or recurrence of diseases.

In addition to Adalimumab, there are three more anti-TNF antibody drugs for treatment of autoimmune diseases. Early detection of ADA-induced drug resistance to Adalimumab can give the physicians the opportunities to prevent the recurrences of diseases by switching to different anti-TNF antibodies, a great benefit to the patients. FDA points out that early detection of ADA can avoid tremendous economic and human costs to patients and health-care providers.

During the clinic trial of Adalimumab and thereafter, a radio-immunoassay was employed to detect the ADA. With a sensitivity of 170 ng/ml, ADAs were detected only in 28% of the patients treated with Adalimumab in a 156-week follow-up study (Bartelds et al., 2011). In the new FDA guideline (U.S. Department of Health and Human Services, 2016), it requires the detection sensitivity of ADA must be $\leq$ 100 ng/ml. Using Meso Scale Discovery (MSD) system, Amgen's group improved the detection sensitivity to 20–40 ng/ml for ADA to Adalimumab. ADAs to Adalimumab were detected in 68–75% of the 263 Psoriasis patients treated with either Adalimumab or Adalimumab's bio-similar, much higher than previous reported rates. Clearly, ADA to Adalimumab posts a much great problem and routine examination of ADA during the treatment is of importance.

MSD-based assay system requires the equipment costing hundred thousand dollars and its consumables also are very expensive, making it beyond the reach of most clinic laboratories all over the world. In this paper, we reported the establishment of a very simple and cost-effective

* Corresponding author at: AbMax Biotechnology Co., Ltd, 99 Ke chuang 14th St, BDA, Beijing 101111, China.
E-mail address: sunl@antibodychina.com (L. Sun).
[1] The first two authors, Maohua Li and Hongzhen Li, contributed equally to this paper.

http://dx.doi.org/10.1016/j.jim.2017.10.003
Received 3 July 2017; Received in revised form 25 September 2017; Accepted 12 October 2017

Bridging ELISA assay for detection of ADA against Adalimumab.

We successfully developed mouse monoclonal antibody against the Adalimumab as the standard and Cynomolgus monkey polyclonal antibodies as the positive quality controls. The limit of detection (LOD) was determined to be 22–80 ng/ml of ADA, meeting the requirement of ≤ 100 ng/ml in new FDA guideline (U.S. Department of Health and Human Services, 2016). The application of the assay kits for human clinical study was further validated by screening the ADAs in 192 serum samples collected from 32 monkeys in pre-clinical study of Adalimumab bio-similar. The ADA-positive rate from the Adalimumab-treated monkeys was 50.0%, in great agreement with the finding of 43–51% positive rates in healthy human subjects reported by Amegn's group in Phase I clinic trials. The assay kit is based on colorimetric reaction, takes only 3 h and can be carried out in any clinical laboratories at very reasonable cost. Further study with human clinic samples soon will be carried out.

2. Materials and methods

2.1. Materials

Adalimumab bio-similars were obtained from several Chinese Biopharmaceuticals. Freund's complete adjuvant (CFA), Freund's incomplete adjuvant (IFA), Polyethylene glycol 4000 (PEG4000), DMSO, TMB substrates were purchased from SIGMA, USA. 4–6 weeks-old Female BALB/c mice were obtained from Vital River Co., Beijing, China. Cynomolgus monkeys were hosted at Ke rui wei de Biotech., Beijing, China. DMEM and FBS were from HyClone, USA. Goat anti-mouse IgG Fc secondary antibody is from Jackson Immun, USA. Horseradish peroxidase (HRP) conjugation kit was purchased from Pierce (IL, USA).

2.2. Generation of mouse monoclonal and monkey polyclonal antibodies

As previously described(Li et al., 2009), 4–6 weeks-old Female BALB/c mice or 10 months-old Cynomolgus monkeys were first immunized with Adalimumab in Complete Freund's Adjuvant and boosted with Adalimumab in Incomplete Freund's Adjuvant. Two to four weeks after the first immunization, bleeds were tested for titers by either indirect or Bridging Enzyme-linked Immunoassay (ELISA).

The spleenocytes from the mice with good titers were fused with the mouse myeloma cell line SP2/0. Culture supernatants from individual hybridoma clones were screened by ELISA. To produce antibodies, the hybridoma clones were seeded in stationary bioreactors in DMEM plus 10% low-IgG FBS. The bioreactor fluids were collected every 3 days, and IgG fractions were affinity-purified using protein G agarose columns (GE). The concentrations of purified IgGs were determined by their absorbance at 280 nm.

