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Increased rheumatoid factor interference observed during immunogenicity assessment of an Fc-engineered therapeutic antibody

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ABSTRACT

Protein therapeutics may elicit an anti-therapeutic antibody (ATA) response in patients. This response depends on a number of factors including patient population, disease state, route of delivery or characteristics specific to the product. Therapeutics for immunological indications often target relatively young and healthy patients with hyperactive immune systems who have periodic flares and remissions. The hyperactive immune system of these patients can add several levels of bioanalytical complexity due to the presence of cross reactive molecules such as autoantibodies. In addition, the long-term chronic dosing regimen often necessary in this patient population can increase their risks of immunogenicity against the therapeutic and lead to safety concerns. Therefore, development of a sensitive and drug-tolerant ATA method is important. Bridging ATA assays are usually very sensitive and drug-tolerant methods for immunogenicity assessment; however these methods are particularly vulnerable to any factor that is able to bridge the conjugated therapeutics used as reagents and can generate false positive signal. Although there are many potential interfering factors in serum, rheumatoid factors (RFs), autoantibodies associated with rheumatoid arthritis (RA), are of particular concern in this type of assay. MTRX1011A is a non-depleting anti-CD4 monoclonal antibody therapeutic that was clinically tested in RA patients. This paper will discuss the bioanalytical challenges encountered during development of a clinical ATA assay for MTRX1011A. These challenges highlight interference due to patient disease state, in this case presence of RF in RA patients, as well as specific molecule-related interference caused by an engineered mutation in the Fc region of MTRX1011A designed to enhance its binding to the neonatal Fc receptor (FcRn). We will discuss the characterization work used to identify the cross-reactive epitope and our strategy to overcome this interference during development of an effective ATA assay to support clinical evaluation of MTRX1011A.

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1. Introduction

Humanized antibody therapeutics are often a safe and effective targeted approach for the treatment of a variety of diseases. Increasing numbers of antibody therapeutics (many approved or under development), are targeting a younger patient population with debilitating diseases which are not necessarily life-threatening such as rheumatoid arthritis (RA), allergic rhinitis, and psoriasis [1–3]. Therefore, there are more stringent safety and efficacy requirements for these therapeutics [4]. Immunogenicity is one of the major safety concerns in the treatment of this patient population as they often have hyper-active immune systems and require chronic dosing. These factors increase their risks of developing antibodies to the therapeutic. Therefore, sensitive drug-tolerant methods are required as part of the therapeutic evaluation in the clinical phases [4–6]. A tiered strategy is typically utilized to assess immunogenicity and often comprises a screening method, a confirmation/specificity step and a characterization step. Here we describe our challenges with the development of a clinical anti-therapeutic antibody assay (ATA) for MTRX1011A, which was investigated as a potential therapeutic treatment for RA.

MTRX1011A is a humanized non-depleting anti-CD4 antibody of the immunoglobulin G (IgG) 1 subclass based on a previously described TRX1 antibody [7]. An amino acid substitution of asparagine 297 to alanine (N297A) was introduced to impair its binding to Fcγ receptors and consequently prevent Fc effector function [8,9], rendering the antibody non-depleting *in vivo* [10,11]. An additional single amino acid substitution of asparagine 434 to histidine (N434H) was made in the Fc portion of MTRX1011A to improve its binding to the neonatal Fc receptor (FcRn), thereby prolonging its half life [12–18].

Abbreviations: ATA, anti-therapeutic antibody; CDR, complementarity determining region; DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; FcRn, neonatal Fc receptor; IgG, immunoglobulin gamma; RA, rheumatoid arthritis; RF, rheumatoid factor.

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A human ATA assay was developed using a homogenous bridging enzyme-linked immunosorbent assay (ELISA) format to monitor patients' immune response to MTRX1011A during clinical trials (Fig. 1). To differentiate between a positive and a negative ATA response to MTRX1011A treatment, an assay threshold or cutpoint needs to be established. The screening cutpoint is determined based on the signal variability from a drug-naïve target patient population and it is set to have about a 5% false positive rate to minimize the potential of false negatives in the study [19]. In our initial assessment, serum samples from RA patients demonstrated baseline reactivities that were much higher and more variable than those observed in healthy subjects. The high signal variability was hypothesized to be attributable to rheumatoid factor (RF) interference in RA patient samples. RFs are characteristic auto antibodies associated with RA. They recognize epitopes on the Fc region of immunoglobulin gamma (IgG) and are often of the IgM isotypes, although IgG and IgA isotypes have also been reported [20,21]. RF values in a normal population are very low and the prevalence is about 1–2%. However, in the RA population the RF values can be significantly elevated and the prevalence can be as high as 70–90% [22]. Initial observations indicated that RF in serum from RA subjects interfered in our bridging ATA assay method. Further characterization work revealed that this RF-Fc binding was enhanced by the FcRn mutation introduced to the MTRX1011A Fc domain. This paper describes our challenges with RF interference in MTRX1011A clinical ATA assay development, and our strategy to characterize and overcome RF interference to identify "true" immunogenic response to MTRX1011A. We have successfully applied this strategy in Phase I exploratory clinical sample analysis.

2. Materials and methods

2.1. Reagents

MTRX1011A is a humanized anti-CD4 antibody with IgG1 heavy chain and κ1 light chain frameworks containing N297A and N434H mutations in the Fc region and was produced in Chinese hamster ovary cells at Genentech (South San Francisco, CA). Recombinant monoclonal antibody X (rhuMAb X) is an irrelevant IgG1 molecule containing neither mutation; rhuMAb X (N297A) contains the N297A mutation only; rhuMAb Y (N434H) is a different irrelevant IgG1 molecule which contains the N434H mutation only (Table 1).

Table 1
Summary of Fc mutations in the five antibodies used for assay characterization.

