CHAPTER 1

Introduction
Immunobiology of inhibitor development in haemophilia A

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Scope of this thesis

The congenital bleeding disorder haemophilia A is caused by a deficiency or functional defect of clotting factor VIII. The clinical picture of haemophilia has been recognised since historic times. Adequate treatment of this disease has only become available since half a century, when methods to prepare factor VIII concentrates from human plasma were developed. Intravenous administration of these concentrates made treatment of bleeding episodes possible, thus leading to a significant reduction in mortality and morbidity. In the last decades factor VIII concentrates have become purer and safer. Although this is of great benefit for most haemophiliacs, in about 25% of the severely affected patients treatment is complicated by the formation of inhibiting antibodies (inhibitors) directed towards factor VIII. Clinically, these antibodies result in an increased bleeding tendency that does not respond to factor VIII replacement therapy. Patients that develop inhibitors suffer increased mortality and morbidity, although alternative (albeit expensive) haemostatic therapies are available. Gene therapy for haemophilia is currently under investigation. However, as with conventional therapy, this potential new treatment may give rise to an immune response against the transferred gene product, i.e. factor VIII. The development of inhibitory antibodies therefore remains one of the greatest challenges in the treatment of haemophilia A patients. Until now, limited attention has been directed at inhibitor development in patients mild haemophilia A. Inhibitor formation in mild haemophilia A is rare and this is most likely due to the presence of tolerizing amounts of endogenously synthesised factor VIII in these patients. It has been suggested that inhibitor formation in this group of patients is related to molecular defects in the factor VIII gene located in particular areas in the factor VIII molecule. At present, however, it is still unclear why some mild haemophilia A patients develop inhibitors while others do not. The aim of this thesis is to gain novel insight in the mechanism underlying inhibitor development in mild haemophilia A. We have studied the molecular properties of anti-factor VIII antibodies specific for different epitopes on factor VIII using phage display technology. We have also examined the role of HLA class II molecules in inhibitor formation in a large cohort of mild haemophilia A patients. To enable detailed studies of the immunological background of inhibitor formation, we have developed a new murine model for inhibitor development in haemophilia A. Before describing our own studies, we first review recent progress in the area of factor VIII inhibitors.

Epitope mapping and mode of action of factor VIII inhibitors

Inhibitory antibodies that develop in patients with haemophilia A are usually of mixed subclass with a dominant contribution of IgG4. Current evidence suggests that these antibodies recognise a restricted set of well-defined regions in factor VIII (see Figure 1). Major binding sites for factor VIII inhibitors have been assigned to residues Arg484-Ile508 in the A2 domain and residues Val2248-Ser2312 in the C2 domain.3,4 Results from two independent studies further suggest the presence of an antigenic site within residues Gln1778-Met1823 in the A3 domain of factor VIII.5,6 Functional studies performed with purified IgG derived from patient’s plasma have shown that anti-A2 and anti-A3 antibodies interfere with complex assembly between factor VIIIa and factor IXa.5,6,7 Antibodies directed against the C2 domain have been shown to limit binding of factor VIII to phospholipid membranes.5
Although there is general agreement on the presence of three major binding sites for inhibitory antibodies on factor VIII, a number of reports have challenged the simplicity of the scheme presented in Figure 1A. A new mechanism for inactivation of factor VIII by human antibodies has been proposed that is based on the capacity of human antibodies to proteolytically inactivate factor VIII. These so-called catalytic antibodies inactivate factor VIII by processing at different sites in both the heavy and the light chain. The reaction kinetics of these catalytic antibodies are slow compared to those of proteolytic enzymes like thrombin, factor Xa and activated protein C. Therefore, it remains to be determined to what extent catalytic antibodies contribute to factor VIII inactivation “in vivo”. Strikingly, several groups have been able to detect anti-factor VIII antibodies in plasma of healthy individuals. The clinical relevance of these anti-factor VIII antibodies is at present not entirely clear. A recent report suggested that factor VIII inhibitors occurring in patients with haemophilia A originate from the expansion of pre-existing B cell clones that produce low levels of “natural” anti-factor VIII antibodies.