The monkey bloods were collected from the veins on the legs and the sera were prepared and stored at − 20 °C prior to test (Mi et al., 2016).

2.3. Indirect ELISA

Each well of 96-well high binding EIA plates was coated with 100 ng/well of antigen, such as Adalimumab, overnight at 4 °C in PBS. After two washes with PBS and blocking with 5% skim-milk/PBS for 1 h at room temperature, wells were incubated with either the sera or the culture supernatants or purified mAb in 5% skim-milk-PBS for another 1 h at room temperature. After two washes with PBS, wells were then incubated with HRP-conjugated goat anti-mouse IgG Fc-specific secondary antibodies (Jackson Lab) in 5% skim-milk-PBS for 1 h at room temperature. After five washes with PBS plus 0.1% Tween20 (PBST), HRP substrate 3, 3′, 5, 5′-tetramethylbenzidine (TMB) solution was added. The reaction was stopped with stop solution (0.1 M H_2SO_4) after 30 min and absorbance was measured at 450 nm with a microplate reader.

2.4. Bridging ELISA

Adalimumab bio-similar was conjugated with horseradish peroxidase according to the manufacturer's instructions (IL, USA). The specific activities of the conjugated antibodies were determined by the ratio of OD_{430}/OD_{280}.

Followed the protocols described previously(Ma et al., 2014), each well of 96-well high-binding ELISA plates was coated with Adalimumab overnight at 4 °C in 0.05 M Na_2CO_3, pH 9.6. After two washes with PBS, the plates were blocked with 3% bovine serum albumin (BSA) in PBS, then air-dried and sealed in a plastic bag. Monkey sera were diluted in 20% Calf serum in PBS and incubated in the wells for 1 h at room temperature. After two washes, wells were probed with the HRP-conjugated Adalimumab diluted in 3% BSA-PBS for another hour. After five washes with PBS-Tween20 (PBST), chromogenic HRP substrate 3, 3′, 5, 5′-tetramethylbenzidine (TMB) was added to the wells. The reaction was stopped with Stop solution (0.1 M H_2SO_4) after 30 min incubation at room temperature and absorbance was measured at 450 nm with a plate reader.

3. Results

3.1. Generation of mouse monoclonal antibodies against Adalimumab

Five BALB/c mice were immunized with full length Adalimumab with the help of adjuvant to generate mouse monoclonal antibody against the variable region of Adalimumab.

On day 14, the tail bleeds were collected from the immunized mice and the titers of the sera against Adalimumab were examined using indirect ELISA. Using a Goat-anti-mouse IgG Fc-specific secondary antibodies as detecting reagents, very strong immune responses (IgG titers > 1:5000) were observed in 4 of the 5 mice (80%).

Two Adalimumab-immunized mice were scarified at two different days and the spleenocytes from them were used to fuse with mouse myoloma SP2/0 cells to generate hybridoma cell lines. Total about 1400 monoclonal hybridoma cell cultures were first screened against Adalimumab coated on 96-well plates, and 152 clones (10.8%) of screened were found producing anti-Adalimumab monoclonal IgGs (defined as the signals were two-time higher than the background).

When we double-screened the Adalimumab monoclonal antibodies against Adalimumab and normal human IgGs respectively, it was found that 52.8% (79/152) of mouse monoclonals were Adalimumab-specific (example data shown in Table 1).

In the proceeding study, mouse monoclonal anti-Adalimumab Clone 11D5 was chosen as the ADA standard for Adalimumab ADA measurement since it has the reasonable yield in antibody production with high affinity.

Table 1
Specificity test of mouse anti-Adalimumab mAbs.

Coating	Clone						
	11D5	7G11	2A2	2B4	5A5	5E11	7B2
Adalimumab	> 3.5	> 3.5	> 3.5	> 3.5	> 3.5	> 3.5	> 3.5
Human IgG	0.076	0.342	0.061	0.089	0.063	0.07	0.243

The values are expressed as mean calculated from duplicate samples. Each well was coated with 100 ng of either Adalimumab or normal human IgGs, and then incubated with the culture supernatants diluted in 3% BSA-PBS. After washes, wells were then probed with HRP-conjugated Goat anti-Mouse IgG (Fc 1 + 2a + 2b + 3). HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

