Factor VIII inhibitors in mild haemophilia A

Wendy Bril

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Remmende antistoffen tegen factor VIII bij milde hemofilie A
(met een samenvatting in het Nederlands)

Proefschrift

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Wendy Simone Bril

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Prof. dr. R. A. W. van Lier

Copromotor: **Dr. J. J. Voorberg**

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Contents

CHAPTER 1

Introduction
Immunobiology of inhibitor development in haemophilia A

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Karin Fijnvandraat[1], Wendy S. Bril[2] and Jan Voorberg[2]
[1]Department of Paediatrics, Emma Children's Hospital, Academic Medical Center, University of Amsterdam, Amsterdam;
[2]Department of Plasma Proteins, Sanquin Research at CLB, Plesmanlaan 125, Amsterdam

Scope of this thesis

The congenital bleeding disorder haemophilia A is caused by a deficiency or functional defect of clotting factor VIII.[1] 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.[2] 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 Arg^{484}-Ile^{508} in the A2 domain and residues Val^{2248}-Ser^{2312} in the C2 domain.[3,4] Results from two independent studies further suggest the presence of an antigenic site within residues Gln^{1778}-Met^{1823} 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.[8]

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.[9] 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.[10,11] 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.[12]

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.[13] Recently, the crystal structure of a Fab fragment of 2C11 in complex with the C2 domain has been obtained [14]. 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).[14,15] 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^{2210} and Arg^{2215} 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^{2150} to His in factor VIII.[16] Interestingly, binding of LE2E9 to factor VIII is critically dependent on Arg^{2150} since LE2E9 does not react with a factor VIII light chain molecule in which Arg^{2150} 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^{2150} to His mutation following administration of DDAVP.[17,18] 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^{593} to Cys mutation.[19]

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.[20-24] 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.[21] 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.[22] 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.[25,26] 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.

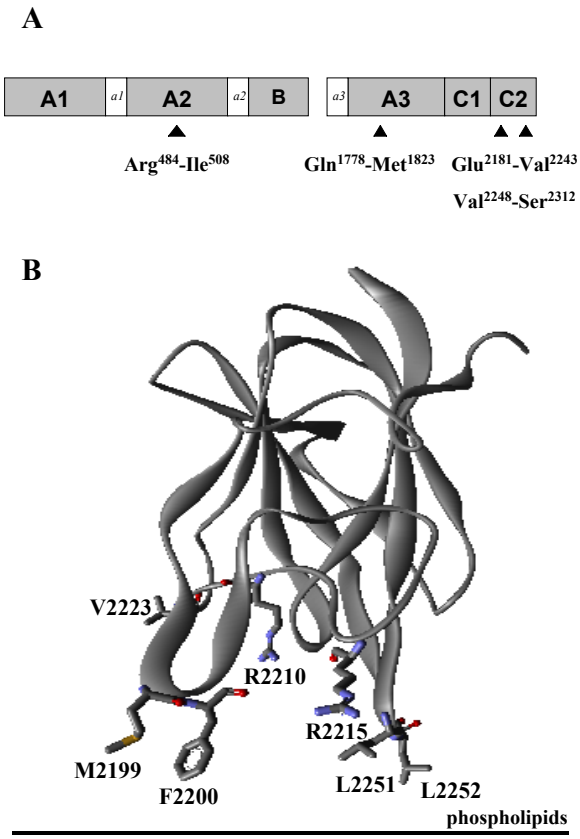

Figure 1. (A) Factor VIII consists of a series of repeated domains (A1-A2-B-A3-C1-C2). Spacer regions that are rich in negatively charged amino acids (*a1*, *a2* and *a3*) are present adjacent to the A1, A2 and A3 domain. Epitopes for factor VIII inhibitors in the A2, A3 and C2 domain are indicated by 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 Val^{2223}, Met^{2199}, Phe^{2200}, Leu^{2251} and Leu^{2252}. Charged residues Arg^{2210} and Arg^{2215} 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.

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.[27] 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.[1] 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.[28-30] 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.[31] The extent of the T cell proliferative responses varied between individual patients and appeared to be related to the inhibitor titre of the patient.[31] 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.[32] 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).[33] Phenotypic analysis showed that these mice bleed to death following clipping of the tail.[33,34] 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.[35] Apparently, insertion of a neomycine-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.[36] 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.[37] 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.[38] 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.[37,39] 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.[39] 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.[39] Another study reported on the production of significant amounts of IL-2, IL-4, IFN-γ and IL-10.[40] 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.[41] 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.[41]

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.[36,37,39] 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.[42,43] 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.[43] 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]

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.[49] 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.[50] Nevertheless, investigation of blocking co-stimulatory interactions in animal models for haemophilia A can yield new insights in the immunobiology of inhibitor development.

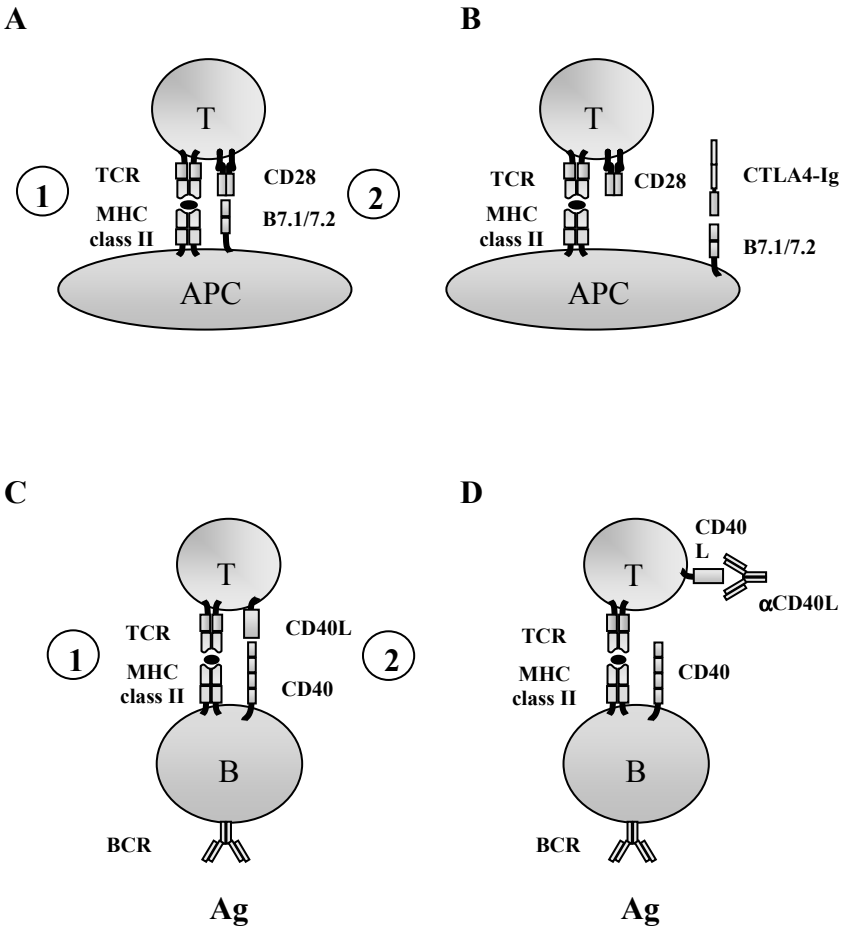

Figure 2. Co-stimulatory blockade using CTLA4-Ig and anti-CD40L antibodies. (A) Activation of T cells by antigen presenting cells (APC) requires multiple contacts between these two cell types. The first signal (indicated by 1) is mediated by the T cell receptor (TCR) that binds to a peptide (indicated in black) present in the groove of major histocompability 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[40]. These newly identified co-stimulatory molecules have not been included in this figure.

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CHAPTER 2

Two classes of germline genes both derived from the VH1 family direct the formation of human antibodies that recognize distinct antigenic sites in the C2 domain of factor VIII

Edward N. van den Brink[1,2], Wendy S. Bril[1,2], Ellen A.M. Turenhout[1], Marleen Zuurveld[1], Niels Bovenschen[1], Marjolein Peters[3], Thynn Thynn Yee[4], Koen Mertens[1,5], Deborah A. Lewis[6], Thomas L. Ortel[6], Pete Lollar[7], Dorothea Scandella[8] and Jan Voorberg[1,2]

[1]Department of Plasma Proteins, Sanquin Research at CLB, Amsterdam, The Netherlands, [2]Laboratory for Experimental and Clinical Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands, [3]Department of Paediatrics, Emma Children's Hospital AMC, Amsterdam, The Netherlands, [4]Haemophilia Centre and Haemostasis Unit, Royal Free Hospital, London, UK, [5]Department of Pharmaceutics, Utrecht University for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands, [6]Departments of Medicine and Pathology, Duke University Medical Centre, Durham NC, [7]Emory University, Atlanta GA, [8]Holland Laboratories, American Red Cross, Rockville MD.

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Abstract

Most plasmas from patients with inhibitors contain antibodies that are reactive with the C2 domain of factor VIII. Previously, we have shown that the variable heavy (V_H) chain regions of antibodies to the C2 domain are encoded by the closely related germline gene segments DP-10, DP-14 and DP-88 which all belong to the V_H1 gene family. Here, we report on the isolation and characterization of additional anti-C2 antibodies that are derived from variable heavy chain gene segments DP-88 and DP-5. Competition experiments using murine monoclonal antibodies CLB-CAg 117 and ESH4 demonstrated that antibodies derived from DP-5 and DP-88 bound to different sites within the C2 domain. Epitope mapping studies using a series of factor VIII/factor V hybrids revealed that residues 2223-2332 of factor VIII are required for binding of the DP-10, DP-14, and DP-88 encoded antibodies. In contrast, binding of the DP-5 encoded antibodies required residues in both the amino- and carboxy-terminus of the C2 domain. Inspection of the reactivity of the antibodies with a series of human/porcine hybrids yielded similar data. Binding of antibodies derived from germline gene segments DP-10, DP-14, and DP-88 was unaffected by replacement of residues 2181-2243 of human factor VIII for the porcine sequence whereas binding of the DP-5 encoded antibodies was abrogated by this replacement. Together these data indicate that antibodies assembled from V_H gene segments DP-5 and the closely related germline gene segments DP-10, DP-14 and DP-88 target two distinct antigenic sites in the C2 domain of factor VIII.

Introduction

Haemophilia A is an X-linked bleeding disorder that is characterized by the absence or dysfunction of blood coagulation factor VIII. Current treatment of haemophilia A consists of infusion of therapeutic amounts of factor VIII that can evoke an immune response in some patients. The presence of neutralizing antibodies to factor VIII, commonly termed factor VIII inhibitors, presents a serious complication of haemophilia care[1]. The biochemical properties of factor VIII inhibitors have been extensively studied with emphasis on the epitope specificity and mode of action of these antibodies. Binding sites for inhibitors have been identified within the A2, A3 and C2 domains of factor VIII.[2-6] In general, heterogeneous mixtures of anti-factor VIII antibodies are present in plasma from patients with factor VIII inhibitors and in more than 80% of inhibitor plasmas antibodies directed against the C2 domain are present.[7] Epitope mapping of anti-C2 domain antibodies revealed the presence of an inhibitor binding site comprising amino acid residues Val^{2248}-Ser^{2312} of the C2 domain.[3] A second inhibitor epitope in the C2 domain has been attributed to region Glu^{2181}-Val^{2243}.[6] Antibodies reactive with the C2 domain prevent factor VIII from binding to phospholipid surfaces and von Willebrand factor.[8,9] Both findings are in agreement with the presence of binding sites for phospholipids and von Willebrand factor in the C2 domain.[10,11] An additional mechanism of factor VIII inhibition has been described for less common human antibodies directed to the C2 domain.[12] These antibodies did not block the binding of factor VIII to von Willebrand factor but reduced the release of activated factor VIII from von Willebrand factor. Although both the factor VIII inhibitory mechanism and epitope specificity of C2-inhibitors are well understood, knowledge about their primary structure of these antibodies is limited.

Recently, anti-C2 antibodies have been studied at the clonal level using phage display technology.[13] The variable heavy chain (V_H) domains of these antibodies were encoded by V_H germline gene segments DP-10, DP-14, and DP-88 derived from the V_H1 gene family. Furthermore, these V_H domains contain hypervariable CDR3 loops that were relatively large (20-23 residues) compared to the average CDR3 length of 14 residues.[14,15] Another human anti-C2 antibody has been isolated from a haemophilia A patient with an inhibitor using classical Epstein-Barr virus immortalization.[16] The V_H domain of this antibody was encoded by germline gene segment DP-5, which is also derived from the V_H1 gene family. This antibody inhibited factor VIII activity by preventing binding of factor VIII to von Willebrand factor and phospholipid surfaces. Interestingly, the anti-C2 antibodies expressed as single-chain variable domain antibody fragments (scFv) composed of DP-10, DP-14 and DP-88-encoded V_H domains did not inhibit factor VIII activity.[13] In the present study, additional antibodies were isolated from the immunoglobulin repertoire of a mild haemophilia A patient with anti-C2 antibodies. The isolated antibodies were encoded by heavy chain germline gene segments DP-5 and DP-88. Epitope mapping studies revealed that antibodies encoded by these two classes of variable heavy chain gene segments bind to distinct antigenic sites within the C2 domain.

Materials and Methods

Materials

DNA modifying enzymes were purchased from Life Technologies (Breda, The Netherlands) and New England Biolabs (Beverly, MA). Immunotubes and microtitre plates were purchased from Nunc (Life Technologies, Breda, The Netherlands). Plasma-derived factor VIII light chain was purified as described.[17] Monoclonal antibodies (mAbs) CLB-CAg 12 and 117 have been characterized previously [4,17]; mAb ESH4[18] was purchased from American Diagnostica Inc. (Greenwich, CT).

Factor VIII assays

Factor VIII activity was measured by a one-stage clotting assay.[19] Factor VIII inhibitor titres were determined by the Bethesda assay.[20] Neutralization experiments and immunoprecipitation assays were performed as described previously.[4,13,21].

Phage display library construction, selection and characterization of selected clones

In this study plasma and peripheral blood mononuclear cells were used from a mild haemophilia A patient with an $Arg^{2163}\rightarrow$His mutation who developed a factor VIII inhibitor.[22] The patient's IgG4-specific V_H gene repertoire was amplified and combined with a V_L gene repertoire of nonimmune origin in pHEN-1-VLrep and displayed as scFv on the surface of filamentous phage.[13] Phages from the library were selected on factor VIII light chain immobilized via antibody CLB-CAg 12-coated microtitre wells as described previously.[13] After 3 rounds of selection for binding to the factor VIII light chain, the factor VIII domain specificity of phages obtained from single infected colonies was determined. Phages were tested for reactivity with factor VIII light chain immobilized to CLB-CAg 12 as described previously.[13] Phages corresponding to clones reactive with factor VIII light chain were selected for further study. V_H and V_L genes were sequenced on an Applied Biosystems 377XL automated DNA sequencer (Foster City, CA) using the BigDye

Terminator sequencing kit. Nucleotide sequences were aligned to their most homologous germline sequences as present in the V-BASE sequence directory.[23] The selected clones were subsequently tested for reactivity with the C2 domain of factor VIII in the following manner. Monoclonal antibodies ESH4 or CLB-Cag117 were immobilized on microtitre wells overnight at 4°C. Subsequently, wells were incubated for 1.5 hour with recombinant C2 domain (3.5 nM in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% (wt/vol) HSA and 0.1% (vol/vol) Tween-20). Wells were washed three times with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20. Recombinant phages derived of single infected clones were then added in 150 mM NaCl, 50 mM Tris, pH 7.4, 3% (wt/vol) human serum albumin, 0.5% (vol/vol) Tween-20 and incubated for 2 hours at room temperature. Wells were washed three times with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20. Bound phages were detected following a 2 hour incubation at room temperature with peroxidase labelled anti-M13 antibody (Pharmacia-LKB, Woerden, the Netherlands) diluted 2000-fold in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% (wt/vol) HSA and 0.05% (vol/vol) Tween-20.

Production and purification of single-chain variable domain antibody fragments (scFv) was performed as described previously.[13] Purified scFv were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentrations were measured spectrophotometrically at A_{280}. Reactivity of scFv with recombinant factor VIII and factor V hybrid molecules was evaluated by immunoprecipitation analysis. Construction and expression of hybrid human factor VIII molecules containing porcine C2 domain substitutions and hybrid human factor V molecules containing factor VIII C2 domain substitutions have been described previously.[6,24,25]. Immunoprecipitation of metabolically labelled factor VIII fragments by scFv/Ni-NTA agarose (Qiagen, Hilden, Germany) complexes was performed as described previously.[13]

Surface plasmon resonance

The kinetics of the interaction between scFv and factor VIII light chain were determined by surface plasmon resonance, using a BIAcore™ 2000 biosensor system (Biacore AB, Uppsala, Sweden). Factor VIII light chain was covalently coupled to the dextran surface of an activated CM5-sensor chip at a density of 88.5 fmol/mm^2, using the amine-coupling kit according to the manufacturer's instructions (Biacore AB, Uppsala, Sweden). As a control, one of the four channels on a chip was activated and blocked in the absence of protein. Binding to coated channels was corrected for binding to non-coated channels (< 5% of binding to coated channels). Binding (association) of scFv was assessed in 100 mM NaCl, 100 mM imidazole, and 50 mM sodium phosphate, pH 7.5 at 25°C for 2 minutes at a flow rate of 20 µL/min. Dissociation was allowed for 2 minutes in the same buffer flow. Association (k_{on}) and dissociation (k_{off}) rate constants were calculated from data sets using the BIAevaluation 3.1 software. Binding data were corrected for bulk refractive index changes and fitted according to a one-site model. Equilibrium dissociation constants (K_d) were calculated from the measured values of k_{off} and k_{on}.

Results

Isolation of anti-factor VIII antibodies using phage display.
The domain specificity of antibodies present in the plasma of an inhibitor patient with mild haemophilia A was determined by immunoprecipitation analysis. The patient's antibodies reacted with metabolically labelled factor VIII light chain and the C2 domain. Approximately 80% of the inhibitory activity originated from anti-C2 antibodies as was determined by neutralization assays (data not shown). These data indicate that the C2 domain is the major target for factor VIII inhibitors present in plasma of this patient. To further examine the patient's anti-C2 domain antibodies we used phage display to isolate factor VIII-reactive antibodies from the patient's immunoglobulin repertoire. Therefore, a phage display library derived from the patient's IgG4-specific V_H gene repertoire and a V_L gene repertoire of nonimmune source was constructed. The library, which consisted of 1.9×10^7 clones was subsequently selected for binding to plasma-derived factor VIII light chain immobilized via antibody CLB-CAg 12. After 3 rounds of selection, phages derived from 19 out of 20 clones were found to react with factor VIII light chain. Nucleotide sequences of the V_H and V_L domains of the 19 clones were determined and aligned to their most homologous germline genes in the V-BASE directory.[23] The V_H domains of 5 clones were encoded by germline gene segment DP-5. Germline gene segment DP-88 could be identified as the most homologous gene segment encoding the V_H domains of the other 14 clones. Both gene segments belong to the V_H1 gene family. In total, 3 unique V_H domains encoded by germline gene segment DP-5 were identified (Figure 1). The V_H domains of WR1 and WR17 are highly homologous, varying in only 2 amino acid residues. At the nucleotide level, 4 substitutions were observed, which makes it unlikely that the observed differences resulted from PCR artefacts. The V_H domains of WR1/17 and WR16 were generated by VDJ-recombination using J_H gene segments J_H4b and J_H3b, respectively. Rearrangement of particular D gene segments could not be determined as a consequence of limited sequence homology with known D genes. CDR3 sequences of WR1/17 and WR16 consisted of 11 and 9 amino acids, respectively.