Antibodies	Fc gamma mutation (N297A)	FcRn mutation (N434H)
MTRX1011A	Yes	Yes
TRX1 (N297A)	Yes	No
rhuMAb X	No	No
rhuMAb X (N297A)	Yes	No
rhuMAb Y	No	Yes

Individual RA serum samples with known RF values and individual and pooled normal human serum samples were purchased from Bioreclamation (Hicksville, NY) and BioChemed (Winchester, VA). Roche StreptaWell high binding plates were acquired from Roche Diagnostics (Indianapolis, IN); Costar polypropylene 96 well round-bottom plates were purchased from Corning Life Sciences (Lowell, MA). A complimentary determining region (CDR) specific mouse monoclonal antibody raised against TRX1 (therefore also against MTRX1011A) was produced at Genentech. In addition, an affinity purified CDR specific rabbit polyclonal antibody raised against TRX1 (and MTRX1011A) was generated at Genentech and used as a positive control (PC) in the assay. Illustra™ Nap-10 columns were purchased from GE Healthcare (Buckinghamshire, UK). Bovine serum albumin (BSA) was purchased from Equitech-Bio Inc. (Kerrville, TX); CHAPS was from Research Organics (Cleveland, OH); ProClin 300 was from Supelco (Bellfonte, PA), and affinity purified rabbit anti-human IgM (anti-hu IgM) polyclonal antibody was purchased from Jackson ImmunoReserach (West Grove, PA).

2.2. Biotin and digoxigenin conjugations of MTRX1011A

Prior to conjugation, MTRX1011A was buffer exchanged into phosphate buffered saline (PBS) using an illustra™ Nap-10 column according to the manufacturers' instructions (GE Healthcare).

Buffer exchanged MTRX1011A was conjugated with biotin for DIG labeling at a challenge ratio of 10:1 (biotin:MTRX1011A) using EZ-Link SulfoNHS-LC-Biotin (Pierce, Rockford, IL), or with 3-amino-3-deoxydigoxigenin hemisuccinamide, succinimidyl ester (Invitrogen Carlsbad, CA) for DIG labeling at a challenge ratio of 10:1 (DIG:MTRX1011A). All conjugations were prepared according

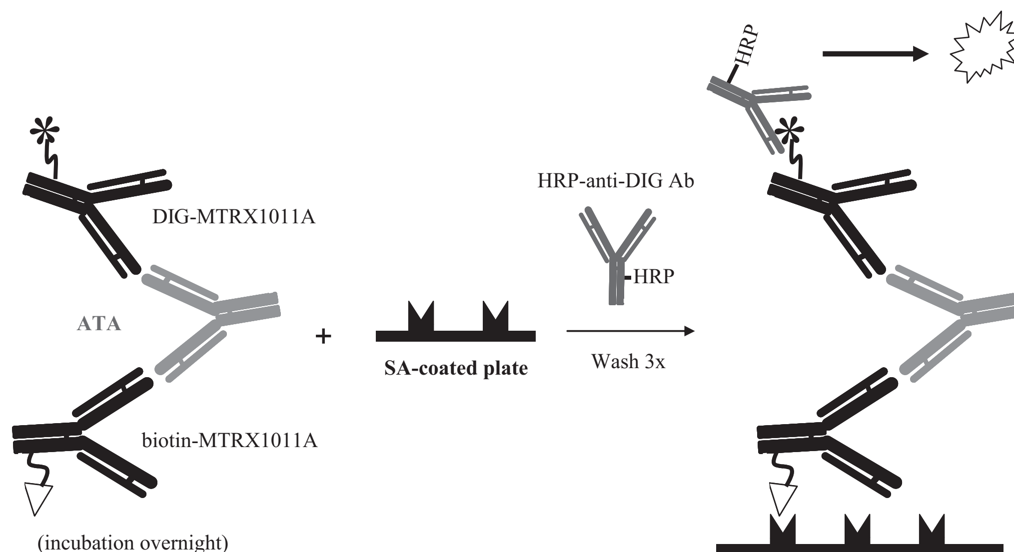

Fig. 1. Homogeneous bridging ELISA: ATA bridges biotin and digoxigenin (DIG) conjugated antibody therapeutics. This immune complex was then captured on a streptavidin (SA) coated plates. An HRP conjugated anti-DIG antibody was used as the detection reagent. Colorimetric signals were generated upon addition of an HRP substrate.