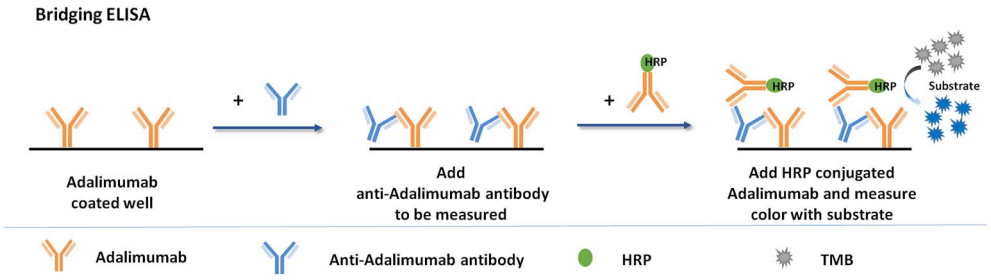

Fig. 1. A brief description of bridging ELISA.(1) Plate is coated with Adalimumab; (2) sample is added, and any anti-Adalimumab antibody present binds to Adalimumab; (3) enzyme-linked Adalimumab is added, and binds to anti-Adalimumab antibody; (4) substrate is added, and is converted by enzyme to detectable form.

3.2. Development of Bridging ELISA assay for screening of anti-Adalimumab ADA

In consideration of throughput, sensitivity and cost of the assay, together with our previous experience (Mi et al., 2016), a Bridging ELISA was chosen for detection of anti-Adalimumab antibodies. Briefly, Adalimumab is used to coat the surface of the wells of 96-well plates, Adalimumab ADA containing samples are allowed to react with coated Adalimumab, and binding of ADA is detected by adding a HRP-labeled Adalimumab (Fig. 1).

To develop the Bridging ELISA, 4 different coating concentrations of Adalimumab, 2 different sample dilution buffers, 4 different concentrations of HRP-conjugated Adalimumab, various washing buffers and procedures have been tested. The best manufacturing and testing key parameters obtained are 1) coating with 200 ng/well of Adalimumab, 2) diluting the samples with 20% Calf serum (CS) in PBS, 3) detecting with 0.2 μg/ml of HRP-conjugated Adalimumab, and 4) final washing with PBS-Tween. The standards were prepared by spiking various amounts of anti-Adalimumab mAb 11D5 (2500, 1250, 625, 312.5, 156, 78, 39, 20, 10, 0 ng/ml) in negative control sera which was prepared by pooling normal human sera from 50 different individuals, then 1:100 diluted with 20% CS-PBS prior to test. As shown in Fig. 2, as plotted using 4-P Fit method, the LOD of ADA in the undiluted human serum samples was 55.8 ng/ml with a linear range between 78–2500 ng/ml, and R is 0.999. The sensitivity of the screening assay meets the new FDA requirement.

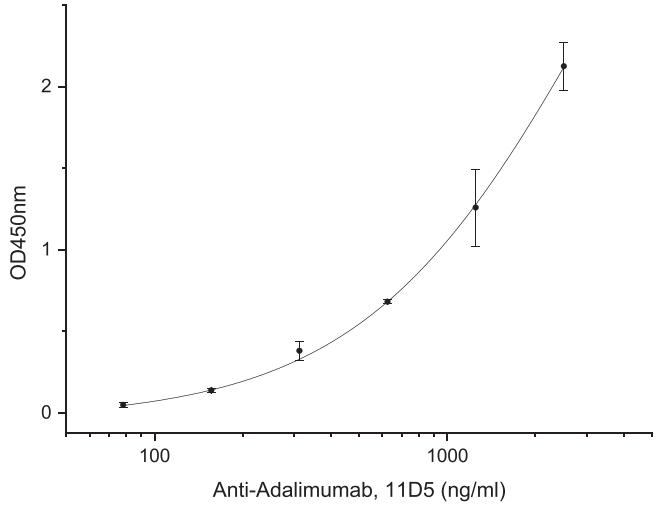

Fig. 2. Stand curve of bridging ELISA with anti-Adalimumab. For each well, 100 μl of 1:100 diluted testing samples containing 0–2500 ng/ml of mAb 11D5 were added and incubated for 1 h. After washes, wells were then probed with HRP-conjugated Adalimumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader. Each point is the average of two readings. The LOD was defined as being the mean of the negative control (0 ng/ml) plus three times the standard deviation. Result was fitted using 4-P Fit method with the equation: $y = (A - D) / (1 + (x / C)^{B}) + D$, whereas $A = -0.04324$, $B = 5.06855$, $C = 3337.33355$, $D = 1.07435$. R was 0.999.

3.3. Determination of minimal required dilution (MRD)

There is a need to dilute testing human sera since matrix components, especially the host antibodies, can contribute to high assay background if undiluted. However, it is also recommended by FDA that the dilution should not exceed 1:100 to preserve the sensitivity (U.S. Department of Health and Human Services, 2016).

