Antibodies against antibodies: immunogenicity of adalimumab as a model
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A novel method for the detection of antibodies to adalimumab in the presence of drug reveals “hidden” immunogenicity in Rheumatoid Arthritis patients

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ABSTRACT

Production of anti drug antibodies (ADA) in adalimumab treated RA patients is associated with reduced serum adalimumab levels and less clinical response. However, most current assays to measure ADA are unable to detect ADA in complex with adalimumab. Thus, ADA is only measured if antibody production exceeds drug levels in the serum, meaning that ADA formation is underestimated. The aim of this study is to develop a method to detect ADA in presence of drug. A pH-shift-anti-Idiotype Antigen binding test (PIA) was used to enable ADA measurement in presence of adalimumab. ADA-adalimumab complexes were dissociated by acid treatment and addition of excess rabbit anti-idiotype Fab before neutralization. Rabbit anti-idiotype Fab blocks reformation of ADA-drug complexes by competing with patient ADA for adalimumab binding. Released ADA are measured by an antigen binding test (ABT). The PIA enabled detection of ADA in presence of large excess of adalimumab and was used to measure ADA in 30 adalimumab treated rheumatoid arthritis (RA) patients during the first 28 weeks of treatment. It revealed ADA in 21 out of 30 tested patients, while the ABT detected ADA in only 5 patients. Indicating that an immunogenic reaction towards adalimumab is present in the majority of adalimumab treated patients.
INTRODUCTION

The introduction of therapeutic monoclonal antibodies has given a major boost to the treatment of several diseases such as rheumatoid arthritis (RA), multiple sclerosis and Bechterew disease. Therapeutic antibodies are known to induce an immune response in part of the treated patients leading to formation of anti drug antibodies (ADA). The production of ADA has been described for several monoclonal antibodies available for the treatment of RA (adalimumab, infliximab), Crohn’s disease (infliximab), multiple sclerosis (natalizumab) and plaque psoriasis (adalimumab).

ADA have been detected in 17% of 121 adalimumab treated RA patients within the first 28 weeks of treatment. Production of ADA is associated with low drug levels and reduced clinical response. However, in currently used assays ADA detection is only possible if the production of ADA exceeds the amount of drug present in patients’ serum due to the formation of ADA-adalimumab complexes. Hence, drug interference will lead to an underestimation of the number of patients producing ADA. Therefore the ability to measure ADA in the presence of adalimumab will provide a better insight in the immune response against adalimumab. Antibodies against adalimumab have been described to become undetectable upon continuation of treatment. This could be the result of drug interference, but it is tempting to speculate that prolonged exposure to the drug might induce tolerance. To discriminate between these two an assay which is able to detect ADA in the presence of adalimumab is needed. Furthermore, such an assay might allow preclinical testing of new monoclonal antibodies without the need of long wash-out periods.

Various groups have reported methods to overcome drug interference. Most of these assays are based on acid dissociation of ADA-drug complexes followed by neutralization in the presence of solid phase bound antigen. Here we present a pH-shift–anti-idiotype (PIA) method where re-association of complexes is prevented by addition of excess fluid phase Fab fragments of rabbit anti-idiotype antibodies that compete with patient antibodies for binding to adalimumab. The result is an assay which is able to detect ADA present in the circulation in the form of immune complexes. Using this assay we observed that the majority of RA patients treated with adalimumab develop ADA.

MATERIALS AND METHODS

Patient material
Sera were obtained in the first 28 weeks of treatment from the first thirty consecutive patients of a prospective observational cohort as previously described by Bartelds et
All patients had a disease activity score in 28 joints (DAS28) of ≥3.2 and fulfilled the American College of Rheumatology 1997 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 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, dose was increased to 40 mg every week. The study was approved by the ethics committee of the BovenIJ Hospital, the Academic Medical Center/University of Amsterdam, Slotervaart Hospital and the Jan van Breemen Research Institute.

Production of adalimumab F(ab’)2
To produce adalimumab F(ab’)2 fragments, 10 µg/ml pepsin (Boehringer, Mannheim, Germany) was incubated with 1 mg/ml adalimumab overnight (o/n) at 37°C in 0.1 M sodium citrate pH 3.5. Afterwards the F(ab’)2 fragments were dialyzed against phosphate buffered saline (PBS). Undigested adalimumab was removed by incubation with sepharose-immobilized protein A (GE healthcare, Chalfont St. Giles, UK).