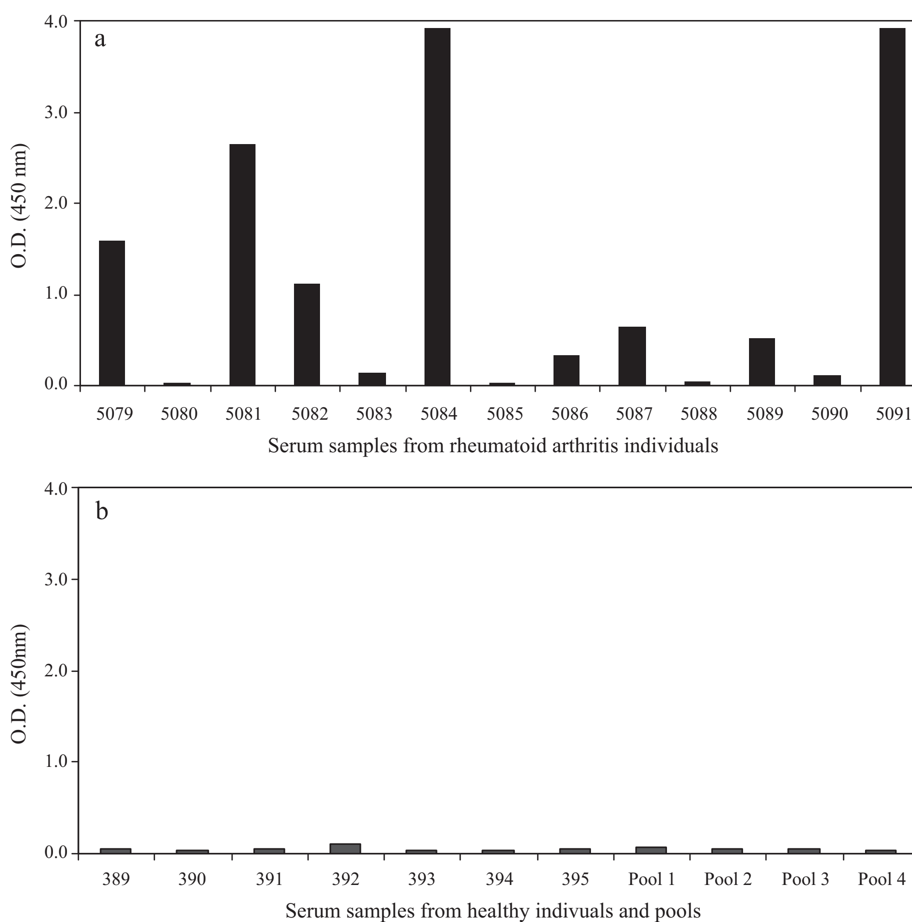

Fig. 2. Significant matrix interferences observed in rheumatoid arthritis (RA) patient samples. Results from MTRX1011A clinical ATA screening assay comparing (a) sera from RA patients and (b) normal human sera.

to the manufacturer's instructions followed by a buffer exchange step into storage buffer (20 mM sodium phosphate, 0.35 M sodium chloride, 6% sucrose, 0.25% polysorbate 20, 0.25% CHAPS, 0.05% Proclin 300, 0.5% BSA, pH 5.2 ± 0.2). The concentration of each conjugate was determined using a BCA protein assay kit (Pierce), prior to addition of BSA.

2.3. Clinical ATA assay

2.3.1. Screening assay

A homogenous bridging ELISA was developed. Equal concentrations (2 μg/mL) of both biotin- and DIG-conjugated MTRX1011A were pre-mixed (master mix) in assay diluent (1× PBS, 0.5% BSA, 0.05% Polysorbate 20, 0.05% (15 PPM) Procline 300, pH 7.4) and added to polypropylene round-bottom plates (80 μL/well). Samples and control preparations were diluted a minimum of 1/20 in assay diluent or sample diluent (assay diluent plus 100 μg/mL anti-hu IgM as a blocker). Samples and controls (80 μL/well) were then added to the plate(s) containing master mix and allowed to incubate for 16–22 h at room temperature (RT) with gentle agitation. The next day, samples/conjugate mix (100 μl/well) were transferred to a high binding streptavidin (SA) coated plate (Roche Diagnostics, Indianapolis, IN) and incubated at RT for 2 h with gentle agitation. Plates were washed three times with washing buffer (PBS, 0.05% Tween-20, pH 7.4) prior to addition of 100 μL per well of 1/2000 diluted (0.8 mg/mL stock) horseradish peroxidase (HRP) conjugated mouse-anti-DIG antibody (Jackson ImmunoResearch) and incubated at RT for 2 h with gentle agitation. Plates were washed three times with wash buffer and 100 μL of tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well, and incubated at RT for 15–20 min. The reaction was stopped by the addition of 100 μL per well of 1 M phosphoric acid and absorbance was measured at 450 nm and at 650 nm for reference on a plate reader (Molecular Devices, Sunnyvale, CA) (Fig. 1).

2.3.2. Confirmatory assay: characterization of screen positive samples

Positive samples identified in the screening assay were pre-incubated with various reagents, including assay diluent (as control), various molecules with IgG1 framework or rabbit anti-human IgM (Table 1) to achieve a final in-well concentration of 100 μg/mL for each antibody prior to analysis. Diluted samples were incubated overnight with the mixture of 2 μg/mL of biotin- and DIG-conjugated MTRX1011A; other assay procedures were the same as the screening assay described above.

2.4. Characterization of high signal specificity observed in RA population

Sera from three RA individuals as well as a positive control (PC) were minimally diluted and incubated with a final in-well concentration of 100 μg/mL of various molecules with IgG1 framework or rabbit anti-human IgM (Table 1). Diluted samples were incubated overnight with master mix; other assay procedures are the same as the screening assay described above.

