Adalimumab immunogenicity and anti-hinge antibody levels in patients with rheumatoid arthritis


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Abbreviations:
ABT: antigen binding test
bELISA: bridge ELISA
CRP: C-reactive protein
ELISA: enzyme-linked immunosorbent assay
GLM: generalized linear model
MFI: mean fluorescence intensity
nMFI: normalized mean fluorescence intensity
NHP: normal human plasma
RA: rheumatoid arthritis
RACER: Rheumatoid Arthritis Comparative and Effectiveness Research
RT: room temperature
TNFα: tumor necrosis factor-alpha

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Abstract

Patients with rheumatoid arthritis (RA) are frequently treated with anti-TNFα immunoglobulin therapy but develop neutralizing antibodies against these drugs, necessitating therapeutic monitoring of drug concentrations and anti-drug antibodies. Patients with RA have multiple factors related to their autoimmune disposition that interfere with conventionally used methods to detect anti-drug antibodies. Currently deployed analytical methods have significant limitations that hinder clinical interpretation and/or routine use, and no method can detect immunogenicity and drug levels simultaneously to provide clinically meaningful recommendations. Given these limitations, the objective of this study was to identify sources of and associations with assay interference in patients with RA. We designed a modular, immunogenicity and drug concentration detection technology to identify the factors that interfere with the detection of adalimumab and anti-adalimumab antibodies in a cohort of 206 patients with RA. Patients were included from the University of Pittsburgh Rheumatoid Arthritis Comparative Effectiveness Research (RACER) registry. In this cohort, we analyzed clinical and plasma factors associated with anti-adalimumab and anti-hinge antibodies. A novel flow cytometry-based assay was developed and validated that simultaneously measures adalimumab and anti-adalimumab antibody concentrations, overcoming many of the interference factors that are limitations of conventional assays, including anti-Fc and anti-hinge antibodies. C-reactive protein (p=0.035), DAS28 score (p=0.002), and disease activity category (p=0.009) were significantly associated with anti-adalimumab antibodies, but not with anti-hinge antibodies (p>0.05). Anti-hinge antibodies were inversely associated with drug neutralizing antibodies (p=0.002). In patients with RA, anti-hinge antibodies may have a potential protective effect against the development of anti-adalimumab antibodies.
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Significance Statement

Using a novel cytometric assay that simultaneously measures drug and anti-drug antibodies, we overcame many interferences that hinder the clinical interpretation of adalimumab immunogenicity testing. Our investigation in patients with RA demonstrated that immunogenicity impaired the pharmacological action of adalimumab via analysis of RA disease severity markers. We also demonstrate that patients with anti-hinge antibodies had lower anti-adalimumab antibody levels and decreased drug neutralization. Our results suggest that anti-hinge antibodies can predict adalimumab immunogenicity before the start of therapy.
1. Introduction

Anti-tumor necrosis factor alpha (TNFα) therapy is essential for the treatment of several immunologic and rheumatologic disease states including rheumatoid arthritis (RA) (Navarro-Sarabia et al., 2005; Aletaha and Smolen, 2018; Mian et al., 2019). Anti-TNFα immunoglobulin therapy such as adalimumab and infliximab are recommended following therapeutic failure of first-line conventional disease-modifying anti-rheumatic drugs (DMARDs), typically methotrexate (Singh et al., 2016; Smolen et al., 2017; Aletaha and Smolen, 2018; Mian et al., 2019). These drugs are highly effective in reducing disease activity or severity (Weinblatt et al., 2003; Keystone et al., 2004; Navarro-Sarabia et al., 2005); however, a major limitation to their use is their immunogenicity (Bendtzen, 2015a). The development of neutralizing antibodies strongly predicts compromised therapeutic efficacy due to reduction in the achieved drug exposure (Radstake et al., 2009; Bartelds et al., 2010; Bartelds et al., 2011; Chen et al., 2015; Jani et al., 2015; Strand et al., 2020). Given the high predictive value of anti-drug antibodies in predicting drug exposure and therapeutic failure, therapeutic drug monitoring of anti-drug antibody titers and drug concentrations is essential for achieving and maintaining disease remission.