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 adjuvans. After four boosts the rabbits were bled and serum was collected. Antibodies were purified from the serum using sepharose-immobilized protein A (GE healthcare). 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 gram sepharose).

Production of rabbit anti-idiotype Fab fragments
Purified rabbit anti-idiotype antibodies were treated with pepsin as described above for adalimumab. Twenty mg of rabbit-anti-idiotype (0.55 mg/ml) was incubated with pepsin (5 µg/ml) (Boehringer). To make monovalent Fab fragments, the F(ab’)2 was reduced by incubation with 10 mM dithioerythritol for 30 minutes. Subsequently free thiol groups were blocked with 20 mM N-ethyl-maleinimide and Fab fragments were purified using size exclusion chromatography.

Biotinylation
Antibodies were biotinylated by incubation of 10 mg antibody with 6.3 mg Sulfo-NHS-LC-Biotin (Thermo scientific, Rockford, USA) in 0.1M NaHCO₃. After two hours of incubation
at room temperature (RT) biotinylated antibodies were dialyzed against PBS.

**Measurement of adalimumab concentration**

To measure adalimumab levels Maxisorp ELISA plates were coated (o/n) at RT with 100 µl 2 µg/ml monoclonal anti-TNF-7 (Sanquin) in PBS. After five times washing with PBS/0.02% Tween (PT), plates were incubated for one hour at RT with recombinant TNF (0.01 µg/ml) (Strathmann Biotech HmbH, Hannover, Germany) diluted in high performance ELISA buffer (HPE, Business Unit reagents, Sanquin). Next, the plates were washed and incubated for one hour with patient serum which was serially diluted in HPE. Subsequently, the plates were washed with PT and incubated for one hour with biotinylated adalimumab specific rabbit anti-idiotype (0.25 µg/ml in HPE). After washing with PT streptavidin-poly-HRP (Sanquin) (1:25,000 in HPE) was added for one hour at 37°C. After washing the ELISA was developed with 100 µg/ml tetramethylbenzidine in 0.11 M sodium acetate (pH 5.5) containing 0.003% (v/v) H₂O₂. The reaction was stopped with 2M H₂SO₄. Absorption at 450 nm was measured with an ELISA reader (Multiskan; TiterTek, Elfab Oy, Finland). Results were related to a titration curve of adalimumab in each plate. The lowest level of detection was 0.002 mg/l.

**Measuring ADA by antigen binding test (ABT)**

The test was essentially carried out as described before.19 One micro liter of serum diluted in PBS/0.3% bovine serum albumin (BSA) (PA buffer) was incubated o/n with 1 mg Sepharose-immobilized protein A (GE healthcare, Chalfont St. Giles, UK) in a final volume of 800 µl. Subsequently the samples were washed with PBS 0.005% Tween and specific ADA binding was detected by o/n incubation with 20,000 dpm (approximately 1 ng) ¹²⁵I labeled F(ab’)2 adalimumab diluted in Freeze buffer (Sanquin). Unbound label was removed by washing, and protein A bound radioactivity was measured. When binding was more than 25% of the input, sera were further titrated. Antibody levels were compared to a standard serum containing ADA levels and expressed in arbitrary units (AU). One AU corresponds to approximately 12 ng.

**In vitro generation of ADA-adalimumab complexes**

Rabbit anti-idiotype or patient serum containing ADA was incubated at RT in PA buffer with different amounts of adalimumab to induce complex formation. After two hours the samples were measured in the ABT or PIA

**pH-shift-anti-Idiotype ABT (PIA)**

30 Microliters of 1:30 diluted (in PA buffer) patient serum was mixed with 30 µl of 0.1 M glycine-HCl (pH 2.5). After 30 minutes at RT 30µl of rabbit anti-idiotype Fab (67 µg/ml)
was added. Then the pH was neutralized by addition of 6 µl of 1M Tris. ADA levels were measured in the ABT.