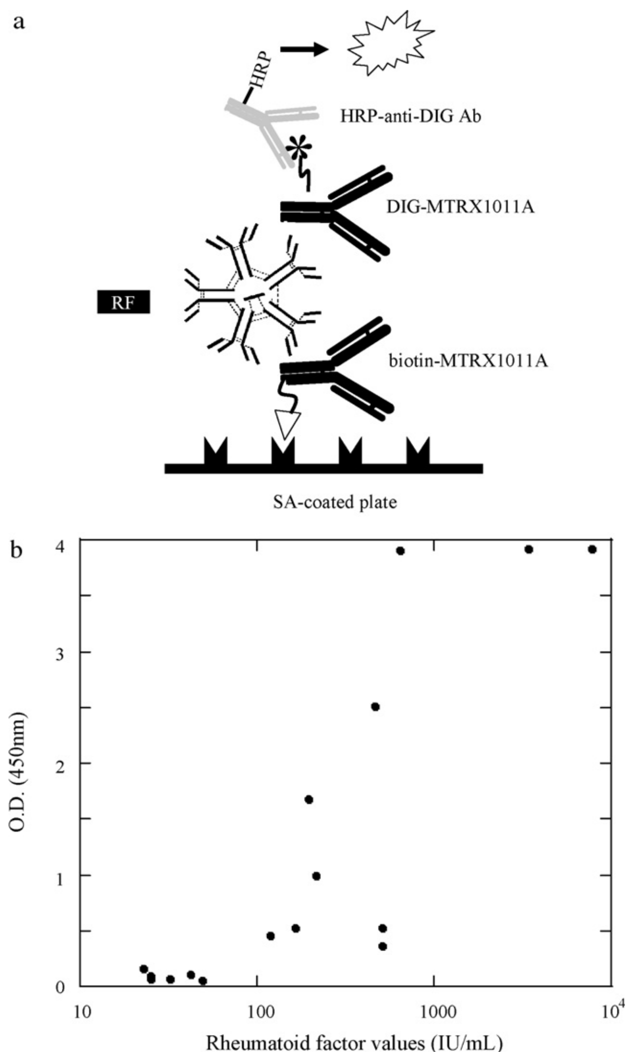

Fig. 3. RF interference: (a) illustration of RF interference in the ATA assay. RF can bridge with the Fc portions of biotin- and DIG-labeled MTRX1011A and generate signal. (b) Correlation between RF and ATA assay signal (OD) in RA individuals. There is a general trend of elevated signals with increased RF values.

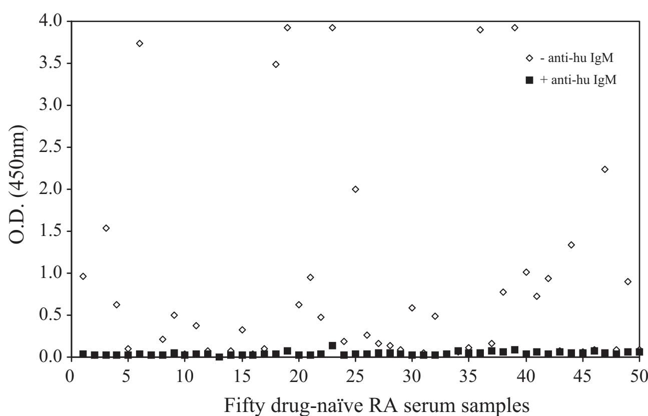

Fig. 4. Cross-reactivity of RA serum in the MTRX1011A ATA assay was dramatically reduced in the presence of anti-human IgM. RA serum samples ($n = 50$) were tested in the clinical ATA assay in the absence and presence of rabbit anti-human IgM antibody. Anti-human IgM antibody effectively blocked unspecific signals.

2.5. Direct RF binding ELISA: preferential binding of RF to MTRX1011A

A direct binding ELISA with a colorimetric detection system was used to demonstrate preferential binding of RF to MTRX1011A compared with TRX1 in human serum. A microtiter plate was coated overnight with 100 μL of 3 μg/mL of a CDR specific monoclonal antibody recognizing both MTRX1011A and TRX1. After a wash step MTRX1011A or TRX1 at 1 μg/mL (100 μL) were added to columns 1–2 and 3–4 of a microtiter plate, respectively and buffer (100 μL) was added to columns 5–6 and 7–8 and incubated for 1 h. The plate was washed and RF (prepared in PBS) (Bio-Rad Life Science, Hercules, CA) at a starting concentration of 4 IU/mL (in well concentration) was added to the first row of columns 1–6 followed by seven subsequent 1:2 dilutions. As a control condition 100 μL of normal human serum pool, at the minimum dilution of 1/25 was added to the first row of columns 7–8 followed by seven subsequent 1:2 dilutions. After a 1 h incubation plates were washed before addition of a HRP conjugated anti-human IgM antibody (Jackson ImmunoResearch) to the plate for detection following a wash step, TMB substrate was added to each well (100 μL), and incubated at RT for 10–15 min. The reaction was stopped by the addition of 100 μL per well of 1 M phosphoric acid and absorbance was measured at 450 nm with 650 nm as background signal on a plate reader (Molecular Devices, CA).

2.6. Assay parameters evaluated

The final cutpoint multiplication factor for the assay was established using a panel of 72 drug naïve RA individual serum samples treated with 100 μg/mL of rabbit anti-hu IgM to eliminate interference from variable pre-existing RFs in the RA patient population. The floating cutpoint methodology was used to set the screening cutpoints. The determination of the screening cutpoint factor follows the guidance described elsewhere [6]. The assay signal values were normalized to the plate's negative control and log transformed. Statistical outliers were removed using the boxplot method. The Shapiro–Wilk test on the data with the outliers removed did not detect a departure from normality so a parametric approach was used to compute the cutpoint factor. The cutpoint multiplication factor was obtained using the formula:

$$10^{(\text{mean}+1.645\ \text{standard deviation})}.$$

The cutpoint of each run was calculated by multiplying the mean of the NC of the plate with the cutpoint multiplication factor. Samples with signals equal to or above the cutpoint were deemed positive; otherwise they were considered negative.

Assay sensitivity and drug tolerance are summarized in Section 3.1.3. Other assay parameters, such as matrix effect, hook effect, and target inference, were also evaluated during assay development according to recommendations described elsewhere [6] but will not be discussed in this paper.