Several methods have been developed to detect anti-adalimumab antibodies. However, when deployed in clinical practice for use in the target patient population, they face significant limitations that can impede interpretation and routine use (Bendtzen, 2015b; Ogric et al., 2017). The most common technique is the enzyme-linked immunosorbent assay (ELISA), such as the bridging ELISA (bELISA). The bELISA requires anti-adalimumab antibodies to simultaneously crosslink the plate-bound adalimumab for capture and the labeled drug for detection (Hart et al., 2011). While easy to perform and inexpensive, the bELISA can yield both false negative and
positive results from multiple sources of interference that can impede interpretation. A major limitation is the inability to measure anti-adalimumab antibodies in the presence of therapeutic drug in the sample, both due to competition with the labeled drug used for detection (Hart et al., 2011) and formation of immune complexes that block the Fab arms of the anti-adalimumab antibody (van Schouwenburg et al., 2013), both of which can lead to false negative signals. Another source of potential false negative results is from undetected IgG subtypes that are not bivalent (i.e. IgG4), which are not negligible with prolonged immunization (van Schouwenburg et al., 2012). False positive signals can result from rheumatoid factors that bridge the Fc regions of the adsorbed and labeled adalimumab, interfering with the desired interaction with the variable domains of anti-adalimumab antibodies (Bendtzen, 2015b). A common alternative is the antigen-binding test (ABT), another bridging assay, typically using radiolabeled bivalent intact Fab arms F(ab’)2 from adalimumab to detect anti-adalimumab antibody captured on a Sepharose-bound substrate (e.g. Protein A) (Rispens et al., 2012; Rispens et al., 2013). One improvement over the bELISA is that the ABT should be free of rheumatoid factor interference given the use of adalimumab fragments lacking the Fc region in place of the intact antibody for detection. However, this assay is still prone to interference, such as from therapeutic adalimumab immune complexes, therapeutic antibody capture on the Sepharose-bound substrate, and other autoantibodies (Hart et al., 2011). Other methods such as the acid-dissociation radioimmunoassay (ARIA) and pH-shift anti-idiotype assays (PIA) have improvements to overcome drug-anti-drug immune complexes but still require the use of radiation, presenting hazards that preclude routine clinical use (Bendtzen, 2015b; Ogric et al., 2017). Currently, there is no validated method to detect both adalimumab drug concentrations and anti-adalimumab antibodies simultaneously.
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Given the various factors that may interfere with the detection of adalimumab and anti-adalimumab antibodies, the objective of this study was to identify potential sources and associations with assay interference in patients with RA. We enrolled patients with RA both naïve and treated with adalimumab and characterized their plasma for sources of interference by designing a modular system that can detect antigen-specific binding as well as interference by autoantibodies to the Fc, hinge, and Fab regions of adalimumab IgG1 antibody. In the process, we developed a high throughput cytometric-based method resistant to many of the identified interferences to simultaneously detect anti-adalimumab antibodies and adalimumab drug concentrations. Finally, we found associations between patient clinical characteristics and anti-adalimumab antibodies and that anti-hinge antibodies inversely associate with neutralizing anti-adalimumab antibodies.
2. Materials and methods

2.1 Patient cohort

The studied patients were enrolled from the University of Pittsburgh Rheumatoid Arthritis Comparative Effectiveness Research (RACER) registry. RACER has continuously enrolled patients older than 18 years who have been diagnosed with RA by rheumatologists at the University of Pittsburgh Medical Center (UPMC). The RACER registry protocol was approved by the University of Pittsburgh Institutional Review Board and all enrolled patients gave informed consent prior to enrollment.

2.2 Adalimumab digestion and biotinylation

2.2.1 Preparation of F(ab')2 and Fab: F(ab')2 and Fab monoclonal antibody fragments were prepared from 4 mg of adalimumab (AbbVie Inc, USA) by pepsin and papain digestion, respectively, using Pierce™ F(ab')2 and Fab Preparation Kit (Thermo Scientific, USA). Enzymatic adalimumab digestion was confirmed by electrophoresis under non-reducing conditions. The final protein concentrations of the generated fragments were determined by BCA kit (Thermo Fisher, USA).

2.2.2 Biotinylation: F(ab')2, Fab and TNFα (Miltenyi Biotec, Germany) were biotinylated using Thermo EZ-Link Sulfo-NHS-LC Biotinylation Kit (Thermo Scientific, USA). The final concentrations were determined by Nanodrop 2000.

2.3 Detection of anti-adalimumab antibodies by F(ab')2-based assay

A total of 1.7 x 10^5 SPHERO™ 6 μm streptavidin-coated polystyrene beads (Spherotech, USA) were added to 96 well microtiter plates (Corning, USA) and washed (0.1% Tween 20/PBS). 100 μL/well of 2 μg/mL biotinylated digested adalimumab F(ab')2 fragments in buffer (Tris/10% BSA/0.05% Tween 20) was added to washed beads and incubated for 15 minutes at room
temperature (RT) on a shaker. Beads were washed and blocked with 100 µL/well of 5% BSA in PBS for 15 minutes at RT on a shaker. Samples were diluted 1:100 and added to the plate, incubated for 1 hour at RT on a shaker, and washed. For detection, 100 µL (1:100 diluted) anti-human IgG Fc-APC (Biolegend, USA) was added and incubated overnight on a shaker at 4°C, washed, and resuspended in 200 µL of running buffer/PBS and acquired by flow cytometry (MACSQuant analyzer 10 flow cytometer, Miltenyi Biotec, Germany). Negative controls were beads alone or wash buffer only; positive control was 300 ng/mL of human anti-adalimumab IgG1 (Biorad-HCA204, USA) prepared in 1% NHP (normal human plasma). Mean fluorescence intensities (MFI) were recorded. Normalized MFI (nMFI) was calculated by subtracting off the background MFI, transforming logarithmically, and dividing the transformed MFI by the transformed MFI of the negative control. Samples were considered positive for anti-adalimumab antibodies if nMFI exceeded 3 times the standard deviation of the adalimumab naïve training set.

