Immunogenicity of therapeutic antibodies
Immunological mechanisms & clinical consequences
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Chapter 4

The antibody response against human and chimeric anti-TNF therapeutic antibodies primarily targets the TNF binding region


Karin A. van Schie, Margreet H. Hart, Els R. de Groot, Simone Kruithof, Lucien A. Aarden, Gert-Jan Wolbink & Theo Rispens
ABSTRACT

**Background:** In a subset of patients, anti-TNF therapeutic antibodies are immunogenic, resulting in the formation of anti-drug antibodies (ADA). Neutralizing ADA compete with TNF for its binding site and reduces the effective serum concentration, causing clinical non-response. It is however unknown to which extent ADA are neutralizing.

**Objectives:** To study which proportion of antibodies to human(ized) anti-TNF (adalimumab, golimumab, certolizumab) as well as chimeric anti-TNF (infliximab) is neutralizing.

**Methods:** Neutralizing capacity of ADA was assessed using a TNF competition assay in ADA-positive sera of patients treated with adalimumab (n=21), golimumab (n=4), certolizumab (n=9), or infliximab (n=34) send in to our diagnostic department.

**Results:** In 34 sera with ADA to adalimumab, golimumab, or certolizumab, >97% of the antibodies were neutralizing. In 34 sera with ADA to infliximab >90% of the antibodies were neutralizing. Further characterization of the broader antibody response to infliximab revealed that non-neutralizing antibodies to infliximab do not target murine domains, but may bind infliximab-unique domains not involved in TNF binding (located outside the paratope).

**Conclusions:** Our study shows that ADA to human(ized) as well as chimeric anti-TNF therapeutic antibodies are largely neutralizing. This highly restricted ADA response suggests an immunodominant role for the paratope of anti-TNF therapeutics.
INTRODUCTION

Anti-TNF therapeutic antibodies are increasingly used to treat inflammatory disorders like rheumatoid arthritis and inflammatory bowel disease. Unfortunately, in some patients the therapeutics are immunogenic, resulting in the formation of anti-drug antibodies (ADA) that can lead to loss of clinical response. The percentage of patients that develop ADA to TNF blockers varies strongly between immunogenicity studies. For adalimumab, using a highly drug-tolerant antigen binding test it was found that around 53% of adalimumab-treated patients made ADA to some extent. In this context, two kinds of ADA can be discriminated, namely non-neutralizing antibodies that bind to the drug simultaneously with TNF, and neutralizing antibodies that compete with TNF for the antigen-binding site (paratope). Neutralizing antibodies can therefore immediately inhibit the working mechanism of the drug.

In patients treated with anti-TNF therapeutics, measuring the ADA titer as well as the drug level provides an objective view on the patient’s clinical response. However, one could argue that not only the ADA titer should be monitored, but also its neutralizing capacity. For example, for multiple sclerosis patients treated with interferon-β, neutralizing antibodies rather than binding antibodies are routinely assessed, since the former better correlate with clinical impact. However, the neutralization capacity is usually monitored using a bioassay, which is difficult to standardize and cumbersome to carry out.

Previous work on the antibody response to adalimumab indicates that this response is restricted to the TNF binding site (the paratope), meaning that all ADA are neutralizing. The antibody construct of the fully human adalimumab might explain this restricted response, since typically the majority of foreign determinants is localized in the complementarity determining regions (Figure 3B). Chimeric antibodies like infliximab differ from humanized antibodies as the entire variable domain is of murine origin which potentially induces a broader immune response. Moreover, the variable domains of infliximab contain additional determinants that are not involved in TNF binding and are neither mouse nor human germline which might be immunogenic (Figure 3A). For the anti-TNF therapeutic antibodies infliximab, certolizumab (humanized) and golimumab (human) the neutralizing capacity has never been investigated.