At first, a panel of 6 individual normal human sera was tested at various dilutions to experimentally determine the assay's MRD. A pool of the 50 individual normal human sera was made as the negative control. As shown in Table 2, all of the six normal sera at 1:40 dilutions had readings below 0.200 so the assay's MRD was determined to be 1:40.

Since it has been reported that the C_{max} of Adalimumab in patients was between 9 and 16 μg/ml (Bartelds et al., 2011), proceeding studies were carried out at 1:100 dilutions to minimize the potential interference of residual Adalimumab in the testing materials during the assay.

3.4. Development of ADA-positive Cynomolgus monkey sera against Adalimumab

In this study, we did not have the ADA-positive sera from Adalimumab treated-patients, so we immunized one Cynomolgus monkey with full length of Adalimumab to generate positive polyclonal control.

Four weeks after the first immunization, the titer of the serum was examined by the Bridging ELISA established above. As shown in Table 3, the monkey anti-Adalimumab titer reached1:300 (defined as the reading of OD450 twice of the negative control).

Three dilutions (1:7.5, 1:60, 1:300) of the monkey serum with human negative control serum were prepared as the quality controls (QC1, QC2 and QC3), representing high, medium, and low values in the anti-Adalimumab ADA screening assay. They will be used in pre-study validation and patient sample testing to ensure the assay is operating within desired assay ranges at the time the assays are performed.

3.5. Preliminary validation of screening assay of anti-Adalimumab

Assay preliminary validation included but not limited to 1) sensitivity, 2) cut-point.

To fulfil the requirements, more than two batches of the Anti-Adalimumab ADA Screening Bridging ELISA kits were manufactured at different times.

3.5.1. Validation of sensitivity

The Screening assays, also known as binding antibody (BAb) assays, are used to detect all antibodies that bind to the therapeutic protein product. Assay sensitivity represents the lowest concentration at which the antibody preparation consistently produces either a positive result or readout equal to the cut point determined for that particular assay. FDA recommends that screening ADA assays achieve a sensitivity of 100 ng/ml or less.

Assay sensitivity should be determined by testing serial dilutions of

Table 2
Determination of MRD.

Dilution	OD reading						
	1#	12#	23#	32#	45#	50#	Negative control
1:10	0.101 ± 0.004	0.112 ± 0.007	0.120 ± 0.031	0.102 ± 0.005	0.120 ± 0.001	0.250 ± 0.006	0.203 ± 0.017
1:20	0.126 ± 0.003	0.133 ± 0.006	0.139 ± 0.012	0.129 ± 0.006	0.132 ± 0.001	0.200 ± 0.000	0.197 ± 0.012
1:40	0.125 ± 0.008	0.126 ± 0.013	0.121 ± 0.004	0.120 ± 0.005	0.126 ± 0.001	0.173 ± 0.004	0.169 ± 0.011
1:80	0.124 ± 0.026	0.117 ± 0.025	0.089 ± 0.008	0.084 ± 0.003	0.097 ± 0.005	0.109 ± 0.006	0.130 ± 0.021
1:100	0.141 ± 0.008	0.124 ± 0.006	0.145 ± 0.001	0.138 ± 0.008	0.144 ± 0.014	0.145 ± 0.010	0.159 ± 0.008

The values are expressed as mean ± SD, calculated from duplicate samples. 100 µl of the normal human sera at various dilutions were added to the wells coated with Adalimumab and incubated for 1 h. After washes, wells were then probed with HRP-conjugated Adalimumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

Table 3
Establishment of Cynomolgus monkey polyclonal positive controls.

Serum dilution	OD reading
	Monkey serum
1:7.5	2.018 ± 0.218
1:30	1.070 ± 0.052
1:60	0.816 ± 0.005
1:300	0.248 ± 0.015
1:600	0.212 ± 0.016
1:1500	0.143 ± 0.008
Pre-bleed	0.1325 ± 0.022

The values are expressed as mean ± SD, calculated from duplicate samples. For each well of the plate coated with Adalimumab, 100 µl of the monkey sera at various dilutions were added and incubated for 1 h. After washes, wells were then probed with 0.2 µg/ml of HRP-conjugated Adalimumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

Table 4
Validation of the sensitivity of the assay.