Of 14 V_H domains encoded by the DP-88 germline gene segment, 8 unique V_H domains were identified (Figure 1). All V_H domains were heavily hypermutated compared to the DP-88 germline. Seven of 8 V_H domains shared homologous patterns of somatic hypermutation suggesting that these V_H domains share an identical B-cell precursor. It should be noted that amino acid replacements in WR3, WR7, WR11 and WR15 were caused by single nucleotide substitutions. Consequently, it cannot be excluded that the nucleotide substitutions in these V_H domains were introduced during amplification of the patient's V_H gene repertoire. All V_H domains, except that of WR10, have a similar CDR3, which is partially encoded by the largest known J_H gene segment, J_H6b. The V_H domain of WR10 has a different pattern of somatic mutation compared to the other DP-88-encoded V_H domains (Figure 1). Furthermore, the CDR3 of WR10, which comprises 20 amino acid residues, has been assembled using gene segments D3-10 and J_H3b. Different V_L domains encoded by germline gene segments of V_κ and V_λ gene families were paired with single V_H domains derived from DP-5 and DP-88 germline gene segments (Table 1).

```
                FR1                CDR1       FR2              CDR2

          ------------------------------ ----- -------------- -----------------

                   1         2         3           4          5          6
          123456789012345678901234567890 12345 67890123456789 012a3456789012345


DP-5      QVQLVQSGAEVKKPGASVKVSCKVSGYTLT ELSMH WVRQAPGKGLEWMG GFDPEDGETIYAQKFQG
WR1       ----q----------S-------------- ----- -------------- -SN-----I-----L--
WR17      ----q------------------------- ----- -------------- -SN-----I-----L--
WR16      e----e----MQ------------P-HSV- -I-F- -------------- --A-----P--------

DP-88     QVQLVQSGAEVKKPGSSVKVSCKASGGTFS SYAIS WVRQAPGQGLEWMG GIIPIFGTANYAQKFQG
WR10      e----e----------------R---D--- -S--- -------------- -V-----A------L--
WR13      ------------------------T---D-- --GVT -------------- ------NA-T-------
WR2       e----e-----------------T---D-- --GV- -------------- V---V----S-T-----
WR3       ------------------------T---D-- --GV- -------------- ----V----S-T-----
WR7       -----------R-----------T---D-- --GV- -------------- V---V----S-T-----
WR8       ------------------------T---D-- --GV- -------R------ V---V----S-T-----
WR11      ------------------------T---D-- --GV- -------------- V---V----S-T-----
WR15      ----q-------------------T---D-- --GV- -------------- V---V----S-T-----

                  FR3                        CDR3             FR4

          -------------------------------- -------------------- ----------
                                                  1                     1
              7         8             9           0                     1
          67890123456789012abc345678901234 567890abcdefghijkl12 3456789012

DP-5      RVTMTEDTSTDTAYMELSSLRSEDTAVYYCAT
WR1       -----A------------------------T- GDLEVTGAY.........DF WGQGTLVTVSS
WR17      -----A------V-----------------T- GDLEVTGAY.........DF WGQGTLVTVSS
WR16      -----------------R--------M----- SDIGNGL...........DI WGQGTMVTVSS

DP-88     RVTITADKSTSTAYMELSSLRSEDTAVYYCAR
WR10      --S---------------N-T-D--G------ GSEGALRYSDGFLPTDAFAI WGQGTMVTVSS
WR13      --S---------I----TG-----A---F--S ASKPNYYGSTDFYS..DMDV WGQGTTVTVSS
WR2       --S---------V----TG-----A---F--S ASKPNYYGSTDFYS..DMDV WGQGTTVTVSS
WR3       --S---------V----TG-----A---F--S ASKPNYYGSTDFYS..DMDV WGQGTTVTVSS
WR7       --S---------V----TG-----A---F--S ASKPNYYGSTDFYS..DMDV WGQGTTVTVSS
WR8       --S---------V----TG-----A---F--S TSKPNYYGSTDFYS..DMDV WGQGTTVTVSS
WR11      --S---------V----TG-----A---F--S ASKPNYYGSTDFYS..DMDV WGQGTTVTVSS
WR15      --S---------V----TG-----A---F--S ASKPNYYGSTDFYS..DMDV WGQGTTVTVSS
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Figure 1. Deduced protein sequences of scFv that bind to the C2 domain. Sequence numbering is according to Kabat et al.[31] Sequences are available from Genbank under accession numbers:AY050689 (VHWR1); AY050690 (VHWR10); AY050691 (VLWR10); AY050692 (WR11); AY050693 (VLWR11); AY050694 (VHWR13); AY050695 (VHWR14); AY050696 (VHWR15); AY050697 (VLWR15); AY050698 (VHWR16); AY050699 (VLWR16); AY050700 (VHWR17); AY050701 (VLWR17); AY050702 (VHWR19); AY050703 (VLWR1); AY050704 (VHWR2); AY050705 (VHWR20); AY050706 (VHWR3); AY050707 (VLWR3); AY050708 (VHWR7); AY050709 (VHWR8); AY050710 (VLWR10); AY050711 (VHWR9); AY050712 (VLWR9). FR, framework region; CDR, complementarity determining region. Dashes indicate sequence identity to germline. Amino acid substitutions that are most likely introduced by the use of V_H gene family-specific oligonucleotide primers during amplification of V_H repertoires are indicated in lowercase.

Table 1. Most homologous germline genes segments used by human antibodies directed against the C2 domain.

V_H and V_L germline gene use and nomenclature according to V-BASE.[23] All observed V_H germline genes belong to the V_H1 gene family. ND (not determined).

Clone	V_H domain	V_L domain		Reference
	germline	germline	family	
WR1	DP-5 (1-24)	DPK8 (L8)	$V_\kappa I$	This study
WR17		DPL5 (V1-19)	$V_\lambda 1$	This study
WR16		DPK3 (L11)	$V_\kappa I$	This study
BO2C11		DPK22 (A27)	$V_\kappa III$	16
YK3-3-38		-	-	27
YK3-3-40		-	-	27
YK3-3-50		-	-	27
EL-14	DP-10 (1-69)	DPK5 (L5)	$V_\kappa I$	13
EL-5	DP-14 (1-18)	L12a	$V_\kappa I$	13
EL16		DPK8 (L8)	$V_\kappa I$	13
EL-25		DPK7 (L15)	$V_\kappa I$	13
EL-9	DP-88 (1-e)	DPK8 (L8)	$V_\kappa I$	13
WR10		DPL11 (V1-4)	$V_\lambda 2$	This study
WR2/7/13		ND	ND	This study
WR3		DPK3 (L11)	$V_\kappa I$	This study
WR8		DPK24 (B3)	$V_\kappa IV$	This study
WR9		V1-2 (V1-2)	$V_\lambda 2$	This study
WR11		DPL11 (V1-4)	$V_\lambda 2$	This study
WR15		DPL16 (V2-13)	$V_\lambda 3$	This study

Epitope specificity of scFv reactive with the C2 domain.

Similar to clones WR2, 3, 7, 8, 10, 11, 13, and 15, the V_H domain of the previously described scFv EL-9 is also derived from germline gene DP-88 (Table 1). Competition experiments have shown that the binding site for EL-9 in the C2 domain overlapped with that of monoclonal antibody CLB-CAg 117.[13] Therefore, phages derived from the newly isolated clones were tested whether they also competed with CLB-CAg 117 for binding to the C2 domain. Phages bearing a V_H domain derived from germline gene segment DP-88 did not bind to the C2 domain that was immobilized via CLB-CAg 117 (Figure 2).

Interestingly, phages corresponding to clones consisting of a DP-5-encoded V_H domain bound readily to the C2 domain that was immobilized via CLB-CAg 117. We also tested reactivity of phages with C2 domain immobilized via ESH4. Previously, we have shown that ESH4 and CLB-CAg 117 bind nonoverlapping epitopes in the C2 domain of factor VIII.[4] Phages derived from clones using germline gene segment DP-5 did not bind to ESH4-immobilized C2 domain. In contrast, the C2 domain immobilized via ESH4 exclusively allowed for binding of phages derived from clones consisting of a DP-88-encoded V_H domain (Figure 2). These data suggest that DP-5 and DP-88 germline gene segments generate antibodies with distinct binding sites in the C2 domain of factor VIII.

Previously, we have shown that scFv EL-5, 9 and 14 did not inhibit factor VIII activity.[13] The capacity of scFv isolated in this study to inhibit factor VIII activity was assessed in a Bethesda assay. None of the scFv significantly inhibited factor VIII activity (titre < 15 BU/mg scFv). Subsequently, we determined the affinity of the scFv for the factor VIII light chain using surface plasmon resonance. All scFv readily associated with factor VIII light chain and for all but three scFv the rate of dissociation was extremely slow (Table 2). The relative high dissociation rate observed for EL-5, EL-9, and WR16 results in a reduced affinity for factor VIII light chain when compared to the other scFv (Table 2).

To further explore the epitope specificity of the isolated scFv, their reactivity with labelled hybrid factor VIII/factor V molecules was evaluated by immunoprecipitation analysis. Three previously isolated scFv EL-5, EL-9 and EL-14 (encoded by VH germline gene segments DP-14, DP-88 and DP-10) were also included in this analysis.[13] Factor VIII/factor V hybrids in which (part of) the C2 domain of factor V was replaced by factor VIII did not react with scFv VK-34 that is directed to the A2 domain of factor VIII. (Figure 3A, lane 1).[28] All scFv tested reacted with the factor VIII/factor V hybrid in which the C2 domain of factor V was replaced by that of factor VIII (panel 3A). The scFv composed of V_H domains derived from germline gene segments DP-10, DP-14, and DP-88 (scFv EL-5, EL-9, EL-14, WR8, WR10, WR11, and WR13) reacted with the factor V hybrid 2A, which indicates that the epitope for these scFv is located within residues 2223-2332 of factor VIII. This group of scFv did not react with factor VIII/V hybrids in which residues 2223-2281 or 2282-2332 of factor VIII were present. Binding of scFv containing VH domains encoded by germline gene segment DP-5 solely react with a hybrid that contained residues 2173-2222 and 2282-2232 of factor VIII (lane 8A). Apparently, residues contained in both these regions contribute to the epitope of this class of scFv (panel 8A). Taken together these results suggest that DP-5 encoded scFv and DP-10, DP-14, and DP-88 encoded scFv bind to distinct antigenic sites within the C2 domain.

We also evaluated the reactivity of the scFv with a series of human-porcine hybrids that have been described previously.[6] All scFv reacted with B domain deleted factor VIII whereas no reactivity was observed with hybrid HP20 in which the human C2 domain was replaced by the corresponding part of porcine factor VIII (figure 3B). Based on the lack of reactivity with HP28, residues within amino acid sequence 2207-2321 are likely to contribute to the epitopes of scFv derived from heavy chain germline gene segments DP-10, DP-14, and DP-88 (Figure 3). Interestingly, both hybrids HP23 and HP25 are not recognized by scFv EL-5, 9 and WR10 suggesting that residues 2253-2311 contain amino acids crucial for binding of these scFv. The pattern observed for scFv derived of germline gene segment DP-5 is clearly different. Hybrid HP24 and HP26 are not recognized by WR1 and WR16 suggesting that residues located between 2181 and 2199 are crucial for binding of these scFv to the C2 domain of factor VIII. Overall the data suggest that the amino-

terminal portion of the C2 domain is involved in binding of DP-5 encoded scFv. Binding of scFv derived from heavy chain segments DP-10, DP-14 and DP-88 requires the presence of residues contained within the central and/or carboxy-terminal part of the C2 domain.

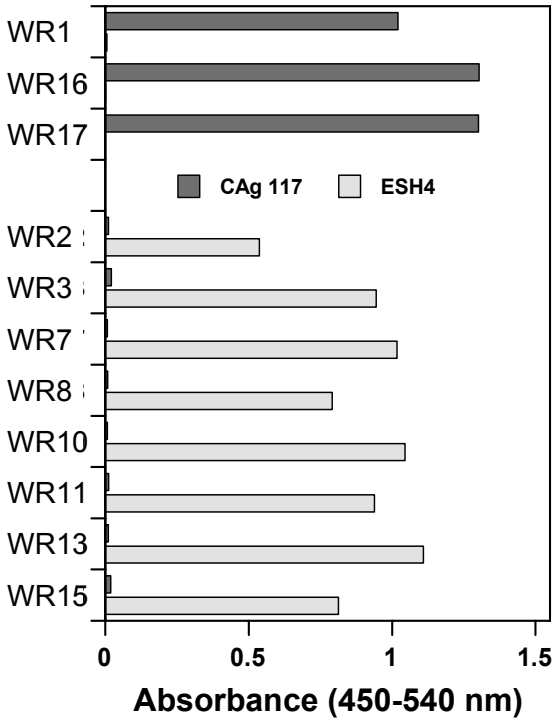

Figure 2. Specificity of phages isolated by selection on plasma-derived factor VIII light chain for the C2 domain. Binding of phages to recombinant C2 domain that was immobilized via monoclonal antibodies CLB-CAg 117 or ESH4 was determined by ELISA. The results were corrected for binding to both antibodies in the absence of C2 domain. Clones were divided in 2 groups, DP-5 and DP88 respectively, based on the variable heavy gene segment that encode the V_H domain.

Table 2. Binding kinetics of isolated single-chain variable domain antibody fragments.
K_d, k_{on}, and k_{off} were determined by surface plasmon resonance as described in "Materials and methods".

ScFv	$k_{on}(M^{-1}s^{-1})$	$k_{off}(s^{-1})$	K_d (nM)
EL5	1.6×10^{4}	3.6×10^{-3}	226
EL9	3.1×10^{4}	1.3×10^{-3}	42
EL14	3.7×10^{4}	4.2×10^{-5}	1.1
WR1	2.7×10^{4}	3.9×10^{-4}	14
WR8	1.2×10^{4}	5.3×10^{-5}	4.2
WR10	4.1×10^{4}	4.9×10^{-4}	12
WR11	8.8×10^{3}	4.2×10^{-5}	4.8
WR13	3.0×10^{5}	6.4×10^{-4}	2.1
WR16	1.6×10^{4}	1.9×10^{-3}	115

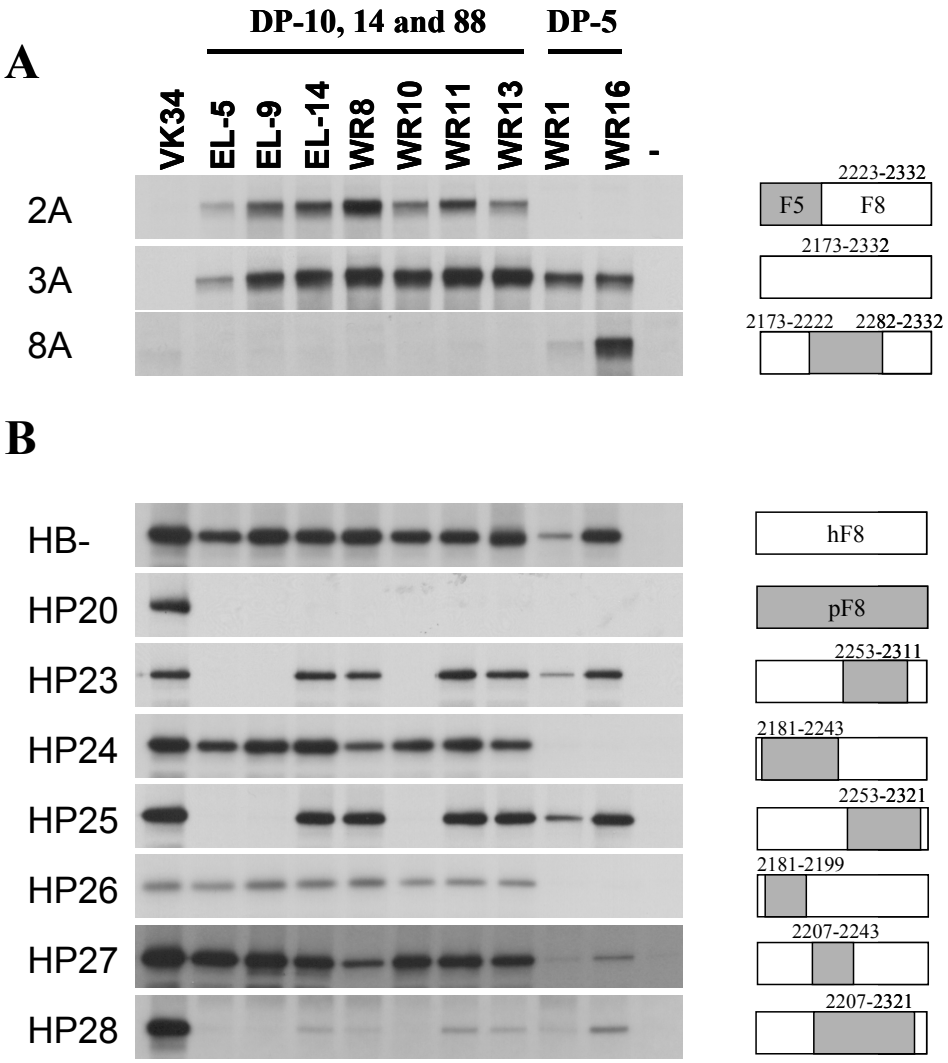

Figure 3. Reactivity of isolated scFv with factor V/factorVIII and human/porcine factorVIII hybrids. Binding of scFv to recombinant factor V/VIII (panel A) and porcine/human factor VIII (panel B) hybrids was assessed by immunoprecipitation. (Lane 1) positive control (34, anti-A2 scFv VK34); (lanes 2-10) 5, 9, 14, 8, 10, 11, 13, 1, and 16; scFv corresponding to clones EL-5, EL-9, EL-14, WR8, WR10, WR11, WR13, WR1, and WR16; (lane 11) negative (-) control. On the right, the fragments used are indicated. Sequences corresponding to human factor VIII are depicted as white boxes. At the top of the figure the two classes of variable heavy chain gene segments that encode the different clones are given. Clone EL-5, EL-9, EL-14, WR8, WR10, WR11 and WR 13 are derived from V_H gene segments DP-10, DP-14 and DP-88 whereas clone WR1 and WR16 are derived from V_H gene segment DP-5.

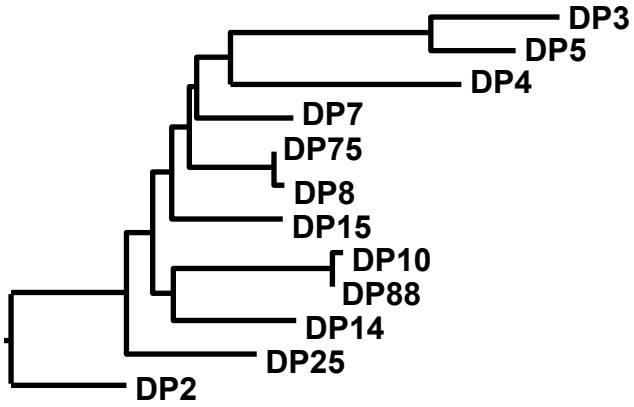

Figure 4. Phylogeny of the V_H1 family germline gene family. Dendrogram of all human V_H1 family germline genes shows relatedness of DP-10, DP-88 and DP-14. The nucleotide sequences were aligned using the CLUSTAL alignment of the DNAstar MegALIGN application. The evolutionary distance between the different gene segments correlates with the length of the closed lines (-).

Discussion

Antibodies directed to the C2 domain of factor VIII are frequently found in plasma of haemophilia A patients with an inhibitor.[7] In this study, we defined the primary structure of human C2 domain-specific antibodies. Two classes of anti-C2 antibodies directed to distinct epitopes were isolated from the immunoglobulin repertoire of a mild haemophilia A patient with an inhibitor. The isolated antibodies consisted of V_H domains encoded by germline gene segments DP-5 and DP-88 from the V_H1 gene family. Previously, we reported on the isolation of a panel of anti-C2 domain antibodies encoded by germline gene segments DP-10, DP-14 and DP-88. [13] The nonmutated germline genes DP-10 and DP-88 are highly homologous and vary by only 1 nucleotide. The DP-14 and DP-88 genes are more distantly related and vary by 29 nucleotides, which lead to 17 amino acid substitutions. In two recent studies anti-C2 antibodies derived from V_H gene segment DP-5 were isolated (Table 1) [16,27]. The nonmutated germline gene DP-5 differs substantially from DP-10, DP-14, and DP-88 (46-47 nucleotides which results in 21-22 amino acid substitutions), although all these genes belong to the V_H1 gene family. Phylogenetic analysis of germline gene segments of the V_H1 family confirms the close relation between germline genes DP-10, DP-14, and DP-88 (Figure 4). In contrast, DP-5 appears to be present in a different branch of the phylogenetic tree of the V_H1 family. Interestingly, the V_H domains encoded by DP-10, DP-14 and DP-88 all contain a large CDR3 consisting of 18-23 residues whereas the CDR3-length of DP-5 derived V_H domains ranges between 8-11 amino acids .[13,16] It cannot be excluded that antibody fragments selected by phage display are not fully representative of the complete spectrum of antibodies present in patient's plasma. Additional anti-C2 antibodies with different characteristics then reported in this study may exist. The common features of human anti-C2 antibodies described in this and

other studies however suggest that these antibodies are derived from a limited proportion of the human immunoglobulin repertoire

Both epitope mapping studies and competition experiments suggest that scFv derived from DP-10, DP-14, DP-88 and DP-5 germline genes bind to distinct antigenic sites within the C2 domain of factor VIII. Based on the results with human/porcine hybrids it is likely that residues 2181-2243 contribute to binding of DP-5 encoded antibodies. This observation is consistent with the identification of a major epitope for factor VIII inhibitors in this part of factor VIII.[6,29] Based on their reactivity with factor VIII/V hybrid 8A, it is likely that residues contained within the carboxy-terminus of the C2 domain also contribute to binding of DP-5 encoded scFv (see Figure 3). Recently, the three-dimensional structure of DP-5 encoded human monoclonal antibody BO2C11 in complex with the C2 domain has been determined.[30] A number of amino acid residues within 2181-2243 has been implicated in binding of BO2C11 to the C2 domain. However, also residues His^{2315} and Gln^{2316} present at the carboxy-terminus of the C2 domain have been shown to interact with BO2C11. From the three-dimensional structure, it appears that the interactive surface of BO2C11 is acidic whereas the antigenic surface of the C2 epitope contains a number of basic residues. In contrast to other germlines, the CDR1 and CDR2 of DP-5 harbour 5 negatively charged Glu (E) and Asp (D) amino acid residues (Figure 1). These residues contribute to the net negative charge of V_H domains encoded by this particular germline gene segment. The average calculated isoelectric point (pI) of nonmutated germline V_H segments is 8.74 ± 1.06. In a previous study the average pI of rearranged V_H domains of 39 randomly picked clones was 9.24 ± 0.8.[31] Conversely, germline DP-5, being one of the 3 V_H segments with a net negative charge, has a predicted pI of 4.84. It can be anticipated that surface Ig molecules on immature B cells carrying a DP-5-encoded V_H domains may more readily interact with a complementary positively charged site in the C2 domain. This may result in selective amplification of these B cell clones and explain the presence of the DP-5 gene segment in a subset of human antibodies directed against the C2 domain.

Human anti-C2 antibodies encoded by VH gene segments DP-10, DP-14, and DP-88 compete with binding of CLB-CAg 117 but not ESH4 for binding to the C2 domain. In contrast DP-5 encoded clones compete with binding of ESH4 to the C2 domain. This observation suggests that DP-10, DP-14, and DP-88 encoded clones bind to an antigenic site within the C2 domain that does not overlap with the epitope of the DP-5 encoded scFv. Epitope mapping studies support this notion and clearly indicate that the C2 domain contains at least two antigenic sites. DP-10, DP-14, and DP-88 encoded scFv bind to HP24 whereas DP-5 encoded scFv do not bind to this hybrid molecule. These data are in agreement with the results obtained for factor V/VIII hybrid 2A and suggest that residues contained within 2243-2332 contribute to binding of DP-10, DP-14 and DP-88 encoded scFv to the C2 domain of factor VIII. More detailed information on the epitope specificity of this group of scFv can be derived from the results obtained with hybrids HP23 and HP25. ScFv EL-5, EL-9, and WR10 most likely bind to an epitope contained within residues 2253-2311 since these scFv do not react with human porcine hybrid HP23 (Figure 3). Interestingly, the related scFv EL-14, WR8, WR11 and WR13 do bind to hybrid HP23. Only when the replaced region was extended from residues 2207-2321 reduced binding of these scFv was observed (see hybrid HP28; Figure 3). The results obtained with hybrid HP23 and 25 suggest that within amino acid sequence 2207-2321 different amino acid residues may contribute to binding of human antibodies to the C2 domain of factor VIII. Inspection of the primary sequence of DP-10, DP-14, and DP-88 derived scFv does not

reveal characteristics that may explain different reactivity of the scFv with hybrids HP23 and HP25. Since the overall structure of the heavy chain of the scFv is likely to be similar, the observed differences may reflect a modulating role of the CDR3-region on the epitope specificity. Alternatively, amino acid changes introduced by somatic hypermutation may result in the formation of additional contacts between antibodies and the C2 domain. At present, we cannot discriminate between these two possibilities. Future studies aim at defining amino acid residues within 2207-2321 that contribute to binding of DP-10, DP-14 and DP-88 encoded human antibodies to the C2 domain of factor VIII.

In a previous study we have shown that scFv encoded by germline gene segments DP-10, DP-14 and DP-88 do not inhibit factor VIII in a Bethesda assay (Van den Brink et al., 2000). Here, we have isolated additional scFv encoded by germline gene segment DP-88 (Table 1). In accordance with previous data these scFv do not inhibit factor VIII activity. The lack of inhibition may be explained by the epitope of this subset of human antibodies, which may not overlap with a functional site within the C2 domain. Alternatively, the affinity of the scFv may be too low to compete for binding of factor VIII to phospholipids. In this study we also show that DP-5 encoded scFv do not inhibit factor VIII activity. At first sight these findings do not correspond with the properties of human monoclonal BO2C11, a DP-5 encoded antibody that does inhibit factor VIII with a specific activity of 7000 BU/mg (Jacquemin et al., 1998). However, the affinity of BO2C11 for factor VIII is 1000-10000 fold higher then observed for the DP-5 encoded scFv WR1 and 16 (0.014 nM versus 14, respectively, 115 nM). Apparently, high affinity binding is required for human antibodies to inhibit the interaction of factor VIII with phospholipid membranes. Therefore, we favour the view that the inability of the isolated scFv to inhibit factor VIII is primarily due to their reduced affinity for the C2 domain compared to that of complete antibody molecules.

Our data show the presence of two distinct antigenic sites within the C2 domain of factor VIII that are recognized by two classes of human antibodies. The first class of antibodies is encoded by V_H gene segments DP-10, DP-14, and DP-88 whereas the second class of antibodies is encoded by V_H gene segment DP-5. As argued previously, the preferential use of V_H gene segment DP-5 for the assembly of anti-C2 antibodies may be explained by its overall negative charge, which can complement positively charged residues in the C2 domain. Yet, we do not have an explanation for the presence of DP-10, DP-14, and DP-88 gene segments in human antibodies that target the C2 domain. A common property of this class of human antibodies is their exceptionally large CDR3, which ranges from 20-23 amino acids. Selection of the DP-10, DP-14, and DP-88 for the assembly of anti-C2 antibodies can be related to their ability to incorporate a large CDR3 segment. In addition, other common structural elements shared between these closely related V_H gene segments might explain their presence in anti-C2 antibodies. Collectively, our data suggest that two classes of antibodies recognize two distinct sites in the C2 domain of factor VIII. Identification and subsequent modification of the interactive surfaces in the C2 domain that reacts with these two classes of scFv can potentially reduce both the antigenicity and immunogenicity of this portion of factor VIII.

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CHAPTER 3

Analysis of factor VIII inhibitors using phage display

Jan Voorberg, Wendy S. Bril, Edward N. van den Brink*

Department of Plasma Proteins, Sanquin Research at CLB, Amsterdam, The Netherlands
* Current address: Crucell BV, Leiden, The Netherlands

In press

Abstract

Inhibitory antibodies that develop in patients with haemophilia A bind to restricted regions in the A2, A3 and C2 domain of factor VIII. Functional studies have shown that anti-A2 and anti-A3 antibodies interfere with assembly of the factor VIIIa-factor IXa complex. Binding of inhibitors to the C2 domain precludes binding of factor VIII to phospholipids. We have used phage display to isolate a large number of human monoclonal antibodies from the immunoglobulin repertoire of haemophilia A patients with an inhibitor. Epitope mapping studies suggest that the majority of human monoclonal antibodies bind to previously identified epitopes on factor VIII. Inspection of the amino acid sequence of the variable heavy chain (VH) domains of human anti-factor VIII antibodies reveals some striking features. Anti-A2 and anti-A3-C1 antibodies incorporate VH gene segments that are frequently used for assembly of human IgG molecules. This may explain the presence of antibodies with this specificity in a large number of inhibitor patients. Anti-C2 antibodies are derived from two classes of VH gene segments both belonging to the VH1 family that bind to two distinct antigenic sites in the C2 domain. Our findings suggest that B cells expressing immunoglobulin molecules that comprise VH gene segments with the above-mentioned characteristics are selectively amplified from the total repertoire following exposure to antigenic determinants in the C2 domain of factor VIII.

Introduction

Factor VIII participates in the intrinsic pathway of blood coagulation. The physiological importance of factor VIII is illustrated by the X-linked bleeding disorder haemophilia A which is due to functional absence of factor VIII. The bleeding tendency in patients with haemophilia A can be corrected by the administration of factor VIII concentrates. In approximately 20-40% of patients with severe haemophilia A inhibitory antibodies develop usually after 5-12 exposure days.[1] Inhibitor formation hampers further treatment of patients with factor VIII. Activated prothrombin complexes concentrates and activated factor VII are used for treatment of bleeding episodes in inhibitor patients.[1] Based on internal sequence homology factor VIII is divided in a series of homologous domains which are interspersed by short spacer regions that are rich in acidic amino acids.[2] In plasma factor VIII circulates as a metal-ion linked heterodimer. The heavy chain consists of the domains A1-*a1*-A2-*a2*-B whereas the light chain is composed of the domains *a3*-A3-C1-C2. Due to proteolysis at various sites in the B domain the size of factor VIII heavy chain ranges between 90 and 220 kDa. In plasma factor VIII is bound to von Willebrand factor (VWF) which protects factor VIII from proteolytic degradation.[2] Upon activation by thrombin or factor Xa, factor VIII dissociates from VWF and assembly of the factor VIIIa-factor IXa complex on phospholipid surfaces can occur.[2] Activation of factor X by factor IXa is significantly enhanced by the non-enzymatic cofactor factor VIII. Functional sites on factor VIII involved in binding to factor IXa and phospholipids have been defined in considerable detail.[3] Concomitantly, knowledge on the binding sites for factor VIII inhibitors on factor VIII has grown rapidly during the last five years.[4] Based on different experimental approaches three major epitopes have been defined on factor VIII. In the A3 domain of factor VIII a binding site for factor VIII inhibitors has been localized to residues Gln^{1778}-Met^{1823}.[5,6] Binding of inhibitory antibodies to this site in the A3 domain interferes with

complex assembly of the factor IXa-factor VIIIa which is mediated by this part of the factor VIII light chain.[5-7] Residues Arg^{484}-Ile^{508} comprise a major determinant of a binding site for factor VIII inhibitors in the A2 domain.[8] Single alanine replacements in this part of the A2 domain suggested that Tyr^{487} is a residue critical for binding of inhibitory antibodies to this part of the A2 domain of factor VIII.[9] Antibodies directed toward residues Arg^{484}-Ile^{508} prevent the stimulatory effect of isolated A2 domain on the catalytic activity of factor IXa.[10] The inhibitory properties of anti-A3 and anti-A2 antibodies are in agreement with our current model of the assembly of the factor VIIIa-IXa complex. Initial high affinity binding is mediated by interaction of the factor VIII light chain with residues present in the EGF1-and EGF2 domains of factor IXa whereas stimulation of factor VIII cofactor activity results from interaction of the A2 domain with the protease moiety of factor IXa.[3] Apparently, factor VIII inhibitory antibodies can block the interaction of factor VIIIa with factor IXa via two distinct mechanisms.

Conflicting data have been reported on the epitope for factor VIII inhibitors in the C2 domain. Recombinant factor VIII fragments expressed in *Escherischia coli* revealed the presence of a binding site within region Val^{2248}-Ser^{2312}.[11] Evaluation of the functional inhibition of a panel of anti-C2 inhibitors against a series of human/porcine hybrids suggested that residues Glu^{2181}-Val^{2243} are involved in binding of factor VIII inhibitors.[12] The apparent discrepancies between the two studies may be explained by the different approaches that have been used to characterize factor VIII inhibitors. Antibodies directed toward the C2 domain interfere with binding of factor VIII to phospholipids.[11,13] Current data suggest that a restricted number of 3-4 major binding sites for inhibitory antibodies are present on factor VIII. At present it is not clear why only a limited number of sites on factor VIII is targeted by the immune system. This may be explained by the use of only a selected portion of the total available immunoglobulin repertoire for the generation of antibodies that interact with immunodominant regions on factor VIII. To explore this issue we have analyzed the anti-factor VIII repertoire of a number of well-characterized inhibitor patients by phage display.

Assembly of human immunoglobulin repertoires

Immunoglobulins are composed of a light and a heavy chain that contain both variable and constant regions. The variable or V-regions are encoded by two and three gene segments for the light and heavy chain respectively. The heavy chain locus is present on chromosome 14 whereas loci encoding κ and λ light chains are present on chromosome 2 and 22, respectively. The individual loci contain large numbers of different gene segments that are assembled into functional antibody molecules during maturation of B cells. The heavy chain locus consists of over 120 variable heavy chain gene segments, 27 diversity (D) segments and 6 joining (J) segments which are followed by gene segments that encode constant parts of immunoglobulin heavy chains (μ, δ, γ3, γ1, α1, γ2, γ4, ε, α2).[14,15] The κ light chain locus on chromosome 2 comprises 91 variable light (VL) chain gene segments and 5 J segments. The λ light chain on chromosome 22 contains >45 variable light chain and four J segments. Not all variable heavy and light chain segments are used for assembly of human antibodies. Approximately 50-75% of VH and VL segments are non-functional. This may be due to the absence of an open reading frame or lack of recombination sites flanking the variable segments.[16] Assembly of immunoglobulin molecules proceeds in a

highly regulated manner. The first event that occurs is the joining of a D segment with a J segments in the variable region of the immunoglobulin locus. Subsequently, a VH gene segment and a μ constant region is added giving rise to a functional heavy chain which is co-expressed together with a surrogate light chain on a B cell precursor in the bone marrow.[17] Next, a VL segment is fused with a JL segment resulting in a functional light chain segment that replaces the surrogate light chain. Following negative selection of self reactive B cells in the bone marrow, immature B cells are transported to the periphery where they express both IgM and IgD.[17] Internalization of antigen via surface immunoglobulins on B cells is followed by proteolytic processing of antigens into peptides that are amenable for presentation by MHC class II molecules on the surface of B cells. T helper cells specifically recognizing the presented peptide then help B cells to proliferate. Proliferating B cells migrate to the lymph nodes where germinal centres arise that provide an environment for adequate T cell help for the generation of high affinity antibodies. Affinity maturation proceeds via amino acid replacements in the variable parts of the antibody, a process termed somatic hypermutation. Additionally, class switching from IgM to IgA, IgG or IgE occurs at this stage. Following affinity maturation, B cells develop into plasma cells that produce antibodies that are present in plasma. Alternatively, B cells develop into memory B cells, which are present for extended periods in the periphery thereby facilitating secondary responses to incoming antigens.

Analysis of factor VIII inhibitors by phage display; general outline

Using peripheral B lymphocytes as a source of RNA we have isolated immunoglobulin repertoires of patients with an inhibitor using a series of PCR reactions that target the variable domains of the immunoglobulin heavy chain.[18] Several restrictions have been applied to preferentially amplify the anti-factor VIII repertoire of these patients. The majority of factor VIII inhibitors are of subclass IgG4 therefore an IgG4 specific amplification step was introduced in our experimental protocol. The concentration of IgG4 is approximately 5% of the total quantity of IgG in human serum. Although this number does not necessarily correspond to the percentage of IgG4 positive B cells in the peripheral blood, it is anticipated that the resulting immunoglobulin repertoires are enriched for anti-factor VIII antibodies. A further simplification involves the use of a immunoglobulin light chain repertoire that has been amplified from peripheral blood lymphocytes of healthy individuals.[19,20] Different immunoglobulin light chains can pair to a single heavy chain and therefor it is anticipated that VH domains derived from patients with haemophilia A will pair with a suitable light chain from the available non-immune repertoire. Because of our focus on the variable part of the heavy chain we can only determine the characteristics of this part of immunoglobulin molecules that bind to factor VIII. The variable heavy chain locus encodes 51 functional variable heavy chain segments that can be classified into seven families (see Figure 1). Some families like VH2, 5, 6 and 7 contain only one or a few members whereas others (VH1, VH3 and VH4) harbour more then 10 members. Usage of the different families of VH germline gene segments in the peripheral repertoire roughly corresponds to the number of VH gene segments present within a single family (see Figure 1; data based on data in ref. n. 21). It has been shown that the variable heavy chain gene segments contribute to the overall fold of the VH domain of immunoglobulins.[22] Fusion of a VH gene with a D and a J segment, a process that involves addition and deletion of

nucleotides at the sites of junction, creates antigen-independent diversity on this initial fold. Suitably assembled IgG molecules make up the naïve B cell repertoire. The presence of antigen then stimulates selective outgrowth and maturation of B cells that express surface immunoglobulins that can interact with antigen. We have assessed the characteristics of the variable heavy chain domains of human antibodies directed against factor VIII. At present we have isolated human antibodies directed against major inhibitor epitopes in the A2, A3-C1 and C2 domain of factor VIII.[23-27] In the following paragraphs an initial analysis of the characteristics of the variable heavy chain (VH) domains of these antibodies will be presented.

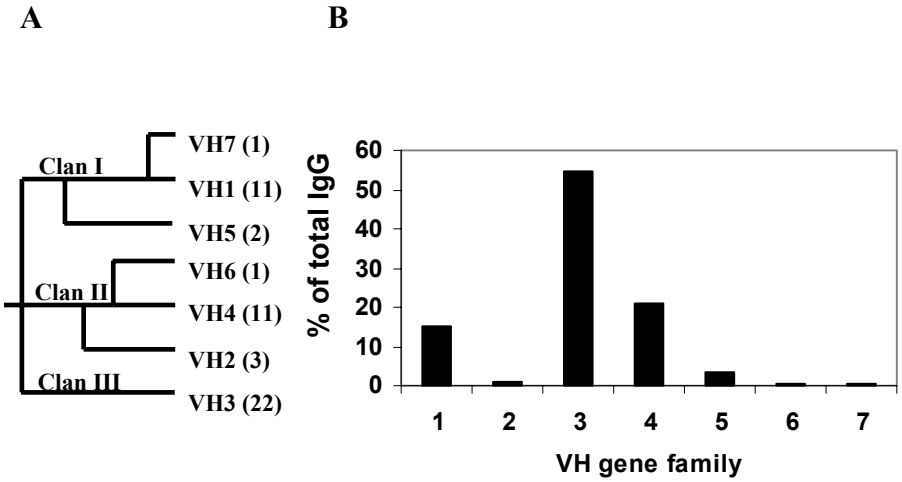

Figure 1. (A) Schematic representation of phylogenetic tree of human variable heavy (VH) gene segments. Based on sequence homology human VH gene segments can be divided into seven families. The number of individual gene segments present within each family is given in brackets (adapted from ref. n.16). **(B) Variable heavy chain segments found in peripheral IgG-positive B cells.** VH gene segments are classified into seven families (x-axis). On the y-axis the percentage of IgG-positive B cells expressing an IgG with a VH gene segments from one of these families is depicted (data based on ref. n. 21).

Characteristics of anti-A2 and anti-A3-C1 antibodies obtained by phage display

Since the characteristics of anti-A2 and anti-A3 antibodies display some common features we will first discuss this subset of human anti-factor VIII antibodies. Four different human antibodies directed against the A2 domain have been isolated of which three bind to the residues Arg^{484}-Ile^{508} an immunodominant region on factor VIII.[24,27] One anti-A2 antibody, designated VK41, bound to the acidic region that follows the A2 domain. Two out of four antibodies were derived from VH segments DP-47 (3-23) whereas the other ones were encoded by DP-10 (1-69) and DP-58 (gene segment not yet mapped). Among the six human antibodies directed against the A3-C1 domains of factor VIII that have been isolated 5 were directed against residues Gln^{1778}-Met^{1823}, a major binding site for factor VIII

inhibitors in the A3 domain of factor VIII.[5,6,25] All isolated antibodies were derived from germline gene segments of the VH1 and VH3 family. Two of the anti-A3-C1 antibodies were derived from germline gene segment DP-49 (3-30), two of DP-77 (3-21), one of DP-14 (1-18) and one of DP-15 (1-8). At first sight there is little to be learned from the origin of variable heavy chain of the anti-A2 and anti-A3 antibodies. Germline gene segments used are derived from both the VH1 and VH3 family which does not come as a surprise since these VH segments from this families are used by 70% of peripheral IgG+ B cells (see Figure 1). Closer inspection of the occurrence of the different VH gene segments in the normal repertoire yields some remarkable features (see Figure 2). About 25% of the human IgG+ repertoire is derived from VH gene segments DP-47 (3-23), DP-49 (3-30) and DP-77 (3-21). These findings show that anti-A2 and anti-A3 antibodies use VH gene segments that are preferentially expressed in IgG molecules in the normal repertoire. The observed preference suggests that epitopes present in the A2 and A3-C1 domain of factor VIII are accessible for surface immunoglobulins that have incorporated VH segments DP-47 (3-23), DP-49 (3-30) and DP-77 (3-21). The high concentration of IgG+ B cells in the periphery that are derived from these VH gene segments may be due to some inherent flexibility of IgG molecules with these VH gene segments to bind to antigenic sites on a variety of proteins. Alternatively, IgG molecules containing these VH gene segments may more efficiently cope with selective processes that occur during maturation of B cells. A relatively large number of immature B cells containing surface IgG derived from these VH gene segments is then available for incoming antigen. Independent of the underlying mechanism the presence of DP-47 (3-23), DP-49 (3-30) and DP-77 (3-21) gene segments in anti-A2 and anti-A3-C1 antibodies suggests that antibodies with this specificity are frequently observed in plasma of inhibitor patients. Indeed a large study concluded that anti-A2 or anti-A3 antibodies are present in virtually all patients with factor VIII inhibitors.[28] The above analysis provides an attractively simple explanation for the frequent occurrence of anti-A2 and anti-A3 antibodies in inhibitor patients. However, some caution is warranted. Sofar only a limited number of patients has been included in our analysis. Also, our studies suggest that the epitopes in the A2 and A3-C1 domain are more complex then previously anticipated.[24,25] More detailed studies on epitope specificity and VH gene usage are required to unequivocally determine whether a particular VH gene segment is preferentially incorporated into an IgG molecule binding to an antigenic site in the A2 or A3-C1 domain of factor VIII.

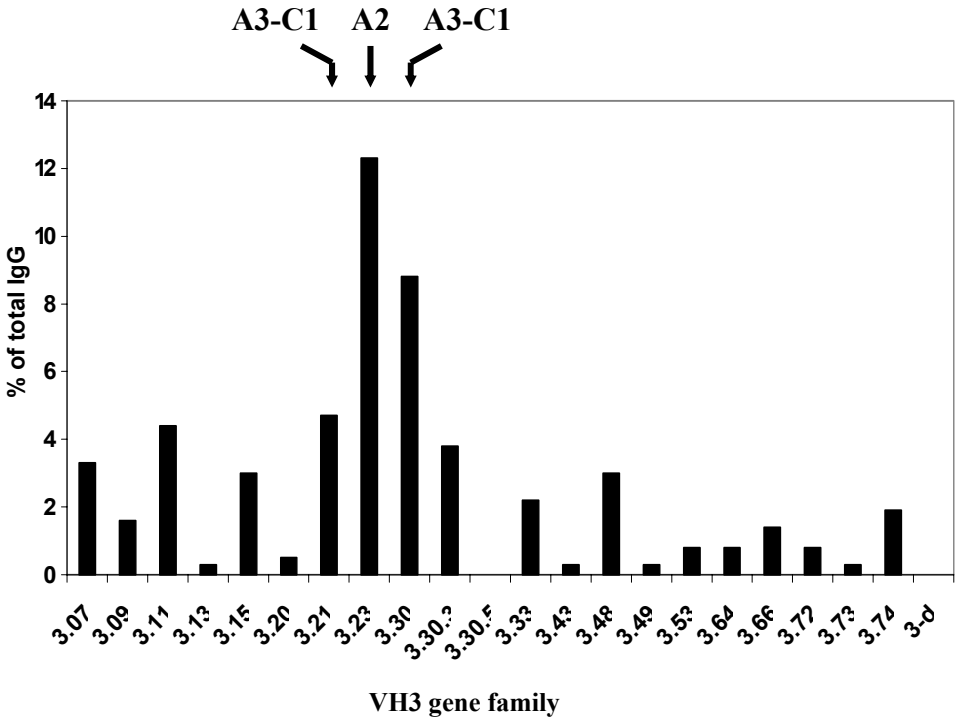

Figure 2. Variable heavy chain gene segments of the VH3 family present in peripheral IgG-positive B cells. On the x-axis the 22 gene segments belonging to the VH3 family are listed. On the y-axis the percentage of IgG-positive B cells expressing an IgG containing one of these gene segments is depicted (data based on ref. n. 21). Anti-A2 and anti-A3-C1 antibodies are derived from gene segments 3-21, 3-23 and 3.30 which are commonly expressed in peripheral IgG positive B cells.

Characteristics of anti-C2 antibodies obtained by phage display

The plasma of most inhibitor patients contains antibodies that react with the C2 domain of factor VIII.[28] At present we have isolated 16 different scFv reactive with this domain from the repertoire of patients with mild and acquired haemophilia A.[23,26] Independently, Arai and co-workers have isolated 3 human antibodies from a phage library of an unrelated patient.[29] Epstein Barr immortalization of peripheral B lymphocytes was used to isolate a human monoclonal antibody directed against the C2 domain of factor VIII.[30] Sequence analysis of the VH domain of these clones reveals some interesting features. Germline gene segment DP-5 (1-24) of the VH1 family is used by about half of the anti-C2 antibodies that have been isolated sofar.[23,26,29,30] The VH domain of the remainder of human antibodies directed against the C2 domain is derived from germline gene segments DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e).[23,26] Phylogenetic analysis of gene segments belonging to the VH1 family shows that DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e) are present within one branch of this family.[26] Consistent with the more extensive differences in nucleotide

sequence, DP-5 (1-24) is present within a distinct branch of the VH1 family (see Figure 3). The available data suggest that based on the characteristics of their VH domains, anti-C2 antibodies can be subdivided in two different classes. In a previous section it was proposed that anti-A2 and anti-A3 antibodies are derived from VH gene segments that are commonly used in the immunoglobulin repertoire. Both DP-10 (1-69) and DP-14 (1-18) are used in approximately 3-5% of peripheral IgG+ B cells whereas DP-5 (1-24) and DP-88 (1-e) occur in less then 1% the total IgG+ repertoire (data extracted from ref. n. 21). These percentages indicate that these germline gene segments are not dominantly expressed in IgG+ peripheral B cells. The restricted usage of VH gene segments DP-5 (1-24), DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e) for assembly of anti-C2 antibodies suggests that B cells expressing human antibodies with these VH gene segments are positively selected following exposure of naïve B cells to factor VIII.

Structural elements present within these VH gene families may facilitate interaction of antibodies containing these gene segments with the C2 domain. Based on the three-dimensional structure of a large collection of antibodies an overall topology of antigen binding sites has been proposed by Chothia and co-workers.[22] Six antigen binding loops derived from both heavy (H1, H2, H3) and light chains (L1, L2, L3) have been defined that partially overlap with the hypervariable complementary determining regions (CDR's) (Figure 4A).[15,31] Both the H1 and H2 region are contained within the VH gene segment of an antibody. For region H1 three so-called "canonical structures" have been defined that differ in the number of amino acids contained within the H1 region. Similarly, the existence of four different canonical structures for the H2 region has been proposed.[22] Interestingly, both germline gene segments DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e) possess the H1-H2 canonical structure 1-2. This combination of canonical structures is observed in only 7 out of 51 VH gene segments. Four of them [DP-3 (1-f), DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e)] belong to the VH1 family whereas DP-73 (5-51), 5-a and DP-21 (7-4.1) belong to the VH5 and VH7 family.[15] Although region H1 and H2 determine only part of the architecture of an antigen binding site the presence of canonical structure 1-2 may facilitate interactions of VH domains with antigenic determinants present in the C2 domain. A canonical structure for the H2 region of germline gene segment DP-5 (1-24) could not be defined due to the presence of an aspartic acid instead of the more usual threonine, alanine or leucine at position 71.[22,32] Interestingly, in three of out of four human antibodies that are encoded by the germline gene segment DP-5 (1-24) amino acid substitutions have taken place at this position (Figure 4A).[26,30] Replacement of the glutamic acid for an alanine at position 71 may affect the canonical structure of the VH domains of the DP-5 (1-24) encoded antibodies which now becomes identical to that of the VH domains of the other anti-C2 antibodies (i.e. 1-2). The above analysis suggests that restricted usage of VH segments may endow anti-C2 antibodies with unique structural features that may play a role in creating an appropriate paratope.

In the previous section evidence has been obtained for the existence of two classes of human antibodies that bind to the C2 domain of factor VIII. The first class contains VH domains that are derived from the closely related VH gene segments DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e) whereas the second class of human antibodies is characterized by the presence of germline gene segment DP-5 (1-24).[26] Epitope mapping studies using a series of human/porcine and factor V/VIII hybrids reveals that these two classes of antibodies bind to distinct sites within the C2 domain of factor VIII. Residues contributing to the epitope of human antibodies encoded by VH segments DP-10 (1-69), DP-14 (1-18)

and DP-88 (1-e) resides in the carboxyl terminal part of the C2 domain (residues 2243-2332). In contrast human antibodies containing VH segment DP-5 (1-24) interact with residues at the amino- and carboxyl terminal part of the C2 domain. Recently, the three-dimensional structure of the C2 domain of factor VIII has been determined.[33] Based on the structure it was proposed that three loops containing hydrophobic residues intersect the phospholipid bilayer whereas a layer of positively charged residues can interact with negatively charged phosphate moieties of phosphatidylserine.[33] In a follow-up study the three dimensional structure of a complex of the human monoclonal antibody BO2C11 and the C2 domain was determined at 2.0Å (Figure 4B).[34] The VH domain of BO2C11 is encoded by VH gene segment DP-5.[30] Figure 4A shows an alignment of the amino acid sequence of BO2C11 with the DP-5 (1-24) encoded antibodies we have recently isolated.[26] All four DP-5 (1-24) encoded antibodies have a relatively small CDR3 which comprises 8-11 amino acids. The pattern of somatic hypermutation varies considerably between BO2C11 and the clones isolated by phage display, which is not surprising, since these antibodies have been derived from different patients. A common replacement between 3 of the 4 antibodies is a replacement of glutamic acid at position 71 for an alanine. This suggests that clones harbouring this amino acid replacement are preferentially selected from the total pool of C2 domain reactive B cells. A possible structural basis for this selective enrichment has been discussed in a previous paragraph. Based on the three dimensional structure of BO2C11 and the C2 domain multiple atomic contacts have been detected between the heavy chain of BO2C11 and the C2 domain (Figure 4A/4B).[34] Comparison of the amino acid sequence of the WR clones with that of BO2C11 reveals that the majority of contact residues are conserved in the sequence of the WR clones (see Figure 4A). This suggests that the interactive surface of the complex between BO2C11 and the C2 domain is representative for other DP-5 (1-24) encoded anti-C2 antibodies. Small differences between binding of individual DP-5 (1-24) encoded antibodies to the C2 domain can however occur. The DP-5 (1-24) encoded residue Asp^{52} of BO2C11 that interacts with Arg^{2215} in the C2 domain has been substituted for an asparagine or alanine in the VH domains of the WR clones (see Figure 4A). Also residue Asp^{99} present in the CDR3 of BO2C11 (indicated by an arrow; Figure 4A) is not conserved among the WR clones. Since several additional negatively charged amino acids are present in both CDR2 and CDR3 of WR1, WR16 and WR17, the effect of these substitutions on the overall architecture of the interactive surface is most likely limited. Single amino acid substitutions in the C2 domain have recently been evaluated for their reactivity with factor VIII inhibitors.[35] A reduction in functional inhibition of human antibodies was observed when residues Met^{2199}/Phe^{2200} (Loop I; Figure 4B) and Leu^{2252} (Loop II; Figure 4B) were substituted for an Ile, Leu and Phe, respectively.[35] Combination of these substitutions resulted in a further decrease in the antigenicity of the C2 domain. The significance of electrostatic interactions mediated by Arg^{2210} and Arg^{2215} for binding to factor VIII inhibitors has not yet been assessed. Residues Arg^{2210} and Arg^{2215} are not critical for binding of factor VIII to phospholipids.[36] Factor VIII variants harbouring substitutions at these sites may therefore display a reduced antigenicity while maintaining their phospholipid binding properties.

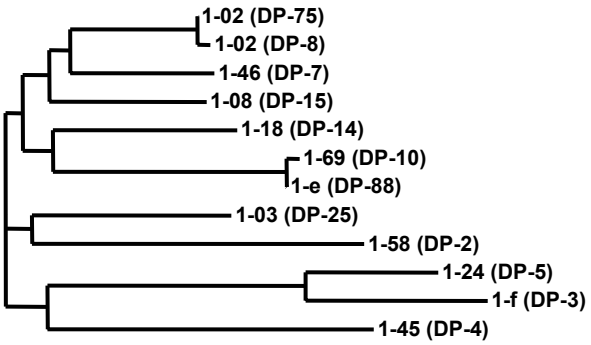

Figure 3. Phylogenetic tree of the VH1 family. Anti-C2 antibodies are derived from two classes of VH gene segments. The first class comprises VH gene segments DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e). The second class of anti-C2 antibodies incorporates VH gene segment DP-5 (1-24).

