Antibodies against antibodies: immunogenicity of adalimumab as a model
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Functional analysis of the anti-adalimumab response using patient-derived monoclonal antibodies

Manuscript in preparation

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ABSTRACT

The production of anti drug antibodies (ADA) in auto-immune patients treated with adalimumab severely diminishes treatment efficacy. We have previously shown that the binding specificity of the antibody response to adalimumab is very restricted and directed against the TNF binding region. Here we investigate whether this restricted response is the result of outgrowth of a limited number of B-cell clones or multiple clones with the same specificity. By producing eleven different adalimumab specific monoclonal antibodies derived from two patients we show that the immune response against adalimumab is a broad response involving multiple B-cell clones each using different combinations of V(D)J segments. We investigated the affinity of the different monoclonal antibodies and studied how the affinity influences the detection of antibodies in various assays. We show that the influence of affinity varies between different assays. The bridging ELISA depends on the affinity of the monoclonal antibody while there is no correlation between measurements in the antigen binding test (ABT) and the affinity. This can partly be explained by the bivalent interaction possible in the ABT.
INTRODUCTION

The introduction of therapeutic monoclonal antibodies has given a major boost to the treatment of Rheumatoid Arthritis (RA) patients. However, part of the patients develops an immune response against the therapeutic resulting in the formation of anti drug antibodies (ADA). The formation of ADA has been linked to lower serum drug levels and reduced clinical response.\textsuperscript{1-7} ADA formation can influence treatment efficiency via two possible mechanisms. First the formation of ADA can lead to enhanced clearance of the drug. Second, the formation of neutralizing antibodies can prevent the therapeutic from binding to its target.

In case of adalimumab, a fully human anti-TNF agent, recent data indicate that essentially all ADA, as measured in the bridging elisa or in the antigen binding test, are directed against the TNF binding region of adalimumab, resulting in neutralization of the drug.\textsuperscript{8} We have shown before that more than half of the adalimumab treated RA patients develop ADA. Only when ADA levels exceed drug levels, antibody production leads to clinical non-response (manuscript in preparation). Together these data show that the magnitude of the immune response determines the clinical significance of ADA formation. A minor ADA response will result in neutralization of only part of the adalimumab leaving sufficient functional drug.

The observation that the vast majority of ADA against adalimumab are neutralizing suggests that the immune response against adalimumab is very restricted, targeting one or a set of epitopes that overlap the TNF binding site on adalimumab. This is confirmed by inhibition experiments showing that a single monoclonal antibody against adalimumab completely prevents binding of ADA in 50 different patient sera.\textsuperscript{8} The most likely explanation of this restriction is that only limited numbers of B-cell clones participate in the ADA response. To investigate this hypothesis we have cloned the antibody specific sequences of 16 patient-derived adalimumab specific B-cells. We have expressed 11 of these fully human monoclonal antibodies in HEK293 cells to confirm the previously observed restriction of the binding site. In addition, we determined the affinity of the obtained monoclonal antibodies and investigated the effect of affinity and binding-specificity on the different assays available for the detection of ADA.

MATERIALS AND METHODS

Patients
Monoclonal ADA were derived from two adalimumab (Abbot, Illinois, USA) treated patients: Patient 1 was treated for Ankylosing Spondylitis, patient 2 was treated for RA.
Both patients had high titers of ADA as detected in the ABT (2960 AU/ml and 8570 AU/ml, respectively). Of both patients informed consent was obtained. The study was approved by the local ethics committee.\textsuperscript{1,9}

**Generation of F(\text{ab}')\text{2} and Fab fragments**

F(\text{ab}')\text{2} and Fab fragments were generated using pepsin, and subsequent reduction and alkylation with dithioerythritol and N-ethylmaleimide respectively, as described before.\textsuperscript{10}

**Bridging ELISA for the measurement of ADA**

Measurement of ADA in the bridging ELISA was essentially carried out as described before.\textsuperscript{8} In short, ELISA plates were coated with adalimumab and after addition of the ADA containing sample, ADA binding was detected using biotinylated adalimumab and streptavidin-poly-HRP. The specificity of the monoclonal antibodies for adalimumab was demonstrated by using a bridging ELISA with another therapeutic anti-TNF monoclonal antibody, infliximab (Remicade®), as a coat and conjugate (detection limit 1 ng/ml of anti-infliximab).