2.3.1 Determination of rheumatoid factor interference

To determine if rheumatoid factor (autoantibodies directed against the Fc region of IgG) interferes with detection of anti-adalimumab, the extent to which anti-human IgG Fc labeled with APC bound to streptavidin beads coated with adalimumab F(ab’)_2-biotin, TNFα-biotin, buffer, or to protein G beads coated with adalimumab was determined.

2.3.2 Detecting autoantibody interference during the capture of anti-adalimumab using adalimumab F(ab’)_2 and estimating anti-hinge antibody levels

After blocking the beads, 20 µg/mL of human IgG F(ab’)_2 recombinant protein (Rockland 009-0104, USA) in PBS was added and incubated for 1 hour at RT on a shaker and subsequently detected as above. Samples with greater than or equal to 50% decrease in MFI following the addition of F(ab’)_2 were considered anti-hinge positive.
2.4 Detection of anti-adalimumab antibodies by Fab-based assay

The procedure was identical to F(ab’)2-based assay above except for the substitution of the biotinylated digested adalimumab F(ab’)2 fragment with the Fab fragment. No IgG F(ab’)2 competition was performed.

2.5 Simultaneous detection of adalimumab and anti-adalimumab antibodies by flow cytometry

A total of 1.7 x 10^5 SPHERO™ streptavidin-coated polystyrene 6 and 2 µm size beads (Spherotech, USA) were added to 96 well microtiter plates (Corning, USA) and washed (0.1% Tween 20/PBS). 50 µL of 2 µg/mL biotinylated digested adalimumab F(ab’)2 fragment or 2.5 µg/mL biotinylated TNFα prepared in buffer (Tris/10% BSA/0.05% Tween 20) was added to 6 µm and 2 µm beads, respectively. After incubation for 15 minutes at RT on a shaker, beads were washed with buffer and blocked with 100 µL/well 5% BSA in PBS for 15 minutes at RT on a shaker. Different concentrations of adalimumab (7.5 to 1000 ng/mL) were prepared and spiked with 10 or 100 ng/mL of anti-adalimumab antibody in PBS. Samples were added to the prepared beads and incubated for 1 hour at RT on a shaker. Adalimumab and anti-adalimumab antibodies were detected using anti-human IgG Fc-APC and flow cytometry as described for Detection of anti-adalimumab by F(ab’)2-based assay. Unbound beads alone were used as negative control.

2.6 Detection of anti-adalimumab antibodies by bridge ELISA

Microtiter plates (Corning, USA) were precoated overnight at 4°C with 100 µL/well of adalimumab 1µg/mL capture antibody (AbbVie Inc, USA) in PBS. After washing (0.1% Tween 20 in PBS), plates were blocked with 5% BSA in PBS for 1 hour at RT. After washing, 50 µL/well of samples and controls were added (10% NHP in PBS as negative and 0.625 µg/mL anti-adalimumab IgG1 as positive controls, respectively) and incubated for 1 hour at RT. After
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2.7 Ex vivo adalimumab neutralization
Adalimumab concentration was measured with and without 300 ng of adalimumab spiked into patient plasma samples diluted 1:100 in PBS. Percent neutralization was calculated as 1 – (adalimumab concentration after spike/adalimumab concentration before spike) x 100. Negative control was 300 ng/mL of human anti-adalimumab IgG1 (Biorad-HCA204, USA) in 1% NHP and positive control was the mixture of 300 ng/mL of adalimumab, human anti-adalimumab IgG1, and 20 µg/mL of human IgG F(ab’)2.

2.8 Demonstrating interference by anti-hinge antibody
Adalimumab F(ab’)2 and Fab fragment bound beads were prepared as described above. Anti-human IgG1 hinge antibody (SouthernBiotech-9052, USA) was added, incubated, washed, and detected with anti-human IgG Fc antibodies labeled with APC.

2.9 Statistical analysis
Baseline characteristics were evaluated by unpaired two-sample t-test or Wilcoxon Rank-Sum for continuous variables, depending on whether the data were normally distributed or not. Wilcoxon Rank-Sum for ordinal variables, and Fisher’s exact test for nominal variables. Differences between groups were analyzed with Wilcoxon Rank-Sum for continuous and ordinal variables for two groups, Kruskal-Wallis with Dunn posttest for multiple comparisons for more than two groups, Fisher’s exact test for categorical variables, and Pearson correlation coefficient ($r^2$) to compare anti-adalimumab signals measured by Fab vs. F(ab’)2-based assay. The univariate analyses were conducted using the generalized linear model (GLM) for assessing the association between clinical outcomes and anti-adalimumab and anti-hinge signals for the covariates RF factor status, DAS28 score, disease activity category, log$_{10}$ C-reactive protein (CRP), and anti-adalimumab antibody status. The effect size was calculated as the natural exponentiation of the beta-coefficient. R statistical software (version 2.13.2) and GraphPad Prism 8.4 (GraphPad Software, California, USA) were used. The criterion of significance was $\alpha=0.05$. All values in text and figures are means ± SD unless specified otherwise.
3. Results