```
                                    H1                          H2
                                 --------                    ------
                 FR1                    CDR1       FR2             CDR2

                ------------------------------ ----- -------------- -----------------

                         1         2         3          4          5          6
                1234567890123456789012345678901 12345 67890123456789 012a3456789012345


DP-5            QVQLVQSGAEVKKPGASVKVSCKVSGYTLT ELSMH WVRQAPGKGLEWMG GFDPEDGETIYAQKFQG
WR1             ----q----------S-------------- ----- -------------- -SN-----I-----L--
WR17            ----q------------------------- ----- -------------- -SN-----I-----L--
WR16            e----e----MQ------------P-HSV- -I-F- -------------- --A-----P--------
BO2C11          QVQLVQSGAEVKKPGASVKVSCKVSGYTLT ELPVH WVGQAPGKGLEWVG SFDPESGESIYAREFQG


                                          H3
                                       -----------
                        FR3                 CDR3         FR4

                ------------------------------- ------------ -----------
                                                   1               1
                    7         8           9        0               1
                67890123456789012abc345678901234 567890abcde12 3456789012

DP-5            RVTMTEDTSTDTAYMELSSLRSEDTAVYYCAT
WR1             -----A------------------------T- GDLEVTGAYDF WGQGTLVTVSS
WR17            -----A------V-----------------T- GDLEVTGAYDF WGQGTLVTVSS
WR16            -----------------R--------M----- SDIGN..GLDI WGQGTMVTVSS
BO2C11          SVTMTADTSTDIAYMELSSLRSDDTAVYYCAV PDPD...AFDI WGQGTMVTVSS
```

Figure 4 (A). Deduced amino acid sequences of variable heavy chain domains of DP-5 (1-24) encoded human anti-C2 antibodies. FR, framework region; CDR, complementary-determining region; H1, H2 and H3, antigen binding loops defined based on structural homology of immunoglobulins.[22] Dashes indicate sequence identity to germline gene segments. Lower case letters indicate amino acid substitutions originating from the PCR primer. The crystal structure of a complex of BO2C11 in contact with the C2 domain has been resolved.[34] Residues in BO2C11 in contact with the C2 domain are underlined.

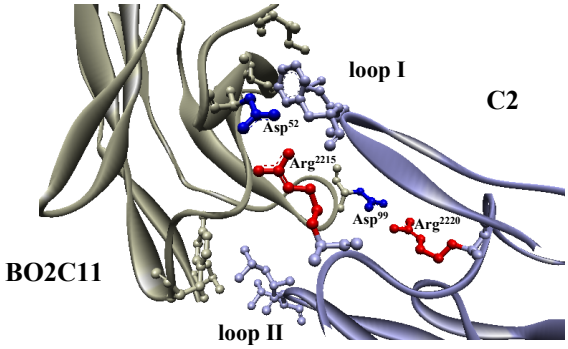

Figure 4. (B) Three-dimensional model of a complex between BO2C11 and the C2 domain of factor VIII. At the left site part of the VH domain (residues 1-113) of BO2C11 is depicted, the C2 domain is positioned at the right. The light chain of BO2C11 is not included in this figure. Multiple contacts exist between BO2C11 and the C2 domain.[34] Loop I represent contacts between residues Met^{2199}/Phe^{2200} of the C2 domain with residues Ser^{50}/Ile^{59} of BO2C11. Loop II represents contacts between $Leu^{2251/2252}$ of C2 with residues Val^{2}/Tyr^{98} of BO2C11. Highlighted are residues Arg^{2215} and Arg^{2220} of C2 that interact with negatively charged residues Asp^{52} and Asp^{99} in BO2C11.

Conclusions and implications

Overall, our data show that phage display is a useful technology to isolate large numbers of human monoclonal antibodies from the repertoire of patients with inhibitors. Inspection of the primary amino acid sequence of these antibodies suggest that only a restricted number of variable heavy chain segments is used for the assembly of human antibodies that react with the C2 domain. In contrast, less restriction is observed for anti-A2 and anti-A3 antibodies at least at the level of VH gene segments. The availability of a large panel of human antibodies derived from different patients is likely to increase our knowledge on the number and complexity of B cell epitopes on factor VIII. Subsequent modification of antigenic sites within the A2, A3 and C2 domain may provide a basis for the reduction of the antigenicity and perhaps also immunogenicity of factor VIII.[4] Alternatively, it may be possible to develop antibody-based reagents that interfere with binding of inhibitory antibodies to factor VIII. In this respect it is noteworthy to mention that the majority of variable domain antibody fragments do not interfere with factor VIII cofactor activity. We have previously shown that the non-inhibitory single chain variable domain antibody fragment EL-14 can partially neutralize factor VIII inhibitors in an *in vitro* assay.[18] Further study is required to establish whether the concept of masking of antigenic sites on factor VIII by antibody-derived reagents can be translated into a new therapeutic option for treatment of patients with inhibitors.

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CHAPTER 4

Diversity of human antibodies directed towards an antigenic site in the C2 domain of factor VIII

Wendy S. Bril, Paul H.P. Kaijen, Niels Bovenschen and Jan Voorberg.

Department of Plasma Proteins, Sanquin Research at CLB, Amsterdam, the Netherlands

Manuscript in preparation

Abstract

We report on the isolation of two antibodies from a phage library of a patient with mild haemophilia A. Inspection of the sequence of these two antibodies designated VK29 and VK33 reveals that they are derived from VH germline gene segments DP-25 (1-03) and DP-79 (4-39). ScFv corresponding to clone VK29 and VK33 were isolated and the affinity for factor VIII light chain was determined. ScFv VK29 bound with high affinity to factor VIII (K_d 3.6 nM). ScFv VK33 displayed a somewhat lower affinity (K_d 28.7 nM) due to its relatively large dissociation rate. Epitope specificity of ScFv's VK29 and VK33 was determined by immunoprecipitation with a series of metabolically labelled fragments corresponding to the light chain of factor VIII. ScFv VK29 and VK33 recognized both factor VIII light chain and C2 domain. Binding of scFv VK29 and VK33 to factor VIII was not abolished by replacement of amino acid residues 1778-1823 (in the A3 domain) for the corresponding region of factor V. These findings indicate that the epitope of these scFv is contained within the C2 domain and not within residues 1778-1823 of the A3 domain. Interestingly, the VH segments of the two isolated scFv do not adhere to previously established criteria for anti-C2 antibodies. To obtain more insight into the epitope of these scFv we performed binding experiments with monoclonal antibodies CLB-CAg 117 and ESH4. These murine monoclonal antibodies have previously been shown to bind to two distinct non-overlapping epitopes within the C2 domain. Clone VK29 and VK33 only reacted with factor VIII light chain that had been immobilized via ESH4. No reactivity was observed when binding to factor VIII light chain immobilized via CLB-CAg 117 was evaluated. These data show that VK29 and VK33 bind to an antigenic site within the C2 domain that overlap with the epitope of CLB-CAg 117. Previously, we have shown that this epitope is recognized by scFv derived of VH gene segments DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e). Our results show that the VH gene usage of antibodies binding to the epitope defined by CLB-CAg 117 is less restricted then previously anticipated.

Introduction

Haemophilia A is an X-linked bleeding disorder that results from a deficiency or dysfunction of blood coagulation factor VIII.[1] Treatment of the bleeding tendency in patients with haemophilia A is accomplished by intravenous administration of factor VIII. Treatment is complicated by the development of immune responses to the transfused product in some patients. This complication results in development of antibodies that limit the functional activity of factor VIII. Extensive characterization of panels of human antibodies present in patient's plasma have been performed.[2,3] At present multiple binding sites for these antibodies, that are commonly termed factor VIII inhibitors, are described in the A2, A3 and C2 domain of factor VIII. In addition, the presence of binding sites in the B domain of factor VIII has been suggested by several studies.[4,5] Inspection of the domain structure of factor VIII (A1-*a1*-A2-*a2*-B-*a3*-A3-C1-C2) reveals that the A domains of factor VIII are interspersed by areas that comprise 35-40 amino acids that have been designated *a1*, *a2* and *a3*.[6] These areas contain a relatively large number of negatively charged amino acids that together constitute a consensus sequence for tyrosine sulfation.[7] Human antibodies that react with these so-called acidic regions have been identified in a number of haemophilia A patients with inhibitors.[5,8-10] Together these data suggest that

different surface-exposed areas may account for the immunogenicity of factor VIII. Analysis of the dispersion of epitopes on factor VIII has suggested that several immuno-dominant regions are present.[9] An immunodominant epitope corresponds to residues Arg^{484}-Ile^{508} that accommodates binding of most anti-A2 antibodies.[11] Also inhibitory antibodies binding to the A3 and C2 domains are observed in plasma of the majority of inhibitor patients.[9,12] At present detailed epitope mapping studies on epitopes present in the A3 domain have only been performed for a limited number of samples. Data from these studies suggests that residues 1778-1823 comprise an immunodominant region in the A3 domain.[13,14] Within the C2 domain residues 2181-2243 have been assigned to contribute to binding of inhibitory antibodies[15,16]. Taken together, epitope mapping studies performed with heterogeneous mixtures of factor VIII reactive IgG point at the presence of a limited number of immuno-dominant antigenic surfaces on factor VIII.

Comparison of the antigenicity of factor VIII with model antigens like hen egg lysozyme reveals some interesting differences. Hen egg lysozyme is a relatively small protein with a molecular mass similar to one of the C domains of factor VIII. The surface area on lysozyme covered following binding of an antibody ranges from 500 to 900 $Å^2$.[17] Modelling studies based on the known three-dimensional structure of hen egg lysozyme suggests that only half of the accessible surface area of lysozyme is covered following binding of four antibodies with different epitope specificity.[17] These data illustrate that multiple epitopes can be presence within a relatively small antigen such as hen egg lysozyme. Thus, it is anticipated that multiple binding sites for antibodies can bind to the C1 and C2 domains of factor VIII that have similar dimensions as hen egg lysozyme. Furthermore, the even larger A1, A2 and A3 domains should be able to accommodate binding of human antibodies to multiple surface exposed areas. Nevertheless, as described above only restricted portions areas within the A2, A3 and C2 domains factor VIII are involved in binding of human antibodies derived from plasma of patients with haemophilia A. The restricted epitope specificity of factor VIII inhibitors suggests that human antibodies with a preference for immuno-dominant sites are selected from the total repertoire that is generated from recombination events at the heavy and light chain immunoglobulin locus.[18] Previously, we have described the characteristics of human monoclonal antibodies directed to factor VIII that were isolated by phage display.[8,19-22] This analysis revealed that anti-A2 and anti-A3 antibodies utilize variable immunoglobulin heavy chain (VH) gene segments that are commonly expressed within the normal repertoire.[8,20] Apparently, these VH segments can participate in creation of appropriate paratopes that preferentially interact with antigenic sites within the A2 and A3 domain. The primary structure of human antibodies directed towards the C2 domains reveals some interesting features[19,21] Based on the amino acid sequence of their VH domains, anti-C2 antibodies can be arranged in two different classes. The first class of antibodies is encoded by VH segment DP-5 (1-24) and these antibodies have a relatively short CDR3 region in the heavy chain of around 8 amino acids. Human antibodies belonging to the second class of anti-C2 antibodies are derived from the closely related VH gene segments DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e). In addition, this second class of human antibodies contain an exceptionally large CDR3 that ranges from 18-23 amino acids. Functional importance of the observed differences in primary sequence is suggested by epitope mapping studies that reveal that the two classes of human antibodies can bind to distinct antigenic sites within the C2 domain of factor VIII.[21]

To further assess the proportion of the human immunoglobulin repertoire that is utilized for assembly of factor VIII inhibitors we screened a phage display library for the presence of light chain antibodies. Two novel human antibodies were isolated that bound with high affinity to the C2 domain. Inspection of their primary structure revealed some striking differences with respect to the characteristics of previously characterized anti-C2 antibodies that argue for a modification in the current classification of anti-C2 antibodies.

Materials and Methods

Materials

Factor VIII light chain was prepared from factor VIII concentrates as described previously.[23] Monoclonal antibodies CLB-CAg 12, CLB-CAg A and CLB-CAg 117 have been described previously.[23,24] CLB-CAg 117 reacts with the C2 domain of factor VIII.[13] CLB-CAg 12 recognizes an epitope in the A3-C1 domains of factor VIII whereas residues 1804-1818 in the A3 domain are required for binding of CLB-CAg A.[20] Monoclonal antibody ESH4 was purchased from American Diagnostica (Greenwich, CT).

Selection of phage library

Construction of a phage library of peripheral blood lymphocytes of patient AMC-67 has been described previously.[8] In a previous manuscript we have described the epitope specificity of inhibitory antibodies that develop in this mild haemophilia A patient with an Arg^{593} to Cys mutation.[25] Neutralization experiments revealed that the majority of inhibitory antibodies were directed against the A2 domain. Also, antibodies reactive with factor VIII light chain and C2 domain were present in plasma of this patient at the time of library construction. The library consisted of 1.9×10^7 independent clones.[8] Recombinant phages corresponding to the patient's immunoglobulin repertoire were prepared by infection of a portion of the phage display library with 8×10^9 VSCM13 helper phage. Recombinant phages were collected and selected for binding to factor VIII light chain using three different selection strategies. Factor VIII light chain was directly immobilized on immunotubes at a concentration of 10 μg/ml dissolved in 50 mM $NaHCO_3$ (pH 9,5). Alternatively, factor VIII light chain was immobilized via monoclonal antibodies CLB-CAg 117 and ESH4 in microtitre plates. Selection of phage binding to factor VIII was performed as described previously.[8] After three rounds of selection recombinant phage prepared from single colonies were screened for binding to factor VIII light chain that was immobilized via monoclonal antibody CLB-CAg 12. Bound phages were detected using a peroxidase-labelled polyclonal antibody directed against the filamentous phage M13 (Pharmacia-LKB, Woerden, The Netherlands). Selection on factor VIII light chain immobilized via CLB-CAg 117 and ESH4 did not yield factor VIII reactive clones. Two positive clones were isolated following three rounds of selection on factor VIII light chain directly immobilized on immunotubes. The nucleotide sequence of the VH and VL segment of the two positive clones was determined on an Applied Biosystems 377 XL automated DNA sequencer (Foster City, CA). The obtained nucleotide sequences were aligned to the V-BASE database of variable heavy and light chain immunoglobulin gene segments.

Characterization of scFv

Two clones designated VK29 and VK33 that express phages that interact with the light chain of factor VIII were selected for further study. The Nco1-NotI inserts encoding the single chain variable antibody domains (scFv) were transferred to plasmid pUC119-Sfi/Not-His6. In the resulting plasmids the scFv are expressed with a carboxy-terminal His tag which allows for purification of scFv by immobilized metal chelate-affinity chromatography (Qiagen, Hilden, Germany). ScFv's VK29 and VK33 were purified essentially as described previously. ScFv were eluted in 20 mM HEPES (pH 7.4), 150 mM NaCl and 100 mM Imidazole. Eluted scFv were free of contaminating proteins as assessed by SDS-PAGE.

The affinity for factor VIII light chain was determined using surface plasmon resonance using a BIAcore 2000 biosensor system (Biacore, Uppsala, Sweden). Factor VIII light chain was covalently coupled to the dextran surface of an activated CM5 sensor chip at a density of 88.5 fmol/mm^2. Binding of different concentrations of scFv was evaluated in 20 mM HEPES (pH 7.5), 150 mM NaCl and 100 mM Imidazole for 2 minutes at a flow of 20 µl/min at 25°C. At 2 minutes, channels were incubated with the same buffer in the absence of added scFv. Under these conditions, part of the bound scFv dissociates from the immobilized factor VIII light chain. Dissociation (k_{off}) and association constants (k_{on}) were calculated as described previously. Equilibrium binding constants (K_d) were calculated from experimentally determined k_{on} and k_{off} values.

Immunoprecipitation analysis was performed using metabolically labelled factor VIII fragments corresponding to the light chain and C2 domain of factor VIII. In addition, a factor VIII light chain in which an immunodominant epitope at residues 1778-1823 was replaced by the corresponding sequence of factor V (HV1778-1823) was used in these studies.[20] Immunoprecipitation analysis was performed as follows. Conditioned medium containing metabolically labelled fragments were first precleared by incubation with gelatin Sepharose 4B and Ni-NTA agarose. Preformed complexes of scFv with Ni-NTA agarose were then incubated with metabolically labelled fragments overnight at 4°C. Following extensive washing with immunoprecipatation buffer samples were incubated for 5 min at 95°C in SDS-PAGE sample buffer. Eluted material was analysed under non-reducing conditions by 12.5% SDS-PAGE.

Results

Isolation and sequence characteristics of factor VIII light chain specific antibodies from a phage display library

Previously, we reported on the epitope specificity of an inhibitor in a mild haemophilia A patient with an Arg^{593} to Cys mutation.[25] The majority of inhibitory antibodies in this patient are directed towards residues 484-508 in the A2 domain. Screening of a phage display library for antibodies reactive with the A2 domain yielded two scFv, VK34 and VK41.[8] Clone VK34 reacted with residues 484-508 of the A2 domain whereas clone VK41 was directed against residues 712-736 in the acidic region following the A2 domain. Besides anti-A2 antibodies in plasma of this patient significant levels of anti-factor VIII light chain antibodies have been found.[25] Therefore, we screened a phage library of this patient for antibodies that reacted with factor VIII light chain. Different protocols were employed using factor VIII light chain immobilized directly on immunotubes or indirectly

via monoclonal antibodies CLB-CAg117 and ESH4.[23,24] Two positive clones were obtained following three rounds of selection on factor VIII light chain immobilized on immunotubes. Selection on factor VIII light chain immobilized via CLB-Cag117 and ESH4 did not yield phage reactive with factor VIII. The nucleotide sequence of the two clones was determined and compared to the most homologous immunoglobulin heavy and light chain segments using the V-BASE sequence directory (Figure 1).[26] Clone VK29 was derived from variable heavy chain gene segment DP-25 (1-03). The amino acid sequence of clone VK29 differed at 9 amino acid positions from that of the VH gene segment DP-25 (1-03). The variable light chain of clone VK29 was derived from VL segment DPK22, a member of the VKIII family. Clone VK33 was derived from variable heavy chain segment DP-79 (4-39). Only four amino acid substitutions were observed when compared to germline gene segment DP-79 (4-39). The variable light chain of clone VK33 was most homologous to variable light chain gene segment DPK24, a member of the VKIV family. The variable heavy chain fragments of both clones contained relatively large CDR3 regions of 18 and 21 amino acids, respectively. The joining segments of the variable heavy chain were most likely derived of segments JH4b (VK29) and JH3b (VK33).

Heavy chains