**Isolation, proliferation and identification of adalimumab specific single B-cells**

Antigen specific B-cells were isolated and cultured as described before.\textsuperscript{11} In short, PBMC’s were isolated using a Percoll gradient and B-cells were isolated using anti-CD19 beads. Antigen specific memory cells were sorted using two differently labeled adalimumab Fab fragments and anti-CD27 and sorted single cell per well or seeded 0.5 cells per well. Cells were cultured in the presence of CD40-ligand expressing cells and a cytokine cocktail. After 10-14 days the supernatants were tested for the presence of ADA using the bridging ELISA.

**Production of recombinant human antibodies**

RNA was isolated from wells positive in the bridging ELISA using Trizol (Peqlab, Erlangen, Germany). cDNA synthesis and 5’-RACE PCR were performed using the Clontech SMART cDNA synthesis kit (Clontech, Mountain view, USA, cat 634914). RACE PCR products for VL and VH were cloned into pcDNA3.3 or pcDNA3.1 (Invitrogen, Paisley, UK) expression vectors together with the constant domains of the human Kappa, Lambda and human IgG1 allotype f genes, essentially as described before.\textsuperscript{12} In some cases an additional nested PCR was performed to obtain the VL and VH sequences. Expression vectors were used for transient transfection of HEK293F cells with 293fectin and OptiMEM (Invitrogen), using the Freestyle HEK293F expression system (Invitrogen) according to the instructions supplied by the manufacturer.
Affinity measurement of monoclonal antibodies

Adalimumab Fab was fluorescently labeled with DyLight 488 amine reactive dye (Pierce/Thermo Scientific). Unreacted dye was removed by repeated dilution/concentration using Amicon Centriprep centrifugal filter devices (Millipore, Billerica, MA, US) until no dye could be detected anymore in the filtrate. The average degree of labeling (DOF) was 1.8 molecule dye/molecule Fab.

Serial four-fold dilutions of anti-adalimumab monoclonal antibody (0.05 – 1000 ng/ml) were incubated with a fixed concentration of 63 pg/ml (1.25 pM) of adalimumab Fab-DyLight 488 (ada-Fab-488) in PBS containing 0.1 mg/ml intravenous immunoglobulin (Nanogam, Sanquin, The Netherlands) that was exposed to daylight for 2 days to remove any residual fluorescence activity. Measurements were also carried out at 31 pg/ml (0.625 pM) for anti-adalimumab 2.2 and 2.12. Samples were equilibrated at 25 °C for up to 4 days before analysis.

Samples (1000 μl) were applied using a thermostatted autosampler (25 °C) to a Superdex 200 HR 10/300 column (GE Healthcare, Uppsala Sweden), which was connected to an HPLC system ÄKTAexplorer HPLC system (GE Healthcare, Uppsala Sweden). Elution profiles of ada-Fab-488 were monitored by measuring the fluorescence (excitation/emission 488/525 nm) with a Prominence RF-20Axs on-line fluorescence detector (Shimadzu, Kyoto, Japan). The column was equilibrated in PBS. Molecular weight estimations were based on the elution profile of the monomeric and dimeric IgG fractions of the intravenous immunoglobulin present in each sample, which were monitored by measuring the absorbance at 214 nm.

To calculate dissociation constants, fluorescence at 13 or 17 ml, corresponding to the peak maxima of bound or free ada-Fab-488, respectively, were plotted against the concentration of anti-adalimumab antibody (molar concentration of the number of Fab arms) and a 1:1 binding model was fitted to the data using Microcal Origin software.

