Antibodies against antibodies: immunogenicity of adalimumab as a model
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Long term measurement of anti-adalimumab using pH-shift-anti-idiotype antigen binding test shows predictive value and transient antibody formation

Submitted for publication

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**ABSTRACT**

Therapeutic monoclonal antibodies are effective drugs for many different diseases. However, the formation of anti drug antibodies (ADA) against a biological can result in reduced clinical response in part of the patients. Measurement of ADA in the presence of (high) drug levels is difficult due to drug interference in most assays, including the commonly used antigen binding test (ABT). We recently published a novel method which enables the measurement of complexed antibodies against adalimumab (an anti-TNF antibody) in the presence of drug. Here we use this pH-shift-anti-Idiotype Antigen binding test (PIA) to measure ADA in a group of 99 rheumatoid arthritis (RA) patients treated up to three years with adalimumab. Fifty-three out of ninety-nine RA patients produce ADA. In 50 of these PIA positive patients, ADA could be detected within the first 28 weeks of treatment. Patients in which ADA could be detected in the PIA after 28 weeks of treatment were more prone to declining adalimumab levels (< 5 µg/ml) (p < 0.01) and high ADA levels which could be detected in the ABT (p < 0.05) at later time points. We observed transient ADA formation in 17/53 patients. Our data show that ADA develop early in treatment. However, levels that completely neutralize the drug may be reached much later in treatment. Furthermore, we show that the PIA has a predictive value to indicate which patients are at risk to develop clinical non-response due to immunogenicity. Also, we show that in part of the patients ADA formation is transient.
INTRODUCTION

The introduction of therapeutic monoclonal antibodies has given a major boost to the treatment of different diseases such as inflammatory bowel disease, ankylosing spondylitis, multiple sclerosis and rheumatoid arthritis (RA). In many cases these biological agents have proven to be very successful in clinical practice. However, in some patients clinical benefit diminishes in time, due to the formation of anti-drug antibodies (ADA) as described for adalimumab, infliximab and natalizumab.6-10

In case of adalimumab, the formation of ADA has been extensively studied and linked to lower adalimumab serum levels and loss of clinical response.4;7;8;11-14 In these studies, the reported frequency of ADA detection varies between 0.7% and 31.4%. The large variation in frequencies found in these studies may be caused by differences in patient groups, time of follow-up, timing of blood withdrawal, co-medication and drug dosing. In addition, different assay formats used for the measurement of ADA will also greatly affect results. Most studies on ADA formation use assays which are sensitive for drug interference, therefore leading to false negative results for ADA in the presence of adalimumab and an underestimation of ADA formation.

Moreover, in previous studies using our standard antigen binding test (ABT), which is sensitive for drug interference, some patients appeared to be transiently positive for ADA. This might indicate that some people develop tolerance against adalimumab, as has been previously described for Factor VIII.15 However, the disappearance of these antibodies is most often accompanied by an increase of adalimumab levels, which makes it impossible to distinguish between patients in which ADA truly disappear over time, and false negative results due to drug interference. To obtain insight into transient ADA formation and to enable the study of tolerance induction in these patients an ADA assay is needed that does not suffer from drug interference.

Recently, we reported such a novel assay for the measurement of ADA: the pH-shift-anti-Idiotype Antigen binding test (PIA). The assay is based on acid dissociation of adalimumab-ADA complexes and prevention of reformation by addition of excess rabbit anti-idiotype Fab upon neutralization. This allows for the measurement not only of “free” or excess ADA, but also of so-called “complexed” ADA in the presence of adalimumab (≤ 24 µg/ml). Detection of ADA using PIA in a small group of 30 adalimumab treated RA patients revealed ADA formation in 21 of these patients in the first half year of treatment.16

Here we tested three year follow-up samples of 99 adalimumab treated RA patients to investigate the time course of ADA formation and to study the frequency and clinical significance of complexed ADA. In addition, PIA measurements allow us to study potential tolerance induction in these patients.
MATERIALS AND METHODS