A total of 206 patients from the RACER study enrolled between 2/15/10 and 5/9/11 were included in this sub-study and evaluated. Patients in the adalimumab treated cohort must have received adalimumab for \( \geq 6 \) months (n=91), and patients in the adalimumab naïve cohort had no documented previous exposure to adalimumab (n=115). Clinical characteristics at the time of sample collection are provided in Table 1. Patients were majority female sex. Of the 206 patient samples available for this study, information regarding the use of methotrexate was available for only 44\% of patients and the majority of those were receiving concomitant methotrexate therapy. Overall, patients were generally well-controlled on therapy with a median DAS28 score of 2.8. Patients naïve for adalimumab were older and had lower DAS28 scores.

Given the need to develop a high throughput, clinically meaningful adalimumab immunogenicity testing platform and to design a modular system to assess interference factors, a novel flow cytometry-based detection method was developed to overcome many of the limitations of previous methods. Capture of anti-adalimumab antibodies and adalimumab was achieved using biotinylated adalimumab F(ab')\(_2\) and TNF\(\alpha\), respectively, conjugated to streptavidin-coated beads of two different sizes, allowing for discrimination between and adalimumab and anti-adalimumab antibodies by flow cytometry gated on side/forward scatter (Figure 1 and Supplemental Figure 1). Different assay parameters were optimized during method development, including the concentration of adalimumab F(ab')\(_2\), the number of streptavidin beads, and the detection antibody incubation time (Supplemental Figure 2). Our cytometric methods demonstrated negligible binding by rheumatoid factors to antibody fragments or beads but strong binding to adalimumab, as expected (Supplemental Figure 3). The linear ranges of anti-adalimumab and adalimumab antibody detection were between 1 to 150 and 79 to 600...
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ng/mL, respectively (Figure 2A-B). To demonstrate the ability to detect anti-adalimumab antibodies in the presence of therapeutic adalimumab from the same sample simultaneously, we created an artificial system of adalimumab and anti-adalimumab antibodies spiked into NHP at several concentrations and detected them by flow cytometry (Figure 2C-D). Our data indicate that the effect of anti-adalimumab antibodies on the free adalimumab available for attenuating TNFα is dependent on both the concentrations of adalimumab and anti-adalimumab. We found that low levels of anti-adalimumab can have less than a 25% decrease in free adalimumab concentrations, whereas increasing adalimumab concentrations in the presence of antibodies can rescue drug neutralization. Our results suggest that dose escalations can increase adalimumab levels in patients with low levels of anti-drug antibodies to assure efficacy.

When RACER patient cohort samples were initially assayed using this cytometric method and adalimumab F(ab’)2 for capture, anti-adalimumab signals were detected unexpectedly in patients that were adalimumab naïve (Figure 3A), indicating that potential interference is not solely due to anti-Fc autoantibodies. Upon clinical verification of true naïve status and based on previous studies (Rispens et al., 2012), we hypothesized that patients with rheumatoid arthritis had autoantibodies directed against F(ab’)2, likely the hinge region of the antibody. The assay was initially modified to outcompete the interfering antibodies with human IgG F(ab’). This reduced the false detection (Figure 3A-B), suggesting that the false positive results were due to autoantibodies against a component of F(ab’)2 or rather anti-hinge antibodies. Based on the change in MFI after the addition of IgG F(ab’), we found that 51% of RA patients were positive for anti-hinge antibodies, and there was no significant difference in the proportion of patients with anti-hinge antibodies between the adalimumab naïve versus treated cohorts (Figure 3C, 54 vs 46%, p=0.262).
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Using samples from 86 patients who were adalimumab naïve as a training set (75% of naïve cohort), an antibody positive threshold was established at 2.1458 nMFI. This threshold was able to identify 100% of adalimumab naïve test set samples (n=29) as negative for anti-adalimumab antibodies (Figure 4A). We then tested samples from patients receiving adalimumab and found that 4.4% of RACER patients receiving adalimumab met our predefined threshold of anti-adalimumab IgG antibody positivity, consistent with many of these patients receiving concomitant methotrexate (Krieckaert et al., 2012). Given the utility of our cytometric methods, we confirmed that patients with the highest anti-adalimumab antibody nMFI signals were truly anti-adalimumab neutralizing antibody positive by confirming greater adalimumab neutralization ex vivo relative to adalimumab naïve patients and normal human plasma (Figure 4B, p <0.0001). Furthermore, the patient antibody status results from the cytometry method were compared to the standard bELISA. The bELISA failed to discriminate anti-adalimumab antibodies in adalimumab naïve vs treated patients (Supplemental Figure 4). In this patient population, the bELISA only had a specificity of 35% for patients naïve for adalimumab and 23% for patients treated with adalimumab (Supplemental Table 1).