Biotinylation

Antibodies and Fab fragments (100 μg) were biotinylated by incubation with 60 μg Sulfo-NHS-LC-Biotin (Thermo scientific, Rockford, USA) in 0.1 M NaHCO3. After 2 h of incubation in the dark at room temperature (RT) biotinylated antibodies were dialyzed against PBS.

Competition ELISA with recombinant monoclonal antibodies

Maxisorp ELISA plates were coated o/n with 100 μl 0.5 μg/ml adalimumab in PBS. The binding of biotinylated monoclonal antibody was analyzed after incubation of the coat with increasing amounts of different unlabeled monoclonal antibodies or as control an irrelevant human monoclonal antibody (Cetuximab (Erbitux, Merck Serono, Darmstadt, Germany). All incubations were performed in high performance ELISA buffer (HPE)
(Sanquin). Binding of biotinylated antibody was detected using streptavidin-poly-HRP as described before.8

Antigen binding test
The test was essentially carried out as described before.13 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 or Fab of adalimumab diluted in Freeze buffer (Sanquin). Unbound label was removed by washing and protein A bound radioactivity was measured.

TNF bioassay
10^4 TNF responsive endothelial cells (ECRF-24 cells)14 were seeded in a 96 wells plate in IMDM containing 5% FCS (Bodinco), 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME, 20 µg/ml human apo-transferrin (Sigma-Aldrich). After 24 hours of incubation the samples containing TNF, adalimumab and Fab fragments of monoclonal antibody were added. Supernatants were harvested after 24 hrs incubation and tested for IL-8 concentration by ELISA.15

Nucleotide sequence analysis
The nucleotide analysis was performed using JOINSOLVER.

RESULTS

The anti-adalimumab response is a polyclonal response
To obtain the sequences of the VH and VL chain of various anti-adalimumab antibodies, antigen specific memory B-cells that were isolated from peripheral blood of two ADA positive patients, were cultured single cell per well for 10-14 days. Supernatants were screened for ADA production and RNA was extracted from ADA positive wells. After the production of cDNA the IgG specific sequence was amplified, sequenced and expressed in HEK293 cells. This resulted in sequences of three monoclonal antibodies from the first patient and thirteen monoclonal antibodies from the second patient. All recombinant monoclonal antibodies tested positive in the anti-adalimumab bridging ELISA and specificity of the antibodies for adalimumab was confirmed in a similar assay using infliximab as coat and detection (data not shown). The usage of the V(D)J segments was determined using JOINSOLVER and listed in table 1. IGHV1 genes were the predominantly
used for the heavy chains. For the light chains, J4 segments and V3-20 segments were most frequently used. Partly this can be explained by a bias in our methods in favor of kappa antibodies. The majority of the antibodies underwent extensive somatic hypermutation. All the monoclonal antibodies used different combinations of V(D)J segments indicating that they originated from different precursor B-cells. This shows that the ADA response in these patients is very diverse.

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Table 1: All monoclonal antibodies are derived from different precursor B-cells. V(D)J usage of the different monoclonal antibodies, the CDR3 length and the number of mutations leading to amino acid replacements (R) or silent mutations (S). *This antibody was originally found as an IgG1 antibody and for further experiments expressed as an IgG4 antibody. *These antibodies were originally identified as either IgG1 or IgG4 antibodies, and expressed as IgG1 antibodies. The original isotype of anti-adalimumab 2.11 was not determined (N.D.) due to limited amount of supernatant.