In this study, we systematically investigated the proportion of neutralizing and non-neutralizing antibodies to infliximab, adalimumab, certolizumab, and golimumab.
METHODS

Details about the methodology can be found in the online supplementary Materials and Methods. Briefly, patient sera from our diagnostic department were selected for ADA positivity for either adalimumab, golimumab, certolizumab, or infliximab. To determine the neutralizing capacity of these ADA, a TNF competition assay was set up in which TNF is used to block the antigen binding site of the labeled drug. In this assay, neutralizing antibodies compete with TNF for their binding to the drug, resulting in a decreasing signal with increasing amounts of TNF. In contrast, non-neutralizing antibodies can bind the labeled drug simultaneously with TNF and its binding is not affected when TNF is added. In some experiments, polyclonal mouse IgG was added as additional blocker to examine reactivity to mouse determinants. The biological activity of anti-infliximab was analyzed using the WEHI bioassay. TNF-sensitive WEHI-164 cells were incubated in the presence of TNF, infliximab, and samples containing anti-infliximab, and viability was measured.

RESULTS

ADA to all anti-TNF drugs are >90% neutralizing

Twenty-one patients producing ADA to the fully human therapeutic antibody adalimumab were tested in a TNF competition assay (described in Figure 1). Upon an increasing concentration of TNF, all patients showed an almost identical reduction in binding in a dose-dependent manner, with the highest concentrations of TNF reducing the binding to background levels (Figure 2A). These results indicate that >97.7% of ADA to adalimumab are directed to the paratope of the drug and are thus neutralizing (Figure 2B). This is congruent with our previous findings which suggested that anti-adalimumab antibodies are highly restricted to the paratope of adalimumab. Similarly, for sera containing anti-golimumab or anti-certolizumab antibodies, resp. >98.3% and >97.2% inhibition of binding to golimumab and certolizumab Fab’ was observed when the highest concentration of TNF was added. Thus, in 34 cases of ADA to a human(ized) anti-TNF, >97% of the antibody response is neutralizing.
Figure 1. Schematic representation of the TNF competition assay. To determine the neutralizing capacity of ADA, a TNF competition assay was set up in which TNF is used to block the paratope (or antigen binding site) of the labeled drug. Serum IgG including ADA is bound by protein A sepharose, and radiolabeled drug Fab’ will subsequently bind to ADA. However, upon addition of TNF, the paratope of the radiolabeled drug Fab’ is blocked by TNF. Consequently, ADA that bind (close to) the paratope of the drug cannot bind anymore and are classified as neutralizing ADA. ADA that can bind simultaneously with TNF are classified as non-neutralizing.

For the chimeric infliximab, of which the entire variable domains are of murine origin, the results from the TNF competition assay were more varied. The ADA of one group of patients showed a decrease in signal with an increase in TNF leading to essentially complete suppression of ADA binding to the drug on the endpoint of the TNF titration. In contrast, a second group of patients showed incomplete inhibition of ADA binding to the drug when the highest concentration of TNF was added. The sera of this latter group thus contain ADA that are able to bind the drug simultaneously with TNF and are therefore non-neutralizing. Nevertheless, all patient sera tested contained >90% neutralizing ADA to infliximab. In line with these observations, ADA titers to infliximab determined in the WEHI bioassay, which only measures the effect of neutralizing ADA, are in good correlation with those determined in the ABT, which measures all ADA (Figure 2C). No relationship was found between ADA levels and the percentage of neutralizing antibodies (online supplementary Figure S2). In conclusion, for all TNF blockers, the vast majority of ADA neutralize the drug, not only in case of human(ized) antibodies but also for the chimeric infliximab.
Figure 2. More than 90% of ADA to all anti-TNF therapeutic antibodies target the paratope of the drug. A) Radiolabeled drug preincubated with increasing amounts of TNF gives a decrease in binding of ADA to the drug. ADA to all four anti-TNF therapeutic antibodies were >90% neutralizing. Each line represents a single patient. In case of adalimumab, certolizumab and infliximab, a selection of representative patients is shown. B) Percentage of AU inhibited by 5000 ng of TNF. Each dot represents a single patient. Median and range of adalimumab 98.9 (97.7-100), n=21; golimumab 98.9 (98.3-99.5), n=4; certolizumab 98.17 (97.2-98.8), n=9; infliximab 98.6 (90.2-99.3), n=34. C) Anti-infliximab titers were measured using the ABT and the WEHI bioassay. The ABT measures all ADA whereas the WEHI bioassay only measures neutralizing ADA. The strong correlation indicates that all ADA are neutralizing. Spearman’s Rho was \( r=0.9362 \) \((p=0.0002, n=10)\).
Incomplete inhibition of ADA to infliximab is not caused by ADA-drug complexes

