General discussion

Part of this work has been submitted for publication
Anti-TNF treatment has given a major boost to the treatment of different auto-immune diseases. Most patients on anti-TNF agents benefit from treatment. However, part of the patients develops anti-drug antibodies (ADA) against the therapeutic. In case of infliximab and adalimumab ADA formation has been linked to lower serum drug levels and a reduced clinical response. In this thesis various aspects of the antibody response against adalimumab were investigated.

Clinical non-response to anti-TNF treatment
Two types of non-response to anti-TNF therapy have been described; patients in whom response to treatment was not observed from the start of treatment (primary non-responders) and patients losing response in time (secondary non-responders). It is suggested that in primary non-responders disease is TNF independent, while secondary non-responders lose clinical response due to the formation of ADA. In clinical practice the definition of primary and secondary non-responders has not been standardized yet. We propose defining primary non-responders as patients not responding to treatment despite sufficient drug levels. Secondary non-responders are patients that do not respond to treatment due to diminished serum drug levels (possibly due to the formation of ADA).

Defining primary and secondary non-responders based on the mechanism will be helpful in the clinic. Recent studies show that the reason for non-response to a first TNF inhibitor has implications for the clinical response to a second anti-TNF agent. RA patients with antibody formation against a first anti-TNF agent (secondary non-responders) had a clinical response to the second anti-TNF agent that did not differ from anti-TNF naive patients. In contrast, primary non-responders to the first anti-TNF agent responded significantly worse to treatment with a second anti-TNF agent compared to either anti-TNF naive patients or patients with ADA formation against their first anti-TNF agent. This suggests that determining immunogenicity can be helpful for clinicians to decide on the appropriate follow-up of therapy.

Measuring ADA
The reported percentage of ADA producing patients varies greatly between different studies. This can partly be explained by differences in patient groups, treatment strategies, length of follow-up and co-medication. Moreover, the type of assay used for ADA detection can also greatly influence results. One major issue in the detection of ADA is drug interference. Since patients are continuously exposed to high levels of drug, drug interference will often lead to false negative results and therefore an underestimation of ADA formation. Most data available on the immunogenicity of adalimumab are generated using assays that are sensitive for drug interference such as the antigen binding test (ABT)
and the bridging ELISA.

In chapter 3 we describe the pH-shift-anti-Idiotype Antigen binding test (PIA) which allows for detection of ADA against adalimumab in the presence of drug. In chapter 4 we use this novel assay to measure ADA in a cohort of 99 consecutive RA patients which are treated with adalimumab. Results show that the majority of adalimumab treated patients develop ADA (53%), but that only high levels of ADA lead to clinical non-response (Figure 1).

In chapter 6 we investigated the effect of affinity of ADA on their measurement in different assays. In this chapter we show that in the bridging ELISA affinity greatly influences ADA detection. In contrast, the ABT is much less influenced by affinity. This can be explained by the fact that two monovalent interactions are required for ADA detection in the bridging ELISA since both the catching and the detecting antibody should be bound by a single Fab arm of the ADA. In contrast, in the ABT only a single interaction enables detection. We compared radiolabeled adalimumab Fab and adalimumab F(ab’)2 fragments for detection in the ABT and observed decreased binding to Fab while using the same amount of ADA. This suggests that all antibodies have higher binding avidity in a bivalent interaction. In case of the Fab-ABT there is a correlation between affinity and ADA detection. This indicates that the effect of affinity is dependent on the assay used.

<table>
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<td>Positive</td>
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<tr>
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<td>ADA detection (PIA)</td>
<td>Negative</td>
<td>Low</td>
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Figure 1: The level of ADA production determines the clinical effect. If only low levels of ADA (black) are produced, small amounts of immune complexes are formed between the drug (grey) and the ADA. The majority of drug is unbound and still functional. As ADA formation increases, the amount of ADA-drug complexes increases. Eventually all drug is present in the form of immune complexes and free ADA are present. The ability to detect ADA is dependent on the assay used.
for ADA detection. In patients sera ADA of different affinities will be present, probably the average affinity will determine the signal in the different assays.

