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Research paper

Immunogenicity screening assay development for a novel human-mouse chimeric anti-CD147 monoclonal antibody (Metuzumab)

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ABSTRACT

The clinical effect of patient immune responses to therapeutic antibodies affect product safety and efficacy, which makes the development of valid, sensitive immune assays a key aspect of antibody drug development. In this paper, we reported the generations of mouse monoclonal and Cynomolgus monkey polyclonal antibodies against the anti-CD147 antibody (Metuzumab) as the internal standards and the positive controls. Seven mouse monoclonal antibodies were shown to recognize both $(Fab)_2$ and full length of Metuzumab, but not the control normal human IgGs, and monoclonal anti-Metuzumab, Clone 2D9 was chosen to be used as the internal standard for anti-Metuzumab study. A Bridging ELISA assay was developed by coating the wells with the antibody drug, and the anti-drug antibody (ADA) in the animal sera were detected by enzyme-labeled antibody. Its limit of detection (LOD) was determined to be 0.39 ng/ml of anti-Metuzumab antibody (ADA) with linear range between 0.39–50 ng/ml and $R^2 = 0.994$. For normal monkey sera, a minimal dilution was determined to be 1:80. However, very different from peptide or other protein drugs, strong interferences from the residual antibody drugs were observed from most of the testing monkey sera in the preclinical study. It was experimentally determined that the concentration of the residual antibody drug in the assay have to be lower than 1 μg/ml, so the assays were carried out at 1:100 dilution of the monkey sera. In the pre-clinical study, 32 monkeys were treated with escalating doses of Metuzumab between 0, 10, 50, 200 mg/kg for 13 times over 13 weeks of time period. 16 of them were terminated right after the last injection, while the other 16 were rested for additional 4 weeks before termination. Afraid to miss any positive response to antibody drug, sera samples were collected at six time points, including 2-, 6- and 10-weeks post 1st dose, prior to last dose, and 2-, 4-weeks into recovery. The highest positive rates were seen with the Medium- and High-dose group 2-weeks post the first injection, 6 out 8 monkeys in the High-dose were positive for free ADA. However, no significant pathologic and clinic adversary effect was observed in those monkeys.

1. Introduction

CD147 is a highly glycosylated transmembrane protein and is involved in physiologic and pathologic tumorigenesis (Li et al., 2005; Nabeshima et al., 2006). Over-expression of CD147 has been observed in many epithelial malignancies including lung cancer, breast cancer and liver cancer (Yu et al., 2009; Sabine et al., 2006). A mouse anti-CD147 monoclonal antibody (HAb18) was generated by our group, and a ^{131}I-labeled HAb18 $F(ab')_2$ (Licartin) was successfully launched for treatment of patients with liver cancers (Chen et al., 2006; Xu et al., 2007). Although safe and tolerable, its immunogenicity resulted in the development of human-anti-mouse antibodies (HAMA) in a fraction of Licartin-treated patients (unpublished data). It led to the development of Metuzumab, an affinity-optimized and nonfucosylated human-mouse chimeric IgG1 monoclonal antibody with enhanced antibody-dependent cellular cytotoxicity (Jiang et al., 2004). Recently, it has been shown that Metuzumab is highly potent against lung cancer cells both in vitro and in vivo (Zhang Z, et al., 2015). In the same study, it was also shown that even with a single dose of 25 mg/kg or multiple doses of 5 mg/kg, Metuzumab do not induce any distinct or novel adverse effect in Cynomolgus monkeys (Zhang Z., et al., 2015).

The clinical effect of patient immune responses to therapeutic proteins has ranged from no effect at all to extreme harmful effects to patient health (Ross et al., 1990; Coutinho et al., 1995; Meide and Schellekens, 1997; Boes, 2001). The potential for such varied immune

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responses affects drug safety and efficacy. Because this range exists, clinicians rely on the immunogenicity section of the package labeling that contains immunogenicity rates observed during pre-clinical and clinical studies. This makes the development of valid, sensitive immune assays as a key aspect of product development (Mire-Sluis et al., 2004; Gupta et al., 2007; U.S. Department of Health and Human Services et al., 2009).