Although no free infliximab was detected in any of the sera, the possibility exists that complexes of infliximab and anti-infliximab are present. Hypothetically, if these complexes dissociate, a false-positive signal may result, since infliximab can bind protein A sepharose and TNF can subsequently form a bridge between bound infliximab and radiolabeled infliximab Fab. To exclude the possibility that the non-inhibitable signals were the result of drug-ADA complexes, monomeric IgG was separated from antibody complexes using FPLC (online supplementary Figure S1A). However, no differences in neutralization were found between the monomeric fraction compared to patient serum (Figure S1B). This indicates that the incomplete inhibition of ADA to infliximab by TNF observed in some patients is not caused by ADA-drug complexes, but in fact are non-neutralizing anti-infliximab antibodies.

Figure 3: Non-neutralizing ADA to infliximab do not target mouse determinants. Crystal structure of infliximab (A) or adalimumab (B) Fab (orange) in complex with tumor necrosis factor (TNF) (purple). Residues that differ from human germ line are highlighted in blue. Residues that are unique to infliximab (i.e., differ from mouse germ line) are highlighted in green. Figures rendered from PDB coordinate files 4G3y (infliximab) and 3WD5 (adalimumab), aligned using DomainGapAlign (available from the ImMunoGeneTics website). (C and D) Sera of four patients that showed incomplete inhibition by TNF were tested for antibodies to mouse determinants of infliximab. Polyclonal mouse IgG was added to the TNF competition assay to block anti-mouse antibodies. No inhibition could be observed. ns=not significant using paired t-test.

Non-neutralizing ADA to infliximab do not target mouse determinants

To investigate whether the non-neutralizing anti-infliximab antibodies target predominantly infliximab unique determinants outside the paratope or mouse germ line determinants foreign to humans (Figure 3A), we modified the TNF competition assay by the addition of polyclonal BALB/c mouse IgG. In theory, non-neutralizing ADA that target mouse germ line epitopes are blocked by mouse IgG, thereby inhibiting binding of radiolabeled infliximab Fab seen as a decrease in signal. However, in serum samples containing non-neutralizing antibodies, we observed no additional inhibition when mouse IgG was added (Figure 3C,D); this suggests that non-neutralizing anti-infliximab...
antibodies are not directed to mouse germline determinants of infliximab, but target
determinants outside the paratope that are unique to infliximab.

DISCUSSION

We demonstrated that ADA to the human(ized) antibodies adalimumab, golimumab
and certolizumab were highly confined to the paratope (>97%). Infliximab elicited a
slightly broader immune response, although in all patients at least 90% of all ADA were
neutralizing. In addition, a strong correlation was found between the anti-infliximab titer
determined by ABT, in which all ADA are detected, and the WEHI bioassay, which only
measures neutralizing ADA. These results indicate that conventionally measured titers of
binding antibodies closely resemble titers of neutralizing ADA. Therefore, measurement
of neutralizing antibodies to these TNF blockers, e.g. by means of a bioassay, is not
useful.

Sera containing low ADA levels (below 100 AU/ml) were not investigated, because the
low levels preclude accurate quantification of the fraction of neutralizing antibodies. The
sera that were tested comprise a broad range of ADA levels that do not correlate with
the percentages of neutralizing antibodies (Figure S2). However, a possibly broader
response in patients with low ADA levels cannot be excluded, although absolute titers
of non-neutralizing ADA will necessarily be low in such cases.

This study focused on IgG antibodies, since these were previously found to represent
the predominant class of antibodies formed to infliximab or adalimumab.6,7 Moreover,
IgM antibodies typically have low affinity and are poor in neutralizing the drug, while IgG
antibodies have high affinity and are therefore more potent drug inhibitors.