So far, most studies on the properties and presence of factor VIII inhibitors have relied on heterogeneous mixtures of IgG molecules present in plasma of inhibitor patients. From a biochemical point of view the use of polyclonal populations of IgG presents an undesirable complexity that may interfere with proper characterisation of these antibodies. Two complementary approaches have been used to circumvent this potential problem. Epstein Barr virus immortalization has been used to isolate two human monoclonal antibodies directed against the light chain of factor VIII. The epitope of one of these antibodies designated 2C11 has been mapped to C2 domain of factor VIII. Antibody 2C11 has been shown to interfere with binding of factor VIII to phospholipids. Recently, the crystal structure of a Fab fragment of 2C11 in complex with the C2 domain has been obtained. Residues in the C2 domain that interact with monoclonal antibody 2C11 have been defined by both site-directed mutagenesis and co-crystallisation studies (see figure 1B). The side-chains of exposed hydrophobic amino acid residues of the factor VIII C2 domain interact with complementary sites in the variable part of 2C11 (Figure 1B). Electrostatic interactions mediated by residues Arg and also contribute to binding of 2C11 to the C2 domain. This report elegantly illustrates that it is now possible to assess the contribution of individual amino acids to the antigenicity of factor VIII.

Human monoclonal antibody LE2E9 has been isolated from a patient mild haemophilia A carrying a substitution of Arg to His in factor VIII. Interestingly, binding of LE2E9 to factor VIII is critically dependent on Arg since LE2E9 does not react with a factor VIII light chain molecule in which Arg is replaced by a histidine. This observation suggests that LE2E9 selectively recognises exogenous factor VIII whereas no reactivity is observed with endogenous factor VIII. This pattern of reactivity is in agreement with the rise in (endogenous) factor VIII observed in inhibitor patients with an Arg to His mutation following administration of DDAVP. A similar discrimination between self and non-self has been reported for a human antibody present in plasma of a mild haemophilia A patient with an Arg to Cys mutation.

In a complementary approach, phage display technology has been used to isolate a large number of different of human monoclonal antibodies from the immunoglobulin repertoire of patients with an inhibitor. Epitope mapping studies showed that the majority of these human antibodies bind to previously identified binding sites for factor VIII inhibitors (see Figure 1A). Within the total repertoire, however, individual antibodies exist that bind to areas on factor VIII that are not contained within these three binding sites.
A human monoclonal antibody generated by phage display binds within the acidic region that follows the A2 domain. Mutagenesis studies suggest that binding of this antibody depends on sulfation of tyrosine residues present in this part of factor VIII. Analysis of anti-A3-C1 antibodies by phage display have shown that multiple binding sites for factor VIII inhibitors are present in the A3 domain of factor VIII. This observation is supported by two recent studies that demonstrate binding of factor VIII inhibitors to the a3-region and the binding site for APC in the factor VIII light chain. Further studies, preferably at the clonal level, are required to describe more adequately the characteristics of anti-factor VIII antibodies directed towards these and other sites in the light chain of factor VIII. Similarly, more extensive characterisation of catalytic antibodies and human anti-factor VIII antibodies that appear in normal individuals seems warranted. These studies may provide new insights into the complexity of the humoral response to factor VIII.

Figure 1. (A) Factor VIII consists of a series of repeated domains (A1-A2-B-A3-C1-C2). Spacer regions that are rich in negatively charged amino acids (a1, a2 and a3) are present adjacent to the A1, A2 and A3 domain. Epitopes for factor VIII inhibitors in the A2, A3 and C2 domain are indicated by closed arrowheads. Stretches of amino acid residues that contribute to the inhibitor epitopes in these domains are indicated. (B) Three dimensional structure of the C2 domain of factor VIII. Side-chains of residues that contribute to binding of human inhibitory monoclonal antibody 2C11 are depicted. Hydrophobic interactions are mediated by Val2223, Met2199, Phe2200, Leu2251 and Leu2252. Charged residues Arg2210 and Arg2215 interact with aspartic acid residues present in the variable parts of 2C11. Amino acids involved in binding of 2C11 are oriented towards the phospholipid surface. This is consistent with the inhibitory effect of 2C11 on binding of factor VIII to phospholipids.
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Immunological aspects of inhibitor formation

Whereas considerable progress has been made at the level of epitope specificity and mode of action of factor VIII inhibitors, it still not clear why some patients do develop inhibitors and others do not. Inhibitors occur much more frequently in patients affected by the severe form of haemophilia than in patients with moderate or mild haemophilia. This is not surprising since moderately or mildly affected patients do have low levels of circulating, endogenous factor VIII, whereas in most severely affected haemophiliacs no factor VIII can be detected in the circulation. In general, patients with intron 22 inversions, nonsense mutations and large deletions are more prone to develop inhibitors than patients with missense mutations and small deletions. It is likely that the association between genotype and inhibitor development is related to the presence of tolerizing amounts of factor VIII in the circulation. Besides factor VIII genotype other yet unidentified determinants contribute to inhibitor development in patients with haemophilia A.