**Isotypes involved in the immune response against adalimumab**

We found that IgG1 and IgG4 are the main isotypes involved in the antibody response against adalimumab (unpublished data, chapter 2). This is confirmed in chapter 5, in which 15 out of 16 of the human monoclonal antibodies against adalimumab that we produced originated from the IgG1 or IgG4 isotype. The isotype of the last monoclonal antibody could not be determined because of lack of sufficient supernatant. Since IgG4 has been linked to long term antigen exposure and RA patients are chronically treated with adalimumab, it is not unexpected to find IgG4 as a major isotype in the immune response against this drug.\(^\text{21}\) In chapter 2 we describe an ABT to specifically detect adalimumab specific IgG4 (IgG4-ABT). The IgG4-ABT appeared to be more resistant to drug interference than the regular ABT (that detects total adalimumab specific IgG) which makes it very difficult to calculate the relative contribution of IgG4 to the total IgG response against adalimumab. In samples in which drug interference is expected to be limited (because of undetectable adalimumab levels) the percentage IgG4 was highly variable between patients. In addition, the kinetics of IgG4 production varied between the patients that we studied: in some patients the percentage IgG4 increased in time, while in other patients the percentage IgG4 was stable or decreased. Since IgG4 has little effector function and is known to form small immune complexes (IC), it would be interesting to link the percentage IgG4 produced to clinical response.

Further research should reveal whether the high levels of IgG4 as found in chapter 2 are a hallmark of the immune response against adalimumab or other anti-TNF agents. Since the absence of TNF may induce a more tolerant micro-environment and IgG4 is considered an immunotolerant antibody, anti-TNF therapy might promote switching towards IgG4. In concordance with this, IgG4 production in the immune response against infliximab has been described.\(^\text{22}\) However, also in case of long term exposure to other types of antigen such as factor VIII and natalizumab (a therapeutic antibody against α4-integrin) IgG4 has been described to play a dominant role.\(^\text{23,24}\)

Currently, mainly IgG1 and IgG4 production against adalimumab is detected. However we have occasionally found low levels of IgG2 and IgG3 (unpublished data). Also it should be expected that IgM would be produced against adalimumab. However, until now there are no data available on this. This could be because IgM antibodies are usually low affinity and therefore difficult to detect in the assays available for ADA detection. In addition, detection of IgM may be challenging because IgM is formed early in the immune response when ADA production is expected to be low therefore drug interference will most probably be a serious problem in the detection of IgM ADA. Setting up an assay that
detects low affinity IgM in the presence of high drug levels would allow the study of the role of IgM in immunogenicity of adalimumab.

**Kinetics of the immune response**

As described above, standard assays to detect ADA against adalimumab are influenced by drug interference. Since drug levels may vary during treatment, it is difficult to study antibody formation over time. To obtain a better insight in the kinetics of the immune response against adalimumab we used the PIA to measure antibodies against adalimumab in longitudinally collected samples of RA patients during long term treatment as described in chapter 4. We show that 94% of the patients that develop ADA at a certain time point during three years of treatment produce ADA within the first 28 weeks of treatment (t=28). Patients positive for ADA in the PIA at t=28 are at increased risk of free ADA (which can be detected in the standard ABT) and low functional adalimumab levels (<5 µg/ml) compared to ADA negative patients in the PIA. This indicates that it might be clinically beneficial to measure ADA in the PIA after 28 weeks of treatment to identify the patients that are at risk for clinical non-response due to the formation of ADA against adalimumab.

The long term immunogenicity data obtained by the PIA also show that in 17 of the 53 ADA producing patients ADA disappear in time, pointing at a transient antibody response in these patients. Further investigation of the clinical relevance of transient ADA production is difficult due to the length (mean length 50 weeks) of transient ADA production and the large number of patients that dropped out of our cohort. It would be interesting to identify factors that influence continuous ADA formation in some patients and transient ADA production in other patients. This could lead to the induction of tolerance in patients as described for factor VIII treatment.\(^{25}\) This type of research might not be cost-effective because of the availability of several anti-TNF agents. In the clinic this allows physicians to switch patients to another anti-TNF agent.