Specificity of monoclonal antibodies

We were able to express eleven of the above described monoclonal antibodies in large enough quantities to investigate their binding affinity and specificity. First we confirmed that the monoclonal antibodies obtained here compete with each other for the binding of
adalimumab. Therefore binding of biotinylated anti-adalimumab to coated adalimumab was inhibited with all the other monoclonal antibodies. An example was shown in figure 1A which shows that all antibodies can inhibit binding of anti-adalimumab 2.4 to adalimumab with some variation in the efficiency of inhibition. The variation in inhibition might be explained by differences in affinity between the monoclonal antibodies (further discussed below). The experiments confirmed that each monoclonal antibody can

Figure 1: All monoclonal antibodies compete with anti-adalimumab 2.4 and TNF for the binding to adalimumab. A) Binding of biotinylated anti-adalimumab 2.4 to adalimumab was inhibited by the addition of unlabeled monoclonal anti-adalimumab antibodies. The control monoclonal antibody cetuximab did not inhibit binding. B) In response to 1 ng of TNF ECRF-24 cells produce IL-8 in the linear range of the titration curve. Adalimumab can neutralize TNF, thereby preventing IL-8 production. 0.1 µg of all antibodies (except anti-adalimumab 2.4) was sufficient to completely restore IL-8 production in the presence of adalimumab. Results represent mean and SEM (error bars).
Functional analysis of the anti-adalimumab response using patient-derived monoclonal antibodies

compete with all other antibodies for the binding to adalimumab (data not shown). All antibodies could prevent binding of adalimumab to TNF as tested with the TNF-sensitive ECRF cell line (figure 1B). Thus, all antibodies are neutralizing. In initial experiments, anti-adalimumab 2.4 seemed unable to neutralize adalimumab. However, a higher concentration of anti-adalimumab 2.4 could restore IL-8 production (data not shown).

Affinity measurement of anti-adalimumab monoclonal antibodies

To determine the affinity of the obtained monoclonal antibodies a fixed concentration of fluorescently labeled adalimumab Fab fragments was incubated with different concentrations of the monoclonal antibodies. The samples were incubated until equilibrium was reached, after which the amount of bound and free adalimumab Fab was determined using size exclusion chromatography. An example of elution profiles is given in figure 2A (anti-adalimumab 2.6). To calculate dissociation constants, the peak maxima

![Affinity measurement](image)

Figure 2: Determination of the affinity of the different monoclonal antibodies. A) The affinity of anti-adalimumab 2.6 was determined by incubation of labeled adalimumab Fab fragments with different amounts of monoclonal antibody (0.013-13.333 nM). After equilibrium was reached the amount of bound and free adalimumab Fab was measured using size exclusion chromatography. (Larger IC appear left on the graph) B) To determine the affinity of the monoclonal antibodies the amount of free adalimumab Fab was plotted against the concentration of anti-adalimumab 2.6. C) A list of the affinities of the different monoclonal antibodies. All measurements are performed in duplicate unless otherwise reported.
free ada-Fab-488 was plotted against the concentration of anti-adalimumab antibody and a 1:1 binding model was fitted to the data (an example shown in figure 2B). The affinities of all monoclonal antibodies are listed in figure 1C. The affinities vary between 0.6 pM and 77 nM. There was no link between the number of hyper mutations in the sequence and the affinity of the antibodies (data not shown).

Next we investigated whether the difference in inhibition potential in figure 1 was the effect of the different affinities of the monoclonal antibodies. Indeed there was a correlation between the affinity of the monoclonal antibodies and their inhibitory

![Figure 3](image-url)

**Figure 3:** The measurement of ADA in the bridging ELISA is influenced by the affinity of the antibodies. **A)** The measurement of the eleven monoclonal antibodies in a bridging ELISA for the detection of ADA against adalimumab. **B)** The EC50 values of the measurement of the monoclonal antibodies in the bridging ELISA correlate with the affinity of the monoclonal antibodies ($r=0.91; p<0.0005; n=3$, error bars mean and SEM). The grey dot represents the measurement of anti-adalimumab 2.1.

![Figure 4](image-url)

**Figure 4:** Measurement of the different monoclonal antibodies in the ABT is influenced by the affinity of the antibodies. **A)** A dose response curve of the detection of 11 monoclonal antibodies in the ABT using labeled adalimumab F(ab’)2 for detection. **B)** The EC50 value of the ABT measurements do not correlate with affinity of the monoclonal antibodies (Spearman $r=0.58; p<0.06; n=3$, error bars mean and SEM).
potential in the competition experiment (data not shown (Spearman r=0.80 p<0.005)).