The immunological mechanisms leading to inhibitor development are now slowly being unravelled. T cells play an important role in the humoral response to protein antigens and several observations demonstrate that inhibitor formation in haemophilia patients is T cell dependent. Analyses performed on peripheral blood mononuclear cells that were depleted for B cells revealed that factor VIII-reactive T cells were present in the circulation of haemophilia A patients with an inhibitor. The extent of the T cell proliferative responses varied between individual patients and appeared to be related to the inhibitor titre of the patient. Surprisingly, factor VIII-dependent proliferation of T cells was also observed in patients without an inhibitor and in normal individuals. The responses in these groups were significantly lower than in the inhibitor positive group. In a recent study Reding and co-workers also observed factor VIII-dependent T cell responses in healthy subjects. Longitudinal analysis revealed that T cell responses in healthy subjects were lower than in haemophiliacs and transient in nature, whereas they persisted at fluctuating levels in haemophilia A patients. The extent of T cell proliferative responses differed slightly for peptide pools derived from the different domains of factor VIII. These findings suggest that multiple T cell epitopes are present scattered over the A, B and C domains of factor VIII. The above mentioned studies provide an initial view on the complexity of the immune response against factor VIII at the T cell level. It is likely that differences in factor VIII genotype, (immuno) genetic background and treatment regime results in considerable heterogeneity of T cell responses in patients with haemophilia A.

Much of our current knowledge on the role of T cells in inhibitor development stems from studies performed in mouse models for haemophilia A. Two similar strains of haemophilia A mice have been developed by targeted disruption of the murine factor VIII gene by insertion of the neomycin phosphotransferase gene into exon 16 (E16KO) or exon 17 (E17KO). Phenotypic analysis showed that these mice bleed to death following clipping of the tail. Consistent with their haemophilic phenotype no factor VIII activity is present in plasma of these mice. Unexpectedly, factor VIII heavy chain has been detected in the circulation of KO mice. Apparently, insertion of a neomycin-cassette in exon 16 or 17 of the murine factor VIII gene does not affect biosynthesis of factor VIII heavy chain.

These murine models of haemophilia A offer an opportunity to study the immunological aspects of inhibitor development after administration of human factor VIII. After two to three intravenous injections with human factor VIII inhibitory antibodies develop in haemophilic mice. Epitope mapping studies revealed that in addition to the
factor VIII light chain both the A2 and B domain were recognised by antibodies developing in this model. Concurrent with the appearance of antibodies in plasma, anti factor VIII antibody-secreting cells are detected by Elispot assay in the spleen after two doses of factor VIII and in the bone marrow after three doses of factor VIII. The antibody-secreting cells in the bone marrow probably originate from the spleen. Persistence of this population of antibody-secreting cells does not require continuous exposure to factor VIII since these cells remain detectable for up to 22 weeks after intravenous administration of factor VIII.