**Sucrose gradients**
Sera were analysed by isokinetic sucrose gradient centrifugation. Sucrose gradients (5-32.9%, w/v, sucrose; Merck) were prepared in PBS, pH 7.4 containing 5 mM EDTA and 0.1% Tween (PET). 100µl serum or 500 AE of patient ADA was diluted in 200 µl PET and layered onto the sucrose gradient. The gradients were centrifuged in a Beckman swing out rotor type SW 41 at 36,000 rpm for 16 hours at 20°C. Fractions of 500 µl were collected and ADA levels were detected using the ABT and the PIA. IgG levels were determined by ELISA. Briefly, Maxisorp ELISA plates were coated o/n with 1 µg/ml Sheep-anti-human IgG (SH16-1) (Sanquin) in PBS. For detection 1µg/ml biotinylated monoclonal anti human IgG (MH16-1) (Sanquin) in HPE was used. Afterwards bound MH16-1 was detected using steptavidin-poly-HRP as described above.

**RESULTS**

**Drug interference in ABT for ADA measurement.**
To assess the drug tolerance of the ABT for ADA detection, a model system was set up using polyclonal rabbit anti-idiotype antibodies against adalimumab. Increasing amounts of adalimumab (0-24 ng; corresponding to a serum concentration of 24 µg/ml) were incubated with rabbit anti-idiotype in a volume of 50 µl and tested in the ABT. Figure 1A shows that adalimumab dose-dependently interfered with the measurement of rabbit anti-idiotype. Addition of as little as 6 ng adalimumab completely inhibited rabbit anti-idiotype detection. A control monoclonal antibody (infliximab) did not influence adalimumab binding (data not shown).

**The pH-shift-anti-Idiotype ABT (PIA) has higher drug tolerance**
To increase drug tolerance, ADA-adalimumab complexes were dissociated by pre-treating sera with low pH. Reformation of complexes was prevented by addition of an excess of rabbit anti-idiotype Fab before neutralization. To test the drug tolerance of the PIA, *in vitro* complexes were generated by incubating increasing amounts of rabbit anti-idiotype with 24 ng adalimumab. As shown in figure 1B, rabbit anti-idiotype was measured dose-dependently in the ABT and addition of 24 ng adalimumab abrogated detection. Addition of rabbit anti-idiotype Fab without acid treatment recovered part of the signal, while the PIA enabled detection of a larger part of the ADA in the presence of adalimumab in a dose dependent matter. Detection of rabbit anti-idiotype in the absence of adalimumab
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Figure 1: The PIA enables measurement of rabbit anti-idiotype in the presence adalimumab. A) Detection of 50 ng of rabbit anti-idiotype against adalimumab in the presence of increasing amounts of adalimumab. B) Detection of rabbit anti-idiotype in the ABT (closed circles). The detection of rabbit anti-idiotype after acid treatment (closed diamonds) and detection of rabbit anti-idiotype in the presence of 24 ng adalimumab in the ABT (open circles), in the ABT in the presence of rabbit anti-idiotype Fab (open diamonds) and the PIA (triangles). C) Detection of 27 ng rabbit anti-idiotype in the absence of adalimumab (open circles) or presence of either 6 ng (closed circles) or 24 ng (closed squares) adalimumab in the PIA with increasing amounts of rabbit anti-idiotype Fab.

is not altered in the PIA.

We investigated how much rabbit-anti-idiotype Fab is required to prevent re-association of complexes. As shown in figure 1C acid dissociation without the addition of rabbit anti-idiotype Fab fragments does not allow the measurement of ADA, while the addition of 2 µg/ml rabbit anti-idiotype Fab enables maximum recovery. As to be expected, the amount of Fab needed depends on the amount of drug present in the samples (figure 1C). In the absence of adalimumab the recovery of rabbit-anti-idiotype is not influenced by the amount of Fab added. We decided to use 2 µg/ml in further experiments.

Increased drug tolerance in patient samples
To investigate whether the PIA enables detection of patient ADA in complex with adalimumab, a clinical serum sample with no detectable adalimumab and high ADA levels
as measured in the ABT was used. This serum was spiked with 24 µg/ml adalimumab to generate in vitro complexes, resulting in complete elimination of the signal in the ABT (figure 2). Similar to the model with rabbit anti-idiotype, a large part of the signal is recovered by measurement in the PIA. When 11 different ADA-positive and adalimumab-negative sera were tested in the PIA without the addition of drug, recovery of ADA varied between 37% and 330% when compared with the ABT.

