Antibodies against antibodies: immunogenicity of adalimumab as a model
van Schouwenburg, P.A.

Citation for published version (APA):

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General introduction

*Part of this work has been submitted for publication*
In the last decade there have been major developments in the field of therapeutic monoclonal antibodies. At this point over thirty different therapeutic monoclonal antibodies have been approved by the FDA. These therapeutics are used for the treatment of cancer, auto-immune diseases and cardiovascular diseases. The majority of the patients respond very well to the treatment, however in some patients the therapeutic gets recognized by the patients’ immune system. This leads to antibody formation against the therapeutic, which has been linked to clinical non-response in these patients.

Recognition by the immune system
Our immune system protects us against the pathogens we are exposed to every day. It consists of the interplay between the innate and the adaptive immune system. The innate immune system is the first line of defense and consists of the complement system and broadly recognizing pattern recognition receptors on innate immune cells such as macrophages and neutrophils. If the innate immune system fails to clear the pathogen swiftly, the adaptive immune system is activated. Upon the first encounter with a pathogen, the adaptive immune system develops immunological memory against this specific pathogen allowing it to respond more rapidly upon a second round of antigen exposure. The adaptive immune system consists of B and T cells each expressing its own antigen specific receptor. T cells proliferate and differentiate in response to antigen presentation to the T cell receptor (TCR) by antigen presenting cells. B cells recognize pathogens directly via their B cell receptor (BCR) and proliferate, maturate and differentiate upon target binding. Moreover, activated B cells produce antibodies with the same specificity as their BCR leading to neutralization of the pathogen and facilitating its clearance.1

Immunoglobulins
To be able to respond to all different types of pathogens, B cells require a large variation in BCR’s. Every BCR contains two heavy and two light chains, both consisting of a constant and a variable domain. The heavy chain variable region consists of a single variable (V), diversity (D) and joining (J) segment, which are combined during V(D)J recombination2,3. In total there are ~40 V segments, ~25 D segments and ~6 J segments which can be combined to form a great variety of heavy chains. The light chain can be formed by recombination of either the kappa gene, in which ~40 V segments can be combined with one of ~5 J regions, or the lambda encoding region consisting of ~30 V regions and ~4 J regions (figure 1).4

V(D)J recombination leads to an enormous variation between BCR’s. This diversity is even further increased by random nucleotide incorporation in the joining regions during recombination and by a process called somatic hyper mutation.5 Somatic hyper mutation
Antigen binding can not only induce affinity maturation of the BCR, but it can also initiate isotype switching. In naive cells all antigen receptors expressed by B cells are of the IgM and IgD isotype. However, after antigen recognition, rearrangement in the heavy chain coding region can lead to the expression of IgG, IgGA or IgE. The class of antibody produced in an immune response depends of the type of antigen the body is exposed to and the microenvironment of the B cell. IgA is mainly produced to protect mucosal surfaces, while IgE is involved in anti-parasitic immune responses and allergy. The IgG isotype consists of four subtypes numbered according to the frequency in peripheral blood. IgG1 is mainly produced against bacterial pathogens; IgG2 is often directed against polysaccharides on bacterial surfaces. IgG3 is made as a response against viruses and IgG4 is produced after long term exposure to allergens.

Figure 1: V(D)J recombination. The great variation between antibodies is, among other mechanisms, the result of V(D)J recombination. The two heavy chains consist of a variable (V), diversity (D) and joining (J) segments. Both light chains are formed by recombination of a V and a J segment.

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Effector functions of immunoglobulins

The production of antibodies can trigger several downstream effector mechanisms. Antibodies can activate complement by binding to the C1 complex via their Fc tails. Activation of the complement system ends in the formation of the membrane attack complex leading to lysis of the target cell. Next to that, complement factors can opsonize pathogens facilitating phagocytosis, and induce the release of anaphylatoxins, thereby promoting inflammation. IgG1, IgG3 and to a lesser extent IgG2 are able to activate the complement system, in contrast to IgG4 which is unable to activate the complement system.