```
                FR1                    CDR1       FR2             CDR2
          ------------------------------ ----- -------------- -----------------

                   1         2         3             4          5          6
          12345678901234567890123456789 0 1ab2345 67890123456789 012a3456789012345


DP-25     QVQLVQSGAEVKKPGASVKVSCKASGYTFT S..YAMH WVRQAPGQRLEWMG WINAGNGNTKYSQKFQG
VK29      ----Q------R-----------------S N..HV-- -------------- ---------E-------

DP-79     QLQLQESGPGLVKPSETLSLTCTVSGGSIS SSSYYWG WIRQPPGKGLEWIG SIY.YSGSTYYNPSLKS
VK33      -v----------------------------- ------- -------------- ---.-----S-----E-


                    FR3                          CDR3              FR4
          ------------------------------- ---------------------- ------------
                                                 1                      1
              7         8             9          0                      1
          67890123456789012abc345678901234 567890abcdefghijklm12 345678901234

DP-25     RVTITRDTSASTAYMELSSLRSEDTAVYYCAR
VK29      --IM-----------F--------------- GMLSDYDSSGYYFDYF...DY WGQGTLVTVSSG

DP-79     RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR
VK33      ---M-----Q---------------------- RSRSFPLRYFDWLPDGGAFDI WGQGTMVTVSSG
```

Light chains

```
                FR1                    CDR1              FR2          CDR2
          ------------------------ ----------------- --------------- -------

                   1         2          3                 4          5
          12345678901234567890123 45678901abcdef234 567890123456789 0123456

DPK22     EIVLTQSPGTLSLSPGERATLSC RASQSVSSS-----YLA WYQQKPGQAPRLLIY GASSRAT
VK29      --------S---A-L-D-V-IT- -----I---.....H-- ---------L----- -T----P

DPK24     DIVMTQSPDSLAVSLGERATINC KSSQSVLYSSNNKNYLA WYQQKPGQPPKLLIY WASTRES
VK33      --Q--------------------- ----------------- ----------R---Y -------


                    FR3                  CDR3              FR4
          -------------------------------- --------- --------------------
                                                  1         1
             6         7         8          9      0         1
          78901234567890123456789012345678 901234567 89012345678901234567
DPK22     GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC QQYGSSP
VK29      -------------------------------- -------RT FGQGTKVEIKRAAAEQKLIS

DPK24     GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC QQYYSTP
VK33      -------------------N------------ -H---Y-LT FGQGTKLEIKRAAAEQKLIS
```

Figure 1. Amino acid sequence of variable heavy and light chain of scFV VK29 and VK33. Sequences are aligned against the most homologous VH and VL gene segments using the V base directory. Lower case indicates amino acid substitutions encoded by the PCR primer. Dashes indicate sequence identity to germline gene segments. Gaps introduced for optimal alignment are indicated by dots. FR, indicates framework regions.

Binding characteristics of scFv VK29 and VK33.

Variable antibody domain fragments VK29 and VK33 were expressed as scFv in E. coli and purified to homogeneity by immobilized metal chelate-affinity chromatography by virtue of a carboxy-terminely inserted 6 x His-tag.[8] The resulting scFv were tested for their ability to inhibitor procoagulant activity of factor VIII in a one-stage clotting assay. Addition of increasing amounts of scFv did not result in inhibition of factor VIII activity (< 10 BU/mg). Subsequently, we determined the affinity of clone VK29 and VK33 for immobilized factor VIII light chains using surface plasmon resonance, using a BIAcore2000 biosensor system. Increasing concentrations of scFv were incubated with immobilized factor VIII. Both scFv VK29 and scFv VK33 rapidly associate with factor VIII light chain (Figure 2). Fitting of this part of the curve yielded a k_{on} of 9.2 x 10^4 for scFv VK29 and 5.7 x 10^4 for scFv VK33 (Table I). Dissociation of scFv was initiated after 2 minutes. The rate of dissociation of scFv VK29 was extremely low whereas scFv VK33 dissociates more rapidly from immobilized factor VIII light chain. A dissociation constant K_d of 3.6 nM was derived from the experimentally determined values of k_{on} and k_{off} for scFv VK29. Largely due to its increased k_{off} ScFv VK33 bound to factor VIII light chain with a much lower affinity compared to scFv VK29 (K_d = 28.7 nM). The affinity for factor VIII light chain is similar to that of other scFv directed against factor VIII light chain.[20,21]

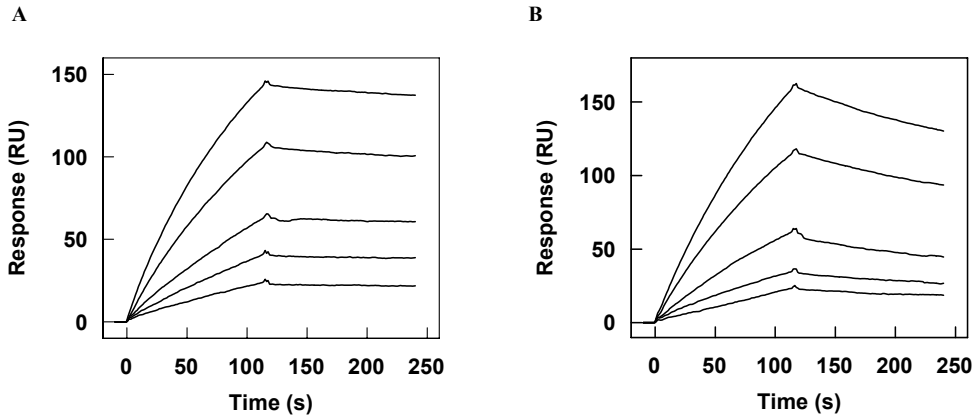

Figure 2. Kinetic analysis of the interaction of scFv VK29 and VK33 with factor VIII light chain. Binding of different concentrations of scFv VK29 (A) and VK33 (B) to factor VIII light chain was determined by surface plasmon resonance. Response is depicted in response units and is corrected for non-specific binding. On the x–axis time is depicted in seconds. Association of scFv with factor VIII light chain was followed for 2 minutes; dissociation was also allowed for 2 minutes. Concentration of scFv added were 10, 20, 40, 80 and 120 nM.

Table I. Binding kinetics of scFv VK29 and VK33.

Values for k_{on}, k_{off} and K_d were determined as outlined in Materials and Methods.

scFv	k_{off} (s^{-1})	k_{on} ($M^{-1}s^{-1}$)	K_d (nM)
VK33	1.6 (± 0.2) x 10^{-3}	5.7 (± 0.7) x 10^{4}	28.7 ± 5.1
VK29	3.3 (± 0.6) x 10^{-4}	9.2 (± 1.4) x 10^{4}	3.6 ± 0.8

Epitope mapping of scFv VK29 and VK33.

To determine the epitope specificity of scFv VK29 and VK33 we determined their reactivity with a set of metabolically labelled fragment derived of factor VIII light chain. Monoclonal antibody CLB-CAg 117, directed against the C2 domain and CLB-CAg A, directed against residues 1803-1818 were included in these studies. First, reactivity of scFv VK29 and VK33 with intact factor VIII light chain was assessed. Similar to CLB-CAg A and 117, both scFv VK29 and VK33 interact with metabolically labelled factor VIII light chain (Figure 3). Replacement of residues 1778-1821 by the corresponding residues of factor V did not abrogate binding of scFv VK29 and VK33 to factor VIII light chain (Figure 3). In contrast, CLB-CAg A does not react with this hybrid molecule. In agreement with previous findings, CLB-CAg 117 retains its ability to interact with hybrid factor VIII molecule HV1778-1821. These data indicate that the epitope of scFv VK29 and VK33 is not contained within residues 1778-1821 of the A3 domain. Subsequently, we evaluated reactivity of different antibodies with metabolically labelled C2 domain. CLB-CAg A did not react with metabolically labelled C2 domain whereas binding of CLB-CAg 117 to this fragment is observed. Both scFv VK29 and VK33 are able to interact with the C2 domain. These results indicate that scFv VK29 and VK33 are directed towards the C2 domain of factor VIII. Previous results have shown that two distinct antigenic sites are present within the C2 domain.[21] This classification is based on competition experiments with monoclonal antibodies ESH4 and CLB-CAg 117 that bind to two non-overlapping sites in the C2 domain. To obtain information on the epitope specificity of scFv VK29 and VK33 we evaluated the binding of phage expressing scFv VK29 and VK33 to factor VIII light chain immobilized via ESH4 and CLB-CAg 117. As a control, we utilized the A3-C1 specific antibody CLB-CAg 12. Phage expressing scFv VK29 and VK34 both interact with factor VIII light chain immobilized via CLB-CAg 12 (Figure 4). Also, binding to factor VIII light chain immobilized via ESH4 was observed under these conditions. In contrast, phage expressing scFv VK29 and VK33 were not capable of binding to factor VIII light chain immobilized via CLB-CAg 117. These data show that scFv VK29 and VK33 bind to an epitope in the C2 domain that overlaps with that of CLB-CAg 117.

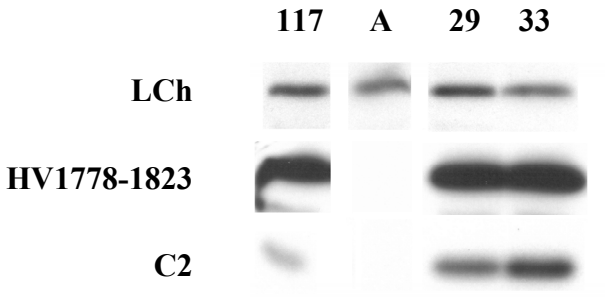

Figure 3. Reactivity of scFv VK29 and VK33 with different factor VIII light chain variants. Reactivity of scFv VK29, VK33, MoAb CLB-CAg117 and CLB-CAgA with factor VIII light chain (LCh), HV1778-1823, in which residues 1778-1823 have been replaced by the corresponding residues of factor V (HV1778-1823) and C2 domain (C2).

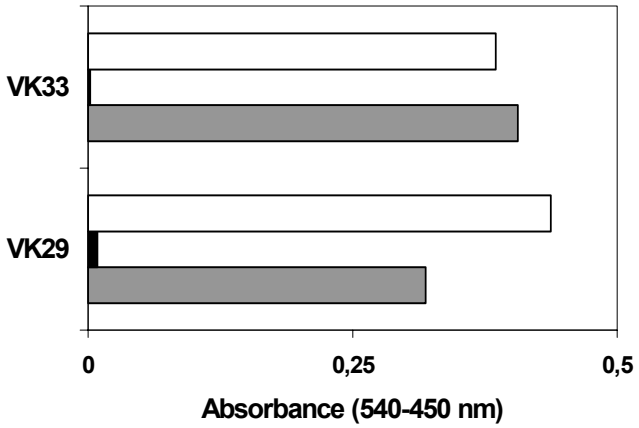

Figure 4. Competition between scFv and murine monoclonal antibodies for binding to factor VIII light chain. Factor VIII light chain was immobilized via monoclonal antibody CLB-CAg12 (white bars), CLB-CAg117 (black bars) and ESH4 (grey bars). Subsequently, it was determined whether VK29 and VK33 could still interact with immobilized factor VIII light chain. On the x-axis absorbance at 540-450 nm is indicated.

Table II. Characteristics of anti-C2 scFv's specific for the epitope defined by monoclonal antibody CLB-CAg 117.

The most homologous VH germline gene segments, total number of somatic hypermutations in the heavy chain and the length of the CDR3 region of the heavy chain (H-CDR3) are listed. Canonical structures for the first (H1) and second (H2) antigen binding loop as defined by Chotchia *et al* and the affinity for factor VIII light chain (K_d in nM) are shown.[32] SvFv's were isolated from three different patients by phage display analysis; [1)] This study, [2)] van den Brink et al Blood 2000, [3)] van den Brink et al Blood 2002.[19,21]

ScFv	VH domain germline	Som. hyperm.	H-CDR3 length	Canonical structure	LCh affinity (K_d in nM)
VK29 [1)]	DP-25 (1-03)	9	18	1-3	3.6
VK33 [1)]	DP-79 (4-39)	4	21	3-1	28.7
EL-14 [2)]	DP-10 (1-69)	12	21	1-2	1.1
EL-5 [2)]	DP-14 (1-18)	10	23	1-2	226
EL-16 [2)]	DP-14 (1-18)	11	23	1-2	*nd*
EL-25 [2)]	DP-14 (1-18)	8	23	1-2	*nd*
WR2 [3)]	DP-88 (1-e)	15	18	1-2	*nd*
WR3 [3)]	DP-88 (1-e)	14	18	1-2	*nd*
WR7 [3)]	DP-88 (1-e)	16	18	1-2	*nd*
WR8 [3)]	DP-88 (1-e)	16	18	1-2	4.2
WR10 [3)]	DP-88 (1-e)	11	20	1-2	12
WR11 [3)]	DP-88 (1-e)	15	18	1-2	4.8
WR13 [3)]	DP-88 (1-e)	15	18	1-2	2.1
WR15 [3)]	DP-88 (1-e)	15	18	1-2	*nd*
EL-9 [2)]	DP-88 (1-e)	16	20	1-2	42

Discussion

One of the initial steps during an immune response requires the functional interaction of a naive B cell expressing surface Ig molecules with a foreign antigen. Several factors may contribute to whether productive binding of antigen to immunoglobulin receptors on naive B cells will occur. On the antigen the number and density of antigenic determinants is important for eliciting an appropriate immune response. Equally relevant is the generation of a broad repertoire of immunoglobulin receptors with a high propensity to interact with surface exposed areas on an antigen. Diversification is created by recombination of different gene segments of the variable heavy and light chain locus.[18] These recombination events generate diversity at the level of the hypervariable antigenic binding loops in the heavy and light chain. Generation of a functional immunoglobulin then requires productive pairing of heavy and light chain, a process that can further modify antigen binding properties of immunoglobulin surface receptors. Additional diversity is provided by ongoing processes as somatic hypermutation, receptor editing and receptor revision.[27-29] Thus a broad antibody repertoire is created which can potentially interact with a virtually unlimited number of foreign antigens.

Recognition of antigen by B cell receptors is driven by the presence of antigenic surfaces that are recognized by the antigen binding loops present on surface

immunoglobulins. Previously, we have shown that antigenic sites present within the C2 domain of factor VIII, are recognized by immunoglobulin molecules that incorporate a selective group of VH gene segments that are derived from the VH1 family.[19,21] Antigenic sites contained within the amino terminus and carboxy terminus of the C2 domain contribute to binding to human antibodies that have incorporated the VH gene segment DP-5 (1-24). ScFv that incorporate this VH segment compete with monoclonal antibody ESH4 for binding to factor VIII. The epitope of DP-5 (1-24) derived antibodies has been mapped to residues 2181-2199.[21] Additional data concerning the epitope specificity of DP-5 (1-24) encoded human anti-C2 antibodies are derived from co-crystallization and epitope mapping studies of the human monoclonal antibody BO2C11.[30,31] Multiple contacts exist between BO2C11 and the C2 domain. Mutagenesis studies reveal an important role for residues Met^{2199} and Phe^{2200} present in one of the phospholipid binding loops of the C2 domain.[31] These amino acids are in close proximity to residues 2181-2199 that are required for binding of DP-5 (1-24) derived scFv's to the C2 domain. [21]

A second class of human anti-C2 antibodies that has been isolated by phage display is characterized by competition with murine monoclonal antibody CLB-CAg 117.[21] Residues contained within the carboxy-terminal part of the C2 domain are involved in binding to scFv belonging to this second class of anti-C2 antibodies. VH domains of this class of scFv are derived from closely related germline gene segments DP-10 (1-96), DP-14 (1-18) and DP-88 (1-e) (Table II). In this report we describe two scFv VK29 and VK33 that are not derived from these germline gene segments. ScFv VK29 is most homologous to germline gene segment DP-25 (1-03), a germline gene segment belonging to the VH1 family. Inspection of a family tree of the VH1 family shows that segment DP-25 (1-03) is present in another branch of the VH1 family as DP-10 (1-96), DP-14 (1-18) and DP-88 (1-e) (see Figure 4, Chapter 2). Remarkably, scFv VK33 is derived from germline gene segment DP-79 (4-39) belonging to a different VH family (VH4). Based on collection of data from a large number of antibody structures, canonical structures have been defined that describe the main-chain fold of antigen binding loops in the CDR1 and CDR2 region (H1 and H2).[32] VH germline gene segments DP-10 (1-96), DP-14 (1-18) and DP-88 (1-e) display canonical structure 1-2 for H1 and H2 (see chapter 3). The variable heavy chain segment DP-25 (1-03) renders scFv VK29 with a different canonical structure (1-3). The observed differences predict a different presentation of the second antigen binding loop between scFv VK29 and the previously identified scFv's. Even more pronounced differences are observed for scFv VK33 that displays canonical structure 3-1 (Table II). Competition experiments with monoclonal antibodies CLB-CAg 117 and ESH4 showed that scFv VK29 and VK33 bind to the same epitope as scFv encoded by DP-10 (1-96), DP-14 (1-18) and DP-88 (1-e). Our results indicate that VH gene segment and predicted canonical structures of the anti-C2 antibodies competing with CLB-CAg 117 can not entirely account for the observed binding specificity of this class of anti-C2 antibodies. Remarkably, all scFv's that compete with CLB-CAg 117 for binding to the C2 domain contain an unusually large CDR3. The CDR3 region of fifteen scFv's isolated from the repertoire of three different patients ranges from 18 to 23 amino acids (Table II). The hypervariable CDR3 region of the heavy chain provides a major contribution to the creation of appropriate paratope.[32] Previously, it has been suggested that long CDR3 loops are relatively flexible and are prone to so-called "conformational isomerism".[33-35] Support for this notion is gained from the molecular properties of polyreactive human antibodies that contain a relatively long CDR3.[36,37] These findings raise the possibility that human antibodies directed against the C2 domain

originate from a pool of polyreactive antibodies that can interact with multiple antigens by virtue of their unusually large CDR3. Subsequent exposure to factor VIII provides a platform for affinity maturation yielding anti-C2 antibodies that can bind specifically and with high affinity to an epitope that is defined by its interaction with the murine monoclonal antibody CLB-CAg 117.

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CHAPTER 5

Analysis of factor VIII inhibitors in a haemophilia A patient with an Arg^{593} to Cys mutation using phage display.

Wendy S. Bril[1], Ellen A.M. Turenhout[1], Paul H.P. Kaijen[1], Edward N. van den Brink[1,*], Maria M.W. Koopman[2], Marjolein Peters[3] and Jan Voorberg[1].

[1]Department of Plasma Proteins, Sanquin Research at CLB, Amsterdam, The Netherlands
[2]Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands
[3]Department of Paediatrics, Emma Children's Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
*Current address: Crucell Holland BV, Leiden, The Netherlands

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Abstract

We have characterized anti-factor VIII antibodies in a mild haemophilia A patient with an Arg^{593} to Cys mutation in the A2 domain using V gene phage display technology. All isolated single-chain variable domain antibody fragments were directed against residues Arg^{484}-Ile^{508}, a binding site for factor VIII inhibitors in the A2 domain. After a further period of replacement therapy, a transient rise in inhbitor was observed. These antibodies were directed against the A2 domain. Activation of a pre-existing pool of B cells, which express antibodies directed towards residues Arg^{484}-Ile^{508}, could explain the rapid anamnestic response.

Introduction

A serious complication in haemophilia A treatment is the development of antibodies that neutralise factor VIII activity (inhibitors). The prevalence of factor VIII inhibitors in patients with severe haemophilia A is approximately 25 %.[1] Factor VIII inhibitors are infrequently observed in patients with mild or moderate haemophilia A.[2] The lower risk of inhibitor formation in this group of patients can be explained by the presence of tolerising amounts of circulating endogenous factor VIII. Inhibitor development in these patients commonly arises after intensive factor VIII replacement therapy and is usually a transient event.[3]

Interestingly, some genetic defects are frequently observed in patients with mild haemophilia A who developed inhibitors. Amino acid substitutions located at the junction between the C1 and C2 domain or located in the A2 domain of factor VIII may predispose towards the development of factor VIII inhibitors.[3] We have previously reported on the presence of human alloantibodies in a patient with an Arg^{593} to Cys mutation.[4] In this patient (AMC-92), the anti-factor VIII antibodies are directed to wild-type A2 domain and not to the A2 domain containing the Arg^{593} to Cys mutation when analysed at a late stage of inhibitor formation. These findings indicate that the missense mutation Arg^{593} to Cys is related to antibody development.

In the present study, phage display technology is used for the characterisation of anti-factor VIII antibodies in patient AMC-92. Our findings show that peripheral IgG^{+} B cells can express human antibodies reactive with residues Arg^{484}-Ile^{508}. These antibodies are distinct from the alloantibodies found in plasma. Following a period of intensive treatment with FVIII concentrate after a surgical intervention, the patient developed a transient low inhibitor that cross-reacts with endogenous factor VIII. We propose that activation of pre-existing factor VIII-specific B cells underlies the rapid appearance of cross-reactive antibodies.

Materials and Methods

Expression, metabolic labelling and immunoprecipitation of A2 domain variants.
Wild-type factor VIII A2 domain, A2 domain containing the Arg^{593} to Cys mutation (A2-R593C) and A2 domain in which the residues Arg^{484}-Ile^{508} were replaced by the corresponding sequence of factor V (A2-FV484-508) have been described previously.[4,5] Recombinant factor VIII fragments were expressed and metabolically labelled in insect cells and immunoprecipitation was performed as described.[4]

Phage display library construction, selection and characterisation of selected clones.
Peripheral blood mononuclear cells were used from a mild haemophilia A patient (patient AMC-92) with a factor VIII inhibitor. Approximately half of the anti-factor VIII antibodies in plasma of this patient are of subclass IgG4.[4] RNA was isolated from the peripheral blood cells and used for the construction of a phage display library as described previously.[5] The patient's IgG4-specific V_H gene repertoire was amplified and combined with a V_L gene repertoire of nonimmune origin in pHEN-1-VLrep and displayed as scFv on the surface of filamentous phage.

Phages from the library were selected for binding to the factor VIII heavy chain essentially as described previously.[5] V_H and V_L genes of clones reactive with factor VIII heavy chain were sequenced and compared with non-mutated germline V genes in the V-BASE sequence database.[6] To produce scFv's, V genes were subcloned into the vector pUC119-Sfi/Not-His6. ScFv's were expressed and subsequently purified by immobilised metal chelate-affinity chromatography as described before.[5] Inhibition of factor VIII activity by scFv was measured in the one-stage clotting assay.

Results and Discussion

Longitudinal analysis of factor VIII inhibitors in patient AMC-92.
Mild haemophilia A patient AMC-92 with an Arg^{593} to Cys substitution in factor VIII had a baseline factor VIII activity of 0.20 IU/ml. He developed an inhibitor after a period of peri-operative replacement therapy in 1980.[4] At that time an inhibitor of 22 BU/ml was observed and the patient's endogenous factor VIII level dropped below 0.01 IU/ml. The inhibitor gradually declined. A low level (< 1 BU/ml) of inhibitor persisted for fifteen years. Epitope mapping studies revealed that antibodies present in plasma samples of 1992 and 1995 bind exclusively to wild-type A2 domain and not to A2 containing the Arg^{593} to Cys mutation (A2-R593C) (Fig. 1B; lane 1 and 2). These findings suggest that antibodies are present that solely react with exogenous, administered factor VIII. In 1999 the patient underwent vasectomy under the protection of factor VIII and DDAVP. Following surgery, he developed a transient rise in inhibitor , which resulted in a decrease of plasma levels of factor VIII. Initially, the patient's factor VIII level decreased below 0.02 IU/ml and an inhibitor of 2.6 BU/ml was measured. Within three months the inhibitor gradually decreased and the circulating factor VIII level returned to a value of 0.15 IU/mL (Fig. 1A). The transient decline in factor VIII suggests cross reactivity of the inhibitor with endogenous factor VIII. To address this issue we evaluated the reactivity of the inhibitor with recombinant fragments corresponding to A2 and variant A2-R593C domain. Epitope mapping studies were performed using plasma samples collected at different time points

during inhibitor development. Five days following surgery an increase in reactivity of the inhibitor towards the A2 domain could be detected (Fig. 1B; lane 3, 4), this persisted for 6 months. At later time points lower reactivity was observed (Fig. 1B; lane 13-15). Immediately following peri-operative treatment binding of antibodies to A2-R593C was observed (Fig. 1B; lane 3, 4). Concomitantly, the factor VIII plasma level of the patient decreased. After 10 weeks, factor VIII plasma levels returned to 0.15 IU/ml, which coincided with a decrease in antibodies directed against A2-R593C (Fig. 1B; lane 11-13).

Our data indicate the existence of two populations of antibodies directed against the A2 domain in this patient. The first class of antibodies binds to a B cell epitope that overlaps with Arg^{593} and persists for an extended period of time. These antibodies do not bind to endogenous factor VIII and consequently plasma factor VIII levels are not affected. Following peri-operative factor VIII replacement therapy, a transient rise in a second class of antibodies is observed that cross react with endogenous factor VIII. Cross reactive antibodies directed towards residues Arg^{484}-Ile^{508}, a major binding site for factor VIII inhibitors in the A2 domain, have been described in two other unrelated inhibitor patients with the same genetic defect.[7,8] In these patients antibodies reactive with Arg^{593} have not been observed. These data suggest that the region Arg^{484}-Ile^{508} constitutes an immunodominant epitope in patients with the Arg^{593} to Cys mutation.

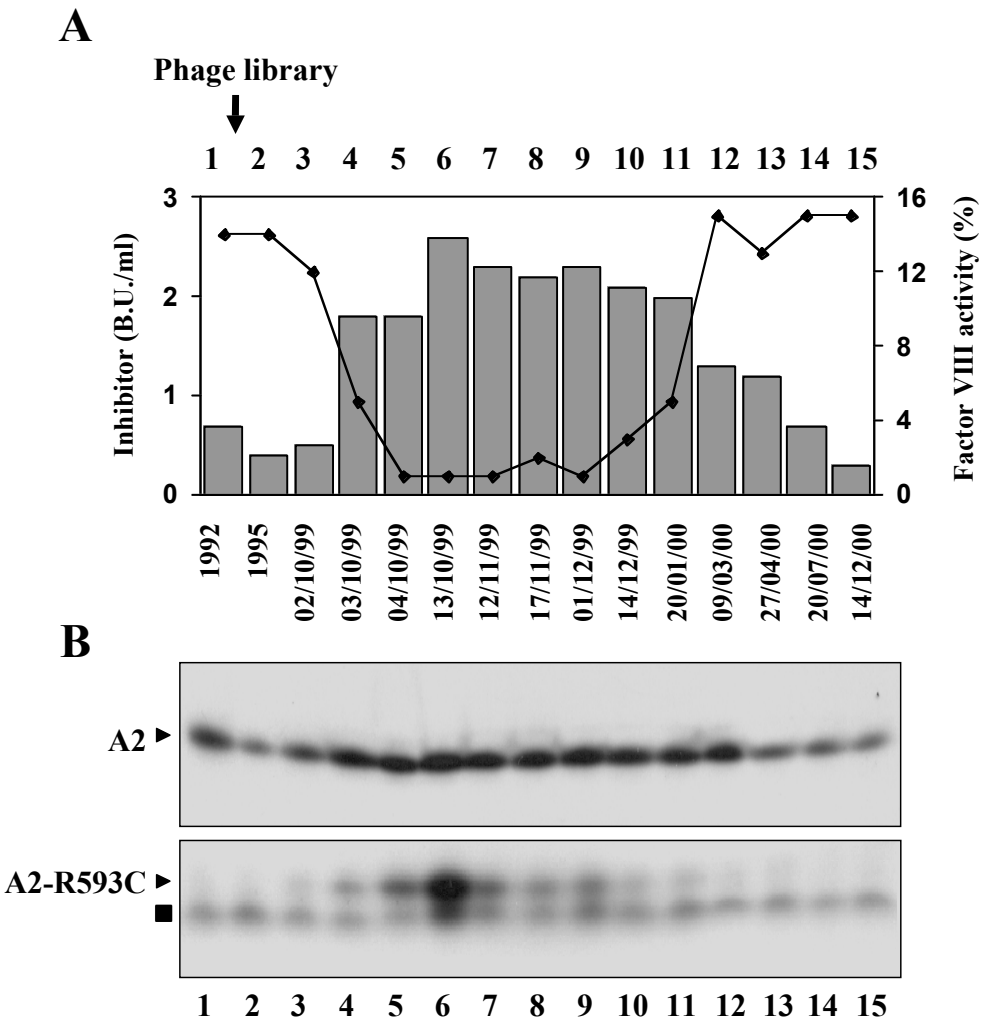

Figure 1. Longitudinal analysis of factor VIII inhibitors in patient AMC-92. (A) On the left y-axis the inhibitor is given in BU/mL (grey bars). On the right y-axis factor VIII activity is depicted in IU/mL (◆). The numbers at the top of the figures represent plasma samples that are obtained at different time point of inhibitor development. At the bottom of this panel the dates at which these samples were taken is indicated. On 28-09-1999 the patient underwent a surgical intervention under the protection of factor VIII and DDAVP. From 04-10-1999 till 11-10-1999 the patient was treated with Novoseven®. A lymphocyte sample of 1993 was used for the construction of the phage display library (indicated by the arrow). **(B)** Binding of anti-factor VIII antibodies present in the patient's plasma to recombinant wild-type A2 domain, A2 domain containing the Arg^{593} to Cys mutation (A2-R593C) was assessed by immunoprecipitation (arrowheads). A non-specific band is present below the A2-R593C fragment (indicated by ■) The numbers at the bottom correspond to plasma samples obtained at different time-points of inhibitor development (see panel A).

A

```
                FR1                   CDR1       FR2             CDR2
 ------------------------------ ----- -------------- -----------------

          1         2         3           4          5          6
 12345678901234567890123456789012 12345 67890123456789 012a3456789012345

DP-47    EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS WVRQAPGKGLEWVS AISGSGGSTYYADSVKG
92-102   q---q------------G--------R-- DF--T ---HS--R------ T---G-D..-----Q-

DP-58    EVQLVESGGGLVQPGGSLRLSCAASGFTFS SYEMN WVRQAPGKGLEWVS YISSSGSTIYYADSVKG
92-137   q----q----------------T------- A---- -------------- ------T-K-H------