Detection of ADA in 30 adalimumab treated RA patients
Sera obtained from thirty RA patients during the first 28 weeks of adalimumab treatment were tested in the PIA. To determine the cut-off of the PIA, ADA levels were measured in sera obtained from these patients before start of adalimumab treatment. From these thirty RA patients in total 53 pretreatment samples were tested, either obtained two weeks before or directly before the start of treatment. The cut off of the PIA was determined at 2.7% binding (mean+3xSD). Addition of 5 µg/ml adalimumab to pretreatment sera did not affect these results (data not shown). Next we tested serum samples obtained 4, 16 and 28 weeks after start of therapy. Figure 3A shows a representative example. This patient showed average adalimumab levels which increased over time. In the ABT no ADA were detected. In the PIA ADA were detected after 16 and 28 weeks of treatment. During 28 weeks of follow-up, ADA were detected in five patients using the ABT (17%). With the PIA ADA were detected in 21 patients (figure 3B). Figure 3B also shows that over time not only the number of patients positive for ADA increased over time, but that ADA levels increased as well. These results indicate that the majority of patients treated with adalimumab developed ADA in the first 28 weeks of treatment. Repeated measurements (in duplicate) of all samples collected after 16 or 28 weeks of treatment revealed identical results.
The PIA allows the detection of ADA in complex.

To show that the PIA enables ADA measurement which is present in complex with adalimumab, sera were fractionated by sucrose gradient centrifugation and fractions were tested in the ABT and the PIA. Figure 4A shows a gradient loaded with serum from a patient on adalimumab treatment with ADA and no detectable adalimumab levels. Measurement of ADA in the ABT revealed a single peak at the position where monomeric IgG is sedimenting. Surprisingly, ADA detected by the PIA revealed a shoulder at a higher molecular weight, indicating the presence of complexes between ADA and adalimumab. Analyzing serum from another patient with high levels of adalimumab marginally positive in the ABT and strongly positive in the PIA we observed that all ADA activity had a substantial higher sedimentation velocity (figure 4B). This shows that the ADA detected with the PIA are present in the serum as complexes.

**DISCUSSION**

In general, assays for ADA detection are disturbed by the presence of drug. Various groups have developed assays to overcome drug interference. Most of them are based
on acid dissociation and neutralization in the presence of solid phase drug.\textsuperscript{12-17} Here we present a study in which we combine acid treatment with the addition of liquid phase rabbit anti-idiotype Fab to prevent re-formation of ADA-adalimumab complexes. In our experience low pH treatment without the addition of Fab fragments does not enable measurement of ADA in the presence of drug. During the development of the PIA we observed that the use of intact IgG-anti-idiotype leads to a high background since intact rabbit IgG binds to protein-A-sepharose. Also F(ab')\textsubscript{2} fragments did not work, either because some F(ab')\textsubscript{2} fragments still bind to protein A or because of the ability of the F(ab')\textsubscript{2} to crosslink between protein A bound adalimumab from the patient serum and the labeled adalimumab F(ab')\textsubscript{2} used for detection. Only by using Fab fragments we succeeded in measuring ADA in the presence of drug.

A potential problem concerns the use of protein A. Protein A binds IgG1, IgG2, IgG4 and only subfractions of other Ig classes and subclasses. Using Ig class and subclass-specific monoclonal antibodies we have analyzed the incidence of ADA of the various classes. We observed only IgG1 and IgG4 antibodies. Remarkably no IgM antibodies were found. This could be due to the low affinity of IgM which might not enable catching labeled adalimumab F(ab')\textsubscript{2} from the fluid phase. It could also be the result of wrong timing of the samples. It is to be expected that IgM-ADA formation will be an early phenomenon. In this early phase the immune response is probably not capable of clearing all drug from the circulation. Therefore IgM-ADA will probably be present in complexes, although IgM might lead to formation of larger complexes which are swiftly removed from circulation. Therefore it would be interesting to look at a PIA with anti-IgM sepharose in the early phase of treatment.