Antibodies can also bind to Fc receptors via their Fc tail leading to activation of macrophages, monocytes, myeloid cells and dendritic cells. Activation of these receptors can lead to phagocytosis, endocytosis, antibody mediated cellular toxicity and cytokine release. Binding of Fc receptors also plays a role in the clearance of immune complexes. IgG1 and IgG3 are able to bind Fc receptors. IgG2 and IgG4 can also bind, but with a lower affinity.

IgG4 is considered an odd antibody, not only is it limited in its effector functions, but it is able to exchange half molecules in vivo, leading to bispecific antibodies. Because of their bispecificity IgG4 antibodies are unable to cross-link identical antigens resulting in the formation of small immune complexes. Even though in general the percentage of IgG4 in serum is low, it has been described that in some immune responses IgG4 can be the main subclass produced.

Therapeutic monoclonal antibodies

When the development of monoclonal antibodies evolved, soon these antibodies were also developed for therapeutic purposes. Because of their specific targeting and powerful effector mechanisms to remove unwanted agents, monoclonal antibodies are considered very good therapeutics. In 1986, the first FDA approved monoclonal antibody was OKT3 which was used to prevent transplant rejection. OKT3 was a mouse monoclonal antibody and one of the major problems in the clinic was immunogenicity. All patients treated with OKT3 developed anti-drug antibodies (ADA) against the therapeutic and these antibodies were linked to loss of clinical response. It was assumed that this immunogenicity could be reduced by making therapeutic monoclonal antibodies that resembled human antibodies more closely. Therefore, a lot of effort was taken to humanize therapeutic monoclonal antibodies.

Humanization of monoclonal antibodies

The first step in the humanization process of therapeutic monoclonal antibodies was the development of chimeric antibodies (figure 2) of which the constant domain is fully
human whereas the variable region is murine. One example of a chimeric therapeutic antibody is infliximab, an anti-TNF agent used for the treatment of auto-immune diseases such as Rheumatoid arthritis (RA) (figure 3). For infliximab, reported immunogenicity numbers vary between 8-61%. The development of ADA is linked to lower infliximab levels and clinical non-response.

Later, humanized antibodies were developed; here the murine framework sequences around the complementarity determining regions (CDR’s) are replaced with human sequences and only the CDR’s are murine. One example of humanized therapeutic monoclonal antibodies is natalizumab, a monoclonal antibody directed against α-4 integrin, which is used for the treatment of multiple sclerosis. Despite humanization of the antibody, immunogenicity against natalizumab is found in 4-11% of the treated patients.

As techniques developed further, this enabled the production of fully human antibodies using phage display or humanized mice. Two examples of fully human antibodies are golimumab and adalimumab, both anti-TNF antibodies used for the treatment of auto-immune diseases (figure 3). The hope was that these fully human antibodies would not elicit an immune response in patients. However, for both golimumab and adalimumab, immunogenicity has been reported. Although little data are available for golimumab, ADA were detected in two studies in 6.5-8.0% of the treated patients. For adalimumab immunogenicity was reported in 1-44% of adalimumab treated patients. As was found with other therapeutic proteins, ADA production is linked to lower adalimumab serum levels and reduced clinical response.

Next to fully human monoclonal antibodies, the development of other types of therapeutic proteins is emerging, hoping to reduce immunogenicity and increase specificity. Examples are certolizumab, a PEGylated Fab fragment directed against TNF and

![Figure 2: Humanization of monoclonal antibodies](image)

The first monoclonal antibodies available for treatment were murine antibodies. Immunogenicity was a major problem in the treatment with these antibodies, which led to the development of antibodies with increased homology with human antibodies. Chimeric antibodies have a human (gray) constant domain, and a murine (black) variable region. In humanized antibodies only the CDR regions of the antibody are of murine origin. Currently, also fully human monoclonal antibodies are available for treatment.
etanercept, a fusion protein between TNF receptor II and the Fc part of an IgG antibody (figure 3). For both drugs ADA formation has been described. Against certolizumab, ADA have been found in 5.1-8.1% of the patients, and for etanercept reports vary between 0-16%.