2.7. Phase I clinical trial in RA patients

The Phase I clinical trial was a randomized, double-blind, placebo-controlled, single ascending-dose (SAD) stage and multiple ascending-dose (MAD) stage as described previously [18]. The SAD stage enrolled patients with stable RA without pre-specified disease activity who were on stable therapy. The SAD stage consisted of single IV and SC dosing. Serum samples from selected patients in the intravenous (IV) dose groups (0.3 and 1 mg/kg) at baseline, day 15 and day 36 post dose as well as the subcutaneous (SC) dose groups (1.0 mg/kg) at baseline and day 15 and for 3.5 mg/kg at baseline were evaluated in the ATA assay in the

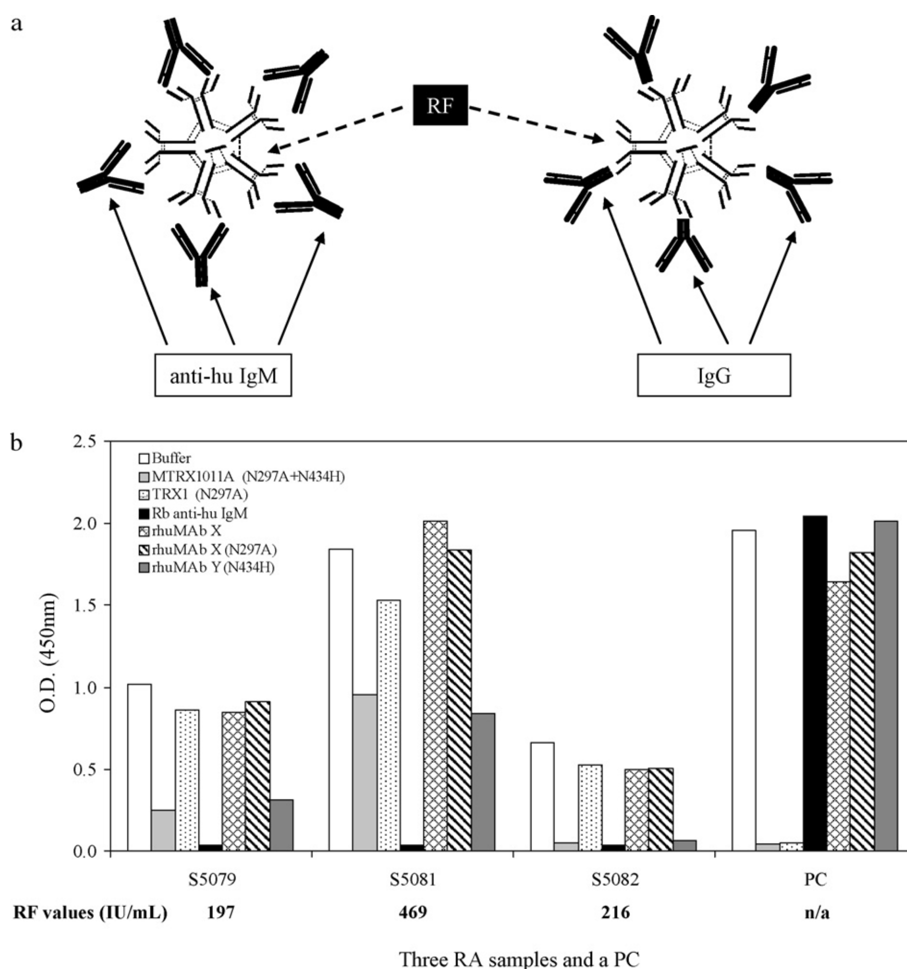

Fig. 5. Characterization of the epitope recognized by IgM. (a) Anti-human IgM and human IgG bind to and prevent RF from bridging the MTRX1011A conjugates in the ATA assay eliminating false positive ATA signals. (b) Various monoclonal antibodies including MTRX1011A (N297A + N434H), TRX1 (N297A), rhuMAb X, rhuMAb X (N297A) and rhuMAb Y (N434H) as well as anti-human IgM (anti-hu IgM) were tested for their ability to inhibit RF interference in the ATA assay in sera from three RA individuals with varying RF values (196, 216 and 469 IU/mL) as well as a positive control (PC).

presence and absence of 100 μg/mL of affinity purified rabbit anti-hu IgM antibody. Samples that screened positive in the presence of anti-hu IgM blocker were further evaluated in the confirmatory assay.

3. Results

3.1. MTRX1011A clinical ATA assay development

3.1.1. RF interference observed during assay development

Clinical screening and confirmatory ATA assays for MTRX1011A were developed on the homogenous bridging ELISA platform (Fig. 1). Initial screening data revealed unexpected high reactivity of samples from the drug naïve RA population as compared to the drug naïve normal healthy donor samples (Fig. 2a, b). In the RA population the signal ranged between 5- and 50-fold above assay background, whereas the normal population had very low signal in the assay (<0.1 OD) as expected. Assay optimization work such as various minimum dilution or more stringent assay buffers were evaluated and found to be ineffective in eliminating the high reactivity observed in RA samples (data not shown). The high signal variability observed in RA population was suspected to be caused by the binding of RF to the Fc regions of the MTRX1011A conjugates thereby forming a bridge in the assay (Fig. 3a). To verify our hypothesis, a panel of 16 RA serum samples with known RF values was evaluated in our assay. A general trend between the RF values (IU/mL) and assay signal (OD) was observed (Fig. 3b).

3.1.2. Anti-human IgM eliminated RF interference

To ensure that the assay cutpoint was independent of RF levels in the RA population, steps were taken to eliminate signals due to RF interference. Since the majority of RF are of IgM isotype, 100 μg/mL of an affinity purified rabbit anti-human IgM antibody was included in the sample diluent to overcome the cross reactive IgM antibody interference in RA samples. When compared side by side, the anti-hu IgM proved to be an effective blocker to eliminate all cross-reactivity in the RA individuals (Fig. 4). Therefore anti-hu IgM was included in the qualified MTRX1011A clinical ATA assay.