	Batch 1/ Tech1	Batch 2/ Tech1	Batch 2/ Tech2	Batch 2/ Tech3
LOD (ng/ml)	22	36	80	68
Assay range (ng/ml)	78–2500	78–2500	78–2500	78–2500
R	0.9957	0.9983	0.9889	0.9879
%CV	15.80	15.78	12.82	19.01

100 µl of the 1:100 diluted ADA standards were added to the wells coated with Adalimumab and incubated for 1 h. After washes, wells were then probed with 0.2 µg/ml of HRP-conjugated Adalimumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

a positive control antibody of known concentration in pooled negative control matrix. In this study, ADA standards were prepared by adding various amounts of mAb 11D5 (0–2500 ng/ml) to the pooled human negative control sera. All of the samples were further diluted 100-fold with 20% CS-PBS prior to test. The validation of the assay sensitivities were carried out by three different technicians at three different days using the kits from two different lots. As shown in Table 4, the LODs of the assays determined by three tests were between 22–80 ng/ml of ADA and the assay ranges were from 78 to 2500 ng/ml. The sensitivity of this screening assay meets the requirement of the 2016 FDA guideline.

3.5.2. Validation of cut-points

The cut point of the assay is the level of response of the assay that defines the sample response as positive or negative. The cut point should be statistically determined using samples from treatment-naïve subjects. In this report, 50 individual normal human sera, one human negative control serum, and three monkey positive controls were first diluted by 100-fold using 20% CS-PBS, examined using the Bridging ELISA. The validations were performed on two different days by two different technicians. As shown in Fig. 3, the cut-points were 0.111 and 0.122 respectively. FDA recommends that when the mean varies between assays, plates, or analysts but the variance around the mean is constant, a normalization factor can be statistically determined and applied in-study. This is also known as a floating cut point. The floating factor for the cut-point is calculated by the cut-point readings divided by the NC readings. For this assay, the floating factor for cut-point is determined to be 1.325.

3.6. Drug-tolerance of assay

It has been reported that the serum concentrations of Adalimumab in patients were between 3 and 16 µg/ml (Bartelds et al., 2011). Adalimumab presented in the serum will interfere with the sensitivity of the assay. Specifically, complexes formed between ADA and Adalimumab, also called ADA-drug complexes, that prevents the detection of ADA in the test format. The assessment of assay sensitivity in the presence of the expected levels of interfering Adalimumab, also known as the assay's drug tolerance, is critical to understanding the suitability of the method for detecting ADA in dosed patients.

To examine drug tolerance of this assay, we deliberately added 100 ng/ml of mAb 11D5 to the human negative control serum in the absence or presence of different quantities of Adalimumab (0, 0.03, 0.1, 0.3, 1, 3, 10, 30 µg/ml) under consideration and determining quantitatively whether the therapeutic protein product interferes with ADA detection. As shown in Fig. 4, even in the presence of 10 µg/ml of Adalimumab, the OD reading of the samples was still above the cut-point, suggesting the drug-tolerance of the assay is 10 µg/ml. It is enough to detect 100 ng/ml of ADA in most of the patients treated with Adalimumab.

3.7. Screening of ADA in Adalimumab-treated monkeys

In the pre-clinical study, 32 monkeys were separated in four groups and injected with four escalating doses of Adalimumab (0, 17.5, 35, and 70 mg/kg) for 13 times over 12 weeks of time period. Total of 192 sera were collected at various time points. The samples were examined for Adalimumab ADA using the assay developed above. In the assay, two QC positive controls (High and Low) and one negative control were included on each 96-well plate. The average reading for QC1 was 2.972 and for QC3 was 0.312. Using the floating factor of 1.325, cut-points for every plate were individually determined by timing the average reading of NC readings by the floating factor.

As shown in Table 5, two out of 48 monkey serum samples collected from the Control group were positive for ADA. The false positive rate was calculated to be 4.2%, close to 5% false positive rate recommended by FDA.