Influence of affinity on ADA measurements
To find out how affinity of the monoclonal antibodies influences their detection, we tested the monoclonal antibodies in the bridging ELISA and the ABT. In the bridging ELISA ADA crosslink adalimumab on the solid phase with labeled adalimumab in solution. There is a large variation in the detection of the different monoclonal antibodies (figure 3A). Remarkably, some monoclonal antibodies reach a lower plateau. Figure 3B shows that there is a good correlation between the half maximal effective concentration (EC50) and affinity of the antibodies (Spearman r=0.91; p<0.0005). In case of anti-adalimumab 2.1 (grey dot in graph) the EC50 is higher than would be expected based on the affinity of the monoclonal antibody. Experiments using Biacore suggest that this can be explained by a low on-rate (kon) value of anti-adalimumab 2.1 (data not shown).

In addition to the bridging ELISA, we also measured the monoclonal antibodies in the antigen binding test commonly used in our department. In this assay ADA are bound to sepharose-coupled protein A and detected using radiolabeled adalimumab F(ab’)2. Again, a large variation in response was observed for the eleven monoclonal antibodies that were tested (figure 4A). Surprisingly in the ABT there was no significant correlation between the EC50 values and the affinity of the monoclonal antibodies, although a clear

Figure 5: Monovalent detection of 11 monoclonal antibodies in the ABT increases the effect of affinity on the detection of ADA. A) A dose response curve of the detection of monoclonal anti-adalimumab antibodies in the ABT using adalimumab Fab fragments for detection. B) The EC50 values in the Fab fragment ABT correlate with the affinity of the monoclonal antibodies (spearman r=0.73; P=0.011 n=3, error bars mean and SEM). C) The fold difference in EC50 values between the measurement of the monoclonal antibodies in the ABT using F(ab’)2 and Fab label.
trend could be observed (figure 4B (Spearman r=0.58; p=0.06)). To investigate whether this could be explained by a difference in preference between monoclonal antibodies to engage in a bivalent interaction, we repeated the measurements using radiolabeled adalimumab Fab for detection (figure 5a). There is a significant correlation between EC50 values in the Fab ABT and the affinity (figure 5b; Spearman r=0.73; P=0.011). Comparing EC50 values of the F(ab’)2 ABT and the Fab ABT shows that all antibodies have a lower EC50 value in the F(ab’)2 ABT (figure 5c) suggesting that all antibodies bind adalimumab F(ab’)2 with an higher avidity compared to Fab. This suggests that all antibodies can bind bivalently, so with two Fab arms at the same time, to adalimumab. However, the increase in avidity differs between antibodies.