We next verified that the interfering antibodies directed against F(ab’)2 were anti-hinge autoantibodies. We modified the detection method by preparing adalimumab Fab fragments lacking the hinge region for anti-adalimumab capture in place of F(ab’)2. As expected, anti-hinge antibodies did not bind to Fab adalimumab fragments, whereas they bound to F(ab’)2 adalimumab (Supplemental Figure 5). Consistent with these data, we measured anti-adalimumab levels using Fab capture in all RACER patients included in this study and found a strong correlation between both capture methods (Figure 4C, r=0.75, p <0.0001). This supports our
hypothesis that much of the false positive assay interference was due to anti-hinge autoantibodies.

Using the clinical anti-adalimumab data collected in the RACER cohort, we performed exploratory analyses to identify RA patient clinical characteristics and markers of disease progression associated with anti-adalimumab antibodies and anti-hinge antibodies (Figure 5 and Supplemental Table 2). Our results indicate that patients with higher anti-adalimumab antibody levels were enriched for rheumatoid factor (Figure 5A, p=0.057) and had positive correlations with CRP (Figure 5B, p=0.012) and DAS28 scores (Figure 5C, p=0.002). Consistent with greater disease severity being associated with greater anti-adalimumab antibodies, patients with moderate to severe disease activity had higher anti-adalimumab antibody levels than patients in remission or with low disease activity (Figure 5D, p=0.009). Unexpectedly, among a subset of samples with anti-hinge antibody and neutralization data, we found that patients with more anti-hinge antibodies had less adalimumab neutralization (Figure 6A, p=0.002), in contrast to our previous finding of a positive correlation between anti-adalimumab levels and adalimumab neutralization (Figure 4B). Based on the association between anti-hinge antibody levels and anti-adalimumab neutralization, we next assessed whether there were additional associations between anti-hinge antibody levels and other markers of immune responses. Consistent with the association between anti-hinge antibodies and anti-adalimumab neutralization, we found a negative correlation between anti-hinge and anti-adalimumab antibody levels (Figure 6B, p=0.028) and with anti-adalimumab antibody positivity (Figure 6C, p=0.026). However, there was no association between anti-hinge antibody levels and rheumatoid factor, disease activity, or CRP levels (Supplemental Figure 6A-D, p>0.05). Altogether, our data indicate that our novel cytometric adalimumab immunogenicity method can detect clinically meaningful antibodies and
that anti-hinge antibodies may be able to identify patients more likely to develop immunogenicity to adalimumab.
4. Discussion

In this clinical study of patients with RA, we demonstrate the presence of several factors that interfere with the detection of anti-adalimumab antibodies. We describe a novel flow cytometry-based assay that overcomes many of these interferences and simultaneously measures adalimumab drug concentrations and anti-adalimumab antibodies in patient plasma samples. Consistent with previous studies, we found that patients with RA, and no prior adalimumab exposure, inherently have factors that can cause false-positive anti-adalimumab signals, or rather that bind to the adalimumab molecule and are recognized by the secondary antibody used for detection. Our approach using papain to enzymatically digest adalimumab and capture anti-adalimumab antibodies with Fab fragments confirmed that RA patients have anti-F(ab’)_2 autoantibodies directed primarily against the hinge region of the molecule. Our analysis in both naïve and adalimumab-treated patients demonstrates that these autoantibodies are present before adalimumab exposure. Furthermore, we found that markers of worse RA disease severity (higher DAS28 score and CRP) and autoimmunity (RF positivity) were associated with higher anti-adalimumab antibody levels. Furthermore, we recapitulated the limitations of the conventional bELISA, which can cause false-positives in 70-90% of patients with RA with rheumatoid factor (Dorner et al., 2004; Tatarewicz et al., 2010), by demonstrating poor specificity for detecting anti-adalimumab antibodies in both patients with and without prior adalimumab exposure.

Even though the antigen-binding tests (ABT) are slightly more robust alternatives to the bELISA, the anti-hinge antibodies present in patients with RA will also lead to false positive signals if radiolabeled F(ab’)_2 fragments are used and bound on the Sepharose-bound substrate for anti-adalimumab capture (Rispens et al., 2012). We demonstrate in our cytometric assay that this false positive anti-adalimumab signal can be reduced by outcompeting the non-specific
Adalimumab immunogenicity and anti-hinge antibody binding with human IgG F\(\text{ab'}\)\(_2\). Furthermore, the lower limits of detection for the bELISA and ABT have been reported as approximately 20 ng/mL and 120 ng/mL, respectively (Hart et al., 2011). The method developed here is more specific and sensitive, allowing quantification of anti-adalimumab antibody concentrations between 1 to 150 ng/mL, with a lower limit of detection of 0.5 ng/mL in plasma samples from patients with RA while also providing simultaneous quantification of adalimumab concentrations from the same sample (Figure 2). Our modular system can also be readily modified to measure the immunogenicity and drug concentrations of other anti-TNFα therapeutics. The benefit of our system versus other methods is that analysis of drug concentrations along with anti-drug antibodies facilitates clinical decision making. Nevertheless, further studies are needed to determine the antibody to drug levels requiring treatment interventions for the clinical implementation of this system. Due to the lack of a “gold standard” assay comparison for validation, correlation with clinical response and adalimumab concentrations in patient cohorts would be needed (Williams et al., 2012).