Because these assays are critical when immunogenicity poses a high-risk and real time data concerning patient responses are needed, it was recommended by US FDA to implement preliminary validated assays early (preclinical and phase 1). In this paper, we reported the generations of mouse monoclonal and monkey polyclonal antibodies against the Metuzumab as the internal standards and the positive controls, as well as the development of a Bridging ELISA assay for screening the anti-drug antibody (ADA) in the animal sera.

2. Materials and methods

2.1. Materials

Metuzumab was produced as reported earlier (Jiang et al., 2004). 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. 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 monkey polyclonal and mouse monoclonal antibodies

As previously described (Li et al., 2009), 4–6 weeks-old Female BALB/c mice or Cynomolgus monkeys were first immunized with Metuzumab in complete Freund's adjuvant and boosted 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 then 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.

2.3. Indirect ELISA

Each well of 96-well high binding EIA plates (Corning) was coated with 100 ng/well of antigen 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 one hour at room temperature. After two washes with PBS, wells were then incubated with HRP-conjugated goat anti-mouse IgG Fc-specific or goat anti-human IgG 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

Metuzumab was conjugated with horseradish peroxidase according to the manufacturer's instructions. The specific activities of the conjugated antibodies were determined by the ratio of OD430/OD280.

Followed the protocols described previously (Ma et al., 2014), each well of 96-well high-binding ELISA plates was coated with Metuzumab 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 3% BSA-PBS and incubated in the wells for 1 h at room temperature. After two washes, wells were probed with the HRP-conjugated Metuzumab diluted in 3% BSA-PBS for another hour. After five washes with PBS-Tween 20(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 Metuzumab

For therapeutic monoclonal antibodies, it is recommended by FDA to obtain mouse monoclonal antibody (mAb) against the variable regions of the antibody drugs. Such a positive control may be more relevant for the anticipated immune response in human patients where the response to humanized or fully human mAb is mainly to the variable regions.

To obtain mouse monoclonal antibodies against Metuzumab, three BALB/c mice were immunized with full length of Metuzumab. Two weeks later, the titers of the tail bleeds were examined by ELISA against both the full length and Fab of Metuzumab, and found that all of them show strong immune responses against the $(Fab)_2$ of Metuzumab (data not shown).

The spleenocytes from one of the mice were used to fuse with myoloma SP2/0 cells to generate hybridoma cell lines. Twenty days later, the culture supernatants of about 700 monoclonal hybridomas were double screened by ELISA against Metuzumab and normal human IgGs respectively, and only the Metuzumab-specific mouse monoclonals were selected for further expansion. As shown in Table 1, all of the seven selected monoclonals recognized both $(Fab)_2$ and full length of Metuzumab, but not the control normal human IgGs, indicates that those seven mouse mAbs all specifically bind to the variable regions of the Metuzumab. Most of the monoclonals, except clone 2A12, had higher readings against the $(Fab)_2$ than the full length Metuzumab since all of the wells were equally coated with 100 ng of antigens, and the molar concentration of $(Fab)_2$ was twice of the full length IgG. For clone 2A12, it is possible that the antigen epitope it recognized could be also used to bind to the surface of the wells. Monoclonal anti-Metuzumab, Clone 2D9, was chosen to be used as the internal standard for anti-Metuzumab study in the proceeding study.

3.2. Initial development of Bridging ELISA kit

Because of the size of pre-clinical and clinical trials and the necessity of testing samples at various time-points, a multi-tiered approach to the testing of patient sample was suggested by FDA (U.S. Department of Health and Human Services et al., 2009). A rapid and sensitive screening should initially be used to test the samples. Samples testing positive in the screening assay then will be subjected to a confirmatory assay.