The production of ADA against therapeutic antibodies has not only been linked to reduced clinical efficacy, but also is associated with increased frequency of clinical side effects. For example, several recent papers suggest a possible link between immunogenicity and increased risk of tromboembolic events. Together this indicates that immunogenicity of therapeutic monoclonal antibodies is of clinical importance.

There is a large variation in the prevalence of immunogenicity as reported in different studies. This can be explained by differences between patients groups, co-medication, timing of sampling, length of follow-up and drug dosing. Also the different types of assays used for detection of ADA will greatly influence the results as further discussed below.

**Figure 3: The five anti-TNF agents available for the treatment of rheumatoid arthritis.** Infliximab is a chimeric antibody of which the variable domain is murine (black), while the constant domain is human (gray). Adalimumab is a fully human antibody. Etanercept is a fusion protein between a human IgG Fc tail and the TNF receptor. Golimumab is also a fully human antibody and certolizumab is a humanized Fab fragment which is PEGylated (light gray).

**Assessment of immunogenicity**
Many different assays are available for the measurement of ADA. A commonly used assay is a bridging ELISA. In this assay format specific ADA in the test sample crosslink the drug which is used both for coating and detection. Another assay often used for ADA detection is an antigen binding test (ABT). In both mentioned assays drug interference is a major complicating factor in the detection of ADA against therapeutic antibodies. Often the presence of drug levels in patient sera leads to false negative results in these assays. In assays that are sensitive for drug interference ADA are only detected in case the amount of ADA produced exceeds drug levels. Since most patients are continuously treated with therapeutic monoclonal antibodies, drug interference often leads to an underestimation of ADA production. Immunogenicity data should therefore
always be interpreted in context of the assay used for ADA measurement. It is important to realize the discrepancy between the ADA levels produced in the patients and the ADA detected by the different assays. Drug interference also complicates investigation of the kinetics of the immune response against therapeutic monoclonal antibodies. Using an assay less sensitive for drug interference would lead to more reliable results.

Factors influencing immunogenicity

Patient related factors

There are many different factors that are thought to influence the immunogenicity of therapeutic proteins. First, the genetic background of patients will make some patients more prone than others to develop ADA. For anti-TNF agents it was shown that patients developing ADA against their first anti-TNF agent are more likely to develop ADA against the second anti-TNF agent, suggesting that there is an intrinsic factor in these patients contributing to the immune response against therapeutic proteins. Until now, only a single paper on genetic variation and immunogenicity of anti-TNF agents was described. In this paper a correlation between IL-10 genotype and antibody production against adalimumab is shown, but a causal relation was not further investigated. It can be expected that MHC restriction might also predispose some patients for ADA production as described below. Collecting data on patient genetics and ADA formation is difficult due to large patient numbers required for this type of research to reach statistical power.

In addition, the immune status of the patient is thought to influence ADA formation. Patients with a highly active immune system are thought to have an increased risk for ADA development. This is supported by various reports that ADA producing patients show higher baseline disease activity and elevated levels of inflammation markers.

Treatment strategy

The treatment regimen is thought to influence ADA formation as well. It was reported that in case of infliximab, higher dosing reduces immunogenicity. Furthermore, the route of administration seems to be important, since intravenous (IV) administration is thought to be less immunogenic compared to intra muscular or subcutaneous (SC) treatment. In contrast to this, a study comparing SC and IV administration of abatacept (a fusion protein between the IgG1 Fc tail and CTLA-4) showed a mild increase in immunogenicity in the IV treated patients. Other studies suggest that the risk of immunogenicity increases with prolonged treatment.