For monkeys subjected with Adalimumab treatment, the ADA-

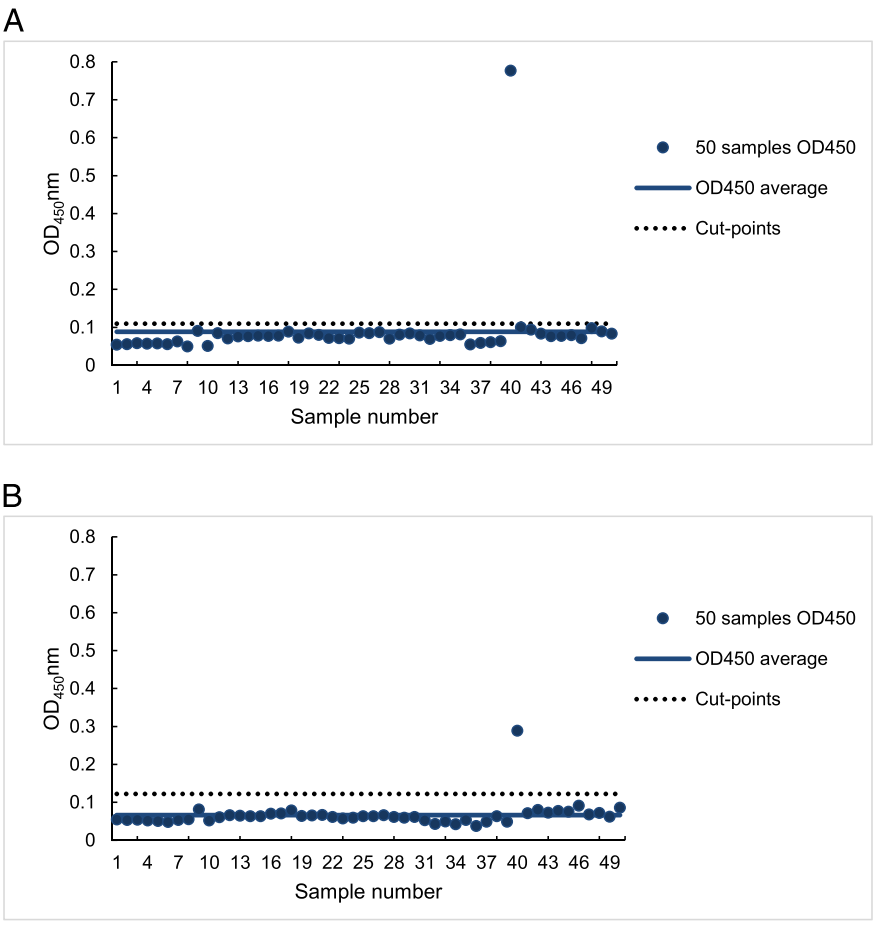

Fig. 3. Results of 50 individual normal human sera sample tested in triplication with bridging assay performed on two different days (A and B). For each well of the plate coated with Adalimumab, 100 µl of 50 individual normal human sera at 1:100 dilutions were added and incubated for 1 h. After washes, wells were then probed with HRP-conjugated Adalimumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader. Each sample was tested in duplicate. The average OD450nm readings were 0.088 (A) and 0.066 (B). The standard deviations were 0.013 (A) and 0.034 (B). Cut-points were 0.111 (A) and 0.122 (B).

positive rates of all three dosing groups reached the peaks on week 2 after the first injection. 50% of the sera from High-dose group tested positive and 37.5% for both Medium- and Low-dose groups. From week 2 to week 10, the ADA-positive rates dropped rather quickly in the High-dose group, and we think that it may be due to the accumulation of Adalimumab in the bloods exceeding the 10 µg/ml of drug-tolerance, led to the false-negative in the ADA assay.

In this study, 6 collections of bleeds from the 24 monkeys treated with Adalimumab were examined for ADA, and 11 Adalimumab-treated monkeys showed ADA-positive at least once. The overall ADA-positive rate was 45.8% of the Adalimumab-treated monkeys. In a recent Phase I human clinic studies of Humira and its bio-similar, Amgen's group reported that the ADAs were detected in 43–51% of the healthy human subjects in three different groups. Clearly, the data from our monkey

Table 5
Screening of ADA from the monkeys treated with Adalimumab bio-similar.

	Control	Low (17.5 mg/kg)	Medium (35 mg/kg)	High (70 mg/kg)	Rate
Prior	0/8	0/8	2/8	0/8	8.3%
Week 2	1/8	3/8	3/8	4/8	41.6%
Week 4	1/8	3/8	3/8	3/8	37.5%
Week 6	0/8	2/8	3/8	1/8	25.0%
Week 8	0/8	2/8	2/8	1/8	20.8%
Week 10	0/8	2/8	2/8	0/8	16.6%
Overall	4.2%	25.0%	29.2%	18.7%	

32 monkeys were treated with escalating doses of Adalimumab between blank (0), low dose (17.5 mg/kg), medium dose (35 mg/kg), and high dose (70 mg/kg) for 13 times over 13 weeks of time period. Sera samples were collected at seven time points.