There are many different formats of screening assays for anti-drug antibody, including indirect binding enzyme-linked immuno sorbent assay (ELISA), Bridging ELISA, radioimmunoprecipitation assays (RIPA), surface plasmon resonance (SPR), etc. Each assay has its advantages and disadvantages as far as rapidity of throughput, sensitivity, and availability of reagents (Boes, 2001; Mire-Sluis et al., 2004; Gupta et al., 2007; U.S. Department of Health and Human Services et al., 2009). But for humanized antibody drug, the indirect ELISA could not be used since there is no suitable anti-monkey or anti-human IgG conjugates which can distinguish the monkey or human anti-antibody drug

Table 1
Specificity test of mouse anti-Metuzumab.

Coating	Clone						
	1E7	1H10	2A12	6C6	2D9	2D11	3H3
Metuzumab $(Fab)_2$	0.899 ± 0.005	1.259 ± 0.012	0.661 ± 0.003	0.894 ± 0.01	>3.5	2.622 ± 0.016	1.276 ± 0.01
Metuzumab IgG	0.359 ± 0.002	0.665 ± 0.008	2.794 ± 0.016	0.344 ± 0.006	3.301 ± 0.127	1.921 ± 0.008	0.482 ± 0.007
Human control IgG	0.052 ± 0.004	0.036 ± 0.003	0.062 ± 0.009	0.046 ± 0.004	0.187 ± 0.005	0.054 ± 0.003	0.055 ± 0.001

The values are expressed as mean ± SD, average and standard deviation were calculated from duplicate samples.
Each well was coated with 100 ng of different antigens, then incubated with the culture supernatants 1:1 diluted in 3% BSA-PBS. After washes, wells were then probed with HRP-conjugated Goat anti-Mouse IgG (Fc-specific). HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

antibodies from the coated humanized antibody drug. Therefore, Bridging ELISA was chosen to detect all anti-antibody drug antibodies for this study. In this assay, antibody drug is used to coat the surface of well, anti-drug antibody containing samples are allowed to react with the antibody drug, and binding is detected by adding a labeled antibody drug.

To produce the detecting reagent, the Metuzumab was conjugated with HRP according to the manufacturer's instruction. To ensure a high sensitivity, by reacting with far excess amount of HRP, the molar ratio of Metuzumab to HRP was achieved at 1:1.3. The HRP-conjugated Metuzumab was also shown could be captured by coated monoclonal anti-Metuzumab, Clone 2D9 (data not shown), demonstrated that the HRP labeling of the detection Metuzumab does not significantly obscure critical antigenic determinants of 2D9.

To develop the Bridging ELISA, each well of 96-well plates was coated with 1 μg/ml of Metuzumab, paired with 2 μg/ml of HRP-conjugated Metuzumab. The assay samples were prepared by spiking 0–1000 ng/ml of anti-Metuzumab mAb 2D9 in 1% Monkey serum-3% BSA-PBS. As shown in Fig. 1, the LOD was 10 ng/ml anti-Metuzumab mAb with a linear range between 10–300 ng/ml. R^2 is 0.993.

3.3. Development of negative and positive Cynomolgus monkey sera

A positive anti-drug antibody from drug-treated patients will be ideal, but such valuable reagent is generally not available in preclinical and early clinical trials. The other option is to hyper-immunize non-human primate with adjuvant to generate a positive control.

In our study, two Cynomolgus monkeys were immunized with full length of Metuzumab for generation of positive monkey polyclonal controls. Four weeks after the first immunization, the titers of the sera were examined by the Bridging ELISA established above. As shown in Table 2, both monkeys produced very high titers (>1:12,800 dilution) of anti-drug antibodies.