Patients
Sera were obtained in the first three years of adalimumab treatment from the first 99 consecutive RA patients of a prospective observational cohort as previously described. All patients had a disease activity score in 28 joints (DAS28) of ≥3.2 and fulfilled the American College of Rheumatology 1987 revised criteria for RA. Despite earlier treatment with two disease-modifying anti-rheumatic drugs (DMARDs), including methotrexate, all patients had active disease at the start of adalimumab (Abbott, Illinois, USA) treatment. This was according to the Dutch consensus statement on the initiation and continuation of TNF blocking therapy in RA. All patients used 40 mg adalimumab every other week by subcutaneous injections. In patients with an inadequate response, as judged by the treating rheumatologist, dose could be increased to 40 mg every week. Blood was withdrawn just prior of an adalimumab injection. The study was approved by the ethics committee of the Slotervaart Hospital and the Jan van Breemen Research Institute | Reade.

Production of adalimumab F(ab')2 and rabbit anti-idiotype Fab fragments
Adalimumab F(ab')2 fragments and rabbit anti-idiotype Fab fragments, were prepared as described before. Adalimumab F(ab')2 fragments and rabbit anti-idiotype Fab fragments, were prepared as described before.

Generation of adalimumab specific polyclonal rabbit anti-idiotype antibodies
Rabbits were injected intramuscularly every four weeks with 1 ml adalimumab F(ab')2 (0.1 mg/ml in PBS) using montanide as adjuvant. After four boosts the rabbits were bled and serum was collected. Antibodies were purified from the serum using sepharose-immobilized protein A (GE healthcare, Chalfont St. Giles, UK). To remove antibodies to F(ab')2 framework determinants the purified antibodies were passed three times over a human IgG-sepharose column (50 mg Nanogam (Sanquin, Amsterdam, the Netherlands) coupled to 2.5 g sepharose).

Measurement of adalimumab concentration
Adalimumab levels were measured by enzyme linked immunosorbent assay (ELISA) as described previously. For short, TNF was indirectly coated via an anti-TNF antibody and adalimumab binding was detected using biotinylated adalimumab specific rabbit anti-idiotype antibodies. Results were related to a titration curve of adalimumab in each plate. The lowest level of detection was 0.002 mg/l.
Long term PIA measurements show predictive value and transient antibody formation

Measuring ADA by antigen binding test (ABT)
The test was essentially carried out as described before.\(^{16,18}\) In short, antibodies were captured using protein A sepharose and ADA were detected using \(^{125}\)I labeled F(ab')2 adalimumab diluted in Freeze buffer (Sanquin). Antibody levels were compared to a standard serum containing ADA levels and expressed in arbitrary units (AU/ml). Patients were said to be positive for ADA if at one time point ADA were above the cut off of 12 AU/ml. All baseline samples were below the cut off of 12 AU/ml.

pH-shift-anti-idiotype antigen binding test (PIA)
Thirty micro liter of patient serum diluted 1/30 in PA buffer (PBS/0.3% bovine serum albumin) was mixed with 30 μl of 0.1 M glycine–HCl (pH 2.5). After 30 minutes incubation at room temperature 30 μl of rabbit anti-idiotype Fab (67 μg/ml diluted in PA) was added. Then the pH was neutralized by addition of 30 μl of 0.2 M Tris. ADA levels were measured in the ABT. Cut-off was determined to be 48 AU/ml, based on the measurements in all available pre-treatment sera of the 99 patients (145 sera) (mean+3xSD). All baseline sera were below the cut-off in the PIA.

Statistical analyses
To reveal differences between groups we used the independent sample T test, Mann-Whitney U or Chi square as appropriate. For estimating the proportion of patients positive in the PIA or ABT we used a log rank test. For calculation of the predictive value of the PIA a Chi square test was used. The threshold for significance was set at p < 0.05.