A study by Maini et al. has suggested a link between increased dosing of infliximab and reduced frequency of ADA formation.\(^{26}\) This suggests that high drug dosing might induce tolerance in these patients. However, another explanation for these results would be that, in patients with high drug dosing, the presence of high residual drug levels hampers ADA detection. ADA measurements using the PIA would allow distinguishing between drug interference and tolerance induction.

Measuring ADA in an assay which allows the detection of ADA in the presence of drug would also be very useful in the earlier stages of drug development. This would yield a better understanding of ADA production and the kinetics of this immune response and therefore might provide a better insight in dose optimization and appropriate treatment regimens.
Neutralization or clearance?

Little data are available on the mechanism by which ADA formation leads to clinical non-response. Two possible mechanisms were proposed; first ADA can prevent the drug from binding to its target, thereby neutralizing its function. Second the formation of IC can lead to increased clearance of the drug. In chapter 5 we show that in case of adalimumab ADA are directed against the TNF-binding region and that all antibodies are able to neutralize adalimumab function. We also show that in many patients’ sera small immune complexes (IC) can be detected. Results from chapter 4 suggest that these IC can be detected in patients’ sera over long periods of time. Together these data suggest that neutralization of the drug is an important mechanism by which ADA formation against adalimumab results in clinical non-response.

To our knowledge, adalimumab is the only therapeutic monoclonal antibody of which such data are available. It might be expected that in case of humanized or chimeric antibodies (such as natalizumab and infliximab, respectively) the immune response is more diverse since there are more immunological foreign epitopes present. This might lead to the formation of larger IC and therefore enhanced clearance of the drug may be the most important mechanism for clinical non-response to these drugs.

The finding that all ADA against adalimumab are neutralizing also suggests that the quantity of the ADA formed determines their clinical relevance. Low levels of ADA neutralize only a fraction of the adalimumab present in the patient, leaving sufficient amounts of functional drug to benefit from treatment. In contrast, in patients with high ADA production, all adalimumab is neutralized leading to clinical non-response. These results emphasize that in case of adalimumab, it would be sufficient to detect functional adalimumab levels to predict the reason of non response.27

Immune complexes

In chapter 5 we show that only small IC can be detected in patients’ sera. However, it is impossible to conclude whether only small IC are formed. It might be that in vivo larger IC are formed as well that are rapidly cleared from the circulation. In the present study only samples were available that were taken just prior to the next administration of adalimumab. Two weeks after the last adalimumab injection, large IC may be cleared already. The formation of IC in vitro using the human monoclonal antibodies described in chapter 6 would provide more insight in the possible size of IC formed in vivo. In addition, taking multiple blood samples with short time intervals of patients just after adalimumab injection would allow the investigation of the formation and clearance rate of IC in vivo as previously described for infliximab.28

Further research on IC formation between adalimumab and ADA would contribute to understanding their clinical effect. On the one hand, permanent exposure to small IC
leads to a kind of pro-inflammatory state by low-level stimulation of effector mechanisms. Recently ADA formation has been linked to increased frequency of thrombo-embolic events which indicates that IC might be harmful for these patients. On the other hand, it can be hypothesized that small IC have a beneficial, anti-inflammatory effect. For dimers of IgG, in particular those present in intravenous immunoglobulin, it has been suggested that they may act as antagonists in FcγR-mediated processes.

The immune response against adalimumab is polyclonal
In chapter 5 we show that a single monoclonal antibody against adalimumab can compete with the entire ADA response of 50 different patients. From these data it is impossible to conclude whether this is the result of one dominant immunogenic epitope present on adalimumab or whether steric hindrance prevents binding of ADA to epitopes in close proximity of each other.