Factor VIII specific T cell proliferative responses could be measured using CD4+ T cells derived from the spleen in haemophilia A mice injected intravenously with factor VIII. Classically, cytokine release patterns are used to subdivide CD4+ (helper) T cells into two subpopulations; IFN-γ and IL-2 are secreted by the Th1 subset that is responsible for many cell-mediated functions. IL-4 and IL-10 are secreted by Th2 cells, which mediate humoral immunity and provide help to B cells. Regulation of the antibody response towards factor VIII in haemophilic mice is very complex and involves both Th1 and Th2 type T cells. In one study, factor VIII specific T cells secreted IL-10, whereas in a few mice also IFN-γ, and IL-4 were detected. No IL-2 was secreted. A factor VIII-specific T cell clone isolated from haemophilic mice injected with factor VIII was able to produce both IFN-γ and IL-4. Another study reported on the production of significant amounts of IL-2, IL-4, IFN-γ and IL-10. Although small differences exist between the studies, the above findings suggest that a mixed Th1 and Th2 response is elicited following a challenge with factor VIII. Kinetic studies of cytokine production demonstrated that concurrent with the appearance of antibodies in plasma, IFN-γ producing T cells, already arising after the first injection in some experiments, were the most prominent type of factor VIII-specific T cells. IL-10 producing T cells were the second most dominant type. Furthermore, IL-2 was produced in all experiments and IL-4 in some experiments. Cytokine co-expression studies identified a subgroup of factor VIII specific T cells producing both IL-10 and IFN-γ. Cytokines secreted by Th2 cells are involved in class switching of immunoglobulins to IgG4 in humans. Since most anti-factor VIII antibodies in human plasma are of subclass IgG4 it is likely that Th2 cytokines participate in an immune response elicited by factor VIII. In the mouse Th2 cytokines result in class switching from IgG2a to IgG1, a subclass that is indeed prominently observed in haemophilic mice following injection of factor VIII. The presence of T cells secreting Th2 cytokines in factor VIII-KO mice is consistent with the strong humoral response induced by factor VIII. At present it is more difficult to explain the co-existence of T cells that secrete Th1 cytokines. Since Th1 cytokines, in particular IFN-γ, inhibit Th2 responses, it is possible that Th1 cytokines have a modulating role during the humoral response to factor VIII. The observation that specific T cells can produce both Th1 and Th2 cytokines emphasises that our current understanding of the dynamics between these subpopulations of T cells is still limited.

For optimal T cell activation at least two signals are needed. Signal one requires the interaction of the antigen specific T cell receptor (TCR) with a peptide bound to the major histocompatibility complex. The peptide (T cell epitope) is obtained after proteolytic degradation of factor VIII by the antigen presenting cell. Signal two is delivered through the interaction of co-stimulatory molecules, present on the T cell and the antigen presenting cell. At present multiple co-stimulatory interactions have been identified that may promote or down-regulate proliferation of T cells. CD28, a surface molecule that is constitutively expressed on T cells, interacts with CD80 (B7-1) and/or CD86 (B7-2) present
on activated professional antigen presenting cells (Figure 2A). The ligands for B7 are CD28 and cytotoxic T lymphocyte antigen 4 (CTLA4), which act antagonistically. Signalling through CD28 delivers a positive co-stimulatory signal to the T cell, but signalling through CTLA4 is inhibitory and down regulates T cell activation. Synthesis of CTLA4 by T cells is upregulated following TCR engagement. Activation of T cells also upregulates a number of molecules like CD40L (CD154). This molecule acts as a ligand for the co-stimulatory molecule CD40 that is expressed on B cells. The interaction between T and B cells facilitated by CD40-ligation results in production of antibodies by B cells (Figure 2C).

Based on our current knowledge of the regulation of immune responses by co-stimulatory molecules a number of reagents have been evaluated for their suppressing effect on the immune response. A fusion protein consisting of soluble CTLA4 and part of the heavy chain of IgG1, designated CTLA4-Ig, has been shown to interfere with B7-CD28 cross-linking in vivo (Figure 2B).44 Recently, the effect of CTLA4-Ig on inhibitor formation in factor VIII knock out mice was evaluated.45 When CTLA4-Ig was administrated on the day before and the day after factor VIII administration inhibitor formation in haemophilia A mice was delayed after additional administration of three doses of factor VIII. Moreover, inhibitor formation could be blocked completely by co-administration of CTLA4-Ig with each of six doses of factor VIII. In mice with a pre-existing low titre inhibitor the secondary immune response to factor VIII is suppressed. In the same study it was shown that haemophilic mice lacking the co-stimulatory molecule B7-2 did not develop an immune response to intravenously infused factor VIII whereas inhibitor development did occur in B7-1 deficient mice.45 Apparently, B7-2 serves an essential role in humoral responses to infused factor VIII in this animal model for haemophilia A. These results suggest that it may be worthwhile to evaluate the effect of monoclonal antibodies that block the activity of the co-stimulatory molecule B7-2 on inhibitor development.