Consistent with previous studies that found associations with anti-adalimumab antibodies and DAS28 scores (Bartelds et al., 2010; Chen et al., 2015; Jani et al., 2015), we identified that more severe RA disease was associated with higher anti-adalimumab antibody levels. As we demonstrated \textit{ex vivo}, these antibodies neutralize adalimumab activity, which likely decreases active drug exposure and compromises therapeutic efficacy \textit{in vivo}. However, it is also possible that more severe RA immunopathology and autoimmunity could contribute to anti-adalimumab antibody formation, as we observed that RF positive patient samples had higher anti-adalimumab MFIs than those that were RF negative. It is also possible that the recognition of adalimumab’s Fc region (i.e. in RF positive patients) facilitates the development of a true anti-adalimumab response in patients with RA.
Consistent with other studies, we demonstrated the presence of anti-hinge antibodies in patients with RA (Rispens et al., 2012; van de Stadt et al., 2014). Interestingly, we found that patients with more anti-hinge antibodies were less likely to neutralize adalimumab (Figure 6A) and that anti-hinge antibodies inversely correlate with anti-adalimumab antibodies (Figure 6B). However, unlike anti-adalimumab antibodies, the presence of anti-hinge antibodies was not associated with markers of disease severity or rheumatoid factor positivity in our study, suggesting that the potential protective effect of anti-hinge antibodies against the development of anti-adalimumab antibodies does not protect against other markers of RA autoimmunity. It has been suggested that anti-hinge antibodies may suppress B-cells and antibody production (Terness and Opelz, 1998; Terness et al., 2002), but whether this extends to anti-drug antibodies is unknown. Others have found anti-hinge antibodies in the synovial fluid of patients with RA (Ryan et al., 2008), suggesting that they may exacerbate overall RA pathophysiology by restoring Fc-mediated antibody effector function (Brezski et al., 2008; van de Stadt et al., 2014; Falkenburg et al., 2017; Trouw et al., 2017). Our results may, nevertheless, indicate that it may be possible to predict TNFα inhibitor immunogenicity from preexisting anti-hinge antibody levels.

Although adalimumab is a fully human antibody, anti-adalimumab antibodies still develop frequently, with a wide range in reported prevalence across studies from 6-87% of patients with RA (Emi Aikawa et al., 2010; Krieckaert et al., 2010). The methods used to detect anti-adalimumab antibodies in these studies varied (Gorovits et al., 2018), and as we have shown in this study, the presence of various unaccounted interference factors likely contributes to the reported variability. Additionally, the concomitant administration of methotrexate with adalimumab is associated with a decreased incidence of anti-adalimumab antibodies (Bartelds et
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al., 2007; Bartelds et al., 2010; Krieckaert et al., 2012), possibly due to the immunomodulatory effects of methotrexate via the adenosine pathway (Krieckaert et al., 2010; Jani et al., 2014). Theoretically, methotrexate could also reduce the levels of other interfering antibodies. Our findings that 4.4% of the RACER patients included in this study develop anti-adalimumab antibodies and that worse RA disease severity and autoimmunity associate with anti-adalimumab antibodies are consistent with previous studies (Radstake et al., 2009; Bartelds et al., 2010; Bartelds et al., 2011) and possibly suggest that better disease control could also prevent the formation of anti-drug antibodies or that these factors prime patients to develop TNFα inhibitor immune responses.

Patients with RA have an inherent autoimmune phenotype and therefore, the presence of these interfering antibodies and proclivity towards developing anti-drug antibodies are not unexpected (Volkov et al., 2020). However, our results underscore the need for appropriate testing methodologies in the target population, including the selection of appropriate disease-specific controls, as observed from the interindividual variability in anti-adalimumab nMFI signal (Figure 4A). Clinically, it is imperative to identify the true antibody status of patients because therapeutic decisions are dependent on the source of therapeutic failure: whether the lack of efficacy is driven by neutralization from anti-drug antibodies or lack of pharmacodynamic response to anti-TNFα therapy. By providing timely and accurate drug concentration and neutralizing antibody information, pharmacotherapy for patients with RA can be optimized.