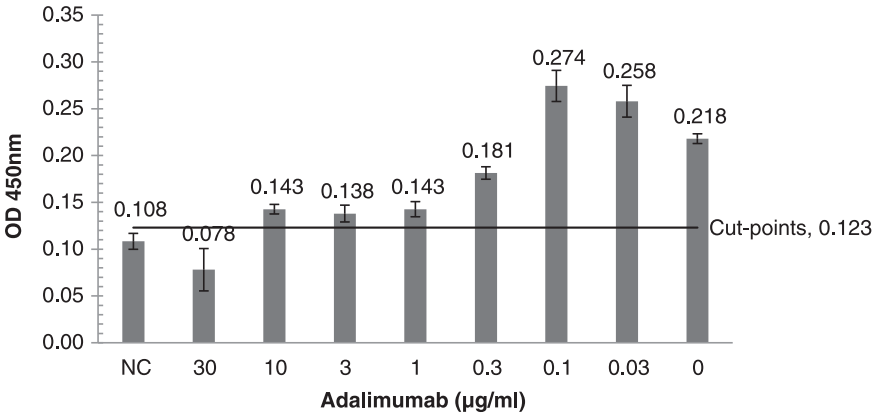

Fig. 4. Drug tolerance of the assay. Various amounts (0–30 µg/ml) of Adalimumab were added to the human negative serum spiked with 100 ng/ml of mAb 11D5. After 5 min incubation, the samples were diluted by 100-fold with 20% CS-PBS, then 100 µl of each was transferred to each well of the Adalimumab-coated plate and incubated for 1 h. After washes, wells were then probed with HRP-conjugated Adalimumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

Table 6
Confirmation of anti-Adalimumab ADA.

	#813 W6		#815 W10		#822 W4		#828 W8	
Adalimumab (500 μg/ml)	−	+	−	+	−	+	−	+
OD reading	> 3.5	2.408	1.365	0.111	0.573	0.176	0.507	0.125
Inhibition				92%		70%		75%

The sera were pre-incubated with or without 500 μg/ml of Adalimumab for 15 mins. 100 μl of 1:100 diluted samples were added and incubated for 1 h. After washes, wells were then probed with 0.2 μg/ml of HRP-conjugated Adalimumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

Table 7
Determination of the titers of Adalimumab ADA.

	Un-diluted	1:5	1:10	1:50	1:100	1:500
#813-W6	> 3.5	> 3.5	> 3.5	2.477	1.897	0.475
#815-W10	1.6	0.726	0.567	0.315	0.212	0.187

ADA-positive monkey sera were first diluted with normal human sera by 5–500 folds, then further diluted with the assay buffer (20% CS-PBS) by 100-fold. 100 μl of the 20% CS-PBS-diluted samples were added to each well of the 96-well plates. After one-hour's incubation, wells were then probed with 0.2 μg/ml of HRP-conjugated Adalimumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

study matched very well with the one from healthy human subjects.

3.8. Confirmation and titering of anti-Adalimumab ADA in Adalimumab-treated monkeys

To confirm the presence of true ADA to Adalimumab, four monkey serum samples (#813-W6 and #815-W10 from low-dose group, #822-W4 from Medium-dose one, and #828-W8 from High-dose one) were first pre-incubated with or without Adalimumab (500 μg/ml), then tested for ADA. As shown in Table 6, for Monkey #813-W6, the ADA signals was partially inhibited with 500 μg/ml of Adalimumab, indicts that there are > 500 μg/ml of ADA existed in the serum. For the other three serum samples, all of the signals were inhibited. The data shows that the ADAs detected by this assay are truly against Adalimumab.

Two of the ADA-positive samples were further examined for their titers, defined as the maximal dilution where a sample still gives a reading just above the cut-point. As shown in Table 7, #813-W6 had a titer > 1:500, whereas #815-W10 had a titer about 1:100.

ADA was also detected in four human clinic samples from the patients treated with Adalimumab using the assay developed here (data not shown). A large scale study with more human patient samples will be carried out soon for predicting the up-coming of drug-resistance to Adalimumab in the patients.

4. Discussion

Today, millions of patients in the world have being treated with Adalimumab. At the same time, up to 75% of the patients treated with Adalimumab in Phase III clinic trials develop ADA against the drug and eventually could lead to the loss of response to the drug. It also has been shown that 67% of the anti-Adalimumab positive patients developed the ADA during the first 28 weeks of therapy, but all of them still been treated with Adalimumab till the regression of diseases became noticeable (Bartelds et al., 2011). It not only wastes huge amount of medical expenses but also denied the patients the opportunities to switch to different treatments for better efficacy.

Methods for measuring anti-Adalimumab ADA used in most of the previous studies were radio immunoassays (Bartelds et al., 2007; Van Schouwenburg et al., 2010; Bartelds et al., 2011). The detection sensitivity was about 170 ng/ml, not sensitive enough to identify the ADA-positive patients. The usage of radio activity also restricted the application in general clinical laboratories. Using Meso Scale Discovery (MSD) system, Amgen's group improved the LOD to 20–40 ng/ml, and showed that up to 75% of the patients developed Adalimumab ADA in Phase III clinic trial. But both the equipment and materials are extremely expensive, making it beyond the reach of most clinic laboratories all over the world.