              FR3                     CDR3         FR4
 ------------------------------- ------------ -----------
                                        1              1
    7         8            9          0              1
 6789012345678901abc345678901234 567890abcde12 3456789012

DP-47    RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK
92-102   --A-------------L--------------- ESFSGNLGDAFDI WGQGTMVTVSS

DP-58    RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR
92-137   -------------------------------- DLEGLFPATL    WGQTMVTVSS
```

B

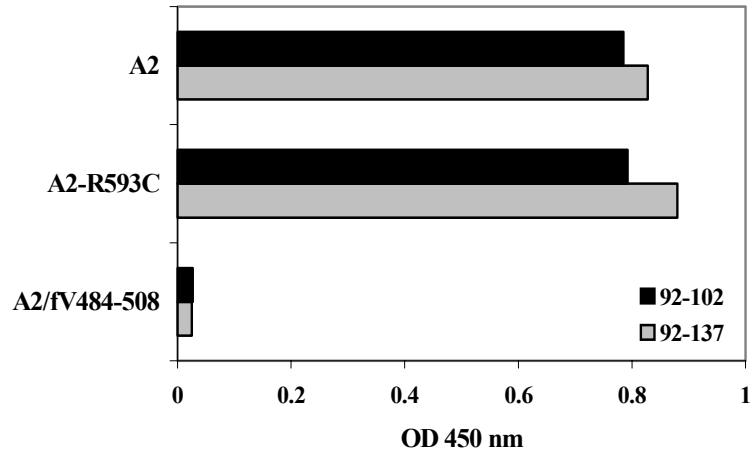

Figure 2. Characteristics of scFv's binding to the A2 domain of factor VIII. (A) Comparison of deduced protein sequence of the heavy chains of isolated human antibodies with variable heavy chain germline gene segments. FR, framework region; CDR, complementarity-determining region. Dashes indicate sequence identity to variable heavy chain germline gene segments. Lower case indicates amino acid substitution encoded by the PCR primers. Sequences are available from Genbank under accession numbers: AY052530 (V_H 92-102); AY052531 (V_L 92-102); AY052532 (V_H 92-137); AY052533 (V_L 92-137). **(B)** Binding of phage expressing 92-102 (black bars) or 92-137 (grey bars) to the A2 domain of factor VIII, A2-R593C and A2-FV484-508 was assessed as described in Materials and Methods.

Isolation and characterisation of antibodies specific for the heavy chain of factor VIII employing phage display.

Complementary to the characterisation of factor VIII inhibitors from plasma we have analysed the anti-factor VIII repertoire of patient AMC-92 by phage display. A V gene phage display library was constructed with a blood sample taken from patient AMC-92 in 1993 (see Fig. 1). The patient's IgG4-specific V_H gene repertoire was combined with a V_L gene repertoire of nonimmune source.[5] The resulting library consisted of 4.7 x 10^6 clones and was used to isolate antibodies binding to the factor VIII heavy chain. After four rounds of selection, phages derived from 23 of 45 clones were specific for factor VIII (data not shown). The nucleotide sequences of V_H and V_L genes of the 23 clones were analysed and it appeared that the sequences of 22 clones were completely identical. The V_H domain of these clones was encoded by germline gene segment DP-47, a member of the V_H3 family. The deduced protein sequence of the heavy chain of one of these clones (92-102) is shown in Fig 2A. One clone (92-137) isolated from the library was encoded by the V_H germline gene segment DP-58 belonging to the V_H3 family as well (Fig 2A). Phages expressing scFv 92-102 and 92-137 were tested for binding to variant and wild type factor VIII A2 domain. Both clones bind to wild-type A2 domain as well as to mutated A2-R593C domain. Phages 92-102 and 92-137 do not react with the A2-FV484-508 (Fig. 2B). Similar results were obtained when epitope mapping studies were performed using purified scFv (data not shown). The inhibitory capacity of scFv 92-102 and 92-137 was evaluated in a Bethesda assay. Only slight inhibition of factor VIII was observed at elevated levels of scFv (specific activity < 5 BU/mg).

Our analysis of patient AMC-92 reveals that B cells express antibodies directed against Arg^{484}-Ile^{508} at a time when antibodies with this specificity are not observed in plasma. Phage display provides information on the immunoglobulin repertoire expressed by peripheral B lymphocytes whereas antibodies in plasma are derived from antibody-secreting plasma cells present in bone marrow and spleen. Only a limited number of plasma cells circulates in the periphery. Virtually all peripheral blood B lymphocytes can be phenotypicaly divided into naive and memory B cells.[9] In view of the large number of somatic hypermutation present in the scFv's it is likely that these antibody fragments originate from the memory B cell pool. Long-living memory B cells differentiate into plasma cells upon stimulation with antigen.[10,11] The rapid anamnestic response observed in patient AMC-92 may be due to stimulation of factor VIII specific memory B cells. Alternatively, antibodies may originate from anergic self-reactive B cells that are activated following a strong immunogenic stimulus during peri-operative treatment.[12] Future studies should address whether human antibodies isolated from peripheral blood lymphocytes by phage display originate from the memory B cell pool.

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CHAPTER 6

HLA class II genotype and factor VIII inhibitors in mild haemophilia A patients with an Arg^{593} to Cys mutation.

Wendy S. Bril[1], Patricia E. MacLean[2], Marleen G. Zuurveld[1], Paul H.P. Kaijen[1], Edward N. van den Brink[1,*], N. M. Lardy[3], Karin Fijnvandraat[2], Marjolein Peters[2] and Jan Voorberg[1].

[1]Department of Plasma Proteins, Sanquin Research at CLB, Amsterdam, The Netherlands
[2]Department of Paediatrics, Emma Children's Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
[3]Department of HLA Diagnostics, Sanquin Diagnostics at CLB, Amsterdam, The Netherlands
*Current address: Crucell Holland BV, Leiden, The Netherlands

Manuscript in preparation

Abstract

Inhibitory antibodies to factor VIII develop as a consequence of replacement therapy in patients with haemophilia A. In this study, we evaluated inhibitor formation in a large group of patients with mild haemophilia A caused by an Arg^{593} to Cys mutation. A remarkably high cumulative inhibitor incidence of 14% over 20 years was observed. Three out of 42 patients developed transient, low titre inhibitors which remained below 2.0 BU/ml. Four patients with the Arg^{593} to Cys mutation developed high titre inhibitors (> 2.0 BU/ml). Three of these four patients have been described previously. In this study, we characterized the anti-factor VIII antibodies in the fourth patient with high titre inhibitors. This patient developed a high titre inhibitor following peri-operative treatment. Epitope mapping studies showed that antibodies were predominantly directed to the A2 domain of factor VIII. In previous studies, similar antibody specificities were found in patients with the Arg^{593} to Cys mutation. We evaluated the role of HLA class II alleles in inhibitor development in patients with the Arg^{593} to Cys mutation by HLA genotyping. We observed raised frequencies of HLA-DQB1*05 and DRB1*01 in the low responder group and a weak association of HLA-DQB1*06 and DRB1*11 or DRB1*13 in the group of patients which developed high titre inhibitors. Overall, our data did not reveal a strong association between inhibitor development and HLA class II alleles in patients with an Arg^{593} to Cys mutation.

Introduction

The X-linked bleeding disorder haemophilia A is caused by a decrease or dysfunction in circulating levels of blood coagulation factor VIII. Treatment of haemorrhages in patients with haemophilia A consists of protein replacement therapy using plasma derived or recombinant factor VIII. An immune response to transfused factor VIII is observed in a significant number of patients with severe haemophilia A. The development of anti-factor VIII antibodies (factor VIII inhibitors) complicates treatment of haemorrhages resulting in an increased mortality and morbidity.[1]

Recent studies have explored the relation between factor VIII genotype and the development of factor VIII inhibitors. Severely affected haemophilia A patients with large deletions, non-sense mutations and inversions in the factor VIII gene showed an inhibitor incidence of 20-35%, whereas patients with missense mutations or small deletions showed a lower incidence.[2,3] Inhibitor formation in mildly or moderately affected patients is relatively rare. The low incidence can be explained by the fact that these patients have tolerizing amounts of circulating factor VIII in their plasma. However, some mild/moderate haemophilia A patients develop anti-factor VIII antibodies after replacement therapy.

It has been suggested that certain missense mutations in factor VIII predispose to inhibitor development in mild/moderate haemophilia A.[4] Mutations in the A2 domain or in the border of the C1 and the C2 domain are frequently observed in patients, who develop an inhibitor. We have studied the properties of inhibitors in a mild haemophilia A patients with an Arg^{593} to Cys mutation in the A2 domain of factor VIII one.[5-7] Previously we demonstrated that antibodies present in one of the patient's plasma were exclusively directed towards wild-type A2 domain and did not bind to A2 domain with the Arg^{593} to Cys mutation.[5] However, when the antibody repertoire of peripheral lymphocytes of this patient was studied using phage display technology, an antibody specific for residues

Arg^{484}-Ile^{508}, a major inhibitor epitope in the A2 domain, was detected.[7] In plasma of another patient antibodies specific for both the A2 domain and the factor VIII light chain were detected.[6] These antibodies did not discriminate between wild type and variant A2 domain with the Arg^{593} to Cys mutation. Thompson *et al.* have observed antibodies specific for residues Arg^{484}-Ile^{508} in the A2 domain in another patient with the same gene.[8] Inhibitor development has also been reported in another family with the Arg^{593} to Cys mutation.[9] Not all patients with an Arg^{593} to Cys mutation develop factor VIII inhibitors. At present, it is not clear why some patients with this genetic defect do develop inhibitors whereas others do not.

In this study, we evaluated inhibitor development in 42 patients with mild haemophilia A with the Arg^{593} to Cys mutation. Seven of these patients developed inhibitory antibodies to factor VIII. In three patients inhibitor titres remained below 2.0 BU/ml. High titre inhibitors occurring in three patients have been described.[5-7] Inhibitory antibodies present in plasma of one additional patient with a high inhibitor titre were characterized. Furthermore, we also investigated whether the presence of a particular HLA class II allele is related to inhibitor formation in patients with the Arg^{593} to Cys mutation.

Materials and Methods

Patients

All non-severely affected haemophilia A patients treated at the Academic Medical Center Haemophilia Treatment Center in Amsterdam were screened for the Arg^{593} to Cys mutation. This genetic defect was found in 56 patients. Seven patients had not yet received factor VIII treatment and seven patients were not available for HLA analysis, resulting in a study group of 42 previously treated patients. The 42 patients belonged to 12 different families and all patients were Caucasians of Dutch origin. Seven patients developed an inhibitor after replacement therapy. Four patients were high responders and three patients developed a low or transient inhibitor. Patient files and laboratory results were checked retrospectively for inhibitor development over a period of 20 years. A high titre inhibitor was defined as an inhibitor titre >5 BU/ml, whereas a low titre inhibitor was defined as an inhibitor titre between 0.1-5 BU/ml concomitant with either an increase in clinical bleeding symptoms or a significant decrease in circulating factor VIII levels. Three high titre inhibitor patients have been described previously.[5-7]

Functional assays

Blood was collected by venapuncture in tubes containing 9 volumes of blood and one volume of 3.2% (w/v) trisodium citrate. Citrated plasma samples were collected as described and stored in aliquots at -70˚C. Factor VIII activity was measured using a one stage clotting assay. Inhibitor titres were determined using the Nijmegen modification of the Bethesda assay.[10]

Immunoprecipiation and neutralization experiments

Expression and metabolic labelling in insect cells of recombinant factor VIII fragments have been described previously.[5] Immunoprecipitation of factor VIII light chain, C2 domain, A2 domain and A2 domain containing the Arg^{593} to Cys mutation (A2-R593C) by anti-factor VIII antibodies was performed using 40 µl of patient's plasma. Binding of

antibodies from the patient's plasma to the ^{35}S-radiolabelled factor VIII fragments was analysed by immunoprecipitation essentially as described previously.[5] Radiolabelled proteins interacting with immobilized IgG derived from patient's plasma were visualized following SDS polyacrylamide gelelectrophoresis under reducing conditions. A mixture of monoclonal antibodies CLB-CAg 9 and CLB-CAg 117 was used as a positive control in these experiments.[11] A pool of normal plasma was used as a negative control. Inhibitor neutralization assays were performed as described using recombinant factor VIII fragments corresponding to the A2 domain, A2-R593C and factor VIII light.[6]

Molecular genetic studies

Peripheral blood lymphocytes were isolated by density gradient centrifugation (Ficoll-Paque, Amersham Pharmacia Biotech BA, Uppsala, Sweden). Genomic DNA was isolated from the lymphocytes using the QIAamp Blood Kit (Qiagen Westburg, Leusden. The Netherlands). Exon 12 was amplified using primer pairs that have been described previously. The presence of the Arg^{593} to Cys mutation was determined using sequence analysis of exon 12 of the factor VIII gene. HLA-DRB1 typing was performed by PCR analysis using the Dynal PCR-SSP DRB1* kit (Dynal, Oslo, Norway). HLA-DQA1 and HLA-DQB1 typing were performed using the Dynal PCR-SSP DQA1* and DQB1* kit (Dynal, Oslo, Norway).

Statistical analysis

Relative risks were calculated and when zero was encountered, we used Haldane's modification of Woolf's formula of relative risk.[12,13] An relative risk was considered to be significant if the value of the lower 95% confidence interval did not fall below 1.00. The significance of an association between HLA allele and inhibitor formation was calculated using Fisher's exact test (two-tailed). A relative risk was considered to be significant when the p-value <0.05.

Results

Seven out of forty-nine previously treated patients carrying the Arg^{593} to Cys mutation developed an inhibitor after factor VIII replacement therapy over the last twenty years, resulting in a cumulative incidence of 14%. Four patients were high responders and three patients developed a low or transient inhibitor. Inhibitor titres were below 2.0 BU/ml in the three patients with low or transient inhibitors. The presence of an inhibitor was further suggested by a decline in endogenous factor VIII levels (Table 1B). In one of the three patients, inhibitor formation resulted in mild clinical symptoms. The characteristics of three high titre inhibitor patients have been described previously (Table I).[5-7] Two of the high responders were monozygotic twins.[5,7] In the present study, the inhibitor of one of these patients (patient D) is characterised. Patient D, aged 70, developed a high titre inhibitor following intensive factor VIII replacement therapy for a surgical procedure. Before undergoing surgery, he had received treatment with factor VIII concentrate on 11 occasions. An inhibitor titre of 62 BU/ml was measured and the patient's endogenous factor VIII activity level dropped below 1%. The inhibitor titre reached a maximum level of 90 BU/ml. Immunosuppression therapy with endoxan was instituted for two months to

suppress the antibody response. One month after endoxan treatment was stopped the patient died from urosepsis. In the present study the characteristics of the inhibitor are evaluated.

Epitope specificities of anti-factor VIII antibodies were evaluated by immunoprecipitation analysis (Fig.1). Antibodies present in the patient's plasma reacted with the light chain and the A2 domain of factor VIII, no binding to the C2 domain of factor VIII was observed. To investigate whether antibody binding to the A2 domain was dependent on the Arg^{593} residue in patient D, immunoprecipitation analysis was performed using A2 domain harbouring the Arg^{593} to Cys mutation. As can been seen in figure 1, antibodies in the patient's plasma reacted both with the A2 domain and with the variant A2-R593C fragment.

Neutralization experiments were performed to assess the contribution of different domain-specific antibodies to the total inhibitor titre. Addition of recombinant A2 domain resulted in almost complete neutralization of inhibitory antibodies (see Figure 2). In contrast, factor VIII light chain could not neutralise the inhibitor. Consistent with the results from the immunoprecipitation experiments, similar levels of neutralization were observed for A2-R593C and A2 domain. Taken together, these data indicate that the majority of inhibitory antibodies are directed towards the A2 domain of factor VIII.

Inspection of the epitope specificity of the four patients with high titre factor VIII inhibitors reveals that the majority of inhibitory antibodies are directed towards residues in the A2 domain (Table 1A). The similar epitope specificity suggests that inhibitor formation proceeds via a common mechanism in these patients. Previously, we hypothesized that inhibitor formation in patients with an Arg^{593} to Cys mutation is dependent on adequate presentation of a peptide overlapping residue Arg^{593} on an appropriate HLA class II molecule.[6] In this model, HLA class II profile potentially influences inhibitor development. Therefore, we determined the HLA class II profile of both inhibitor and non-inhibitor patients. All patients were genotyped for HLA-DQB1 and HLA-DRB1/3/4/5 (see Appendix Table III). Table IIa shows the frequencies of HLA-DQB1 and HLA-DRB1/3/4/5 alleles in inhibitor and non-inhibitor patients. Overall, no strong association between inhibitor development and an HLA-DQB1 or HLA-DRB1/3/4/5 alleles was observed. However, the frequency of HLA-DQB1*05 is raised in the group of inhibitor patients (57.1%) compared to non-inhibitor patients (34.3%). HLA-DRB1*01 was also raised in inhibitor patients (57.1%) compared to non-inhibitor patients (22.9%). HLA-DQB1*05 and HLA-DRB1*01 are known to be in linkage disequilibrium (haplotype HLA-DQB1*0501, DRB1*0101).The increased frequency of this haplotype in inhibitor patients, however, did not reached statistical significance. The group of inhibitor patients can be divided in four patients with a high inhibitor titre and three patients with a low inhibitor titre. In the high responder group the frequency of haplotypes HLA-DQB1*0602/04/09, DRB1*1302 and HLA-DQB1*0602/04/09, DRB1*1101 were raised but the group of patients is too small to reach statistical significance (Table IIb). Remarkably, the frequencies of HLA-DQB1*05 and DRB1*01 are not raised in the group of patient that developed a high inhibitor titre while both alleles are raised in the low responder group (Table IIc). The association of HLA-DRB1*01 with inhibitor formation in low responder patients was statistical significant.

Table I: Characteristics of inhibitor patients included in this study.

(A) High titre inhibitor patients.

Maximum inhibitor titre (BU/ml), number of exposure days (Exp. days) and major inhibitor epitopes are indicated. Three of these patients have been described previously. Patient A has been previously described by Fijnvandraat *et al* (1980) and also by Bril *et al* (AMC-92, 1999). Patient B has been described by Fijnvandraat *et al* and patient C (AMC-67) has been described by van den Brink *et al*. Analyis of the epitope specificity of factor VIII inhibitors of patient B was not performed (*n. p.*).

Patient	Max. titre (BU/ml)	Exp. days	Major inh. epitope
A (1980)	22	10	A2
A (1999)	2.6	32	A2
B	9	6	*n.p.*
C	400	10	A2
D	90	25	A2

(B) Low titre inhibitor patients.

Maximum inhibitor titre (BU/ml), number of exposure days (ED) and presence of clinical symptoms are indicated. Factor VIII levels during and prior to (in brackets) inhibitor episode are given.

Patient	Max. titre (BU/ml)	Exp. days	Clinical sypmtoms	Factor VIII level
E	1.2	14	No	8 (19)
F	0.5	6	Yes	4 (17)
G	0.2	9	No	5 (27)

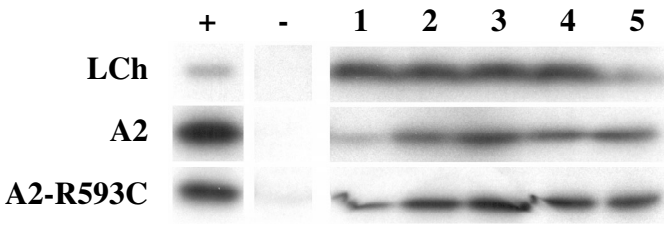

Fig 1. Epitope mapping of factor VIII inhibitors in patient AMC-118. Binding of anti-factor VIII antibodies present in the patient's plasma to recombinant factor VIII light chain, wild-type A2 domain and A2 domain containing the Arg^{593} to Cys mutation (A2-R593C) was assessed by immunoprecipitation. Samples obtained at different times of inhibitor development were analyzed; 10/5/2000 (1), 25/5/2000 (2), 05/6/2000 (3), 19/06/02 (4), 20/07/02 (5), positive control (+), negative control (-).

Table II Allele frequencies of HLA-DQB1 and DRB1/3/4/5 in inhibitor and non-inhibitor patients.

Relative Risks and 95% confidence intervals were calculated as described in the section materials and methods. All associations were not significant exept for the association of HLA-DRB1*01 with inhibitor formation in the low responder group. **(A)** Allele frequencies of HLA-DQB1 and DRB1/3/4/5 in 7 inhibitor and 35 non-inhibitor patients. Allele frequencies in patients with high inhibitor titres **(B)** or patients with low inhibitor titres **(C)** compared to non-inhibitor patients. [(1)]: The association of HLA-DRB1*01 with inhibitor formation in the low responder group was statistical significant (RR = 5,91, 95% confidence interval = 1,36-25,6 and p-value = 0.04).

A

HLA-allele	Inh. pat.		Non inh. pat.	
	n=7	%	n=35	%
DQB1*				
02	2	28,6	9	25,7
03	4	57,1	25	71,4
05	4	57,1	12	34,3
06	4	57,1	24	68,6
DRB1*				
01	4	57,1	8	22,9
03	1	14,3	8	22,9
04	1	14,3	11	31,4
07	1	14,3	6	17,1
09	0	0,00	2	5,71
10	0	0,00	2	5,71
11	2	28,6	6	17,1
12	0	0,00	1	2,86
13	3	42,9	16	45,7
14	1	14,3	0	0,00
15	1	14,3	8	22,9
16	0	0,00	2	5,71
DRB3	4	57,1	25	71,4
DRB4	2	28,6	17	48,6
DRB5	1	14,3	10	28,6

B

HLA-allele	High inh. pat n=4	%	Non inh. pat. n=35	%
DQB1				
05	1	25,0	12	34,3
06	4	100	24	68,6
DRB1				
01	1	25,0	8	22,9
11	2	50,0	6	17,1
13	3	75,0	16	45,7
15	1	25,0	8	22,9

C

HLA-allele	Low inh. pat. n=3	%	Non inh. pat. n=35	5	
DQB1					
02	2	66,7	9	25,7	
03	1	33,3	25	71,4	
05	3	100	12	34,3	
DRB1					
01	3	100	8	22,9	(1)
03	1	33,3	8	22,9	
07	1	33,3	6	17,1	

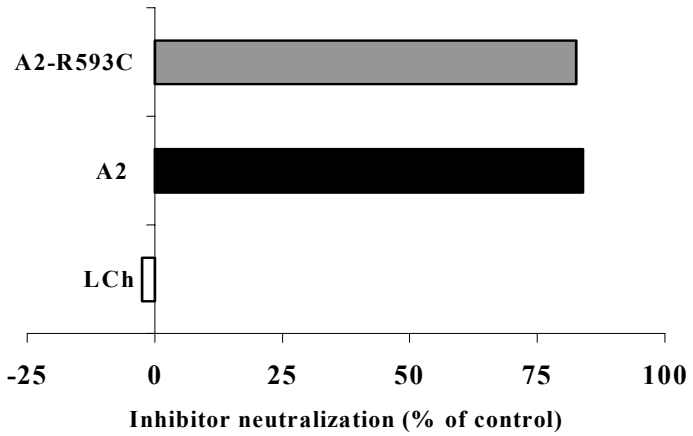

Fig 2.
Inhibitor neutralization by recombinant factor VIII fragments.
Neutralization of anti-factor VIII antibodies in the patients plasma by recombinant A2 domain (gey bar), A2-R593C (black bar) and factor VIII light chain (open bar). Residual factor VIII activity is indicated on the x-axis as a percentage of the control sample.

Discussion

Over the last few years increasing attention has been directed towards inhibitor development in patients with mild haemophilia A. In this study, we describe inhibitor formation in a group of previously treated mild haemophilia A patients with the Arg^{593} to Cys genotype. Patient D developed a high titre inhibitor after a period of factor VIII replacement therapy. Immunoprecipitation analysis revealed antibodies specific for the light chain and the A2 domain of factor VIII. The patient's antibodies recognized both exogenous A2 domain and mutated endogenous A2 domain (A2-R593C). Cross-reactive antibodies directed to the A2 domain have been observed in three other inhibitor patients with the Arg^{593} to Cys mutation . Interestingly, the predominant inhibitor epitope is restricted to the A2 domain in patients with Arg^{593} to Cys mutation. Only limited reactivity of inhibitory antibodies to light chain or C2 fragments has been observed. In general, a heterologous mixture of inhibitors is present in inhibitor plasmas. In more than 60% of inhibitor patients, antibodies against A2 and/or A3-C1 domains are detected while in over 80% of inhibitor patients antibodies specific for the C2 domain are detected.[14] The predominance of anti A2 antibodies in patients with an Arg^{593} to Cys mutation argues for a common mechanism of inhibitor formation in these patients.

Several observations suggest that inhibitor formation in haemophilia A patients is dependent on an adequate T cell response to factor VIII.[15-17] Activation of factor VIII-specific T cells is dependent on appropriate presentation of factor VIII derived peptides by HLA molecules present on the surface of antigen presenting cells. In addition, co-stimulatory signals are required for optimal activation of T cells. Given the nature of the antigen, it seems of particular interest to study the role of HLA class II alleles in inhibitor formation. So far, the relation between inhibitor formation and HLA class II alleles has only been studied in severely affected haemophiliacs. Two studies have shown an increased frequency of HLA-DR4 in patients who did not developed an inhibitor whereas others did not find a relation between HLA class II alleles and inhibitor development.[18-21] Two reports describe the role of HLA class II alleles in patients with the same factor VIII.[22,23] HLA typing was performed in severe haemophilia A patients with intron 22 inversion mutations. A statistical significant association of HLA-DQA1*0102 with inhibitor formation has been observed by one group.[22] The present study shows that one established haplotype (HLA-DQB1*0501, DRB1*0101) is weakly associated to inhibitor development in mild haemophilia A patients with the Arg^{593} to Cys mutation. The frequencies of HLA-DQB1*05 and DRB1*01 are raised in patients which developed a low or transient inhibitor compared to non-inhibitor patients. In contrast, the frequencies of HLA-DQB1*05 and DRB1*01 are not raised in the high responder group (Table IIc). Interestingly, haplotype HLA-DQB1*0501, DRB1*0101 also weakly associates with inhibitor development in severe haemophilia A patients with an intron 22 inversion.[22] Thus, haplotype HLA-DQB1*0501, DRB1*0101 is associated to a high-level inhibitor in severe haemophilia A while this HLA class II may be associated with a low and transient inhibitor titre in mild haemophilia A patients which are partially tolerant to factor VIII. In the high responder group the frequencies of HLA-DQB1*06 and DRB1*11 or DRB1*13 are raised but a study with a larger group of subjects should point out whether this is significant or not (Table IIb). We did not observe an association of HLA-DRB1*04 or DRB1*15 with inhibitor formation as reported by other groups in severe haemophilia A patients .The development of inhibitors in patients with the Arg^{593} to Cys mutation can be explained by the following

series of events. It has been proposed that after uptake and processing of exogenous factor VIII by antigen presenting cells, a T cell epitope containing the Arg^{593} residue may be presented by a matching HLA class II molecule. Subsequent T cell activation may result in loss of tolerance to both exogenous and endogenous factor VIII and stimulation of factor VIII specific B cells to produce antibodies .

In a recent study the molecular background of inhibitor formation in patients with an Arg^{2150} to His mutation in the C1 domain has been addressed.[17] T cell clones generated from patients with inhibitors all bound to synthetic peptides overlapping residue Arg^{2150}. Interestingly, synthetic peptides encompassing Arg^{2150} could interact with multiple HLA class II molecules. These findings suggest that a restricted number of T cell epitopes that promiscuously interacts with HLA class II molecules are involved in initiation of immune responses in patients with an Arg^{2150} to His mutation in factor VIII. Our findings did not reveal a strong association of inhibitor formation and HLA class II molecules in patients with an Arg^{593} to Cys mutation. Potentially, this can be explained by a lack of specificity of peptides overlapping residue Arg^{593} for particular HLA class II molecules.

Additional factors may contribute to the lack of association observed in this study. The number of subjects in the inhibitor group is relatively small. Also, about half of the patients included have received only limited amounts of factor VIII (< 10 exposure days). Inclusion of more patients and follow-up of the patients described in this study is needed to assess whether inhibitor formation in mild haemophilia A patients with Arg^{593} to Cys mutation is linked to one or more HLA class II alleles.

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Appendix

Table III. HLA-DQB1, DRB1, DRB3, DRB4 and DRB5 alleles and inhibitor status of 42 patients with the Arg^{593} to Cys mutation. The 42 patients belong to 12 families, which are shown in Table III. High titre patients have been described elsewhere: (A) patient A, Fijnvandraat et al 1997 and as patient AMC-92, Bril et al 2002; (B) patient B, Fijnvandraat et al 1997; (C) patient AMC-67, van den Brink et al 1999 and (D) patient AMC-118, this study.