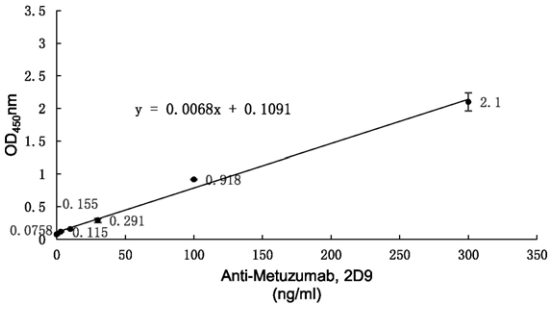

Fig. 1. Stand curve of bridging ELISA with anti-Metuzumab mAb 2D9. For each well, 100 μl of the testing samples containing 0, 3, 10, 30, 100, 300 and 1000 ng/ml of anti-Metuzumab mAb 2D9 were added and incubated for one hour. After washes, wells were then probed with HRP-conjugated Metuzumab. HRP substrate 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 reading at 1000 ng/ml was higher than the test range of plate reader which was not included. The LOD was defined as being the mean of the negative control (0 ng/ml) plus two times the standard deviation.

For each well of the plate coated with Metuzumab, 100 μl of the monkey sera at various dilutions were added and incubated for one hour. After washes, wells were then probed with HRP-conjugated Metuzumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

Based on the above data, we prepared and reserved 1:800, 1:12,800 and 1:50,000 dilutions of the monkey sera for use as quality controls (QC). These dilutions will be representative of high, medium, and low values in the anti-Metuzumab antibody assay. They will be used for validation and patient sample testing to ensure the assay is operating within desired assay ranges at the time the assays are performed.

Sera from 10 Cynomolgus monkeys which never exposed to any antibody drug previously were collected, aliquot into multiple vials of 0.1 ml and used for minimum dilution study. A pool of 0.2 ml from each serum was prepared as the negative control for future anti-Metuzumab antibody assay.

3.4. Determination of minimal dilution

Matrix components, especially the host antibodies, can contribute to high assay background if undiluted. Therefore, there is a need to dilute testing monkey sera to maintain a reasonable ability to detect anti-drug antibodies (sensitivity). Ideally, the minimum dilution is the dilution that yields a signal close to the signal of non-specific binding of assay diluent.

Based on FDA's recommendation (U.S. Department of Health and Human Services et al., 2009), we carried out the experiment to determine the minimum dilution from a panel of 10 sera from the untreated monkey population. As shown in Table 3, while some of the pre-immune sera at 1:20 dilution showed false positives, but all of the pre-immune sera at 1:80 dilutions were negatives. Different dilutions of the monkey sera which were used as quality controls were also performed in this test, the OD reading at 25 ng/ml and 3.125 ng/ml were 2.184 ± 0.19 and 0.59 ± 0.127, respectively (data not shown). So 1:80 dilution was initially defined as the Minimum dilution.

Each well was coated with 100 ng of Metuzumab, then 100 μl of the negative monkey sera at various dilutions were added and incubated for

Table 2
Establishment of Cynomolgus monkey polyclonal positive controls.

	OD reading	
Serum dilution	#1 Monkey serum	#2 Monkey serum
1:200	3.021 ± 0.107	3.286 ± 0.217
1:400	2.948 ± 0.116	2.887 ± 0.098
1:800	2.235 ± 0.103	2.649 ± 0.114
1:1600	1.897 ± 0.086	2.042 ± 0.125
1:3200	1.497 ± 0.094	1.599 ± 0.119
1:6400	0.997 ± 0.065	1.272 ± 0.054
1:12,800	0.740 ± 0.023	1.207 ± 0.087
1:25,600	0.310 ± 0.003	0.632 ± 0.016
1:50,000	0.106 ± 0.008	0.269 ± 0.006
pre-bleed	0.098 ± 0.001	0.088 ± 0.003

The values are expressed as mean ± SD, average and standard deviation were calculated from duplicate samples.

Table 3
Determine the minimum dilution from 10 negative sera samples.