Also the use of co-medication with immunomodulators has been linked to reduced immunogenicity. For azathioprine, mercaptopurine, hydrocortisone, and methotrexate it was described that co-treatment is linked to lower frequency of ADA formation in either RA of Crohns disease (CD) patients treated with anti-TNF agents.
Product related factors
Product related factors can also influence the immunogenicity of a therapeutic protein. First, the presence of B or T cell epitopes in the therapeutic protein will increase their immunogenic potential, as will be discussed more extensively below. Amino acid sequence differences between the therapeutic and endogenous protein may increase immunogenicity. Structural alteration of the therapeutic protein, such as aggregation, has been described to increase the risk of an immune reaction.71-73 The same is true for small particles present in therapeutics.71 These aggregates and particles may be introduced during the production and purification process of the therapeutic protein. It has been suggested that glycosylation of the therapeutic protein can influence the immunogenic potential.74

Target related factors
Therapeutic antibodies form immune complexes (IC) with their targets in vivo. It appears that the type of IC formed between a therapeutic monoclonal antibody and the target protein influences ADA formation. The formation of large IC might enhance uptake of the therapeutic antibody by antigen presenting cells. Large IC can also directly crosslink BCR’s leading to T cell independent B cell activation. Aggregates in the therapeutic can resemble immune complexes and induce immune activation in a similar fashion.75 In case of anti-TNF agents there are reports on IC formation between TNF and some of the known anti-TNF agents.76,77 These studies show that adalimumab and infliximab can form large complexes with TNF of respectively 4,000 kDa and 14,000 kDa. In contrast, etanercept only forms small IC complexes with a maximal size of 300 kDa (consistent with a 1:2 TNF: etanercept complex). In concordance with these data, immunogenicity against etanercept was only reported in few studies whereas others found low or no levels of ADA.

T cell epitopes
After phagocytosis of therapeutic proteins by antigen presenting cells, the protein is digested into peptides which can be presented via MHC molecules. Once the peptide is loaded, MHC is transported to the cell surface, where it can bind to specific T cell receptors (TCR) and induce proliferation of antigen specific T cells. Depending on the peptides available on the therapeutic protein and the HLA type of the patient, antigen presentation is more or less efficient.

To investigate the role of T cell epitopes in the immune response against therapeutic proteins different approaches can be used. First, in silico methods are used to identify T cell epitopes by the prediction of binding affinities of peptides to common HLA types.78 Next to this, proliferation assays are used to test which peptides are able to induce
T cell proliferation *in vitro*. Furthermore linkage studies can indicate whether certain HLA alleles are linked to ADA formation.

Notwithstanding the fact that various groups are setting up systems to investigate T cell epitopes, little published data are available on T cell epitopes in anti-TNF agents. One study identified a T cell epitope associated with the G1m1 allotype, which is able to induce T cell proliferation in healthy donors with a different allotype.\(^7^9\) In case of adalimumab, which is of the G1m1 allotype, this would suggest that patients with a G1m1 allotype are less likely to produce ADA against adalimumab. This is contradicted by a study by Bartelds et al showing increased immunogenicity in patients with the G1m1 allotype.\(^6^0\)

Theoretically, knowing which peptides of a therapeutic protein result in T cell proliferation gives the opportunity to modify the protein to reduce immunogenicity. However, in case of anti-TNF the removal of T cell epitopes might not be cost-effective, since many alternative drugs are available and patients can easily switch to a different anti-TNF agent. However, increasing the knowledge on T cell epitopes will help the further development of *in silico* prediction tools and pre-clinical *in vitro* proliferation assays. These tools could be very valuable in reducing immunogenicity in a next generation of therapeutic proteins.