In chapter 6 we describe the production of 11 human derived monoclonal antibodies against adalimumab. As discussed above, all these antibodies were fully neutralizing adalimumab and competed with each other for the binding to adalimumab, indicating that the antibody response against adalimumab is restricted to the TNF-binding region. Studying the V(D)J segments of a total of 16 monoclonal antibodies showed that all antibodies were the result of different V(D)J recombination, indicating that they originated from different precursor B-cells. This indicates that the restricted anti-adalimumab response is the result of the outgrowth of multiple B-cell clones, showing that the response is polyclonal. It is however impossible to estimate the number of B-cell clones involved based on these data. For the immune response against tetanus toxoid it has been described that as many as 100 different B-cell clones are involved in the immune response. High throughput sequencing of adalimumab specific B-cells would indicate whether a restricted response such as the immune response against adalimumab is also the result of such a broad outgrowth of B-cell clones.

The study of V(D)J segment usage also shows that there is some restriction in the usage of V1 segments for the heavy chain and V3-20 and J4 segments usage for the light chain. Confirmation of this restriction would be possible by purification of ADA from patients sera and analyses of V(D)J segments by protein sequencing using mass spectrometry.

Internal image
Theoretically, ADA formation can not only influence treatment efficacy by neutralizing adalimumab or increasing clearance of the drug. Another mechanism would be when ADA bear an “internal image” of the antigen. Previous studies by others have shown that an antigen bound by the idiotype of an antibody may be mimicked by the idiotype of a secondary antibody directed against the idiotype of the primary antibody. In case of anti-
TNF treatment this would mean that the ADA might mimic TNF and therefore interact with the TNF receptor. This is only possible if adalimumab and the TNF receptor bind to the same epitope on TNF. If this were the case this could be detrimental for the patients.

In chapter 5 we show that two of the monoclonal antibodies that we produced against adalimumab are unable to react with the TNF receptor. So, apparently these antibodies are no internal image of TNF. It would be very interesting to investigate whether one of the other monoclonal antibodies described in chapter 6 is able to bind the TNF receptor. Preliminary data as shown in chapter 6 suggest that some of the monoclonal antibodies may have an activating effect on the TNF receptor. We observed increased IL-8 production upon incubation of TNF dependend endothelial cells with TNF and adalimumab in the presence of some of the monoclonal antibodies. Additional experiments in which these cells are incubated with the monoclonal antibodies in the absence of TNF and adalimumab would provide more insight.

In the seventies of last century Niels Jerne hypothesized the presence of an anti-idiotype network: the idiotype of a novel produced antibody would be recognized as immunologically foreign leading to anti-idiotype antibodies that may be immunosuppressive by dampening the primary antibody response. In case of adalimumab this would mean that there would be anti-idiotype antibodies produced against the ADA. This is a tempting theory that would provide an explanation for the transient antibody response that we observed in some patients. However, the broadness of the immune response against adalimumab on a genetic level argues against the presence of an anti-idiotype network that dampens the immune response. If many different idiotypes are involved this would indicate that the number of secondary antibodies necessary for this would be very large.

Conclusion
In conclusion the following picture emerges. Upon repeated exposure to adalimumab, the majority of RA patients develop ADA against the biological within the first 28 weeks of treatment. The antibody response is polyclonal and mainly consists of the IgG1 and IgG4 isotype. In one third of the patients ADA formation is transient. The formation of ADA results in the formation of ADA-drug complexes. Small IC, the size of dimers, stay in circulation and can be detected in patient sera overtime. All antibodies are directed against the idiotype of adalimumab and result in functional neutralization of the drug. Depending on the amount of ADA produced, there are clinical consequences. Low ADA levels do not influence treatment efficacy because there is still sufficient functional adalimumab in the serum. In contrast high ADA production results in neutralization of all adalimumab and therefore clinical non-response.
REFERENCES


32. Korswagen LA, Bartelds GM, Krieckaert CL, Turkstra F, Nurmohamed MT, van SD et al. Venous