It was already known that anti-infliximab antibodies may neutralize the drug.6 We
however observed that the majority (>90%) of antibodies to infliximab are neutralizing,
clearly demonstrating that the paratope of infliximab is immunodominant. Given the
high number of non-human determinants in the variable domains of infliximab (Figure
3A) this is a remarkable observation. Interestingly, paratope immunodominance was
already suggested in an earlier study of the antibody response to the therapeutic mouse
monoclonal antibody OKT3, where it was estimated that 50-60% of all ADA were
targeting the paratope.8 Moreover, in a more recent study, binding of minipig antibodies
to adalimumab could not be inhibited by human polyclonal IgG F(ab)2, suggesting anti-
idiotypic antibodies.9 These results highlight that reducing immunogenicity in anti-TNF
therapeutic antibodies will remain a challenging endeavor.
Surprisingly, the non-neutralizing fraction of anti-infliximab antibodies that was found in approximately one-third of the patients did not target mouse germline determinants, since addition of polyclonal BALB/c mouse IgG did not reduce binding of ADA to the drug. Aligning the amino acid sequence of the infliximab variable domains with the mouse germline sequence revealed several accessible non-mouse germline (inflimab unique) amino acids that were not part of the paratope but could be targeted by the immune response (Figure 3A). It is therefore likely that non-neutralizing anti-infliximab antibodies target determinants that are unique to infliximab, but not involved in TNF binding.

In summary, only for the chimeric therapeutic infliximab, but not for the human(ized) adalimumab, golimumab and certolizumab, a small amount of non-neutralizing antibodies could be observed, which implies that the closer the drug is to the human germline, the more restricted the antibody response to it is. Nevertheless, in all cases >90% of all ADA were neutralizing, which shows that the TNF binding region of anti-TNF therapeutic antibodies is immunodominant even in case of the chimeric antibody infliximab.

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**Contributorship Statement**

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**Competing interests**
G JW has received a research grant from Pfizer and honoraria for lectures from Abbvie, Pfizer and UCB. LA has received honoraria for lectures from Abbott, Roche, and Pfizer. TR has received honoraria for lecture from Pfizer and Abbvie.
REFERENCES


Chapter 4

Supplemental material

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Figure S1. Incomplete inhibition of ADA to infliximab is not caused by ADA-drug complexes. A) FPLC fractionation of patient serum. To eliminate drug-ADA complexes, fractions corresponding to monomeric IgG were pooled and used to repeat the TNF competition assay. B) Percentage of AU that is inhibited in binding radiolabeled infliximab Fab' after adding 5000 ng of TNF. No significant difference is detected between patient serum and the monomeric IgG fraction. Open dots: patient showing complete inhibition, closed dots: patients showing incomplete inhibition in TNF competition assay, ns = not significant using paired t-test.

Figure S2. There is no relationship between the percentage of neutralizing antibodies and the level of ADA. The percentage of units that is inhibited in binding radiolabeled drug Fab’ after adding 5000 ng of TNF, and the ADA level determined by our diagnostics department. Each symbol represents one patient.
MATERIALS AND METHODS

Patient material
Patient sera from our diagnostic department were selected for ADA positivity for either adalimumab, golimumab, certolizumab, or infliximab measured using the standard antigen binding tests (ABT). In total, serum of 68 patients (adalimumab (n=21), golimumab (n=4), certolizumab (n=9) and infliximab (n=34)) send in to our diagnostic department were analyzed. In all cases, the amounts of antibodies exceeded 100 AU/ml. Of these patients, no further clinical data is available. In case of infliximab, adalimumab and golimumab, no free drug was detected in the serum samples. On the other hand, most certolizumab samples contained free drug. However, since certolizumab is a Fab fragment and does not bind to protein A, free drug will be washed out during the ABT and therefore will not interfere with antibody measurements.1