A limitation of our study is that adalimumab concentrations in patient samples were not measured due to lack of drug administration records in the home setting, although our simultaneous detection assay accurately detected spiked adalimumab. Likewise, measuring
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Adalimumab neutralization for all the patients included in our study was not possible due to sample limitations. While we and others (Rispens et al., 2012) show that the autoantibodies against F(\(\text{ab}'\))\(_2\) are primarily directed to the hinge region of immunoglobulins, we did not directly distinguish between interference resulting from anti-hinge or other possible regions that differ between the Fab and F(\(\text{ab}'\))\(_2\) molecules. Additionally, the anti-adalimumab antibody positive threshold established can be further refined in a larger patient cohort to further capture interindividual variability. However, adding data from the adalimumab naïve testing subset to the training subset to refine the threshold identified only one additional patient as anti-adalimumab antibody positive, demonstrating the robustness of our results. Furthermore, we did not find an association between anti-adalimumab antibodies or anti-hinge interference and methotrexate use, but this was limited by data available and high proportion of reported use in the RACER cohort. Our cohort of 206 patients with RA of which 91 were treated with adalimumab is still sizable and comparable to other trials (Bartelds et al., 2007; Chen et al., 2015) and the point estimate of the effect size of anti-adalimumab antibodies on disease severity (i.e. DAS28 scores) is similar relative to those reported previously (Garces et al., 2013; Chen et al., 2015). While our results suggest that increased adalimumab doses could overcome neutralization in settings of low anti-adalimumab antibody concentrations and has been successfully used previously (Bartelds et al., 2007), consideration needs to be given to potentially increased adverse effects. The threshold at which therapeutic substitution with alternative anti-TNFα therapy or other immunosuppressive agents should occur is unknown and requires further research. Finally, although age was statistically different between the adalimumab treated versus naïve groups, it was not associated with anti-adalimumab nor anti-hinge signals, making it an unlikely clinical or biologic confounder.
Adalimumab immunogenicity and anti-hinge antibody

Altogether, we identified factors in patients with RA that can interfere with conventional technologies used to monitor anti-TNFα therapy. We developed and validated a flow cytometry-based assay that overcomes these factors and can detect adalimumab and anti-adalimumab antibodies simultaneously in plasma from patients with RA. Results using this method may correlate better with patient response to anti-TNFα therapy and improve prediction of clinical efficacy. Finally, we showed that RA disease severity associates with anti-drug antibodies while anti-hinge antibodies were inversely associated with drug neutralization and the development of anti-drug antibodies.
Author contributions

Participated in research design: Hoshitsuki, Rathod, Moreland, and Fernandez
Conducted experiments: Rathod and Ramsey
Contributed clinical samples: Moreland
Performed data analysis: Hoshitsuki, Rathod, Zhu, Fernandez
Wrote or contributed to the writing of the manuscript: Hoshitsuki, Rathod, Fernandez
All authors reviewed and approved the final manuscript.
References


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Footnotes

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Disclosures
The authors declare no conflicts of interest.
Main Figure Legends

Figure 1. Flow cytometry gating strategy identifies adalimumab and anti-adalimumab antibodies simultaneously. A) Adalimumab was captured with TNFα bound to 2 µm streptavidin-coated polystyrene beads, and anti-adalimumab antibodies were captured with adalimumab F(ab’)2 or Fab bound to 6 µm streptavidin beads. (B) Adalimumab and anti-adalimumab antibodies were detected by flow cytometry gated on side/forward scatter.

Figure 2. The cytometric assay measures anti-adalimumab and adalimumab simultaneously over several orders of magnitude. (A) Using anti-adalimumab mAb, anti-adalimumab linearity was established between 1 to 150 ng/mL, with a lower limit of detection of 0.5 ng/mL. (B) Using known spiked concentrations of adalimumab, the linear range of adalimumab detection was established between 79 to 600 ng/mL, with a lower limit of detection of 3.2 ng/mL. (C and D) Anti-adalimumab IgG1 (10 and 100 ng/mL) was spiked into serially diluted adalimumab, and anti-adalimumab and adalimumab antibody concentrations were measured simultaneously by flow cytometry. (C) Percent anti-adalimumab recovery is represented as the anti-adalimumab MFI detected at that adalimumab concentration relative to MFI detected without adalimumab spike. (D) Percent adalimumab recovery is represented as the adalimumab MFI detected relative to MFI detected without anti-adalimumab IgG1 at the serially diluted concentration.

Figure 3. Anti-hinge antibodies interfere with the detection of anti-drug antibodies. A total of 115 samples from patients with no previous adalimumab exposure (A) and 91 samples from patients treated with adalimumab (B) were evaluated by flow cytometry and resulted in high
anti-adalimumab MFIs. The addition of 20 μg/mL of human IgG F(\(\text{ab}'\))\(_2\) reduced the anti-adalimumab MFI signals in both naïve and adalimumab treated patients. (C) There was no difference between adalimumab naïve versus treated groups in MFI fold-change or proportion of patients positive for anti-hinge antibodies. Anti-hinge positive status was defined as \(\geq\)1.5-fold change as shown by the horizontal line.