The Bridging ELISA assay kit for Adalimumab ADA we developed is based on colorimetric reaction, the LOD is between 22 and 80 ng/ml, sensitive enough to meet the new requirement of FDA guideline. It is very simple and cost-effective, comparing to both Radioimmunoassay and MSD. Most importantly, it can be carried out in any clinical laboratories.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

This work was supported in part by grants for Small Business Research from Chinese Ministry of Science and Technology (2014 14C2621110037, to L. Sun) and Beijing Science and Technology Commission (2013 Z131102000113051, to L. Sun).

References

Bartelds, G.M., Wijbrandts, C.A., Nurmohamed, M.T., et al., 2007. Clinical response to adalimumab: relationship to anti-adalimumab antibodies and serum adalimumab concentrations in rheumatoid arthritis. Ann. Rheum. Dis. 66 (7), 921–926.

Bartelds, G.M., de Groot, E., Numohamed, M.T., et al., 2010. Surprising negative association between IgG1 allotype disparity and anti-adalimumab formation: a cohort study. Arthritis Res. Ther. 12, R221.

Bartelds, G.M., Krieckaert, C.L., Nurmohamed, M.T., van Schouwenburg, P.A., Twisk, W.F., Dijkmans, B.A., Aarden, L., Wolbink, G.J., 2011. Development of antidrug antibodies against adalimumab and association with disease activity and treatment failure during long-term follow-up. JAMA 305 (14), 1460–1468.

Ben-Horin, S., Yavzori, M., Katz, L., et al., 2011. The immunogenic part of infliximab is the F(ab)2, but measuring antibodies to the intact infliximab molecule is more clinically useful. Gut 60, 41–48.

Billioud, V., Sandborn, W.J., Peyrinbiroulet, L., 2011. Loss of response and need for adalimumab dose intensification in crohn's disease: a systematic review. Am. J. Gastroenterol. 106 (4), 674–684.

Garces, S., Demengeot, J., Benito-Garcia, E., 2012. The immunogenicity of anti-TNF therapy in immune-mediated inflammatory diseases: a systemic review of the literature with a meta-analysis. Ann. Rheum. Dis. 0, 6–9.

Li, X., Mao, C., Ma, S., Wang, X., Sun, Z., Yi, Y., Guo, M., Shen, X., Sun, L., Bi, S., 2009. Generation of neutralizing monoclonal antibodies against Enterovirus 71 using synthetic peptides. Biochem. Biophys. Res. Commun. 390, 1126–1128.

Ma, S., Mao, Q., Liang, Z., Zhang, C., Yang, W., Sun, Z., Zhang, H., Shen, X., Bi, S., Sun, L., 2014. Development of a sandwich ELISA for the quantification of Enterovirus 71. Cytotechnology 66, 413–418.

Mi, L., Li, W., Li, M.H., Chen, T., Wang, M.Y., Sun, L., Chen, Z.N., 2016. Immunogenicity screening assay development for a novel human-mouse chimeric anti-CD147 monoclonal antibody (Metuzumab). J. Immunol. Methods 433, 38–43.

U.S. Department of Health and Human Services, et al., 2016. Guidance for Industry, Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products. Available at. http://www.fda.gov/RegulatoryInformation/Guidances/default.htm.

Van Schouwenburg, P.A., Bartelds, G.M., Hart, M.H., Aardenn, L., Wolbink, G.J., Wouters, D., 2010. A novel method for the detection of antibodies to adalimumab in the presence of drug reveals “hidden” immunogenicity in rheumatoid arthritis patients. J. Immunol. Methods 362 (1–2), 82–88.

Van Schouwenburg, P.A., Kruithof, S., et al., 2014. Functional analysis of the anti-adalimumab response using patient-derived monoclonal antibodies. J. Biol. Chem. 289 (50), 34482–34488.

vanSchie, K.A., Hart, M.H., de Groot, E.R., Kruithof, S., Aarden, L.A., Wolbink, G.J., et al., 2015. The antibody response against human and chimeric anti-TNF therapeutic antibodies primarily targets the TNF binding region. Ann. Rheum. Dis. 74, 311–314.

Weinblatt, M.E., Keystone, E.C., Furst, D.E., et al., 2003. Adalimumab, a fully human anti-tumor necrosis factor alpha monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrxate: the ARMADA trial. Arthritis Rheum. 48, 35–45.