	OD reading									
Dilution	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
1:20	0.135 ± 0.017	0.087 ± 0.002	0.231 ± 0.018	0.078 ± 0.005	0.083 ± 0.001	0.098 ± 0.003	0.154 ± 0.007	0.09 ± 0.005	0.3 ± 0.03	0.1 ± 0
1:40	0.112 ± 0.005	0.09 ± 0.003	0.146 ± 0.012	0.087 ± 0.002	0.09 ± 0.003	0.11 ± 0.002	0.113 ± 0.006	0.094 ± 0.002	0.228 ± 0	0.093 ± 0.004
1:80	0.097 ± 0.004	0.086 ± 0.001	0.113 ± 0.003	0.08 ± 0.002	0.092 ± 0.001	0.114 ± 0.004	0.12 ± 0.003	0.106 ± 0.008	0.18 ± 0.008	0.102 ± 0.007
1:100	0.089 ± 0.002	0.079 ± 0	0.107 ± 0.001	0.085 ± 0.007	0.082 ± 0.003	0.13 ± 0.007	0.125 ± 0.006	0.11 ± 0.001	0.18 ± 0.013	0.116 ± 0.001

The values are expressed as mean ± SD, average and standard deviation were calculated from duplicate samples.

one hour. After washes, wells were then probed with HRP-conjugated Metuzumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

3.5. Optimization of the bridging assay

Based on the data from completed clinical trials, FDA recommends that screening assays achieve a sensitivity of approximately 250–500 ng/ml as such antibody concentrations have been associated with clinical events. Since the minimum dilution was determined to be 1:80, the preliminary Bridging ELISA's LOD of 10 ng/ml anti-Metuzumab was not sensitive enough.

To increase the sensitivity, the coating concentrations of Metuzumab were increased to 2, 2.5, 5, 10 and 20 μg/ml, and the detecting HRP-Metuzumab concentrations were used at 0.5, 1 and 2 μg/ml. Standards were prepared as 0, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 ng/ml of anti-Metuzumab mAb 2D9 in 1% Monkey serum-3% BSA-PBS. As shown in Fig. 2, it was found that the combination of 2.5 μg/ml of coating concentration and 2 μg/ml of HRP-Metuzumab produced the best sensitivity of 0.39 ng/ml of anti-Metuzumab antibody, with linear range between 0.39–50 ng/ml and $R^2 = 0.994$, as plotted using 4-P Fit method.

3.6. Determination of assay cut point

The cut point of assay is the level of response of the assay at or above which a sample is defined as positive and below which it is defined as negative. In our study, 20 sera from untreated monkeys were tested at 1:100 dilutions. As shown in Fig. 3, the average NC reading was 0.062, and the standard deviation (SD) was 0.010. When we used the NC mean plus 1.645 SD, the calculated cut point was 0.078 and there was one sample showed false positive.

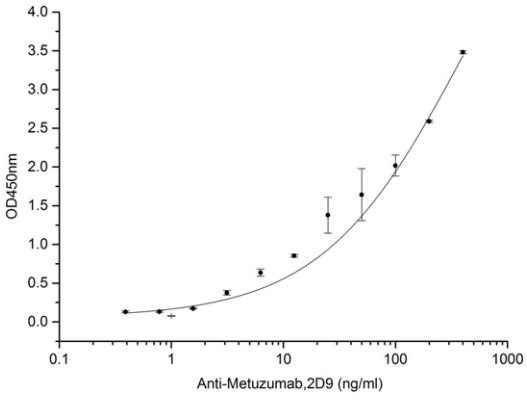

Fig. 2. Standard curve of the optimized bridging assay. Each well was coated with Metuzumab with the concentration of 2.5 μg/ml, then 100 μl of anti-Metuzumab mAb 2D9 at various dilutions in 1% Monkey serum-3% BSA-PBS were added and incubated for one hour. After washes, wells were then probed with 2 μg/ml of HRP-conjugated Metuzumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader. Results was fitted using 4-P Fit method with the equation: y = (A − D) / (1 + (x/C) ^B) + D, A: 0.05521 ± 0.02949, B: 0.68112 ± 0.08039, C: 415.89375 ± 354.80928, D: 6.90961 ± 2.14351. Adj. R-square was 0.994.