**Figure 4.** RACER patients developed neutralizing anti-adalimumab antibodies during adalimumab treatment. (A) Using a subset of samples from patients naïve for adalimumab (naïve training) to estimate the anti-adalimumab antibody threshold (horizontal line), we detected that 4/91 (4.4%) of patients were positive for anti-adalimumab antibodies. Neither the normal human plasma (NHP) nor naïve RACER samples testing set (naïve testing) were positive for anti-adalimumab antibodies. (B) The top 10% of samples with the highest anti-adalimumab MFI were evaluated for adalimumab neutralization and showed greater *ex vivo* adalimumab neutralization relative to samples from naïve patients and normal human plasma controls (\(p<0.0001\)). (C) In patients treated with adalimumab, anti-adalimumab antibodies detected using Fab and F(\(\text{ab}'\))\(_2\) adalimumab fragments yield similar MFI signals. \(n=90\) samples were available for analysis.

**Figure 5.** Anti-adalimumab antibodies are associated with poorer RA disease control. In patients treated with adalimumab, anti-adalimumab nMFI was positively associated with indicators of poorer disease control including plasma (A) rheumatoid factor (RF), (B) C-reactive protein (CRP), (C) DAS28 score, and (D) disease activity category.
Figure 6. **Anti-hinge antibodies are inversely associated with anti-adalimumab immunogenicity.** Anti-hinge antibodies were negatively associated with (A) drug neutralization, (B) anti-adalimumab antibodies, and (C) anti-adalimumab antibody positive status.
Table 1. Characteristics of patients enrolled in study (n=206)

<table>
<thead>
<tr>
<th></th>
<th>Adalimumab treated (n=91)</th>
<th>Adalimumab naïve (n=115)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, n (%)</td>
<td>67 (73.6)</td>
<td>86 (74.8)</td>
<td>0.874</td>
</tr>
<tr>
<td>Age, years, Mean ± SD</td>
<td>55.8 ± 12.0</td>
<td>64.6 ± 14.2</td>
<td>&lt;0.001</td>
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<tr>
<td>Disease duration, years, Median (IQR)</td>
<td>11.7 (5.1-18.0)</td>
<td>10.5 (4.9-20.4)†</td>
<td>0.654</td>
</tr>
<tr>
<td>DAS28 score, Median (IQR)</td>
<td>3.0 (2.1-4.0)</td>
<td>2.7 (1.8-3.5) † †</td>
<td>0.025</td>
</tr>
<tr>
<td>Disease activity category, n (%)</td>
<td>† †</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>Remission</td>
<td>34 (37.4)</td>
<td>51 (45.1)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>14 (15.4)</td>
<td>29 (25.7)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>34 (37.4)</td>
<td>30 (26.5)</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>9 (9.9)</td>
<td>3 (2.7)</td>
<td></td>
</tr>
<tr>
<td>CRP (mg/dL), Median (IQR)</td>
<td>3.0 (1.1-8.4)</td>
<td>4.3 (1.8-9.7) †</td>
<td>0.093</td>
</tr>
<tr>
<td>Rheumatoid factor positive, n/n available for analysis (%)</td>
<td>52/78 (66.7)</td>
<td>29/49 (59.2)</td>
<td>0.450</td>
</tr>
<tr>
<td>Self-reported or physician reported methotrexate use, n/n available for analysis (%)</td>
<td>51/63 (80.9)</td>
<td>73/91 (80.2)</td>
<td>0.999</td>
</tr>
</tbody>
</table>

p-values are from Wilcoxon Rank-Sum for continuous and ordinal variables, except for age where the unpaired two-sample t-test was used. p-values for nominal variables are from Fisher’s Exact test.
†n=114 available for analysis
††n=113 available for analysis
Figure 1
Figure 2

(A) Graph showing the relationship between Anti-Adalimumab (ng/mL) and Anti-adalimumab MFI. The correlation coefficient $r^2 = 0.9965$.

(B) Graph showing the relationship between Adalimumab (ng/mL) and Adalimumab MFI. The correlation coefficient $r^2 = 0.9938$.

(C) Bar chart showing the percentage of anti-adalimumab recovered at different spiked adalimumab concentrations (ng/mL) for 10 ng/mL and 100 ng/mL anti-adalimumab.

(D) Bar chart showing the percentage of Adalimumab recovered at different spiked adalimumab concentrations (ng/mL) for 10 ng/mL and 100 ng/mL anti-adalimumab.
Figure 3

Adalimumab naïve samples

Adalimumab treated samples

Anti-hinge IgG antibody

Naïve  Treated
Figure 6

A

Anti-hinge IgG antibody vs. % Adalimumab Neutralization

B

Anti-hinge IgG antibody vs. Anti-adalimumab antibody status

C

Anti-hinge IgG antibody vs. Anti-adalimumab nMFI