**DISCUSSION**

Previously we have shown that in ADA positive patients all antibodies are directed against the same small region on adalimumab, since the polyclonal antibody response in patients’ sera could be inhibited by a single monoclonal antibody against adalimumab. This finding was extended using the larger panel of mAbs described in this paper. The sequences of the monoclonal antibodies are the result of different V(D)J rearrangements, showing that they derived from different precursor B-cells. This suggests that the antibody response against adalimumab results from outgrowth of multiple B-cell clones all recognizing the same or overlapping regions on the adalimumab molecule. From our data it is impossible to extract the number of different B-cells involved in the immune response against adalimumab, because of the limited number of sequences obtained. For other immune responses, such as the antibody response against tetanus toxoid, it has been described that as many as 100 different B-cell clones can be involved.\(^{16}\) It would be very interesting to obtain more adalimumab specific sequences and investigate the number of B-cells involved in an immune response with restricted antibody binding as described here. However, it is possible that we have a selection in this system. It might be expected that high affinity antibodies are easier to sort antigen specifically and these cells are easier detected in the bridging ELISA used to screen the supernatants after culture. This might also explain why the majority of antibodies presented here bind adalimumab with high affinity (range 0.6 pM and 77000 pM). To get a better insight of the affinity of the total anti-adalimumab response, similar experiments as shown in this paper should be done with polyclonal antibodies derived from patient sera. However, there is a chance that the free antibodies that are present in patient sera are also no good representation of all antibodies produced. It can be expected that the antibodies with highest affinity are bound to adalimumab and cannot be detected or are cleared form the circulation.
The majority of antibodies presented here have kappa light chains, which is the result of a selection in our system. Studying the different V segments used in our monoclonal antibodies shows that the majority of monoclonal antibodies use heavy chain V segments of the V1 family. For the light chain J4 segments and V3-20 segments are commonly used. However, from 16 monoclonal antibodies it is difficult to draw conclusions on V D or J segments usage in the whole antibody response. Presently we try to confirm this V(D)J restriction on the total ADA response using mass spectrometry analyses of purified ADA from different patients and comparing this with V(D)J distribution on the total antibody pool from the same patient.

Previous data from our group have shown that the immune response against adalimumab mainly consists of IgG1 and IgG4 antibodies\(^1\) which is supported by the present data, since all but one monoclonal ADA found were originally identified as IgG1 and IgG4 antibodies. Comparing the affinity of the monoclonal antibodies that were originally identified as IgG4 with the IgG1 monoclonal antibodies suggests that IgG4 antibodies have a higher average affinity (median (range) IgG1; 138.5 (2.6-77000) IgG4; 9.33 (0.64-115)). This is in contradiction to the antibody responses against tetanus toxoid and hepatitis B antigen for which it has been suggested that IgG4 antibodies have lower affinity compared to IgG1 antibodies.\(^17-19\) It would be interesting to further investigate this on the polyclonal ADA response or other immune responses.

Next to accelerating clearance of adalimumab and neutralization of the drug, ADA could interfere with the effectiveness of adalimumab treatment in case the ADA bear an “internal image” of TNF. Others have shown that an antigen bound by an antigen combining region (idiotype) of an antibody may be mimicked by a secondary anti-idiotype antibody. In case of adalimumab this would mean that ADA could functionally mimic TNF and therefore induce signaling via the TNF receptor. In figure 1B we show that some monoclonal antibodies restore IL-8 production to more than 100%. It would be interesting to see whether these monoclonal antibodies are able to induce IL-8 production in the absence of TNF and adalimumab.

Our data clearly show that the affinity of ADA can greatly influence their detection, depending on the type of antigen binding required for detection. The ABT using adalimumab F(ab’\(^2\)) fragments for detection allows for bivalent interactions therefore limiting the effect of affinity of ADA measurement. This was confirmed by the experiments using adalimumab Fab fragments for detection in which there is an effect of affinity on detection. In case of ADA detection in the bridging ELISA, two monovalent interactions are necessary to allow detection. This might explain why there is a better correlation between affinity and EC50 values in this assay.

It would also be very interesting to study the type of immune complexes formed between the different monoclonal antibodies and adalimumab. Previous data have shown that
only small immune complexes between ADA and adalimumab can be detected in patient sera.\textsuperscript{8} Immune complex data using these monoclonal antibodies would provide insight into whether only small immune complexes are formed in these patients or whether all larger immune complexes are cleared from the circulation. Also we observed a lower plateau in the bridging ELISA for the detection of some of the monoclonal antibodies, it would be nice to see whether we could correlate this with antibodies that preferably bind with both Fab arms to the same antibody.

In summary, here we present 16 human monoclonal antibodies against adalimumab all obtained from different precursor B-cells, showing that the restricted binding specificity of anti-adalimumab antibodies is the effect of the outgrowth of multiple B-cell clones. Moreover we measured the affinity of 11 of the monoclonal antibodies and show how affinities influence the measurement of ADA in different assays available.
REFERENCES