\begin{figure}
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\includegraphics[width=\textwidth]{figure4.png}
\caption{PIA enables ADA measurement while in complex. A) ADA measured in a sucrose gradient with serum of a patient with 10344 units of ADA in the ABT and no detectable adalimumab levels. B) ADA measured in the different fractions of a sucrose gradient with patient serum with 16 units of ADA in the ABT, 920 units in the PIA and 11.8 µg/ml adalimumab.}
\end{figure}
Because the acid treatment might damage ADA we were interested in the recovery of ADA activity after PIA treatment. We observed a large variation in recovery ranging from 37-330%. We propose that this is due to two different mechanisms with opposite effects. First, the acid treatment likely damages part of the ADA which will lead to a less than 100% recovery. Second, the more than 100% recovery is probably the result of complexes present in these patients. This notion is supported by the results shown in figure 4A where an adalimumab-negative serum was shown to contain complexes. The presence of varying amounts of complexes makes it extremely difficult to estimate the damage to ADA by the treatment. However we are encouraged by the fact that the acid treatment has not resulted in any false-negative results in thirty patients tested in the PIA. Furthermore, these results indicate that the detection of drug levels is inhibited by the presence of high levels of ADA.

Also, it should be noted that the recovery of ADA is not complete. For in vitro generated complexes with rabbit-anti-idiotypic as well as human ADA recoveries are often less than 100% which means that PIA still underestimates ADA production. One possible explanation for this incomplete recovery is that the rabbit anti-idiotypic Fab is not covering all possible epitopes. However, the recovery of ADA in (the same) rabbit anti-idiotypic was not complete either (figure 1B). Another possibility is that, as discussed, the procedure might partially damage the ADA. Finally it is possible that pH 2.5 is not sufficient to dissociate all complexes.

In the PIA the addition of 2 µg of rabbit anti-idiotypic Fab was sufficient to detect ADA in the presence of at least up to 24 ng of adalimumab in the PIA. This is comparable with a concentration of 24 µg/ml in patient serum. In our experience such high adalimumab concentrations are rare in patient serum hence this amount of anti-idiotypic Fab should suffice for the majority of sera. Sera of RA patients before treatment were negative in this assay. Within 28 weeks of treatment the majority of RA patients became positive in the PIA.

To be sure that we were not measuring an artifact we did a number of extra controls. First we tested if the presence of adalimumab after start of treatment results in false negative results in the assay. Therefore all sera obtained before start of treatment were tested in the presence of 5 µg/ml adalimumab, they all remained negative. Next we reasoned that if ADA were present in sera with high levels of adalimumab, these should have a higher sedimentation rate than IgG. Therefore the ADA activity measurable in the PIA should sediment faster in an ultracentrifuge than IgG. Indeed we found that the ADA measured in the PIA sediment as a di- or trimer of IgG, in agreement with an earlier study where we analyzed immune complexes formed by infusion of 99Tc-labeled infliximab in patients with ADA to infliximab. So we are left with the notion that within 28 weeks the majority of RA patients develop antibodies to the idiotype of a fully human antibody.
will be interesting to find out whether the 30% PIA-negative patients will become positive at a later stage. Alternatively these patients are negative because they clear immune complexes faster.

Our ultracentrifuge experiments also revealed that even a serum containing high levels of ADA (10,000 U/ml equals about 120 µg/ml of anti-adalimumab) and no measurable adalimumab, contained complexes (figure 4A). This indicates that, as was to be expected, ADA interferes with the measurement of adalimumab.

We are left with the question what the clinical significance is of ADA detected with PIA. We showed earlier that ADA levels strongly correlated with clinical efficacy of adalimumab in a variety of diseases.\textsuperscript{7,9,22} In those studies ADA were measured in the ABT. We now show that the majority of these ADA-negative patients do make ADA but not sufficient to clear or neutralize all adalimumab. Further studies should reveal whether the presence of such “hidden” ADA is relevant for the efficacy of adalimumab. The presence of “hidden” ADA is correlated with a minor reduction in adalimumab levels (data not shown). Figure 3B suggests that in time “hidden” ADA levels increase in frequency as well as in magnitude. Moreover our data show that the majority of patients treated with adalimumab are chronically exposed to (small) immune complexes. It is interesting to see what the clinical consequences to this exposure are.

In conclusion, the PIA allows the study of the antibody response against adalimumab in the presence of adalimumab. Therefore it will enable long term follow up of ADA production in order to study tolerance induction in patients while on adalimumab treatment. Moreover application of the PIA might relieve the necessity to include long wash-out periods in preclinical testing of new monoclonal antibodies.

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REFERENCES