3.7. Interference of residual antibody drug and recovery

In general, antibody drug has very long half-life, a true advantage for the drug itself but become a huge issue for measuring the anti-drug antibody. From pharmacokinetic studies of ^{131}I-labeled Metuzumab in mice, we have learnt that its half-life was about 13.5 days. So the antibody drug and the anti-drug antibodies will decay the same rate in the testing objects (Zhang Z., et al., 2015).

The drug interference issue was then addressed by deliberately adding known amounts of purified anti-Metuzumab monoclonal antibody (2D9) into assay buffer in the absence or presence of different quantities of the Metuzumab. Then the pre-mixed samples were diluted 1:100 prior to assay for ADA. As Fig. 4 shows, if there were 300 μg/ml of Metuzumab left in the sample, the recovery was 37.9% for 20 μg/ml of anti-Metuzumab mAb 2D9 presented, and 14.4% for 1.25 μg/ml of anti-Metuzumab mAb 2D9. When the concentration of Metuzumab dropped to 1 μg/ml, the interference of drug itself was minimized.

For peptide or small protein drugs, acidification of the sera followed with ammonia precipitation has been successfully applied to separate drug from the anti-drug antibodies. However, this approach will not work for antibody drug. So the Bridging ELISA assay developed can only measure the free ADA in the presence of antibody drug.

3.8. Screening of anti-Metuzumab antibodies in Metuzumab-treated monkeys

In the pre-clinical study, 32 monkeys were treated with escalating doses of Metuzumab between 0, 10, 50, 200 mg/kg for 13 times over 13 weeks of time period. 16 of them were terminated right after the last injection, while the other 16 were rested for additional 4 weeks before termination.

Based on the dosing and the half-life of the antibody drug, the concentrations of residual antibody drug in the sera were estimated to be between 200–3000 μg/ml (Zheng et al., 2014), so the antibody drug interference is inevitable.

Afraid to miss any positive response to antibody drug, sera samples were collected at six time points, including 2-, 6- and 10-weeks post 1st dose, prior to last dose, and 2-, 4-weeks into recovery. The sera

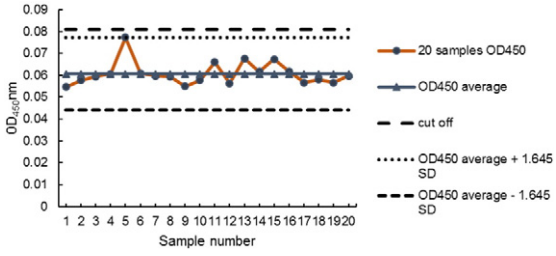

Fig. 3. Results of 20 negative monkey sera sample using bridging assay. For each well of the plate coated with Metuzumab, 100 μl of 20 negative monkey sera with 1:80 dilution were added and incubated for one hour. After washes, wells were then probed with HRP-conjugated Metuzumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader. The average OD450nm reading was 0.062. The standard deviation was 0.010. Cut off was 0.081.

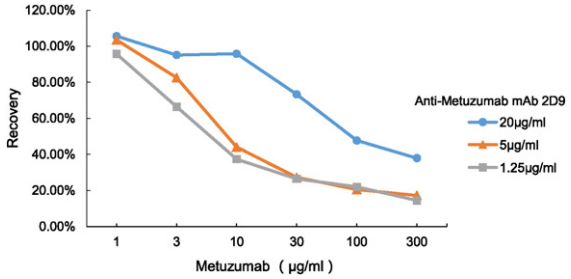

Fig. 4. Interference of recovery under different level of Metuzumab and anti-Metuzumab presented. For each well of the plate coated with the 100 ng of Metuzumab, 100 μl of anti-Metuzumab mAb 2D9 at various concentrations (20, 5 and 1.25 μg/ml) containing 1, 3, 10, 30,100 and 300 μg/ml of Metuzumab were added and incubated for one hour. After washes, wells were then probed with HRP-conjugated Metuzumab. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader. Recovery was calculated as the ratio of the absorbance to the OD reading at the corresponding concentration in the standard curve.

were examined at 1:100 dilutions for the anti-Metuzumab antibodies using the Bridging ELISA kit optimized above.