Pat.	DQB1	DRB1	DRB3	DRB4	DRB5
1 C	06	13	+	-	+
	06	15			
2 E	05	01	-	-	-
	03	01			
3	06	13	+	-	+
	06	15			
4	03	07	+	+	-
	06	13			
5	03	07	+	+	-
	06	13			
6	05	01	-	-	+
	06	15			
7	03	04	-	+	+
	06	15			
8	05	01	+	-	-
	06	13			
9	03	07	-	+	+
	06	15			
10	05	01	-	+	-
	03	04			
11	03	04	-	+	+
	06	15			
12	05	10	+	-	-
	06	13			
13	06	13	+	-	-
	06	13			
14	05	01	+	-	-
	02	03			
15	05	10	+	-	-
	06	13			
16	05	01	+	-	-
	03	11			
17	03	07	+	+	-
	03	03			
18 B	03	11	+	-	-
	06	13			
19 A	03	11	+	-	-
	06	13			
20 D	05	01	-	+	-
	03	04			
21	06	15	+	-	+
	06	13			

Pat.	DQB1	DRB1	DRB3	DRB4	DRB5
22	06	15	+	-	+
	03	11			
23	02	03	+	-	-
	06	13			
24	02	03	+	-	-
	03	12			
25	02	03	+	-	-
	03	11			
26 F	02	03	+	-	-
	05	14			
27 G	05	01	-	+	-
	02	07			
28	05	01	-	+	-
	03	04			
29	02	03	+	+	-
	03	04			
30	05	01	-	-	+
	05	16			
31	02	07	+	+	-
	03	11			
32	03	04	-	+	-
	03	04			
33	02	03	+	+	-
	03	04			
34	05	01	+	-	-
	06	13			
35	03	04	+	+	-
	06	13			
36	03	04	-	+	-
	02	07			
37	03	04	+	+	-
	03	11			
38	06	13	+	-	-
	06	13			
39	06	13	+	-	-
	06	13			
40	05	16	-	+	+
	03	09			
41	06	15	+	-	+
	02	03			
42	03	09	+	+	-
	03	11			

CHAPTER 7

Tolerance to human factor VIII in a mouse transgenic for human factor VIII-R593C

Wendy S. Bril[1], Marleen G. Zuurveld[1], Martine J. Hollestelle[1], Pieter H. Reitsma[2], Jan A. van Mourik[1, 3], Rene A.W. van Lier[4], Koen Mertens[1, 5] and Jan Voorberg[1]

[1]Department of Plasma Proteins, Sanquin Research at CLB, Amsterdam, The Netherlands. [2]Department of Experimental Internal Medicine, AMC, Amsterdam, The Netherlands, [3]Department of Vascular Medicine, AMC, Amsterdam, [4]Department of Experimental Immunology, AMC, Amsterdam, [5]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS) Utrecht, The Netherlands.

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Abstract

Haemophilia A is a bleeding disorder caused by the functional absence of clotting factor VIII. A serious complication of replacement therapy is the formation of inhibitory antibodies to factor VIII in approximately 25% in patients with severe haemophilia A. Although inhibitor formation in mild and moderate haemophilia A is rare, several reports have described inhibitor development in patients with mild haemophilia A caused by an Arg^{593} to Cys mutation. At present, it is not clear why some patients with this genetic defect develop inhibitors and others do not. To mimic the immune response in these patients, a transgenic mouse expressing human factor VIII-R593C was generated (hufVIII-R593C mice). Transgenic hufVIII-R593C mice (in a wild type mouse background) receiving multiple serial intravenous injections of factor VIII or factor VIII heavy chain did not develop anti-factor VIII antibodies. HufVIII-R593C mice were crossed with factor VIII-deficient mice (E-16 KO mice). Factor VIII-deficient E-16 KO mice develop anti-factor VIII antibodies after five serial intravenous injections with human factor VIII while hufVIII-R593C/E-16 KO mice did not develop an immune response. Apparently, hufVIII-R593C mice are tolerant to human factor VIII which is in agreement with the strongly reduced incidence of factor VIII inhibitors in patients with mild haemophilia A. However, anti-factor VIII antibody development was induced in HufVIII-R593C and hufVIII-R593C/E-16KO mice by multiple subcutaneous injections of factor VIII with an adjuvant. Our data show that hufVIII-R593C/E-16 KO mice provide a valuable model for studies directed at the mechanisms underlying inhibitor development in haemophilia A.

Introduction

Haemophilia A is an X-linked bleeding disorder caused by a deficiency of coagulation factor VIII. Severe haemophilia A patients, whose plasma have less than 1% factor VIII activity, suffer from spontaneous joint and muscle bleedings and excessive hemorrhage after trauma or surgery.[1,2] An inversion of DNA sequence within intron 22 of the factor VIII gene is responsible for disruption of the factor VIII gene in almost 50 % of severe haemophilia A patients.[3] Large or small deletions, missense and nonsense mutations that are scattered over the factor VIII gene occur in other patients with severe haemophilia A. Recently, a murine model for severe haemophilia A was developed by gene targeting techniques. Two strains of mice were generated by insertion of a neo cassette into exon 16 (E-16 KO) or exon 17 (E-17 KO) of the mouse factor VIII gene. Both the E-16 and E-17 knock out mice have impaired hemostasis, suffer from spontaneous bleedings and have less than 1% factor VIII activity compared to wild type mice.[4-6] The murine haemophilia A model has been successfully used as a tool for the development of haemophilia gene therapy strategies. Phenotypic correction and sustained factor VIII expression has been achieved in haemophilic mice.[7-10]

Severe haemophilia A patients frequently develop antibodies (so-called factor VIII inhibitors) that neutralize factor VIII activity.[11] Although progress has been made in obtaining information on inhibitors and their epitope specificity, the immunological mechanisms underlying the formation of inhibitory antibodies to factor VIII have not been elucidated. Much of our current knowledge on the immunobiology of factor VIII inhibitors

has been obtained from the murine haemophilia A model. After repeated intravenous injections of human factor VIII, haemophilic mice develop high titres of antibodies which resemble inhibitors in haemophilia A patients.[12] The antibodies detected in haemophilic mice are directed to the heavy chain, light chain and the non-functional B domain of factor VIII.[13] The anti-factor VIII antibodies are mainly IgG1 (the equivalent of human IgG4) but other subclasses are present as well.[13,14] The anti-factor VIII response in the haemophilic mice has been shown to be T-cell dependent resembling the immune response in haemophilia A patients.[12,15,17] Inhibition of T-cell co-stimulation via CD40-CD40L has been shown to suppress the formation of anti-factor VIII antibodies.[18-20] In addition, blockade of the CD28-B7 pathway prevented antibody formation in haemophilic mice.[12] Cytokine profiles of factor VIII-specific T cells indicate that the regulation of the anti-factor VIII antibody response in haemophilic mice involves both Th1- and Th2-type cells.[14,20] Altogether, these results suggest that factor VIII-deficient mice constitute a suitable model for inhibitor formation in haemophilia A.

Whereas inhibitor development occurs in 25% of patients with severe haemophilia A, factor VIII inhibitors are less frequently observed in mild and moderate haemophilia A. Inhibitor formation in these patients commonly occurs after extensive factor VIII replacement therapy.[21,22] Although inhibitor titres are usually low and inhibitors often disappear spontaneously, patients can suffer from severe life-threatening bleedings. Most of these patients have low amounts of circulating factor VIII, which provides tolerance to infused factor VIII. This can explain the lower risk of inhibitor development in mild/moderate haemophilia A patients. Interestingly, the risk of inhibitor development is higher for patients with missense mutations in the A2 domain or around thee C1/C2 junction of factor VIII.[11,21,22] Besides factor VIII genotype, inhibitor formation is probably related to other genetic determinants as well. In addition, different treatment strategies or inflammatory conditions can influence inhibitor formation in this group of patients.

Patients with the missense mutation Arg^{593} to Cys in the A2 domain of factor VIII suffer from mild haemophilia A. Several of these patients have been described who developed anti-factor VIII antibodies.[23-26] To mimic the immune response in these patients, a transgenic mouse expressing human factor VIII with the Arg^{593} to Cys mutation was generated. We evaluated immune responses to administrated factor VIII of transgenic hufVIII-R593C mice in wild type and in E-16 KO background. Our results indicate that transgenic hufVIII-R593C mice provide a useful model for inhibitor development in haemophilia A.

Materials and Methods

Generation and characterization of hufVIII-R593C transgenic mice

Full-length human factor VIII cDNA containing the Arg^{593} to Cys mutation was assembled into pBluescript (Promega, Wisconsin, MI). Subsequently, hufVIII-R593C cDNA was cloned into a plasmid containing a mouse albumin promoter sequence and the 3' part of human β globin gene which includes downstream polyadenylation signals.[27-29] The polylinker of the construct was modified and subsequently the plasmid was linearized by digestion with NotI. The linearized 12.3 kb fragment, which contains hufVIII-R593C under the control of the mouse albumin promoter, was microinjected into pronuclei of fertilized

oocytes of FVB or C57BL/6 mice. Offspring was screened by PCR and Southern blot analysis for the presence of the transgene. DNA was isolated from a 5 mm piece of mouse-tail using QIAGEN DNeasy Tissue Kit (Qiagen, Leusden, The Netherlands). For genotyping of transgenic hufVIII-R593C mice, 50 to 150 ng of genomic mouse DNA was used in one PCR reaction. To confirm the presence of the transgene hufVIII-R593C, the forward primer was derived of the human factor VIII exon 14 sequence (5'GTGCTCTCAAAACCCACC3') and the backward primer was derived from the human factor VIII exon 16 sequence (5'GCCTGATTTCTGAAAGTTACC3'). Amplifications were performed in a total volume of 30 µl containing 1 unit Taq polymerase (incubated for 10 min with Taq start antibodies), reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 100 ng each primer. Taq DNA polymerase and Taq start antibodies were purchased from Life Technologies (Breda, The Netherlands). Reactions were performed at 94° C for 2 min followed by 30 cycles of 10 sec 94° C, 30 sec 58° C and 1 min at 72° C and finally 10 min at 72° C. To confirm the presence of the disrupted exon 16 in mouse factor VIII and the presence of the undisrupted X-chromosome, two PCR reactions were performed using two previously described primer sets.[5] Amplifications were performed as described above using 50 to 150 ng of genomic mouse DNA. Southern blot analysis was performed using genomic DNA isolated from a piece of tail of transgenic mice. Following digestion with proteinase K and phenol/chloroform extraction, genomic DNA was recovered by ethanol precipitation. Twenty microgram of DNA was digested with BamHI and subjected to Southern blot analysis. The blots were probed with a 695 bp fragment of human factor A2 domain containing the R593C-mutation derived of the plasmid pCLB-GP67-A2-R593C.[23] Digestion of the hufVIII-R593C transgene with BamHI yields a 1929 bp product that is complementary to the probe.

Liver and kidney of heterozygous hufVIII-R593C mice were collected and immediately frozen in liquid nitrogen. Total RNA was isolated from the organs and factor VIII mRNA levels were measured by RT-PCR essentially as described previously.[30] Primers for amplification of murine factor VIII mRNA have been described elsewhere.[30] Amplification of human mRNA was performed with a different primer set (forward primer: 5'-GAGGAACCATCGCCAGGCGTCCTT-3', backward primer: 5'-AGGACTGGGACTA TGCTCCCTTAG-3').

Factor VIII protein levels in plasma of heterozygous hufVIII-R593C mice were determined using an ELISA specific for human factor VIII as described previously, except that antibody CLB-CAg 12 was used instead of antibody CLB-CAg A.[30] The monoclonal antibodies CLB-CAg 12 and CLB-CAg 117 against human factor VIII have been described previously.[31,32] Microtitre plates were coated with monoclonal antibody CLB-CAg-12 (0.5 µg/well) in 50 mM $NaHCO_3$ pH 9.8 at 4° C overnight. After washing with PBS/0.1% Tween-20, wells were incubated with plasma samples diluted in 50 mM Tris pH 7.4/0.15 M NaCl/2% HSA at 37° C for 2 h. Wells were washed and incubated with horseradish peroxidase labelled monoclonal antibody CLB-CAg 117 at 37° C for 2 h. The concentration of human factor VIII protein was estimated from a standard curve using a human plasma standard.

Founder T4 (FVB strain) and offspring were crossed with C57BL/6 mice (Jackson Laboratories, Bar Harbor, MN). Adult heterozygous mice from the second to fifth generation were used for experiments. The characteristics of the E-16 knock out (E-16 KO) strain of haemophilic mice (C57BL/6 mice with a *neo* cassette in exon 16) have been described elsewhere.[4,5] Heterozygous transgenic hufVIII-R593C males were bred with homozygous E-16 haemophilic females. Male transgenic hufVIII-R593C/E-16 KO mice and E-16 KO littermates without the hufVIII-R593C transgene were used in our studies.

Administration of human factor VIII

To investigate the immune response in transgenic hufVIII-R593C mice we injected heterozygous hufVIII-R593C mice and non-transgenic littermates with plasma-derived human factor VIII or human factor VIII heavy chain[31,33] Serial injections of factor VIII (1 µg in 100 µl phosphate-buffered saline (PBS)) were administered intravenously (*i.v.*) every week. Plasma samples were obtained before the first injection and subsequently at a two-weekly interval by retro-orbital bleeding. One week following the last injection mice were sacrificed. Blood was collected by cardiac puncture at this time point. Injections of human factor VIII heavy chain (1 µg in 100 µl PBS) were performed at a two-weekly interval. Plasma samples were obtained before each injection and blood samples were collected by cardiac puncture two weeks following the last injections. Immunization of the hufVIII-R593C/E-16KO and E-16KO mice consisted of five *i.v.* injections of 1 µg human factor VIII at two-weekly intervals. The mice were sacrificed three days after the last injection. Spleen cells and plasma samples were collected at this time point.

To induce loss of tolerance to factor VIII, heterozygous hufVIII-R593C mice and non-transgenic littermates were subcutaneous injected with factor VIII (1 µg in 100 µl PBS) and 100 µl Freund's adjuvant (Difco Laboratories, Detroit, Michigan, USA) every week. For the first injection, Complete Freund's adjuvant was used whereas subsequent injections were performed with Incomplete Freund's adjuvant. Plasma samples were obtained before the first injection and subsequently every two weeks by retro-orbital bleeding. One week following the sixth injection, mice were sacrificed and blood was collected by cardiac puncture. Immunization of hufVIII-R593C/E-16KO and E-16KO mice consisted of four *s.c*. injections with factor VIII and Freund's adjuvant and the mice were bled one week following the last injection.

Detection of anti-factor VIII antibodies

Antibody titres specific for human factor VIII or human factor VIII heavy chain were determined by ELISA. Microtitre plates (Nunc, Roskilde, Danmark) were coated with 6 µg/ml factor VIII or 5 µg/ml human factor VIII heavy chain in 50 mM $NaHCO_3$ pH 9.8 at 4° C overnight. After washing with PBS/0.1% Tween-20, wells were blocked with PBS/0.1% Tween-20/2% gelatin at 37° C for 2 h. After washing, mouse plasma samples diluted in 50 mM Tris pH 7.4/0.15 M NaCl/2% HSA at 37° C for 1 h. Subsequently, the wells were washed and anti-factor VIII antibodies were detected using polyclonal horseradish peroxidase labelled goat anti-mouse immunoglobulin. The concentration of anti-factor VIII antibodies was estimated from a standard curve using a mixture of two monoclonal antibodies in a 1:1 ratio; CLB-CAg 9 specific for factor VIII heavy chain and CLB-CAg 12 specific for the light chain of factor VIII.[31,32]

Factor VIII T cell proliferation assay

Three days after the last factor VIII injection, mice were sacrificed and spleens were isolated. Spleen single-cell suspensions were prepared and after lysis of erythrocytes, the cells were washed twice with serum free X-vivo 10 medium supplemented with gentamycin (BioWhittaker, Walkersville, Maryland, USA). Cells were subsequently cultured (5 x 10^5 cells/well) in six fold in flat-bottom wells in X-vivo medium supplemented with varying amounts of purified human factor VIII. Control wells were cultured in six fold with medium without factor VIII. Cells were incubated at 37 ºC for 72 hours. Two µCi ^{3}H-thymidine (0.074MBq) was added to the cultures and the cells were harvested after 20 hours (Titretek Cell Harvester) and incorporation of ^{3}H-thymidine, expressed in counts per minute (cpm), was measured by liquid scintillation. The rate of T cell proliferation is expressed as stimulation index (SI), which represents the amount of ^{3}H-thymidine incorporated in cultures in the presence of factor VIII divided by the amount of ^{3}H-thymidine incorporated in the absence of factor VIII.

Results

Generation and characterization of hufVIII-R593C transgenic mice

A single nucleotide substitution that resulted in replacement of Arg^{593} by a Cys was introduced in full-length factor VIII cDNA. This modified human factor VIII cDNA (hufVIII-R593C) was placed under control of a mouse albumin enhancer/promoter to direct factor VIII expression to the liver. Polyadenylation signals derived of the β globin gene were inserted at the 3' end of hufVIII-R593C cDNA (Figure 1A). Injection of the plasmid encoding huFVIII-R593C into fertilized oocytes of FVB mice resulted in two founders. Founder T4 (FVB strain) was crossed with C57Bl/6 mice for 5 generations and heterozygous offspring was further characterized. As expected transgenic mice did not show abnormalities in either development or reproduction.

The presence of the human factor VIII transgene in founder T4 was detected by Southern blot analysis (Figure 1B). Offspring was subsequently screened for the presence of hufVIII-R593C cDNA by PCR analysis (Figure 1C). The transgene was found in roughly 50% of both female and male offspring indicating that the transgene is not linked to either the X or Y chromosome. Previously, it has been shown that the albumin enhancer/promoter used in this study, directs liver specific expression.[28] In wild type mice, murine factor VIII mRNA is primarily detected in the liver as well as in the kidney.[30] To assess liver-specific expression of the human factor VIII transgene, RT-PCR was performed on total RNA derived from liver and kidney using primers specific for human factor VIII. As expected, human factor VIII mRNA was detected only in de liver whereas murine factor VIII mRNA could be detected in both liver and kidney (Figure 2). Expression levels of human factor VIIIR593C in murine plasma were measured by using a human factor VIII specific ELISA. In plasma of heterozygous transgenic mice hufVIII-R593C, protein levels are below the detection limit of our ELISA (i.e. 15 mU/ml) and protein levels are consistently negative in wild type C57BL/6 plasma and non-transgenic littermates. Similar results were obtained upon analysis of plasma derived of the second founder. Overall, our findings show that liver-specific expression of the transgene results in undetectable levels of human factor VIII-R593C in the circulation of transgenic mice.

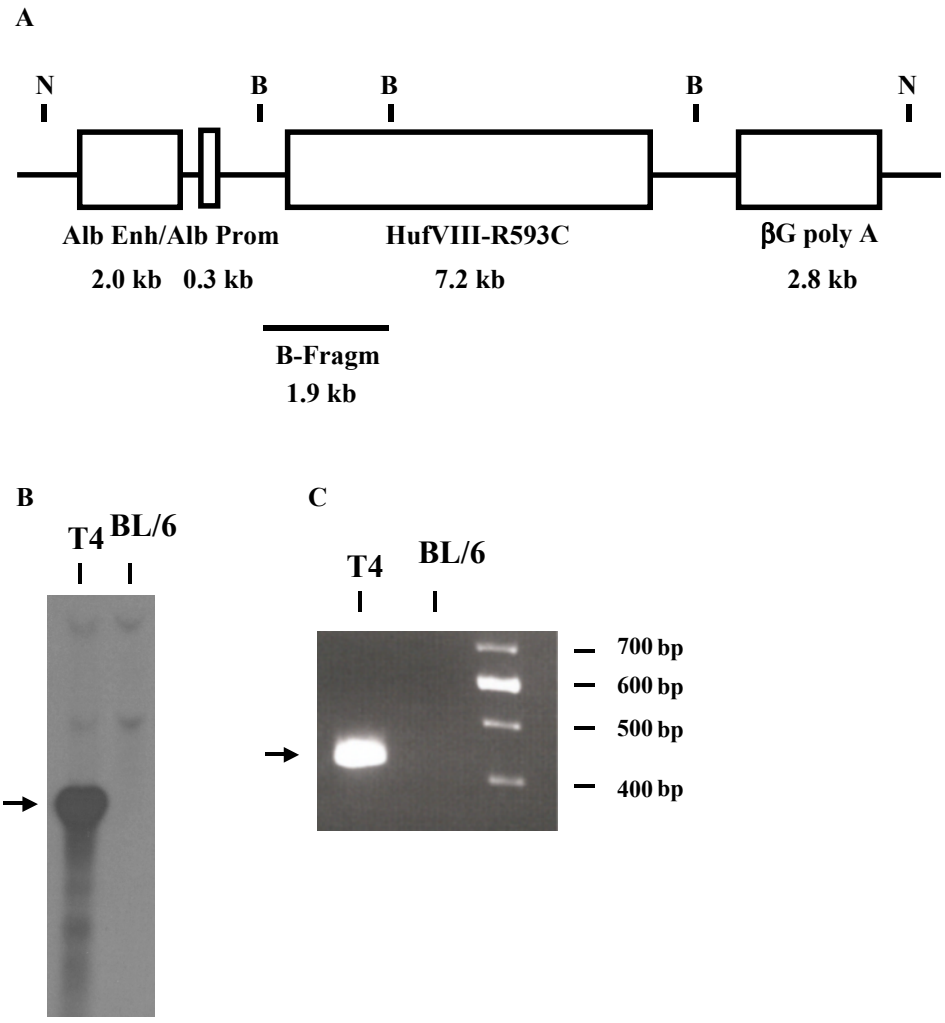

Figure 1. Construction and genotyping of transgenic hufVIII-R593C mice. (A) A plasmid with an insert of 12.3 kb consisting of a mouse albumin enhancer/promoter (Alb Enh/Alb Prom), full-length human factor VIIIR593C cDNA (hufVIII-R593C) and β globin polyadenylation signals (βG poly A) was constructed as described in Materials and Methods. The insert was separated from the vector sequences by digestion with NotI (N) and the linearized fragment was injected into fertilized oocytes of FVB mice. The BamHI (B) restriction fragment (B-Fragm) of human factor VIII-R593C DNA generated by Southern blot analysis is shown. **(B)** Genotyping of the transgenic hufVIII-R593C mouse by Southern blot analysis. Southern blot analysis was performed on BamHI digested genomic DNA of founder T4 and a wild type C57Bl/6 control. The blot was hybridized with a human factor VIII-specific probe. The 1.9 kb BamHI restriction fragment of human factor VIIIR593C DNA that reacts with this probe is indicated by an arrow. **(C)** Genotyping by detection PCR of hufVIII-R593C mice and a wild type C57Bl/6 control mouse. A 432 bp fragment was amplified from genomic DNA of founder T4 (indicated by the arrow) using human factor VIII specific primers that are described in Materials and Methods.

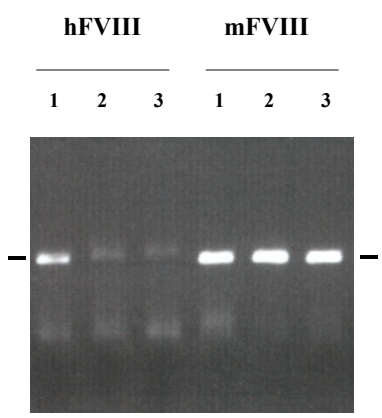

Figure 2. HufVIII-R593C mRNA expression and protein expression in the liver. RT-PCR was performed on total RNA of liver and kidney to detect factor VIII mRNA. Primers specific for human factor VIII mRNA amplified a 362 bp fragment (left panel). Primers specific for murine factor VIII mRNA amplified a 359 bp fragment (right panel). Lane 1: RNA isolated from the liver of a heterozygous hufVIII-R593C mouse in C57BL/6 background, lane 2: RNA isolated from the kidney of a heterozygous hufVIII-R593C mouse in C57BL/6 background and lane 3: RNA isolated from the liver of a C57BL/6 control mouse.

Immune response to human factor VIII in transgenic hufVIII-R593C mice

Previous studies have shown that intravenous injection of human factor VIII in normal C57BL/6 mice results in the appearance of inhibitory antibodies.[12,13] Therefore, we first assessed the effect of the huFVIII-R593C transgene on the immunogenicity of human factor VIII in a background of normal mice. HufVIII-R593C mice were injected six times with 1 µg of plasma derived factor VIII with one-week interval between injections. Figure 3A shows the anti-factor VIII antibody response of four transgenic mice and six non-transgenic controls measured by ELISA. All non-transgenic control mice developed a strong antibody response (> 1 µg/ml) after six intravenous injections of factor VIII. In contrast, two hufVIII-R593C mice showed a low anti-factor VIII antibody response (< 0.7 µg/ml) and two hufVIII-R593C mice did not show any antibody response at all. In a second series of experiments in which we administered 1 µg of plasma derived human factor VIII heavy chain to both wild type and huFVIII-R593C mice at two weekly intervals. The anti-factor VIII heavy chain antibody concentration in plasma was determined by ELISA (Figure 3B). All non-transgenic controls show significant antibody responses after six injections whereas most transgenic mice do not develop anti-factor VIII antibodies. Only two transgenic mice had a low titre of anti-factor VIII heavy chain antibodies after six and eight injections, respectively. Collectively these findings suggest that the humoral immune response to injected human factor VIII observed in wild type mice is markedly suppressed by the introduction of a transgene encoding human factor VIII with the Arg^{593} to Cys mutation.

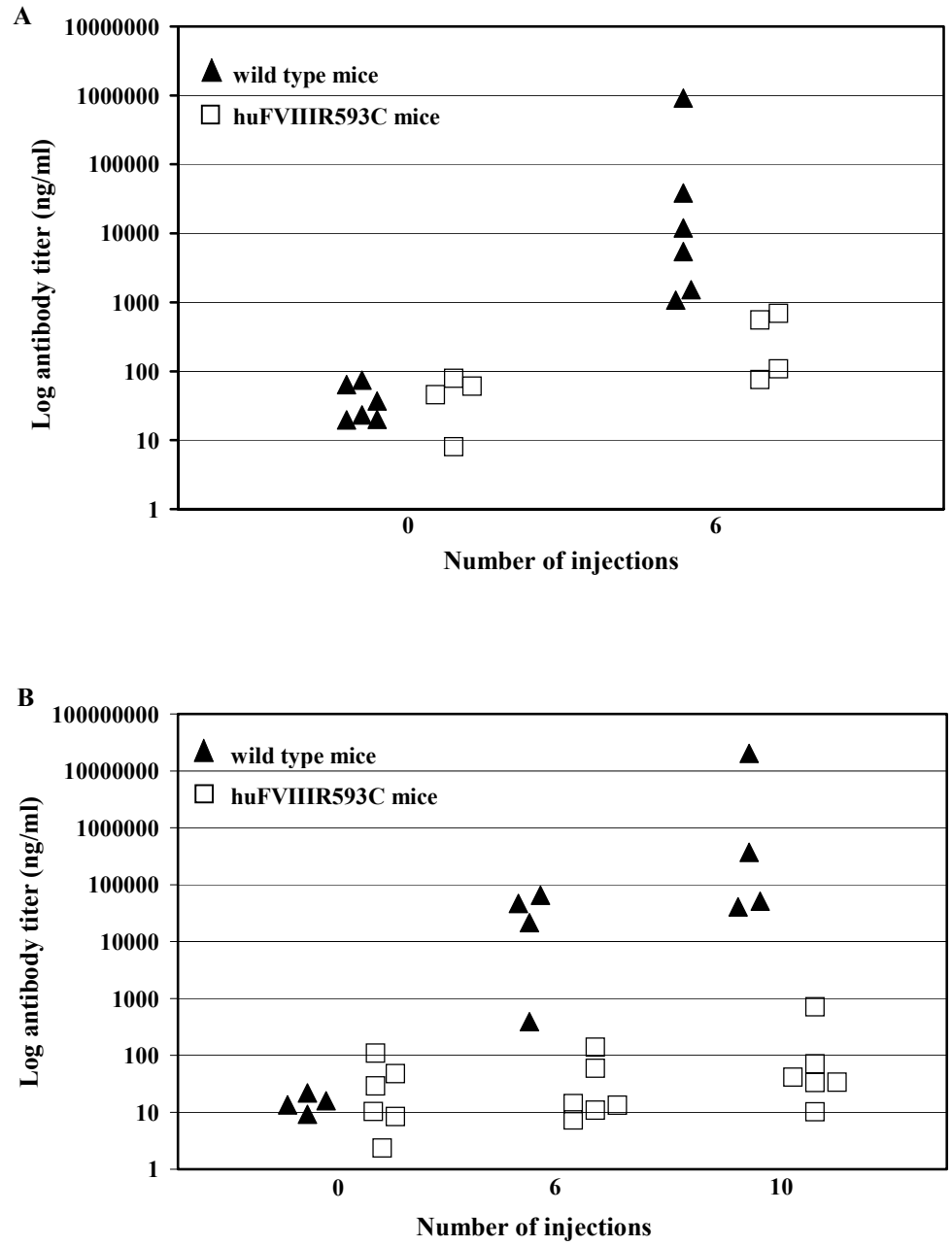

Figure 3. Anti-factor VIII antibody response in hufVIII-R593C mice. (A) Anti-factor VIII antibody formation measured by ELISA in hufVIII-R593C mice (open squares) and littermate controls (closed triangles) after serial *i.v.* injections of 1 µg of factor VIII every week. Plasma samples were obtained before the first injection and subsequently every two weeks. **(B)** Anti-factor VIII antibody formation measured by ELISA in hufVIII-R593C mice (open squares) and littermate controls (closed triangles) after serial *i.v.* injections of 1 µg of factor VIII heavy chain every two weeks. Plasma samples were obtained two weeks after each injection.

Immune response to human factor VIII in transgenic hufVIII-R593C/KO mice

Heterozygous transgenic hufVIII-R593C males were crossed with homozygous E-16 knock out females. Breeding of heterozygous hufVIII-R593C males with homozygous E-16 KO haemophilic females yielded hufVIII-R593C/E-16 KO or E-16 KO male offspring. These males were subjected to five consecutive intravenous injections of factor VIII. Figure 4A shows that in most E-16 KO mice considerable levels of anti-factor VIII antibodies are present. The concentration of these antibodies varied from 200 ng/ml to 73 µg/ml. Remarkably, in none of the four hufVIII-R593C/E-16 KO mice anti-factor VIII antibodies were found. Factor VIII specific T cell proliferation was assessed using spleen derived T cells of hufVIII-R593C/E-16 KO and E-16 KO males, which were isolated three days after the last injection (Figure 4B). The proliferative response of T cells of the E-16 KO mice is heterogeneous and dependent on the amount of factor VIII present in the incubation mixture. The largest responses were observed at a dose of 10 µg/ml of factor VIII (Figure 4B). T cell responses were high in mice with high titres of anti-factor VIII antibodies in their plasma (Figure 4C). In 2 out of 8 E-16 KO mice tested, only a modest increase in T cell proliferation was observed following the addition of factor VIII. In six E-16KO mice, significant responses were observed, resulting in stimulation indexes that ranged from 2.5 until 7.5 (Figure 4B). The hufVIII-R593C/E-16 KO mice did not show a factor VIII specific T cell response after five injections of factor VIII. In none of these mice, a stimulation index above 1.2 was observed. Statistical analysis revealed that T cell responses in hufVIII-R593C mice were significantly decreased when compared to E-16 KO mice (Mann-Whitney U test; $p < 0.005$). The greatly reduced T and B cell responses suggest that hufVIII-R593C/E-16KO mice are tolerant to intravenously administered factor VIII.

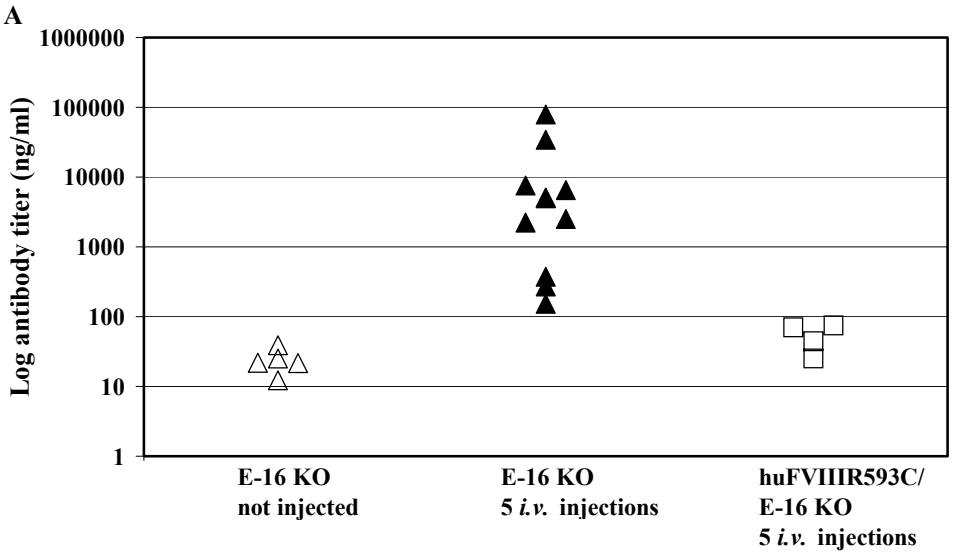

Figure 4 A

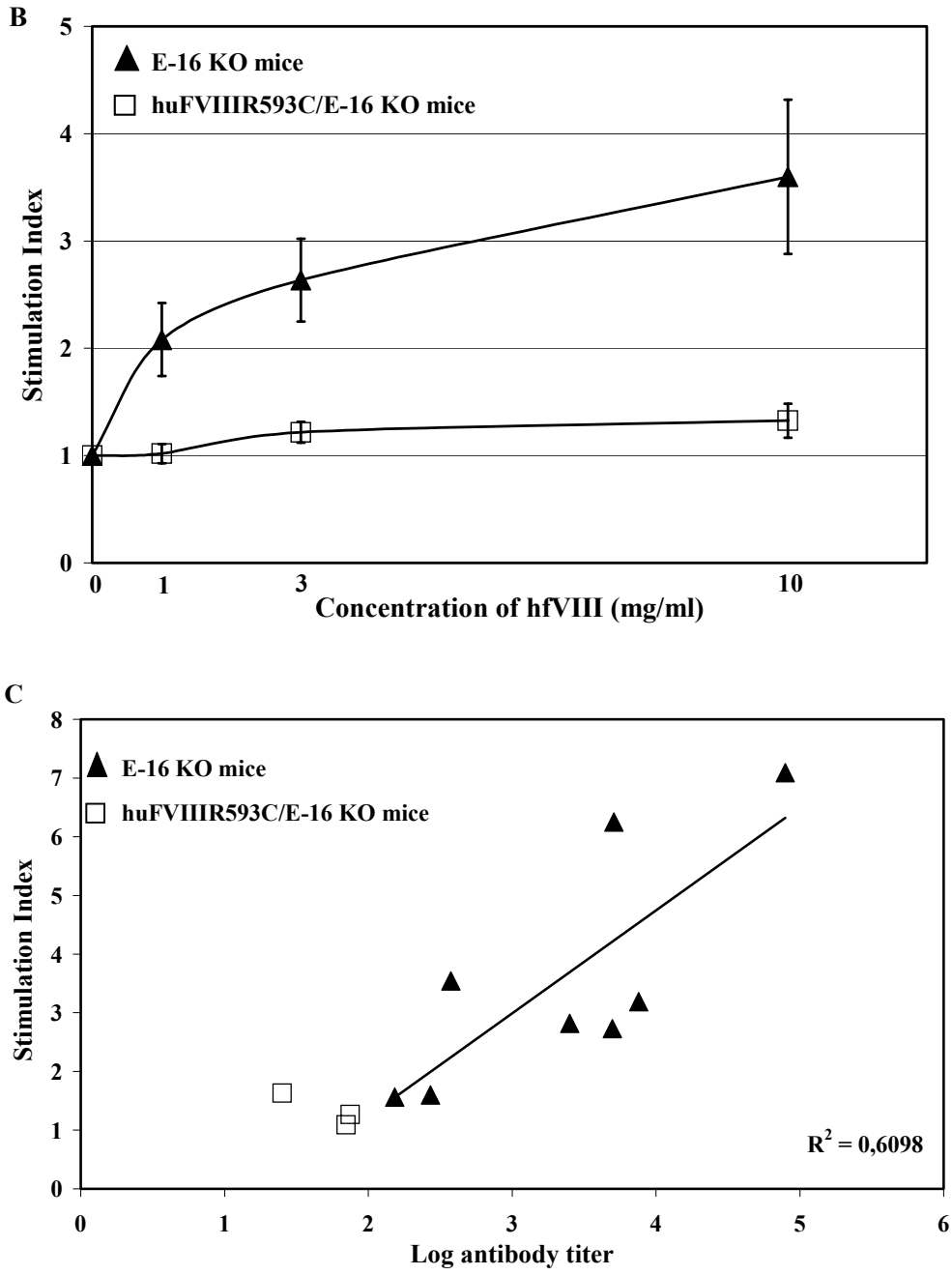

Figure 4. Characterization of the immune response to factor VIII in hufVIII-R593C mice in E-16 KO background. (A) Anti-factor antibody formation measured by ELISA in hufVIII-R593C/E-16 KO mice (open squares) and littermate E-16 KO controls (closed triangles) after five *i.v.* injections of 1 µg of factor VIII at two-weekly intervals. Plasma samples were obtained three days after the fifth injection. **(B)** HufVIII-R593CT cell proliferative responses to factor VIII in hufVIII-R593C/E-16 KO mice (open squares) and littermate E-16 KO

controls (closed triangles) after five *i.v.* injections of 1 μg of factor VIII. T cell responses were measured at different concentration of factor VIII. On the x-axis, the concentration of factor VIII is depicted in μg/ml. On the y-axis the average stimulation index (± SD) obtained for hufVIII-R593C mice (open squares) and littermate E-16 KO controls (closed triangles) is given. **(C)** Plot of T cell responses versus concentration of anti-factor VIII antibodies in plasma. Each symbol represents data obtained for individual hufVIII-R593C/E-16 KO (open squares) and littermate E-16 KO mice (closed triangles).

Loss of tolerance to human factor VIII in transgenic hufVIII-R593C and hufVIII-R593C/KO mice.

Inhibitor formations in mild haemophilia A patients is rare and frequently occurs following a period of extensive factor VIII replacement therapy. Pro-inflammatory conditions and/or increased antigen load may contribute to loss of tolerance. To mimic these conditions we evaluated whether subcutaneous administration of factor VIII in the presence of Freund's adjuvant could evoke an immune response in transgenic hufVIII-R593C mice. Six hufVIII-R593C mice and six non-transgenic control mice were subcutaneous injected every week and anti-factor VIII antibody responses were measured in plasma samples which were obtained every two weeks (figure 5A). Two wild type mice died early in this experiment. High titre antibodies (> 10 μg/ml) developed in wild type mice after 4-6 subcutaneous injections with factor VIII (Figure 5A). Antibody responses were also observed in the transgenic hufVIII-R593C mice. Three transgenic mice developed high antibody titres similar to the levels observed in wild type mice while three mice developed lower antibody titres ranging from 0.5 to 5 μg/ml. These results suggest that partial loss of tolerance in hufVIII-R593C mice occurs following subcutaneous administration of factor VIII in the presence of an adjuvant. These experiments were repeated with the presence of the hufVIII-R593C transgene in an E-16 KO background. Four hufVIII-R593C/E-16KO and eight E-16KO mice received four subcutaneous injections of factor VIII with Freund's adjuvant (figure 5B). All E-16KO mice developed a high antibody titre (> 10 μg/ml) whereas only two hufVIII-R593C/E-16KO mice developed high titre antibodies to factor VIII (figure 5B). The other two hufVIII-R593C/E-16KO mice developed low levels of anti-factor VIII antibodies. These results show that also in an E-16 KO background partial loss of tolerance occurs following subcutaneous injections of factor VIII in the presence of Freund's adjuvant.