**B cell epitopes**

Mapping the binding sites of ADA on therapeutic proteins will enable the identification of B cell epitopes involved in immunogenicity. For golimumab and certolizumab no data are available on the immunogenic epitopes. For infliximab, one study shows that the immunogenic region of infliximab lies within the F(ab’)2 region.\(^8^0\) Another study by Kosmac et al confirmed these results and performed experiments using linear peptides of the F(ab’)2 region to identify the precise B cell epitope. Unfortunately they did not succeed in this, suggesting that the immunogenic epitope is only present on correctly folded infliximab.\(^8^1\)

Until now there are no data available for adalimumab on the identification of B cell epitopes. However, different assays for the detection of ADA use adalimumab F(ab’)2 for detection, indicating that the immunogenic region lies within the F(ab’)2 region of adalimumab. For etanercept, ADA have been described to be non-neutralizing, but the exact binding epitope has not been investigated.\(^4^3;^8^2;^8^5\)

Identification of the precise epitopes involved in ADA binding could potentially allow their elimination from the therapeutic protein or shielding of these epitope using PEG groups or HIS tags.\(^8^6;^8^7\) Of course such alterations would only be possible when small numbers of immunogenic epitopes are involved and they are located outside the antigen binding domain to avoid destruction of target binding.
ADA: neutralizing or binding antibodies?
There are two possible mechanisms by which the formation of ADA against therapeutic monoclonal antibodies can influence treatment efficacy. First, neutralizing ADA can block the binding of the therapeutic to its target. Second, ADA may form immune complexes with the therapeutic antibody, leading to increased clearance and reduced half life of the therapeutic. It is currently unknown which of these mechanisms is most important in clinical non-response to therapeutic antibody treatment.

For infliximab, two different studies show that at least part of the antibodies formed against infliximab have neutralizing capacity. In case of adalimumab, no such data are available yet.

The size of IC formed determines their fate. Large IC will activate complement and will be rapidly cleared in the liver whereas small IC are not efficiently cleared and stay in circulation. Rojas et al. published a study investigating IC formation of infliximab in cynomolgus monkeys. They injected these monkeys with infliximab and a labeled polyclonal anti-infliximab serum obtained from immunized rhesus monkeys and studied the size and clearance of the in vivo formed IC between infliximab and anti-infliximab antibodies. They observed the formation of IC of two different sizes, the largest being 670 kDa in size. As expected, the larger IC were more rapidly cleared from the circulation.

Another study followed the formation of IC in three infliximab treated patients during infusion. In two of these patients only small IC were formed, while in the last patient large IC were found. This last patient also developed a severe infusion reaction. This suggests that the size of IC formed between ADA and infliximab varies between patients. For adalimumab no such data are available.

The treatment of rheumatoid arthritis patients with adalimumab
In this thesis we investigate the role of immunogenicity against adalimumab in the treatment of rheumatoid arthritis (RA) patients with this therapeutic monoclonal antibody. RA is a systemic inflammatory disease that affects approximately 1% of the western population. The disease is characterized by chronic synovitis which can result in destruction of cartilage and bone, leading to functional disability, joint damage, decreased quality of life and shortened life expectancy. Part of the RA patients are treated with adalimumab, and in these patients the immunogenicity has been extensively linked to lower serum drug levels and reduced clinical response.

Scope of this thesis
A variety of studies have been focusing on the clinical aspects of immunogenicity of adalimumab, in this thesis we focus on the immunological aspects. The role of IgG4
in the immune response against adalimumab is elucidated in chapter 2. In chapter 3 a novel assay for the detection of ADA against adalimumab is described, in which ADA can be measured in the presence of high drug levels. In chapter 4 this assay is used for the measurement of ADA in a cohort of 99 adalimumab treated RA patients during three years of treatment. This gives a more complete overview of the kinetics of the anti-adalimumab response. Chapter 5 describes that all antibodies against adalimumab are neutralizing, providing for the first time a mechanism by which ADA formation leads to clinical non-response. In chapter 6 we describe the production of human monoclonal antibodies against adalimumab, which are derived from patient peripheral blood. We study their affinity and the effect of the different affinities in various assay formats. Chapter 7 contains a general discussion and a summary of the results.
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