RESULTS

Patients
In this study we have followed the first 99 consecutive patients of a cohort of RA patients during 3 years of adalimumab treatment. The median follow-up was 156 weeks (interquartile range 40-156 weeks) and in total 58 patients completed the three year follow-up. Forty-one patients dropped out of the study, of which 16 due to treatment failure, 14 due to adverse events, while the remaining 11 stopped treatment due to other reasons such as relocation (n=5), unwillingness to participate (n=3) or loss to follow-up (n=3).

Within six months 54% of the patients develop ADA.
In 54% of the patients ADA were detected at least once by PIA during three year follow-up. Figure 1A shows the number of patients testing positive or negative at the different
time points in the PIA. In figure 1B the cumulative percentage of ADA positive patients is shown for the PIA and the for drug interference-sensitive ABT.\textsuperscript{16,19} The PIA picks up ADA in more patients (54\% vs 29\%) and at an earlier time point. (log rank test p<0.0005).

Furthermore, measurements with the PIA show that 51/53 patients (94\%) producing ADA already do so in the first 28 weeks of treatment (figure 1B). Using the standard ABT, many patients appear to start making ADA at later time points. However, the present results imply that this refers a point in time when ADA production exceeds adalimumab
levels, since only in that case can ADA be measured in the ABT.\textsuperscript{20}

We divided patients into three groups: patients negative for ADA at all time points in both assays, patients with “complexed ADA” (negative in the ABT at all time points but positive in the PIA at least once), and patients positive for “free ADA” (positive in the ABT at least once). All but two patients positive for free ADA were also positive for complexed ADA, as may be expected. These two patients were positive in the ABT at a single time point, but never in the PIA, probably because both had low levels of ADA that were below detection level of the PIA. Baseline characteristics for the three groups described above as well as the total patient population are shown in Table 1. Baseline CRP and DAS28 were statistically lower in patients without ADA compared to patients with ADA. Furthermore, ADA negative patients and patients with complexed ADA had a significantly higher frequency and dose of concomitant methotrexate usage. Also methotrexate dose was lower in free ADA positive patients compared to patients with complexed ADA.\textsuperscript{21,22}

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<th>“Complexed ADA” n=26</th>
<th>ADA positive n=29</th>
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Table 1: Demographic and Clinical Characteristics at Baseline
* p=0.007 (Fisher’s Exact)
# CRP: p=0.006; DAS28: p=0.040; MTX use: p<0.001; MTX dose: p=0.006 (Mann-Whitney U, Fisher’s Exact, independent sample T test)
^ p=0.012 (Mann-Whitney U)
Clinical relevance of ADA measured in the PIA
Twenty-two out of 70 ABT-negative patients (thus either without ADA or having complexed ADA only) reached sustained remission (DAS<2.6) during the follow-up period. In contrast, none of the 29 patients with free ADA did (p<0.001; figure 2A). Comparing PIA positive and PIA negative patients does not reveal a statistically significant difference in the number of patients reaching sustained remission (figure 2B). To further investigate this, PIA positive patients were subdivided into patients with both free and complexed ADA (PIA and ABT positive n=27), and patients with only complexed ADA (PIA positive, ABT negative n=26) (figure 2C). Patients with both free and complexed ADA were less likely to reach sustained remission, compared to PIA-negative patients (p<0.01) and patients with only complexed ADA (p<0.001). There was no significant difference in clinical response between ABT negative patients and patients with complexed ADA (p=0.4).

Predictive value of PIA measurements for future treatment failure
We investigated whether the presence of complexed ADA will eventually result in a stronger immune response capable of clearing adalimumab and leading to the presence of free ADA which can be detected in the ABT. Therefore we looked at all patients negative in the ABT after 28 weeks of treatment, who completed a minimal follow-up of 40 weeks (n=63) (figure 3A). These patients were divided into PIA-wk28 negative (n=38) and PIA-wk28 positive (n=25) patients based on measurements at t=28 weeks. In 3/38 of

Figure 2: The relationship between ADA formation in either the ABT or the PIA and clinical response. The percentage of patients who reached sustained remission (DAS28<2.6) for patients with or without ADA at least one timepoint during follow-up as measured in the ABT (A) and PIA (B) or patients with free and complexed ADA (PIA and ABT positive), only complexed ADA (PIA positive, ABT negative) or no ADA (PIA negative) during three years of treatment (C).
Long term PIA measurements show predictive value and transient antibody formation

Chapter 4

The PIA-wk28 negative patients free ADA can be detected in the ABT during subsequent follow-up. In contrast, 9/25 of the PIA-wk28 positive patients developed free ADA in at least one available serum (p<0.01). This shows that PIA-wk28 positive patients have an increased chance of developing free ADA, which can be detected in the ABT (figure 3B).