3.1.3. MTRX1011A clinical ATA assay qualification parameters

The final MTRX1011A clinical ATA assay conditions are described in Section 2.3.1. The assay cutpoint multiplication factor was established based on signals from a panel of drug-naïve RA patient serum samples ($n = 72$) in the presence of 100 μg/mL of rabbit anti-hu IgM to eliminate the RF dependence of the cutpoint factor calculated to be 1.58. Based on this cutpoint multiplication factor, a false-positive rate of approximately 9.7% was determined for the population tested. The relative sensitivity of the assay is estimated to be 17 ng/mL in the absence of drug and in the presence of 100 μg/mL of MTRX1011A the sensitivity is calculated

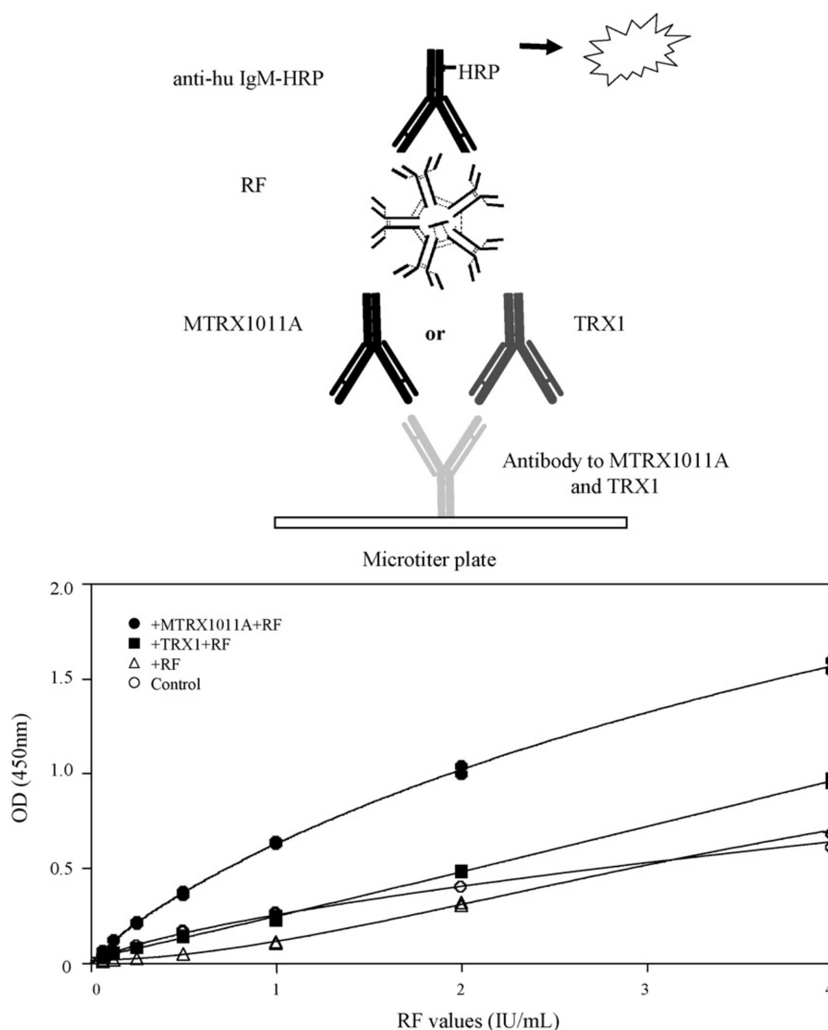

Fig. 6. Direct binding ELISA evaluating differential binding of MTRX1011A (N297A + N434H) and TRX1 (N297A) to RF. MTRX1011A or TRX1 are captured by an anti-idiotype monoclonal antibody (MAb) to MTRX1011A and TRX1. RF recognizes the Fc portion of MTRX1011A and TRX1. The bound RF is detected by HRP conjugated anti-human IgM.

to be 552 ng/mL using an affinity purified polyclonal rabbit anti-MTRX1011A antibody.

3.2. Characterization of specific RF binding to the Fc epitope on MTRX1011A

3.2.1. RF binds to wild type and mutant Fc IgGs with different affinities

Addition of either anti-hu IgM or Fc mutant IgG with high affinity for RF can potentially inhibit RF bridge formation in the ATA assay as illustrated in Fig. 5a. The extent of the signal inhibition depends on the level of RF binding for the added reagents. To determine the specificity of RF binding to the MTRX1011A Fc epitope, sera from three RA individuals with varying RF levels (197, 216 and 469 IU/mL) as well as a PC to MTRX1011A were evaluated. Samples were spiked with either assay diluent, MTRX1011A (N297A + N434H), TRX1 (N297A), an unrelated monoclonal antibody rhuMAb X, rhuMAb X (N297A), a second unrelated monoclonal antibody rhuMAb Y (N434H) or with anti-hu IgM. Results from this study showed that in all three sera, a significant decrease in signal (immunodepletion) was achieved only in the presence of anti-hu IgM and the two antibodies containing the FcRn mutation N434H (Fig. 5b). As expected, the PC was only depleted with MTRX1011A or TRX1 and not the other molecules or anti-hu IgM. These experiments suggest that N434H mutation generates a neo-epitope for RF of IgM isotype to the Fc portion of MTRX1011A.

3.2.2. Preferential binding of RF to N434H mutation on MTRX1011A

A direct ELISA was utilized to compare the RF binding to MTRX1011A (N297A + N434H) and TRX1 (N297A) as depicted in Fig. 6. The data clearly shows increased binding of RF to MTRX1011A as compared to TRX1. This data further confirms N434H as the critical residue in generating a neo-epitope for RF binding to the Fc epitope on MTRX1011A.