Antigen binding test (ABT) and TNF competition assay
To determine the neutralizing capacity of ADA, the TNF competition assay was used. First, the optimal serum dilution was determined for every patient using the ABT, an assay routinely used by our diagnostics department, validated for each TNF-blocker. Patient serum was serially diluted in PBS/0.3% bovine serum albumine (BSA, Millipore). Fifty µl of diluted serum (or 50 µl of the optimal concentration in case of the TNF competition assay) was added to 1 mg of Sepharose-immobilized protein A (GE Healthcare) in PBS-AT (PBS containing 0.3% BSA, 0.2% tween-20 (Merck) and 0.01M EDTA) in a final volume of 800 µl and incubated overnight on a rotator. The samples were subsequently washed five times with washing buffer (PBS/0.005% Tween-20) and incubated with 125I labeled Fab’ fragments of adalimumab, certolizumab or infliximab, or biotinylated golimumab Fab’ (no significant difference was observed between directly radiolabeled drug or indirect radiolabeling via biotinylation), all diluted in PBS-FAT (PBS-AT containing 40 µg/ml IVIG F(ab’)2, Sanquin) in order to avoid false-positive results due to anti-hinge antibodies.2 In case of the TNF competition assay, the labeled drugs were preincubated with a titration of TNF (Human TNF-alpha cct premium, Active Bioscience), ranging from 0-5000 ng per tube. Labels were added in a final volume of 800 ul, and incubated for 5 h. Unbound labeled Fab’ was washed away and in case of golimumab 125I labeled streptavidine was added, incubated overnight and subsequently washed. Sepharose-bound radioactive label was measured using Wallac 1260 Multigamma II (LKB). Sample measurements were normalized to the percentage of total radioactively labeled input (% binding). For the TNF competition assay, the % binding was converted into arbitrary units (AU) using a standard curve (pooled serum of ADA-positive patients for adalimumab and infliximab, polyclonal rabbit-anti-drug for
certolizumab and golimumab). Percentage of inhibition was calculated by dividing the AU of inhibited samples by the AU of non-inhibited samples.

**WEHI bioassay**

The biological activity of ADA was analyzed using the TNF-sensitive WEHI bioassay. Per well 40,000 WEHI-164 cells were plated in IMDM (BioWhittaker) containing 5% FCS (Bodinco), 100 U/ml penicillin, 100 µg/ml streptomycin (both from Gibco), 1 µg/ml Actinomycin D and 50 µM β-Mercapto-ethanol (both from Sigma). Subsequently, a mixture of 100 pg/ml TNF (Strathmann), 30 ng/ml infliximab or adalimumab and a titration (twofold dilution, 1:100 – 1:6400) of sera containing ADA against infliximab or adalimumab were added. As a positive control, a titration of rabbit-anti-infliximab or rabbit-anti-adalimumab was added to the WEHI cultures containing TNF and infliximab or adalimumab, respectively. WEHI-164 cells were incubated for 24 h at 37°C and 5% CO₂ after which cell viability was measured using the MTT-reduction method. MTT (Sigma, diluted in 0.14M NaCl and 0.01M HEPES) was added to the cell cultures in a final concentration of 0.83 mg/ml and incubated for four hours, after which SDS (Gibco, diluted in 0.01M HCl) was added to a final concentration of 4% for overnight incubation. Adsorption was measured at 595 nm and as reference 670 nm using the Multiskan EX (Thermo Scientific).

**Monomer isolation by FPLC**

Serum of five patients was fractionated by Fast protein liquid chromatography (FPLC) to isolate fractions that contain monomeric IgG but no complexes of two or more IgG molecules. Serum was three times diluted in PBS and filtered (0.22 µm filter, Merck Millipore) prior to application. A Superdex 200 10/300 GL column in combination with the FPLC system GE ÄKTA Explorer (Amersham Pharmacia) was used to fractionate the sera. Molecular weight estimations were made using intravenous immunoglobulin (IVIG, 20 mg/ml) that contains both monomeric and dimeric IgG. Fractions of patient serum containing only monomeric IgG were pooled for each patient.

**TNF/mouse IgG competition assay**

Further characterization of the target of non-neutralizing ADA to infliximab was done using the TNF competition assay with addition of polyclonal BALB/c mouse IgG (Molecular Innovations). Per patient, twelve tubes containing 1 mg of CaptureSelect anti-human IgG-Fc Affinity Matrix (Life Technologies) were incubated overnight with 50 µl of the optimal serum dilution. After washing, 20 µg mouse IgG in PBS-FAT was added in six of the tubes, whereas in the other six tubes only PBS-FAT was added. In both sets of tubes, 50 ul of ¹²⁵I labeled infliximab Fab’ preincubated with a titration of TNF, ranging from 0-5000 ng per tube, was added. The tubes were incubated for 5h on
a rotator and subsequently washed. Bound radioactivity was measured as described above.

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