We also investigated whether PIA-wk28 positive patients have an increased chance of having low adalimumab trough levels (<5µg/ml), as detected in the last available serum. As shown in figure 3C, 9/25 of the PIA-wk28 positive patients have low adalimumab levels at the end of treatment, while only 4/38 of PIA-wk28 negative patients have low

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Figure 3: The predictive value of measuring ADA in the PIA after 28 weeks of treatment. A) Flow chart of the 99 patients in which the predictive value of measuring ADA in the PIA after 28 weeks was investigated. B) The percentage of patients in which free ADA are detected during follow-up in PIA positive and negative patients after 28 weeks of treatment. C) The percentage of patients having low adalimumab levels (<5µg/ml) at the end of treatment in PIA positive and negative patients, as measured after 28 weeks of treatment.
adalimumab levels (p<0.05). This suggests that PIA-wk28 positive patients have a higher chance of having low functional adalimumab levels at the last available serum sample.

**Transient ADA production**
Using the ABT for the detection of ADA we have observed transient ADA production in 11 of the 99 patients studied here (4 representative patients shown in figure 4A-B). Antibody formation was considered to be transient if ADA measurements were above the cut off at any time point during treatment and negative at the last available time.

**Figure 4:** The course of adalimumab levels and ADA levels as measured in the ABT and the PIA in single patients. All values below the cut-off of 12 AU/ml in the ABT or 48 AU/ml in the PIA are plotted as 5 AU/ml. A) Two representative patients with transient ADA production measured in the ABT and PIA. B) Two examples of patients with transient ADA production measured in the ABT and persistent ADA positivity in the PIA. C) Two examples of patients negative in the ABT and transiently positive in the PIA.
Long term PIA measurements show predictive value and transient antibody formation

point. This disappearance of ADA in the ABT is often accompanied by an increase of drug levels (n=10). Since the ABT is sensitive for drug interference, the ABT did not enable us to determine whether the disappearance of ADA was the result of a transient immune response, or the effect of false negative results due to drug interference. Measurements using the PIA show that in only four of these patients the ADA response is confirmed to be truly transient (two representative examples shown in figure 4A). In the remaining six patients ADA levels are being masked by increased drug levels (n=6) (representative figures shown in figure 4B). In two of these patients this might be explained by dose increase. The last patient was only weakly positive (12 AU/ml) in the ABT and no ADA were detected using the PIA (not shown).

The PIA also revealed transient ADA formation in thirteen patients in which no antibodies were detected in the ABT (figure 4C). There is no clear link between dose increase in some patients and transient ADA production. Furthermore, in an additional five patients we observed a severe drop in ADA levels (> 66% decrease of ADA). Although ADA levels stayed above the cut off of 48 AU/ml during the period of follow-up, it might be expected that in these patients eventually ADA levels decrease below the cut off (data not shown). In total these data show that in 17/53 patients producing ADA, this antibody response is transient as measured in the PIA.

![Model for the clinical effect of ADA development](image)

**Figure 5: Model for the clinical effect of ADA development.** We propose that ADA development only results in clinical non-response if it results in low functional adalimumab levels.
DISCUSSION
Here, we present long-term data on ADA levels measured in the PIA in a group of 99 adalimumab treated RA patients and show that 53/99 patients produce ADA against adalimumab. We show that complexed ADA measured only in the PIA, are not linked to a reduction in clinical response, while ADA measured in the ABT are. Due to drug interference, the ABT can only detect ADA if adalimumab levels are low. Hence, it seems that the most important variable governing clinical response is the adalimumab concentration (figure 5). However, in patients who continue to make high levels of complexed ADA, functional adalimumab levels drop, eventually allowing for the detection of ADA in the ABT and leading to a loss of clinical response.