As shown in Table 4, one male monkey in the blank group tested positive in five out of the six time points for free anti-Metuzumab antibodies and one female in the blank group tested positive only once, although the reading was just above the cut-off value. The overall positive incidents were 15% (6/40) for the control group. A same free anti-Metuzumab antibodies positive rate was observed with the low-dose group but the readings were all more than double the cut-off value. The highest ADA positive rates, 75% (6/8) in the High-dose group and 62.5% (5/8) in the Medium-dose group, were seen 2-weeks post the first injection. The rates dropped rather quickly thereafter for both Medium- and High-dose groups. We suspect this observation was due to the built up of the antibody drug in the monkey sera to 2 mg/ml which could pre-mask all of the anti-Metuzumab antibodies developed. At the end of 4-week's recovery, one female monkey from the High-dose groups showed positive again.

Metuzumab was well tolerated in all animals, and no Metuzumab-related adverse effects on mortality, clinical observations, body weight, food consumption, local irritation, ophthalmology, heart rate, ECGs, clinical pathology were observed during the pre-clinical study (Zhang Z., et al., 2015).

4. Discussion

Human therapeutic proteins, especially the Fc portion of human or humanized antibody drugs, are frequently highly immunogenic in mouse, rat, dog and other animals. Immunogenicity in animal models is sometime not very predictive of immunogenicity in humans. However, assessment of immunogenicity in animals may be useful to interpret nonclinical toxicology and pharmacology data. In addition, immunogenicity in animal models may reveal potential antibody related toxicities that could be monitored in clinical trials (U.S. Department of Health and Human Services et al., 2009).

Here we reported the generation of mouse monoclonal antibodies against Metuzumab. Monoclonal anti-Metuzumab antibody, Clone 2D9, specifically binds to the variable regions of the Metuzumab, making it a much more relevant control for the detection of the anticipated immune response of humanized or fully human mAb in human patients. However, monoclonal anti-Metuzumab antibody 2D9 is IgG and binds to ONLY one of the many epitopes on the variable region of Metuzumab with one affinity, while the testing animals or the patients could generate polyclonal antibodies with different isotypes, including IgA, IgE, IgGs and IgM, against various epitopes with wide range of affinities. Therefore, anti-Metuzumab antibody 2D9 is used only as the internal controls for establishment of the standard curve and LOD, but ADA levels reported in mass units based on interpolation of data from the standard curves are generally less informative because interpretation is based on the specific control Metuzumab antibody 2D9.

Bridging ELISA was chosen to screen all anti-antibody drug antibodies for this study by coating the wells with Metuzumab, and paired with HRP-conjugated Metuzumab. To ensure the specificity of the assay, the standards were prepared by spiking 0–1000 ng/ml of anti-Metuzumab mAb 2D9 in 1% Monkey serum-3% BSA-PBS. As shown in Fig. 1, the preliminary screening assay system is specific and has LOD of 10 ng/ml anti-Metuzumab mAb with a linear range between 10–300 ng/ml.

For therapeutic monoclonal antibodies, it is recommended by FDA to give special consideration to the selection of a positive control for the assay. If non-primate animals are immunized with a monoclonal antibody (mAb) containing a human immunoglobulin constant region (Fc) to develop a positive control, the antibody response is likely to be against the human Fc and not the variable region. Such a positive control may not be relevant for the anticipated immune response in human patients where the response to humanized mAb is primarily to the variable regions. Ideally, the positive control should reflect the anticipated immune response that will occur in monkeys and humans. A positive anti-Metuzumab antibody from Metuzumab-treated monkeys will be ideal, but such valuable reagent was not available in its pre-clinical trials. In this study, Cynomolgus monkeys were hyper-immunized with Metuzumab in complete and incomplete Freund's adjuvant, and produced very high titers of anti-Metuzumab antibodies. Specific dilutions of the monkey sera were prepared and reserved as high, medium, and low quality controls (QC) for validation and drug-treated monkey sample testing to ensure the assay is operating within desired assay ranges at the time the assays are performed.