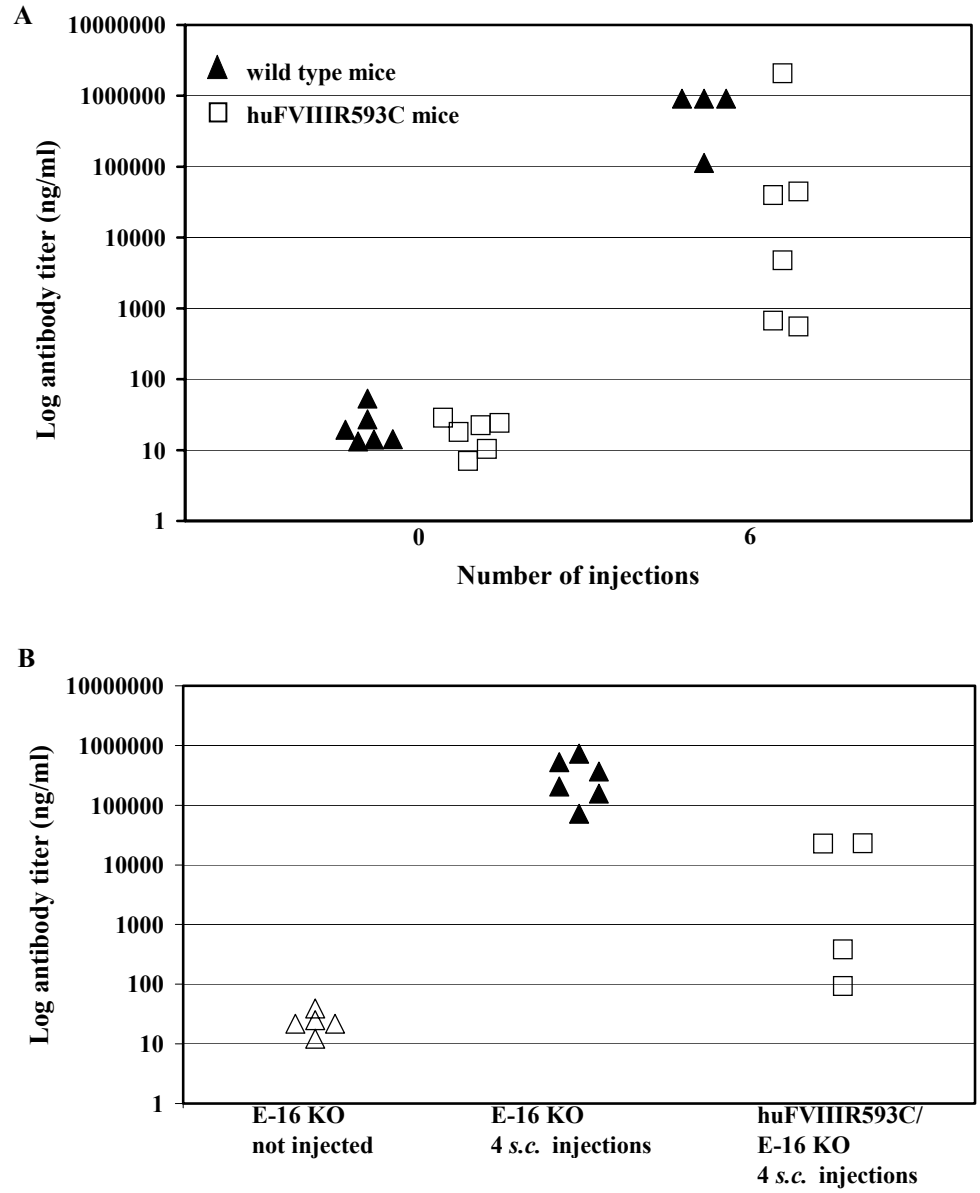

Figure 5. Loss of tolerance in transgenic hufVIII-R593C mice after multiple s.c. injections of factor VIII with Freund's adjuvant. (A) Levels of anti-factor antibodies as measured by ELISA in hufVIII-R593C mice (open squares) and littermate controls (closed triangles) after serial *s.c.* injections of 1 µg of factor VIII with Freund's adjuvant at a one weekly interval. Plasma samples were obtained before the first injection and subsequently every two weeks. **(B)** Anti-factor antibody formation measured by ELISA in hufVIII-R593C/E-16 KO mice (open squares) and littermate E-16 KO controls (closed triangles) after four *s.c.* injections of 1 µg of factor VIII with Freund's adjuvant at a one week interval. Plasma samples were obtained one week following the last injection.

Discussion

The immune response to factor VIII is an important obstacle for treatment of haemophilia A patients with factor VIII concentrates. In the majority of the patients with severe haemophilia A levels of circulating factor VIII are <1% of normal. Therefore, it is not surprising that infused factor VIII is recognized as a foreign substance by the immune system. Patients with mild haemophilia A do have circulating levels of factor VIII. It is likely that tolerance to factor VIII is maintained in these patients by ignorance or anergy of potentially self-reactive T and B cells. Despite the presence of circulating levels of factor VIII, a number of reports have suggested that inhibitor formation in mild haemophilia A does occur. Apparently, conditions can arise that result in loss of tolerance in this group of patients. Although epidemiological studies have not been conducted, it has been suggested that missense mutations in the A2 and C1-C2 domain of factor VIII are frequently found in mild haemophilia A patients with inhibitors.[21]

Here, we describe the characteristics of a transgenic mouse for human factor VIII harboring an Arg^{593} to Cys mutation that causes mild haemophilia A in humans. Both E-16 KO mice and huFVIII-R593C/E-16 KO mice have a severe haemophilia phenotype based on factor VIII levels in plasma (<1% of normal). We have not performed phenotypic analysis of the bleeding tendency in hufVIII-R593C/E-16 KO mice. However, the majority of hufVIII-R593C/E-16 KO mice did not survive tail clipping that was initially used for purposes of genotyping. This observation suggests that the bleeding tendency of hufVIII-R593C/E-16 KO mice is similar to that of E-16 KO mice.

Multiple factors may contribute to the undetectable levels of huFVIII-R593C in the transgenic huFVIII-R593C mice. It is possible that the transgene is integrated at a chromosomal location that is flanked by sequences that negatively affect expression of the transgene. However, both founders showed equally low amounts of circulating factor VIII. In addition, it has been reported that the Arg^{593} to Cys missense mutation is responsible for intracellular accumulation and subsequent degradation of factor VIII.[34] The intracellular degradation of factor VIII may explain the low levels of circulating factor VIII in both patients and transgenic mice carrying this particular genetic defect in the factor VIII gene.

Intravenous administration of factor VIII does not result in formation of a high titre of anti-factor VIII antibodies in huFVIII-R593C mice whereas in non-transgenic E-16 KO and wild type mice an immune response was observed. Apparently, introduction of hufVIII-R593C gene renders the huFVIII-R593C mice tolerant for human factor VIII. These findings are in agreement with a study of Evans and co-workers which describes the induction of immune tolerance to human factor VIII in E-17 KO mice by transplantation of bone marrow transduced with a retroviral vector expressing huFVIII.[35] Although factor VIII was undetectable in plasma, 30-50% of the treated E-17 KO mice were tolerant to factor VIII. It was suggested that low-level exposure of human factor VIII in bone marrow-derived antigen-presenting cells induced tolerance in these mice. In our mouse model, tolerance to factor VIII may be induced by ignorance or anergy of factor VIII reactive T and B cell. Anergic or down-regulated auto-reactive T and B cells are not eliminated from the repertoire and can still participate in immune responses but their activation threshold has been raised (reviewed by Goodnow).[36] Negative selection of factor VIII reactive T and B cells by clone elimination in central or peripheral lymphoid tissues is not likely since loss of tolerance was observed after subcutaneous injections of factor VIII in the presence of an

adjuvant. We are currently investigating whether the observed anti-factor VIII antibody response coincides with increased factor VIII dependent T cell responses. It has been suggested that central T cell tolerance can be induced by promiscuous gene expression of tissue-specific antigens in the thymus.[37-40] To investigate whether T cell tolerance is achieved in the thymus or in peripheral lymphoid tissues, it would be interesting to determine whether the transgene is expressed in the thymus of the hufVIII-R593C mice.

The anti-factor VIII immune responses observed in E-16 KO and wild type mice is similar to the anti-factor VIII antibody and T cell responses reported by others.[12,13,15] In our studies we have used highly purified, plasma derived factor VIII whereas other investigators have used recombinant factor VIII. Our results suggest that these preparations are similar with respect to their immunogenicity. We observed variability in immune response within the different groups of animals, for example, not all E-16 KO developed high titres of anti-factor VIII antibodies following *i.v.* injections of factor VIII. This variability in antibody titres and T cell proliferation may be due to the heterogeneity of the animals used for immunization. HuFVIII-R593C mice have been generated on an FVB background. In our experiments, we used mice that were backcrossed to C57BL6 for at least two generations. Also, our population of E-16 KO mice was only partially inbred into a C57Bl/6 genetic background. This heterogeneity may contribute to a variable anti-factor VIII immune response within the different groups of mice.

The lack of inhibitor formation in huFVIII-R593C mice after intravenous injections of factor VIII is in agreement with the reduced incidence of inhibitor formation in patients with mild haemophilia A. In some patients, loss of tolerance is observed following factor VIII replacement therapy. Genetic defects in the A2 domain and at the C1-C2 junction have been implicated in inhibitor development.[21] Inhibitor formation is not observed in all patients with these genetic defects, which suggest that other factors contribute to the onset of an immune response. Transgenic huFVIII-R593C mice are tolerant to wild-type human factor VIII when injected intravenously. However, subcutaneous administration of factor VIII in the presence of an adjuvant results in loss of tolerance to infused factor VIII. These data suggest that inflammatory conditions and/or antigen presentation are important for initiation of immune responses in patients with mild haemophilia A. Collectively, our findings indicate that hufVIII-R593C/E-16 KO mice provides a useful model for studies directed at mechanisms involved in loss of tolerance in haemophilia A.

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CHAPTER 8

General discussion

Introduction

Haemophilia A is a bleeding disorder caused by a decrease or dysfunction of blood coagulation factor VIII. Bleeding episodes can be prevented or treated by replacement therapy using plasma-derived or recombinant factor VIII. In response to replacement therapy, patients can develop an immune response to transfused factor VIII. The formation of inhibitory antibodies to factor VIII (factor VIII inhibitors) is one of the most difficult problems in treatment of haemophilia A patients.[1]

Whereas inhibitor development occurs in 25% of patients with severe haemophilia A, inhibitor development in mild and moderate haemophilia A is less frequently observed. It is generally believed that the low amount of circulating factor VIII in mild/moderate haemophilia A patients explains the lower risk of inhibitor development. Loss of tolerance and subsequent inhibitor formation in these patients commonly occurs after extensive factor VIII replacement therapy. It has been shown that the risk of inhibitor development is higher for patients with missense mutations in the A2, C1 or C2 domain of factor VIII (Table I).[2] However, not all mild haemophilia A patients with these missense mutations develop inhibitors after administration of factor VIII. This observation suggests that in addition to factor VIII genotype additional factors contribute to inhibitor development in mild haemophilia A.

To investigate the mechanisms underlying inhibitor formation in mild haemophilia A patients we have characterized anti-factor VIII antibodies using phage display technology and immunoprecipitation analyis. We have evaluated the role of HLA class II molecules in inhibitor development in patients with mild haemophilia A caused by an Arg^{593} to Cys mutation. Furthermore, we have developed a small animal model for inhibitor development in haemophilia A which enables studies directed at the immunological mechanism underlying inhibitor development.

Table I. Missense mutations associated with inhibitor development in patients with mild or moderate haemophilia A.

Factor VIII domain	Mutation	Reports of patients with inhibitors
A1 domain	Leu^{198} to His	Pieneman 1995
A2 domain	Gly^{420} to Val	Liu 1998
	Arg^{593} to Cys	Pieneman 1995, Fijnvandraat 1997, Thompson 1997, Hay 1998, van den Brink 1999, Knobe 2000, Bril Chapter 6 this thesis
	Asn^{618} to Ser	Vlot et al 2002
	Val^{663} to Phe	Hay 1998
	Gly^{701} to Asp	Bichandi 1995
A3 domain	Arg^{1941} to Gln	Liu 1998
	Gly^{2009} to Arg	Hay 1998
C1 domain	Thr^{2086} to Asn	Freson 1998
	Tyr^{2105} to Cys	Naylor 1993, Hay 1998, Knobe et al, 2000
	Arg^{2150} to His	Santagostino 1995, Peerlinck 1998, Hay 1998, Laprise 1998, Liu 2002
	Arg^{2163} to His	Hay 1998 (van den Brink et al, 2002: Chapter 2 this thesis), Waseem 1999
C2 domain	Glu^{2181} to Asp	Hay 1998
	Arg^{2209} to Gln	Schwaab 1993
	Trp^{2229} to Cys	Naylor 1991, hay 1998, Cutler 2002
	Val^{2232} to Ala	Liu 2000
	Phe^{2260} to Ile	Hay 1998
	Pro^{2300} to Leu	Liu 2000
	Arg^{2304} to Cys	Liu 2000

Analysis of anti-C2 antibodies of mild haemophilia A patients using phage display

Factor VIII circulates in plasma as a metal-ion linked heterodimer. The heavy chain of factor VIII consists of the domains A1-A2-B and the light chain consists of the domains A3-C1-C2. Previous studies have demonstrated that inhibitory antibodies detected in both severe and mild/moderate haemophilia A patients bind to restricted regions of factor VIII (see Figure 1 Chapter 1). Binding sites have been identified in the A2, A3 and C2 domain of factor VIII. Binding of antibodies to the A2 domain is localized to amino acid sequence Arg^{484}-Ile^{508} and the major epitope in the A3 domain consists of residues Gln^{1778}-Met^{1823}.[3-5] Inhibitors directed towards the A2 and A3 domain neutralize factor VIII activity by interfering with the interaction of factor VIIIa with factor IXa. Residues Glu^{2181}-Val^{2243} and residues Val^{2248}-Ser^{2312} are involved in antibody binding in the C2 domain.[6,7] Inhibitors specific for the C2 domain block the binding of factor VIII to the phospholipid surface or reduce the release of activated factor VIII from von Willebrand factor.[8,9] Usually, a mixture of antibodies neutralizes factor VIII function in the patients' plasma.[10]

Previously, anti-C2 antibodies have been studied in a patient with severe acquired haemophilia A using phage display technology (patient A in Table II).[11] Because the majority of the factor VIII inhibitors are of subclass IgG4, a phage display library was constructed of the IgG4-specific immunoglobulin heavy chain variable (V_H) repertoire of the patient.[11,12] The patient's V_H repertoire was combined with the immunoglobulin light chain variable (V_L) repertoire of a nonimmune donor. From this library, anti-C2 specific single chain variable fragments (scFv's) were isolated and sequence analysis showed that their V_H gene segments were all derived from the V_H1 family. In chapter 2, the isolation of 19 anti-C2 specific scFv's from a library of a mild haemophilia A patient with a missense mutation (Arg^{2163} to His) in the C1 domain of factor VIII is described (patient B in Table II).[13] These anti-C2 scFv's were also derived from the V_H1 gene family. In two recent studies, anti-C2 antibodies encoded by aV_H1 derived germline segment were isolated as well.[14,15] The described anti-C2 antibodies can be divided into two groups derived from two different classes of germline genes. The first class of antibodies is derived from germline gene segments DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e) and the second class is derived from DP-5 (1-24) encoded germline gene segments (see Figure 3 Chapter 3). In chapter 2, we show that these two classes of anti-C2 antibodies bind to two different epitopes in the C2 domain of factor VIII. ScFv's encoded by DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e) compete with monoclonal antibody CLB-CAg 117 for binding to the carboxyl terminal part of the C2 domain while scFv's encoded by DP-5 (1-24) bind to an epitope which overlaps with the binding site of monoclonal antibody ESH4.[16,17] The epitope of the DP-5 (1-24) encoded antibodies involved residues at the amino- and carboxyl terminal part of the C2 domain.

In chapter 3, the characteristics of the V_H domains of scFv's isolated from the immunoglobulin repertoire of different patients are analysed in more detail.[18] V_H domains of antibodies specific for the A2 and A3-C1 domains of factor VIII are encoded by germline gene segments derived from both V_H1 and V_H3 family. Anti-A2 and anti-A3-C1 antibodies are encoded by V_H segments, which are commonly used in the normal IgG repertoire. In contrast, the anti-C2 antibodies described in chapter 2 are encoded by V_H gene segments, which are not preferentially expressed in the normal IgG repertoire.[19] A closer look at the structural elements of de V_H domains of the three DP-5 (1-24) encoded anti-C2 scFv's, scFv's that bind to the epitope defined by monoclonal antibody ESH4, revealed the presence of negatively charged amino acid residues that can react with positively charged residues in the C2 domain.[13] Recently, the three-dimensional structure of a complex of the antibody BO2C11 (a DP-5 (1-24) encoded antibody which has been isolated from a patient using Epstein Barr immortalisation) and the C2 domain was determined.[14,20] Comparison of the three DP-5 (1-24) encoded scFv's with the antibody BO2C11 showed that all antibodies have relatively small CDR3 regions and that the majority of the negative residues that react with the C2 domain of factor VIII are conserved. It is anticipated that more restricted usage of V_H germline segments by anti-C2 antibodies results from a selective recruitment of B cells expressing immunoglobulins with negatively charged V_H domains upon antigen stimulation. Our results indicate that naive B cells expressing immunoglobulins with a negatively charged DP-5 (1-24) encoded V_H domain are preferentially expanded following exposure tofactor VIII. Subsequent somatic hypermutation introduces point mutations in the variable regions, giving rise to high affinity antibodies. Selection of these B cells explains why DP-5 (1-24) encoded human

antibodies were isolated from the repertoire of haemophilia A patients while the DP-5 (1-24) germline gene segment is not dominantly expressed by B cells in the normal repertoire.

The V_H germline gene segment usage by anti-C2 antibodies that compete with monoclonal antibody CLB-CAg 117 is less restricted. We show that scFv's binding to the carboxyl terminal part of the C2 domain, which were isolated from the repertoire of patient A and patient B (Table II) are encoded by the related germline gene segments DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e).[11,13] However, the two scFv's described in chapter 4 are derived from different germline gene segments (patient C in Table II). ScFv VK29 is derived from DP-25 (1-03), a germline gene segment belonging to the V_H1 family that is not related to DP-10 (1-69), DP-14 (1-18) and DP-88 (1-e). ScFv VK33 is encoded by DP-79 (4-39), which belongs to the V_H4 gene family (see Figure 1 Chapter 3). A striking feature of all anti-C2 scFv's, which bind to the epitope defined by monoclonal antibody CLB-CAg 117 is the presence of a long CDR3 region (18-23 aa). Whereas the negatively charged residues in the VH domain determines the epitope specificity of the monoclonal antibody ESH4 competing anti-C2 antibodies, the exceptionally large CDR3 region may determine the epitope specificity of the monoclonal antibody CLB-CAg 117 competing anti-C2 antibodies. It has been postulated that factor VIII inhibitors may originate from the expansion of pre-existing natural anti-factor VIII B cell clones.[21-23] Natural autoantibodies are immunoglobulins reactive with self-antigens and the majority of natural autoantibodies are polyspecific. Because most natural autoantibodies are reactive with a variety of self-antigens, natural autoantibodies might provide the template for specific high-affinity autoantibodies or antigen-induced antibodies in patients with autoimmune diseases. It has also been suggested that polyreactive antibodies have long CDR3 regions in the heavy chain.[24] Aguilera et al. described the characteristics of eight polyreactive autoantibodies with long CDR3 regions in the heavy chains (9-20 aa).[24] These observations suggest that the anti-C2 antibodies specific for the epitope of CLB-CAg 117 may originate from natural anti-factor VIII antibodies with long CDR3 regions.

Table II Overview of the anti-C2 antibodies isolated from the IgG4 repertoire of three haemophilia A patients using phage display.

[1)] Patient A has been previously described as patient AMC-174. Anti-C2 scFv's isolated from the repertoire of this acquired haemophilia patient have been described by Van den Brink et al.[11]

[2)] Patient B is a mild haemophilia A patient with an Arg^{2163} to His mutation. Anti-C2 scFv's isolated from the repertoire of patient B are described in chapter 2.[133)] Patient C is also known as patient AMC-67, a mild haemophilia A patient with the Arg^{593} to Cys mutation described by Van den Brink et al. (see also chapter 6 this thesis).[25] Anti-C2 scFv's isolated from the repertoire of this patient are described in chapter 4.

	Patient A [1)]	**Patient B [2)]**	**Patient C [3)]**
Plasma:			
Percentage of inhibitory anti-C2 antibodies of total inhibitors	20%	80%	10%
Isolated scFv's using phage display:			
V_H germline gene segment	DP-10 (1-69) DP-14 (1-18) DP-88 (1-e)	DP-5 (1-24) DP-88 (1-e)	DP-25 (1-03) DP-79 (4-39)

Analysis of anti-A2 antibodies of mild haemophilia A patients using phage display

In chapter 5, we describe inhibitor development in a patient with mild haemophilia A caused by the Arg^{593} to Cys mutation.[26] Two previous studies have shown that inhibitory antibodies arising in patients with the same mutation were directed towards Arg^{484}-Ile^{508}, a major binding site for factor VIII inhibitors.[27,28] In patient AMC-92, a low level of antibodies with a different specificity was detected fifteen years after the onset of the first inhibitor.[29] The patient's antibodies were directed at residue Arg^{593} and could discriminate between exogenous and endogenous factor VIII. However, our analysis of the immunoglobulin repertoire reveals antibodies specific for residues Arg^{484}-Ile^{508} (chapter 5).[26] Surprisingly, antibodies reactive with Arg^{593} were not isolated from the repertoire of patient AMC-92 by phage display. Peripheral B cells expressing this specificity may be absent in the blood sample of 1993 that was used for construction of the library. However, we cannot exclude that limitations in our experimental protocol have precluded isolation of antibody fragments specific for Arg^{593}. For instance, we have used a variable immunoglobulin light chain repertoire of non-immune source for construction of our phage display libraries. Assembly of human antibodies reactive with residue Arg^{593} may require the presence of a particular immunoglobulin light chain that is not represented in the repertoire we have used.

The isolated scFv's specific for Arg^{484}-Ile^{508} and analysis of antibodies present in the plasma of patient AMC-92 during a second high titre inhibitor period indicated the presence of cross-reactive antibodies specific for residues Arg^{484}-Ile^{508} (chapter 5).[26] Cross-reactive antibodies recognize both administrated (exogenous) factor VIII and the patient's mutated (endogenous) factor VIII. Our immunoprecipitation data suggest that cross-reactive antibodies co-exist with a persisting level of 'true' alloantibodies that selectively recognise a B cell epitope overlapping with amino acid Arg^{593} in patient AMC-92. The latter population of antibodies was not detected in two other unrelated inhibitor patients with the same genetic defect.[27,28] We propose that the region Arg^{484}-Ile^{508} constitutes an immunodominant epitope that may obscure B cell epitopes overlapping Arg^{593}. This may explain the rare occurrence of antibodies reactive with Arg^{593} in patients with the Arg^{593} to Cys mutation. Interestingly, antibodies reacting exclusively with exogenous factor VIII have until now been observed in three patients with the Arg^{2150} to His mutation.[30,31] Apparently, B cell clones reactive with Arg^{2150} dominate the anti-factor VIII repertoire of this group of patients. At present it is not clear why in some patients with mild haemophilia A true alloantibodies exist whereas in plasma of most patients cross-reactive antibodies against the major inhibitor epitopes on factor VIII are found.

We proposed the following mechanism of inhibitor development in patient AMC-92. Fifteen years after the onset of the first inhibitor in patient AMC-92, a low level of antibodies directed against Arg^{593} are still present in the patient's plasma. Our analysis of the immunoglobulin repertoire expressed by peripheral B cells reveals antibodies specific for residues Arg^{484}-Ile^{508} (Figure 1, upper panel). These cross-reactive antibodies are not observed in plasma at this time point but were probably present at the onset of the first inhibitor when the patient had a high inhibitor titre and reduced factor VIII levels. The antibody titre decreased which coincided with a increase of plasma factor VIII levels. However, a low level of antibodies directed against the Arg^{593} epitope present on exogenous factor VIII peristed for an extended period of time. Following a second period of

factor VIII replacement therapy, cross-reactive antibodies, directed against Arg^{484}-Ile^{508}, are present in plasma (Figure 1, lower panel). We suggest that memory B cells expressing antibodies directed against Arg^{484}-Ile^{508} differentiate into antibody-secreting plasma cells upon treatment with factor VIII. This explains the rapid appearance of anti-A2 antibodies and decrease of factor VIII levels in patient AMC-92. The V_H gene library of patient AMC-92 originates from RNA isolated from peripheral blood lymphocytes. The B cell pool in peripheral blood consists of naive B cells (60%) and memory B cells (40%). Peripheral memory B cells are defined by expression of the CD27 surface antigen and the presence of somatic hypermutations.[32] Memory B cells can differentiate into antibody-secreting plasma cells upon stimulation with antigen.[33-35] We suggest that scFv's reactive with residues Arg^{484}-Ile^{508} of patient AMC-92 were isolated from peripheral memory B cells. Further studies should address whether the phage display repertoire of factor VIII inhibitors indeed originates from peripheral memory B cells. The analysis of inhibitor development of patient AMC-92 could be relevant for treatment of other haemophilia A patients. Knowledge on the presence of peripheral factor VIII-specific memory B cells can be helpful in predicting whether an anamnestic response will occur in haemophilia A patients with a history of inhibitor development.

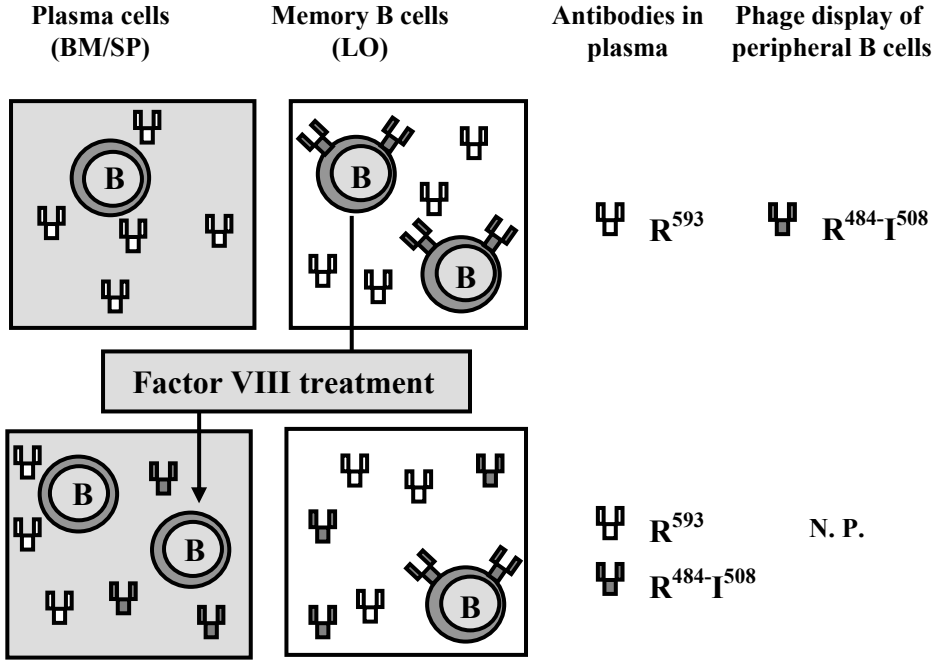

Figure 1. Proposed mechanism of inhibitor development in patient AMC-92.
Factor VIII replacement therapy stimulates the differentiation of peripheral memory B cells expressing surface immunoglobulins directed against Arg^{484}-Ile^{508} into plasma cells that rapidly produce cross-reactive antibodies (indicated in gray). Memory B cells expressing antibodies reactive with Arg^{484}-Ile^{508} are mainly present in secundary lymphoid organs (LO). Plasma cells secreting antibodies directed against Arg^{593} (indicated in white) are present in bone marrow (BM) and spleen (SP) for extended periods (> 8 years). In the right panel, the results from immunoprecipitation analysis (Antibodies present in plasma) and the results from phage display analysis (Phage display of peripheral B cells) are summarized. Phage display analysis after factor VIII treatment was not performed (N.P.).

ScFv's reactive with Arg^{484}-Ile^{508} have been isolated from the immunoglobulin repertoire of patient AMC-92 using phage display.[26] Two different clones reactive with Arg^{484}-Ile^{508} were isolated from the patient's IgG4-specific repertoire. The majority of scFv's, represented by scFv 92-102, were derived from heavy chain gene segment DP-47 (3-23), belonging to the V_H3 family (Table III). The same V_H segment was observed for scFv VK41, an anti-A2 antibody that was derived from a phage library of a different patient (11)with the Arg^{593} to Cys mutation (patient AMC-67).[28] ScFv VK41 is directed against amino acid residues Asp^{712}-Ala^{736} a binding site in the acidic region following the A2 domain. Despite the common of V_H segment DP-47, scFv 92-102 and scFv VK41 recognise distinct antigenic sites in the A2 domain of factor VIII. Inspection of the pattern of somatic hypermutation of the V_H domain of scFv 92-102 reveals a deletion of three amino acids in the CDR2 region (Table III). Small deletions and/or insertions are observed in approximately 6% of the human B cell repertoire.[36] The CDR2 region is a common site for deletions/insertions of amino acids although deletions of three amino acids are infrequently observed.[32,37] Modelling studies suggest that sequence variations in this part of the CDR2 changes the surface properties, however, they may not alter the main-chain structure of the antibody molecule.[38] Only one isolated scFv was encoded by V_H segment DP-58 (clone 92-137). V_H gene segment DP-58 is infrequently used in the immunoglobulin repertoire and Matsuda and co-workers have not mapped this segment to a functional locus.[32,39,40] However, the sequence of clone 92-137 shows several amino acid mutations from the germline DP-58 and is therefore likely to be derived from an antigen stimulated B cell. Previously, another scFv specific for the major epitope Arg^{484}-Ile^{508} was isolated from mild haemophilia A patient AMC-67.[28] This scFv (VK34) was encoded by germline gene segment DP-10 (1-69), belonging to the V_H1 family (Table III). Anti-A2 antibodies in these two inhibitor patients are derived of V_H1 and V_H3 gene segments, which are present in 70% of peripheral IgG+ B cells in the normal repertoire.[19] Apparently, anti-A2 antibodies in mild haemophilia A patients with the Arg^{593} to Cys mutation are encoded by V_H gene segments which are commonly used in the normal repertoire.

Table III. Comparison of deduced protein sequence of the heavy chains of anti-A2 specific human antibodies with variable heavy chain germline gene segments.

FR, framework region; CDR, complementarity-determining region. Dashes indicate sequence identity to variable heavy chain germline gene segments. Lower case indicates amino acid substitution encoded by the PCR primers. Sequences are available from Genbank under accession numbers: AY052530 (V_H 92-102); AF217790 (V_H VK41); AY052532 (V_H 92-137); AF217789 (V_H VK34).