Although not directly linked to reduced clinical response, ADA measurement using the PIA might provide a predictive tool for development of free ADA and loss of therapeutic levels of adalimumab. We find that in 94% of the patients positive in the PIA, ADA can be detected within the first 28 weeks of treatment. The detection of ADA in the PIA at 28 weeks of treatment is indicative for a future decline of adalimumab levels (<5µg/ml) due to potentially increased ADA production. PIA measurements after 28 weeks might provide insight into which patients are at risk of developing free ADA.

It would be very interesting to see whether there is a link between HLA types and ADA formation. Such a correlation has been described for the immunogenicity of factor VIII used for the treatment of hemophilia patients.23,24 Some HLA types might have a higher affinity for adalimumab peptides leading to more efficient peptide presentation and ADA formation in these patients.

Our results suggest that many patients have complexed ADA. Unpublished data from our group show that these are present in form of small immune complexes, which suggests that many patients are continuously exposed to large numbers of small immune complexes. We can only speculate what the effect of these complexes might be in clinical practice. On the one hand, small immune complexes might block FcγR, being too small to signal efficiently yet binding stronger than monomeric IgG. In this respect, it is interesting that a similar mechanism has been suggested for IVIG which contains dimeric IgG25. On the other hand, in systemic lupus erythematosus and serum sickness, long term exposure to small immune complexes leads to an increased risk of type III hypersensitivity reactions. A recent paper has shown a link between ADA production and increased risk on trombo-embolic events.26 It would be interesting to study whether this is correlated to the presence of small immune complexes in these patients.

Our data also shows transient ADA production in 17/53 patients that were ADA positive. The median length between the first ADA positive serum sample and the first ADA negative there after was 50 weeks (range 12-114 weeks). Due to the large drop-out numbers and the extended time of ADA formation in transiently positive patients we
were not able to study the clinical relevance of transient ADA formation. Investigation of transient ADA production in more detail might allow us to investigate the possibility to induce tolerance as described for factor VIII. In factor VIII treatment, tolerance induction is achieved by long term exposure to high levels of factor VIII (~ 15µg/kg every twelve hours (Bonn protocol)). However, because of the differences in dosing between adalimumab (40 mg every other week) and factor VIII (1.5-7.5 µg/kg every 2-3 days, depending on disease severity) such an immune tolerance induction protocol might be difficult to achieve for adalimumab treatment. Moreover due to the possibility of switching towards other types of biological agents, the need of tolerance induction against adalimumab might be limited compared to factor VIII.

A previous study by Maini et al. has shown that increased dosing of infliximab leads to reduced immunogenicity, suggesting that high levels of drug might induce tolerance in these patients. However an alternative explanation for these results could be that, in patients receiving high drug dosing, the presence of residual drug levels might interfere with the detection of anti-infliximab antibodies. It would be very interesting to measure antibodies in these samples using an anti-infliximab PIA to confirm whether high dosing indeed reduces immunogenicity of therapeutic monoclonal antibodies.

In conclusion, this study which follows ADA formation measured by the PIA in 99 RA patients shows that the clinical relevance of measuring ADA in the PIA is limited, although it does have a predictive value. Importantly, PIA measurements give insight in the immune response against adalimumab because it is able to detect ADA in the presence of adalimumab. It reveals that 94% of the patients who produce ADA develop these antibodies in the first half year of treatment. Measurements with the PIA also allow the study of transient ADA formation, which we found in 32% of the ADA positive patients. Moreover, these data underline the importance of the quantity of an ADA response. Only the formation of high levels of ADA leads to low functional adalimumab levels and reduced clinical response.
Chapter 4

REFERENCES


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Long term PIA measurements show predictive value and transient antibody formation.