3.3. Immunogenicity assessment of Phase I exploratory study samples

Representative samples from patients in the Phase I single dose study at baseline, day 15 and where possible day 36 were obtained and evaluated in the clinical MTRX1011A ATA assay. All samples were evaluated in the presence and absence of 100 μg/mL of anti-hu IgM (Fig. 7a). Consistent with what we had observed during assay development, there was significant signal reduction in the presence of anti-hu IgM. During assay development an assay cut-point was determined using the signal obtained in the presence of anti-IgM using a panel of drug naïve samples. Based on this

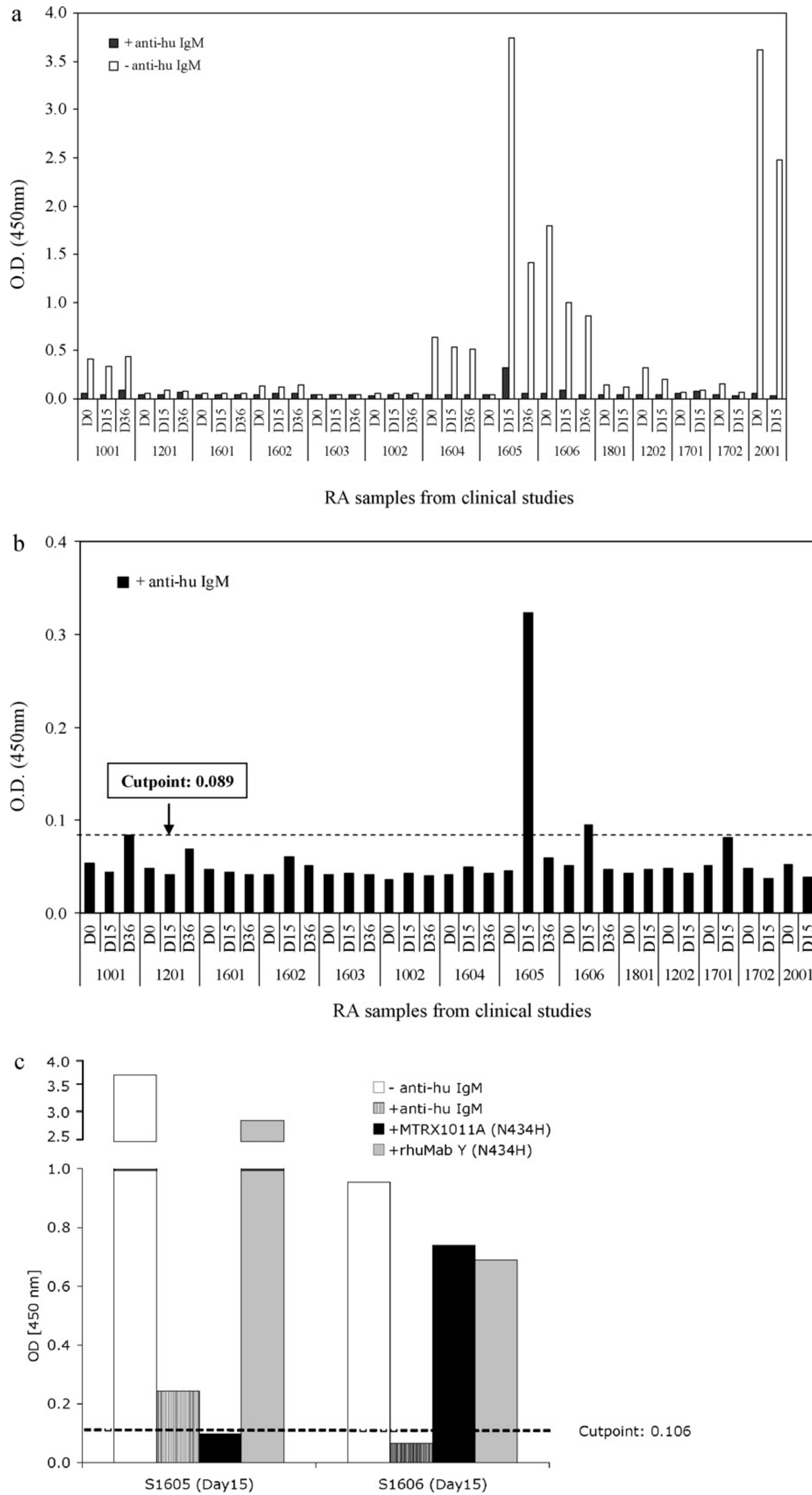

Fig. 7. Evaluation of clinical samples. (a) Clinical study samples screened in the presence and absence of anti-human IgM. (b) Two positive samples were identified based on the cutpoint calculated in the presence of anti-human IgM. (c) Characterization of the two positive samples indicated that sample S1606 was a false positive as the signal was not reduced to below the cut point by the addition of MTRX1011A (N297A + N434H). The signal was only reduced to below the cut point in the presence of the blocker anti-human IgM. However, sample S1605 was a confirmed positive for ATA as only addition of MTRX1011A (N297A + N434H) reduced the signal to below the cut-point.

cutpoint two positive samples were identified in the screening assay during the exploratory clinical sample evaluations (Fig. 7b). These two samples were further characterized in a confirmatory assay by immunodepletion with MTRX1011A, anti-hu IgM, and rhuMAb Y (N434H). Sample 1606 (S1606) at day 15 (D15) was not confirmed as a "true" positive response as only addition of anti-hu IgM but not MTRX1011A reduced the signal to below the assay cutpoint (Fig. 7c). This result illustrates that the "positive" signal observed was due to the presence of RF of IgM isotype in the sample and not specific antibodies generated in response to MTRX1011A treatment. However, we were able to confirm sample 1605 (S1605) at D15 as a "true" positive, as only MTRX1011A was able to immunodeplete the signal to below the assay cut point.

4. Discussion

Multiple types of molecules have been identified in serum and plasma capable of causing significant interference in bioanalytical methods used to evaluate protein therapeutics. RF as well as multimeric soluble targets are of particular concern in immunogenicity assessment using a bridging assay format. In this format the antibody therapeutic is used as both the capture and detection reagent. Bridging assays are widely used for immunogenicity assessment as this format can measure total antibody response regardless of isotype.