Blockade of the CD40-CD40L interaction has been successful in the prevention of renal allograft rejection.46 This method for the suppression of inhibitor formation has been evaluated in several studies using different treatment regimens (Figure 2D). In haemophilic mice that had developed high titre inhibitors anti-CD40L treatment reduced the inhibitor titre, whereas little effect was seen in mice with low titre inhibitors.47 Two other studies confirmed the modulating role of anti-CD40L on inhibitor development.40,48 Surprisingly, in one of these studies, in about half of the animals treated with anti-CD40L long term tolerance (150 days) was achieved whereas in the other half inhibitory antibodies did develop after repeated challenges with factor VIII.40 In the non-tolerant mice factor VIII reactive T cells were mainly of the Th2 type whereas in control mice, receiving only factor VIII, also T cells secreting cytokines characteristic of a Th1 response were observed. As yet no explanation is provided for the fact that only half of the mice has become tolerant to factor VIII. Possibly, under conditions of co-stimulatory blockade of the CD40 pathway, inhibitor formation becomes critically dependent on the expression of other co-stimulatory molecules like B7.2. The narrow and tightly regulated window of B7.2 expression on antigen presenting cells may explain why inhibitor development is not initiated in all treated animals.45 In another study inhibitor formation could be prevented by co-administration of anti-CD40L with factor VIII, but contrary to the former study, no lasting effect was noted. Flow cytometric analysis and analysis of spleen cell supernatants demonstrated that prior treatment with anti-CD40L did not influence Th1/Th2 polarization, nor did it alter subclass distribution of the formed antibodies.48
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A few years ago, a humanised monoclonal antibody directed against CD40L has been assessed for its efficacy of reducing the inhibitor titre in four haemophilia A patients. The results of this study did not show a strong reduction in inhibitor titres in the treated patients, although preliminary results suggest anti-CD40L treatment prevented an anamnestic response to the infused factor VIII in some patients. Due to thrombo-embolic complications associated with the use of anti-CD40L in patients with autoimmune diseases, clinical trials with anti-CD40L were discontinued. Nevertheless, investigation of blocking co-stimulatory interactions in animal models for haemophilia A can yield new insights in the immunobiology of inhibitor development.

Figure 2. Co-stimulatory blockade using CTLA4-Ig and anti-CD40L antibodies. (A) Activation of T cells by antigen presenting cells (APC) requires multiple contacts between these two cell types. The first signal (indicated by 1) is mediated by the T cell receptor (TCR) that binds to a peptide (indicated in black) present in the groove of major histocompatibility complex (MHC) class II molecules on APCs. In case of an immune response against factor VIII the peptide originates from factor VIII which is taken up and proteolytically degraded into small fragments by
APCs. For optimal activation of T cells a second co-stimulatory signal (indicated by 2) is required which involves the interaction between CD28 on T cells with B7.1 or B7.2 molecules expressed on APCs. T cells can now proliferate and exert their biological function. Excessive proliferation of T cells is prevented by cytotoxic T lymphocyte antigen 4 (CTLA4) which is upregulated following activation of T cells. CTLA4 competes with CD28 for binding to B7.1 and B7.2 on APCs. CTLA4-B7.1/7.2 ligation delivers result in the onset of signalling pathways that prevent proliferation of T cells. (B) The modulating role of CTLA4 during initiation of an immune response has been exploited by construction of a recombinant soluble variant of this molecule designated CTLA4-Ig. Binding of CTLA4-Ig to B7.1/7.2 interferes with co-stimulation of T cells by CD28/B7.1/7.2 ligation. As a result activation of T cells is halted and they lose their ability to proliferate in response to the presented antigen. (C) Co-stimulatory interactions also play a role in the interaction between antigen specific B and T cells. Also here signal 1 is delivered via the T cell receptor and peptide containing MHC class II molecules. The peptide is derived from antigen which has been internalized via the B cell receptor (BCR). Following intracellular processing antigen derived peptides are loaded on MHC class II molecules. An important co-stimulatory signal for a productive interaction between B and T cells is mediated by CD40 ligation (indicated by 2). Interaction of CD40 on the B cell with CD40L on the T cell provides a strong activating signal to B cells that ultimately results in the production of high affinity antibodies. (D) Administration of monoclonal antibodies directed towards CD40L provides a co-stimulatory blockade which prevents stimulation of antigen specific B cells. During the last years several additional co-stimulatory molecules have been identified that regulate the interaction between T cells and APCs. These newly identified co-stimulatory molecules have not been included in this figure.

References

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