Components in the matrix other than antibodies can interfere with assay results. Of greatest concern is the presence of Metuzumab in the matrix. From our early study, we learnt that the C_{max} of Metuzumab ranged from 22.33 to 727.34 μg/ml in the sera from the monkeys with single i.v. injections between 1.0–25.0 mg/kg, and the half-life of Metuzumab was about 13.5 days. With such large quantities of Metuzumab are

Table 4
Screening of anti-Metuzumab antibodies in Metuzumab-treated monkeys.

Group	Sex	Reported free ADA rate					
		2-weeks post 1st dose	6-weeks post 1st dose	10-weeks post 1st dose	Prior to last dose	2-weeks in recovery	End of recovery
Blank	Female	0/4	0/4	1/4	0/4	0/2	0/2
	Male	1/4	1/4	1/4	1/4	1/2	0/2
Low dose	Female	0/4	1/4	0/4	0/4	0/2	0/2
	Male	0/4	1/4	1/4	1/4	1/2	1/2
Medium dose	Female	1/4	1/4	1/4	1/4	0/2	0/2
	Male	4/4	1/4	1/4	1/4	0/2	0/2
High dose	Female	3/4	1/4	1/4	0/4	0/2	1/2
	Male	3/4	0/4	0/4	0/4	0/2	0/2

32 monkeys were treated with escalating doses of Metuzumab between blank (0), low dose (10 mg/kg), medium dose (50 mg/kg), high dose (200 mg/kg) for 13 times over 13 weeks of time period. 16 of them were terminated right after the last injection, while the other 16 were rested for additional 4 weeks before termination. Sera samples were collected at six time points (2-, 6- and 10-weeks post 1st dose, prior to last dose, and 2-, 4-weeks into recovery).

present in sera/plasma, for sure it can prevent detection of ADA in the test format by blocking the ADA for binding to either the coating Metuzumab or the detecting HRP-Metuzumab. This issue was examined by deliberately adding known amounts of anti-Metuzumab antibody 2D9 into assay buffer in the absence or presence of different quantities of Metuzumab. Clearly, as shown in Fig. 3, only the concentration of Metuzumab dropped to 1 μg/ml, the interference of drug itself was minimized. Very significant interference was observed if the concentration of residual Metuzumab is 30 μg/ml or more.

In this pre-clinical study, 32 monkeys were treated with escalating doses of Metuzumab between 0, 10, 50, 200 mg/kg for 13 times over 13 weeks of time period. As shown previously, the C_{max} of Metuzumab was 727.34 μg/ml in the sera with single i.v. injection at 25.0 mg/kg, we expect to see many false negative for ADA in the sera from the monkeys this study.

Since the Metuzumab and the anti-Metuzumab antibodies will decay the same rate in the testing objects and there is no feasible way to separate anti-Metuzumab antibodies from Metuzumab, it is impossible to obtain Metuzumab-free samples during the treatment phase or even after the four-week's recovery period. So the Bridging ELISA assay developed can only measure the free ADA in the presence of antibody drug.

Not to miss any positive response to Metuzumab, sera samples were collected at seven time points during the treatment and recovery phases. The highest ADA positive rates, 75% (6/8) in the High-dose group and 62.5% (5/8) in the Medium-dose group, were seen 2-weeks post the first injection.

At this moment, it is impossible to find out what is the molar ratio of polyclonal anti-Metuzumab antibodies to the monoclonal antibody drug in the complex. But we knew that there were 727 μg/ml or more of Metuzumab presented in the sera from the Medium-dose group, so we estimated there will be at least 700 μg/ml of anti-Metuzumab antibodies in the sera which were tested free ADA-positive. However, even with such high anti-Metuzumab antibodies concentrations, no significant pathologic and clinic adversary effect was observed in the monkey (Zhang Z., et al., 2015). Phase I/II clinical trials of Metuzumab in treating NSCLC patients are currently underway.

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