RFs, the characteristic autoantibodies associated with RA recognize epitopes located in the Fc region of IgG molecules [23], and can activate complement and inflammatory responses that lead to tissue damage [24]. High RF levels are associated with poor prognosis, systemic symptoms and erosion of the joints [25]. RFs predominantly belong to the IgM isotype and generally display low to moderate affinity for IgG Fc [26,27]. In subjects with severe disease, the RFs may include IgG and IgA antibodies [28].

MTRX1011A is a humanized non-depleting anti-CD4 antibody of the IgG1 subclass that was clinically tested in RA patients. Unexpected elevated signals were observed in drug naïve serum samples from an RA population as compared with normal human sera during early development of the ATA assay for MTRX1011A (Fig. 2). Further analysis of a set of RA samples with known RF values revealed that there was a clear trend between RF values (IU/mL) and assay signals (OD) (Fig. 3b). This suggested that the elevated signals observed in RA samples were attributable to the binding of RFs to the MTRX1011A conjugates (Fig. 3a). Since IgM is the predominant isotype of RFs in the RA patient population, an affinity purified rabbit anti-human IgM (anti-hu IgM) polyclonal antibody was added to the RA samples as a blocker to greatly reduce or eliminate the RF interference (Fig. 4), allowing for the development of an assay free from RF interference. However, addition of the anti-human IgM blocker would prevent us from detecting antibody of IgM isotype in patient serum. Our strategy for the detection of antibodies of IgM isotype was to screen all samples with and without the blocker and monitor increase in titers during treatment time course. If there is an increase in titers without the blocker and samples screen negative with the blocker this may indicate the presence of antibody of IgM isotype. If there is increase in titer without blocker and samples screen positive with the blocker the signal may be due to both antibodies of IgM or other isotypes. The positive samples from the screening assay would be further characterized in the confirmatory assay as described in Section 2.3.

This assay approach was used to evaluate a subset of samples from a Phase I study (Fig. 7a, b). Two screen positive samples (S1605 at D15 and S1606 at D15) were identified with one of the two (S1605 at D15) confirming as a "true" positive through further characterization. However, this was a transient positive response as the sample tested negative on day 36 post-dose.

Table 2
A common RF epitope is recognized between the gamma subclasses. The FcRn mutation N434H on MTRX1011A is immediately adjacent to the critical RF epitope 435.

Isotype	Motif	Recognition
Amino acid positions	433-*434*-*435*-436-x-438-x-440	N/A
IgG1	H-N-H-Y-x-Q-x-S	+
IgG2	H-N-H-Y-x-Q-x-S	+
IgG3	H-N-*R*-Y-x-Q-x-S	–
ΔIgG3	H-N-*H*-Y-x-Q-x-S	+
IgG4	H-N-H-Y-x-Q-x-S	+
MTRX1011A	H-*H*-*H*-Y-x-Q-x-S	++

Although we had previously experienced RF interference in ATA assays developed for other monoclonal antibody therapeutics targeting the RA population, the baseline signals were much lower. In one assay developed previously, RA samples with RF levels ranging from 1 to 1000 IU/mL generated signals between 0.01 and 0.1 OD at 450 nm in the assay (data not shown); however, the signals ranged from 0.1 to 4.0 OD in the MTRX1011A ATA assay (Fig. 3b). We have found limited literature focusing on RF interference in ATA assays. There has been one other case reported [29], in which RF interference in an ATA assay was specifically attributable to the presence of aggregates in the conjugated detection and capture antibodies. In this paper eliminating aggregates in the conjugated reagents effectively reduced the RF interference. However, reagent aggregation was not an issue in our MTRX1011A ATA assay.

Our observation of an unusually high OD signals in the RA population with our ATA assay, suggested a stronger interaction between RF and the Fc region of MTRX1011A compared with the previous molecules we had evaluated. To understand the high baseline signals (OD up to 4.0) observed with MTRX1011A, further characterization work was performed. As mentioned previously, there were two mutations introduced in the Fc portion of MTRX1011A. An amino acid substitution of N297A was introduced to impair its binding to Fcγ receptor and an additional single amino acid substitution N434H to improve the binding of the molecule to the FcRn. To investigate which of the two mutations were responsible for the high signal observed, caused by RF binding to MTRX1011A, a series of immunodepletion experiments were performed with three individual sera containing known RF levels as well as a positive control (Fig. 5b). A decrease in signal was only observed with anti-hu IgM and the two molecules containing the N434H mutation (MTRX1011A and rhuMAb Y). No immunodepletion was observed with antibodies containing either no mutation (rhuMAb X) or with the N297A mutation alone (TRX1 and rhuMAb X (N297A)). The signal of the positive control was only reduced with MTRX1011A and TRX1 as expected and not with other molecules or with anti-hu IgM. The stronger interaction between RF and the N434H mutation in the Fc portion of MTRX1011A was further confirmed by comparing MTRX1011A and TRX1 side-by-side in a direct binding ELISA (Fig. 6).

But why is N434H mutation so critical in RF binding? The crystal structure of human IgM Fab binding to IgG Fc [23] reveals a conserved epitope in human subclass IgG1, 2 and 4 but not IgG3 Fc region (Table 2) that is recognized by RF. IgG3 differs from the other isotypes by a change from a histidine to an arginine residue at position 435. The importance of this residue in RF binding has been previously established [30,31]. Mutation of this residue from an arginine to a histidine restores the binding of RF. Therefore, we hypothesized that the N434H FcRn mutation on MTRX1011A which is adjacent to the critical RF epitope at residue 435, creates a neo-epitope for RF. This proximity seems to be enhancing the recognition and/or binding affinity of RF to MTRX1011A.

5. Conclusion

Our results highlight the complexities associated with bioanalytical method development to support antibody therapeutic evaluations. These challenges are not only attributable to the patient population, disease state, and route of delivery but also due to characteristics specific to the product, in this case the unforeseen characteristics of an engineered molecule designed to improve its half-life.

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