```
                        FR1              CDR1       FR2             CDR2
                ------------------------------ ----- -------------- -----------------

                         1         2         3           4          5          6
                123456789012345678901234567890 12345 67890123456789 012a3456789012345

DP-47 (3-23)    EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS WVRQAPGKGLEWVS AISGSGGSTYYADSVKG
92-102          q---q-------------G--------R-- DF--T ---HS--R------ T---G-D..-----Q-
VK41            ----v----D--------------------- NF-----------------A --G-RS-T-F-------

DP-58           EVQLVESGGGLVQPGGSLRLSCAASGFTFS SYEMN WVRQAPGKGLEWVS YISSSGSTIYYADSVKG
92-137          q----q----------------T------- A---- -------------- ------T-K-H------

DP-10 (1-69)    QVQLVQSGAEVKKPGSSVKVSCKASGGTFS SYAIS WVRQAPGQGLEWMG GIIPIFGTANYAQKFQG
VK34            ------------------------------ -H--- -------------- D----L--G--------


                          FR3                  CDR3         FR4
                --------------------------------- ------------ -----------
                                                    1               1
                    7         8          9          0               1
                6789012345678901abc345678901234 567890abcde12 3456789012

DP-47 (3-23)    RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK
92-102          --A-------------L--------------- ESFSGNLGDAFDI WGQGTMVTVS
VK41            ------------V--E----------I----- GRGGYKYYGMDV. WGQGTTVTVS

DP-58           RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR
92-137          ------------------------------- DLEGLFPATL... WGQTMVTVSS

DP-10 (1-69)    RVTITADESTSTAYMELSSLRSEDTAVYYCAR
VK34            -----------------T-T---------EL DWFYI........ WGQTMVTVSS
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HLA genotype and factor VIII inhibitors in mild haemophilia A patients with an Arg^{593} to Cys mutation

We have studied the inhibitor development in a cohort of 42 patients with mild haemophilia A. All these patients have a missense mutation in the A2 domain of the heavy chain of factor VIII that causes a substitution of an arginine at position 593 to a cysteine. In this cohort, four patients developed a high titre of inhibitor following factor VIII replacement therapy. Inhibitor development of two high responder patients from our cohort and one high responder patient with the Arg^{593} to Cys mutation from a different study have been described previously.[25-29] In chapter 6, we describe the epitope specificity of the anti-factor VIII antibodies of another high responder patient (patient D). Remarkably, all high responder patients with the Arg^{593} to Cys mutation develop antibodies directed against the A2 domain and three patients develop antibodies specific for the light chain as well (Table IV). The predominant inhibitors in these patients are cross-reactive A2 antibodies, most

likely directed against the major inhibitor epitope Arg^{484}-Ile^{508}, whereas only a minor contribution of anti-light chain antibodies is observed. These data suggest that patients with a missense mutation in the heavy chain of factor VIII are less tolerant for the heavy chain compared to the light chain. One explanation for this phenomenon can be that when exposed to wild-type factor VIII, the patient's immune system recognises the heavy chain as non-self while whereas the light chain is recognised as self. Factor VIII-specific T cells will recognize that portion of factor VIII carrying the Arg^{593} residue and will provide T help for antibody formation predominantly targeting the non-self part of the factor VIII protein. Another explanation can be that after initiation of the immune response to factor VIII, light chain-specific T cells and/or light-chain specific B cells become anergic because of circulating levels of wild-type factor VIII light chain. At present it is not fully understood why high responder patients with the Arg^{593} to Cys develop inhibitory antibodies that predominantly target the A2 domain of factor VIII.

Table IV. Epitope specificities in high responder patients with the Arg^{593} to Cys mutation.

High titre patients have been described elsewhere: [1)] patient AMC-67: van den Brink *et al* 1999 and as patient C in chapter 6 this thesis; [2)] patient F: Thompson *et al* 1997; [3)] patient D: chapter 6 this thesis; [4)] as patient A: Fijnvandraat *et al* 1997 and as patient AMC-92:, Bril *et al* 2002 (chapter 5, this thesis) and also as patient A in chapter 6 in this thesis.[25-27,29]

Patient	Antibody epitope specifities	Major inhibitor epitope
AMC-67 (C) [1)]	cross-reactive A2, LCh, C2	A2
F [2)]	cross-reactive A2, LCh, C2	A2
D [3)]	cross-reactive A2, LCh	A2
AMC-92 (A) [4)]	wild-type reactive A2, cross-reactive A2	A2

Several studies have provided evidence that inhibitor development in haemophilia A patients is T-cell dependent. Anti-factor VIII antibodies are usually extensively hypermutated and of IgG4 subtype which points at a role for T cells in inhibitor formation.[11-14,26.28,41] It has been shown that factor VIII-reactive T cells are present in the circulation of haemophilia A patients with inhibitors.[42] Also, the disappearance of factor VIII inhibitors in haemophilia A patient with declining $CD4^+$ T cell counts caused by HIV infection indicate that inhibitor formation in T cell dependent.[43] Reding et al. observed anti-factor VIII $CD4^+$ T cells in congenital and acquired haemophilia A patients but also in healthy individuals.[44] Recently, three factor VIII-specific T cell clones were isolated from a mild haemophilia A patient.[45] Several studies in haemophilic mice have shown that the antibody response in murine haemophilia A is T cell dependent as well.[46-48] We hypothesize that in patients with the Arg^{593} to Cys mutation the immune response to infused factor VIII is initiated by factor VIII-specific T cells that recognize a factor VIII peptide containing the residue Arg^{593} as non-self (Figure 2). Following factor VIII infusion, antigen presenting cells (APC's) will endocytose and degrade the factor VIII protein. Antigen-derived peptides are bound by HLA class II alleles and presented to $CD4^+$ T cells. As peptides containing the Arg^{593} residues will only bind to the T cell receptor (TCR) in the context of a matching

HLA class II molecule, we investigated whether the presence of a particular HLA class II allele is related to inhibitor development patients with the Arg^{593} to Cys mutation. We observed in three (out of four) high responder patients the presence of haplotype HLA-DQB1*06, DRB1*13. However, it should be noted that two of these patients are twin brothers. In the fourth high responder patient haplotype HLA-DQB1*05, DRB1*01 was found. Interestingly, haplotype HLA-DQB1*05, DRB1*01 was raised in the low responder group compared to non inhibitor patients in our study and has also been associated with inhibitor development in patients with severe haemophilia A.[49]

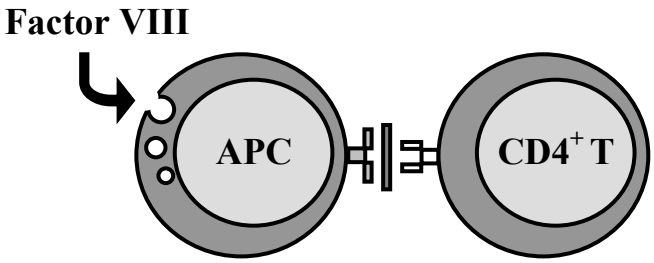

Figure 2. Antigen presentation of a factor VIII peptide bound to an HLA class II allele on the surface of an antigen-presenting cell (APC) to the T cell receptor (TCR) on the surface of a $CD4^+$ T cell.

Analysis of the amino acid sequence of factor VIII reveals that peptide fragments containing residue Arg^{593} are likely to bind to relevant HLA class II molecules in the high responder patients from our study (Figure 3). Peptides encompassing Arg^{593} were predicted to bind to HLA-DRB1*0101, 1101, 1302 and 1501 by an HLA class II binding peptide prediction server based on quantitative matrices.[50] These results indicate that antigenic peptides containing Arg^{593} can be presented to T cells in the context of multiple HLA class II molecules.

Recently, Jacquemin and co-workers have characterised three factor VIII-specific T cell clones from a mild haemophilia A patient with an Arg^{2150} to His mutation in the C1 domain of factor VIII.[45] This patient developed a high titre of inhibitory antibodies specific for wild type and not self factor VIII. The T cell clones recognized a peptide containing residue Arg^{2150} and did not recognize the mutated peptide containing His^{2150}. Interestingly, the peptide encompassing Arg^{2150} was able to bind to a large array of HLA-DR molecules. Binding of antigenic peptides to multiple HLA class II alleles can explain the increased frequency of inhibitor development in patients with the Arg^{2150} to His and Arg^{593} to Cys mutation and can also explain the weak associations observed between inhibitor development and HLA class II alleles.

hufVIII E N R S W Y L T E N I Q R F L P N P A G V Q L

DR*0101/02	**I Q R F L P N P A**
DR*1101	**I Q R F L P N P A**
DR*1101	**W Y L T E N I Q R**
DR*1302	**I Q R F L P N P A**
DR*1302	**Y L T E N I Q R F**
DR*1501	**I Q R F L P N P A**

Figure 3. Peptide prediction of wild type factor VIII to HLA-DR alleles present in high responder patients with the Arg^{593} to Cys mutation by the MHC class II binding peptide prediction server ProPred.[50]

Peptide prediction of factor VIII peptides to HLA-DRB1*04, which is present in one high responder patient was not determined because this allele was not subtyped.

A murine model for inhibitor development in haemophilia A patients

In chapter 7 we present a new murine model for inhibitor development in haemophilia A patients. We have created a mouse transgenic for human factor VIII harbouring the Arg^{593} to Cys mutation, which causes mild haemophilia A in humans. Liver -specific expression of the transgene was directed by the murine albumin promotor. Nevertheless we could not detect human factor VIII in plasma by sensitive ELISA and western blotting techniques. The transgenic hufVIII-R593C mice were crossed with the murine factor VIII knock out mice (E-16 KO) generated by Bi and co-workers.[50,51] E-16 KO mice have less than 1 % factor VIII activity in plasma and display a severe bleeding tendency. Although we did not study the phenotype in detail, we observed that hufVIII-R593C/E-16 KO have an impaired hemostasis and bleed to death after tail clipping as well. Introduction of the hufVIII-R593C gene did not reduce the severe phenotype of the E-16 KO strain in hufVIII-R593C/E-16 KO mice. Injections experiments performed in chapter 7 clearly show that transgenic hufVIII-R593C/E-16 KO mice are tolerant for *i.v.* injected human factor VIII in contrast to E-16 KO mice, which develop anti-factor VIII antibodies following injections of factor VIII. We show in chapter 7 that hufVIII-R593C/E-16 KO do not develop anti-factor VIII antibodies and have no factor VIII-specific T cell proliferation following serial *i.v.* injections of human factor VIII. The observed tolerance to factor VIII in transgenic HufVIII-R593C/E-16 KO mice is in agreement with the low inhibitor incidence in mild haemophilia A patients.

Following factor VIII replacement therapy, inhibitor development has been observed in some mild haemophilia A patients with the Arg^{593} to Cys mutation.[25-27,29] We investigated whether our transgenic mouse model can be used as a model for inhibitor development in haemophilia A patients. We increased pro-inflammatory conditions by injecting factor VIII *s.c.* in the presence of an adjuvant to provoke an immune response to factor VIII. We observed significant anti-factor VIII antibody responses in transgenic hufVIII-R593C mice in both wild type and E-16 KO background. These results show that an anti-factor VIII immune response can be evoked in our transgenic model and we therefore conclude that hufVIII-R593C mice are a valuable model for inhibitor development in haemophilia A.

Inhibitors which arise in haemophilia A patients, bind factor VIII at restricted regions in the A2, A3 and C2 domain of factor VIII.[3-7] In a previous study, a first attempt has been made to investigate whether factor VIII knock out mice exhibit an anti-factor VIII antibody response with epitope specificities similar to that observed in patients[53]. Antibodies obtained from immunized E-17 KO mice (another factor VIII knock out strain) were tested for their reactivity with thrombin cleavages products of recombinant human factor VIII. The antibodies were directed to the heavy chain, light chain and the non-functional B domain of factor VIII.[53] We have investigated the epitope specificity of anti-factor VIII antibodies in immunized E-16 KO mice by immunoprecipitation analysis with radiolabelled recombinant human factor VIII constructs. Preliminary data show that the antibodies were predominantly directed to the light chain whereas limited reactivity was observed with the A2 and C2 domain of factor VIII. Since these results were obtained from one single experiment, we cannot exclude that we underestimate the binding to A2 and C2 due to limitations in our experimental protocol. Both factor VIII knock out strains have been constructed by targeted disruption of part of the factor VIII gene that encodes factor VIII light chain. Surprisingly, both the E-16KO and E-17KO mice do produce some amounts of non-functional factor VIII heavy chain.[54] Despite the presence of circulating murine factor VIII heavy chain, both knock out strains can develop antibodies directed to the heavy chain of human factor VIII. Overall, it can be concluded that the antibody specificities of factor VIII knock out mice resemble the immune response in haemophilia A patients. Future studies will have to address whether the antibody specificities observed in transgenic hufVIII-R593C mice resemble the antibody specificities of mild haemophilia A patients with the Arg^{593} to Cys mutation.

Factor VIII-specific $CD4^+$ T cells which drive the synthesis of inhibitors have been detected in haemophilia A patients as well as in the factor VIII knock out mice.[42,44-48] Classically, $CD4^+$ T cells are divided in Th1 and Th2 cells. Th1 cells secrete pro-inflammatory cytokines such as IFN-γ and IL-2 whereas Th2 cells secrete IL-4 and IL-10. Both $CD4^+$ T cell populations secrete cytokines that drive B cell differentiation and antibody synthesis. Th1 cells mediate the synthesis of IgG subclasses that bind complement (IgG1 and IgG2 in humans) and Th2 cells drive the synthesis of IgG4 antibodies which are associated with inhibitor development in humans.[12] Recently, the distribution of Th1- and Th2-induced anti-factor VIII IgG subclasses in severe haemophilia A patients has been studied.[55] The results from this study indicate that Th1 driven antibody synthesis may be more important than previously appreciated. The authors suggested that Th2-driven synthesis of inhibitors occurs when the anti-factor VIII antibody response in high and intense, while Th1-driven synthesis of inhibitors occurs when the antibody response is low. It was suggested that Th1 cells might be involved in long-term maintenance of anti-factor

VIII antibody synthesis. These observations are in agreement with the observed immune responses in the factor VIII knock out mice. Haemophilic mice developed both Th1 and Th2 cell responses following immunization with factor VIII. Factor VIII dependent T cells produce both Th1- and Th2-specific cytokines. Also, anti-factor VIII antibodies are of subclass IgG1 (Th2-driven) and IgG2 (Th1-driven).[47,48,56] It is of interest to determine cytokine profiles and IgG subclass distribution in transgenic huFVIII-R593C/E-16 KO mice following immunization with human factor VIII.

Tolerance to factor VIII is likely maintained by ignorance or anergy of auto-reactive T and B cells in the hufVIII-R593C/E-16 KO mice and mild haemophilia A patients. Anergic T and B cells are tuned down but are still capable of participating in an immune response (reviewed by Goodnow).[57] Following subcutaneous injections of factor VIII with an adjuvant, an anti-factor VIII antibody response was observed in our mouse model which suggests activation of anergic factor VIII reactive T and B cells. The activation threshold of anergic T and B cells is raised and a strong immunogenic stimulus is required to activate anergic T and B cells. Apparently, subcutaneous immunization with factor VIII in the presence of an adjuvant provided a strong stimulus for anergic T and B cells and partial loss of tolerance to factor VIII was observed. In some mild haemophilia patients loss of tolerance to factor VIII is observed following peri-operative factor VIII treatment. Pro-inflammatory conditions and a high antigen load may provide a strong stimulus for anergic factor VIII reactive T and B cells in humans. Besides inflammatory conditions, HLA class II profile may be related to loss of tolerance to factor VIII.[50,58] In chapter 6, we evaluated the role of HLA class II alleles in inhibitor development in mild haemophilia A patients with the Arg^{593} to Cys mutation. We observed a weak association but more patients should be studied to link a particular HLA class II allele with inhibitor formation in patients with the Arg^{593} to Cys mutation. It is interesting to investigate the role of HLA class II alleles and loss of tolerance to factor VIII in our mouse model. Chimeric human/mouse MHC class II molecules containing the antigen-binding region of HLA-DR alleles found in inhibitor patients can be introduced in the hufVIII-R593C/E-16 KO mice.[59-61] HufVIII-R593C/E-16 KO mice transgenic for chimeric human/mouse MHC class II alleles would provide a powerful tool to study the role of HLA class II alleles in inhibitor development. It is likely that both inflammatory conditions and immune response genes like HLA class II alleles contribute to inhibitor development in mild haemophilia A.

Future directions

The studies presented in this thesis aim at a better understanding of the immunobiology of inhibitor development in mild haemophilia A. Detailed knowledge of the characteristics and epitope specificities of anti-factor VIII antibodies can be applied for the construction of recombinant factor VIII variants with reduced immunogenicity. Recent studies that employ human-porcine hybrids suggest that it is indeed possible to decrease the antigenicity of factor VIII.[62] However, factor VIII is a large molecule with multiple potential T cell and B cell epitopes. At present it is not clear whether elimination of immunodominant regions will result in factor VIII variants with a decreased immunogenicity. Studies directed at the interactions of factor VIII specific B and T cells are perhaps more promising. Prevention of immune responses to factor VIII by interfering with co-stimulatory signals has already been achieved in haemophilic mice. Despite these promising results long-lasting tolerance to

factor VIII could not be established in a reproducible manner. Obviously, there is a need for alternative protocols for downregulation and modulation of inhibitor formation in haemophilia. The results presented in this thesis have provided detailed insight into the molecular characteristics of factor VIII inhibitors. Furthermore, several determinants potentially contributing to loss of tolerance in haemophilia A have been explored both in humans and a newly developed murine model for haemophilia A. Ultimately, a combination of these two approaches will help to identify candidate genes and treatment-related factors that are involved in inhibitor development in patients with haemophilia A. At the same time, it should be emphasized that despite regular treatment the majority of haemophilia A patients do not mount an immune response to factor VIII. Little information is present on (genetic and non-genetic) determinants that prevent immune responses to intravenously administered factor VIII in this group of patients. Clearly, this is an area of interest that given the availability of well-characterized cohorts of patients with identical genetic defects in the factor VIII gene should be actively pursued.

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Summary

The X-linked bleeding disorder haemophilia A is due to a deficiency or functional defect of coagulation factor VIII. The bleeding tendency can be corrected by administration of factor VIII concentrates. A serious complication of factor VIII replacement therapy is the development of anti-factor VIII antibodies (inhibitors) that neutralize factor VIII activity. In recent years, the epitope-specifities and the inhibitory mechanisms of factor VIII inhibitors have gained increasing interest. The generation of factor VIII knock-out mice has opened the possibility of studying the immunobiology of inhibitor formation in murine models of haemophilia A. In spite of these recent developments however, the immunological mechanisms underlying the anti-factor VIII immune response have remained poorly understood so far. Most of our current knowledge is based on studies on inhibitor formation in the severe form of haemophilia. However, inhibitors also occur in patients with mild haemophilia A, in particular after a period of extensive factor VIII replacement therapy. These patients differ from severe haemophiliacs in that they have low levels of circulating factor VIII activity (5-25% of normal). The presence of endogenous factor VIII may have major impact on the immune response to exogenous factor VIII during replacement therapy. The studies presented in this thesis were performed to obtain a better understanding of the immunobiology of inhibitor development in mild haemophilia A.

In the introduction (*chapter 1*), recent studies on the immunobiology of factor VIII inhibitors in haemophilia A patients are summarized and discussed. While factor VIII is a large protein comprising multiple domains (A1-A2-B-A3-C1-C2), the majority of factor VIII inhibitors bind to a few restricted regions in the A2, A3 and C2 domain and their inhibitory mechanism is well characterized. Several studies have addressed the T cell dependence of the immune response to factor VIII. Recent studies in a murine model for severe haemophilia A suggest that inhibition of T cell responses by co-stimulatory blockade provides a potentially promising approach for treatment and/or prevention of inhibitor formation.

We have characterized the anti-factor VIII antibodies in patients with mild haemophilia A employing phage display technology. In *chapter 2*, anti-C2 antibodies were isolated and characterized from the repertoire of a mild haemophilia A patient. Our results provide evidence for the presence of two classes of anti-C2 antibodies that recognize distinct antigenic sites in factor VIII. The characteristics of the anti-C2 antibodies were further analysed in *chapter 3*, and compared to the epitopes of previously described murine monoclonal antibodies. The first class of anti-C2 antibodies bind to the epitope defined by monoclonal antibody ESH4 and these antibodies are encoded by germline gene segment DP-5 (1-24). The second class of antibodies bind to the epitope defined by monoclonal antibody CLB-CAg 117. Antibodies belonging to this second class of antibodies were also isolated from a different patient with mild haemophilia A (*chapter 4*). The VH gene segment usage of the antibodies directed at the epitope defined by CLB-CAg 117 is less restricted compared to the first class of anti-C2 antibodies. This second class of anti-C2 antibodies is striking in that its CDR3 region, which is one of the antigen binding loops in the antibody molecule, is exceptionally large. Based on this property we argue that this class of anti-C2 antibodies originates from a pool of polyreactive human antibodies.

In *chapter 5*, we describe the inhibitor development of a patient with mild haemophilia A caused by an Arg^{593} to Cys mutation. We have isolated and characterized anti-A2 antibodies using phage display and we have performed epitope-mapping studies of anti-factor VIII antibodies in plasma using immunoprecipitation analysis. We show that in this patient cross-reactive antibodies specific for a major epitope in the A2 domain of factor

VIII co-exist with a persisting level of antibodies exclusively recognizing wild-type (and not endogenous) factor VIII. The data presented in *chapter 5* provide a possible explanation for anamnestic responses observed in patients with a history of inhibitor development. We propose that activation of a quiescent pool of memory B cells underlies the rise in inhibitor titre observed in haemophilia A patients with a history of inhibitor development.

Chapter 6 describes the epitope specificities of anti-factor VIII antibodies in another patient from our cohort of mild haemophilia A patients with the Arg^{593} to Cys mutation. Results from this chapter and previous studies show that high responder patients with the Arg^{593} to Cys substitution develop inhibitory antibodies predominantly directed at the A2 domain of factor VIII. This suggests that inhibitor formation proceeds via a common mechanism in these patients. The role of HLA class II alleles in inhibitor formation was investigated by HLA genotyping of 42 patients with the Arg^{593} to Cys mutation. We observed in three (out of four) high responder patients the presence of haplotype HLA-DQB1*06, DRB1*13. In the low responder group, a weak association of haplotype HLA-DQB1*05, DRB1*01 with inhibitor formation was observed. These data suggest a weak association between inhibitor development and HLA class II alleles in mild haemophilia A patients with the Arg^{593} to Cys mutation.

In *Chapter 7*, we present the characteristics of a mouse transgenic for human factor VIII with the Arg^{593} to Cys mutation (hufVIII-R593C mouse). The anti-factor VIII immune response was analysed in transgenic hufVIII-R593C mice crossed with factor VIII-deficient mice (exon 16 knock out, or E-16 KO mice). Serial intravenous injections of human factor VIII do not evoke an immune response in hufVIII-R593C/E-16 KO mice. The introduction of the human factor VIII–R593C transgene renders the mice tolerant to human factor VIII. However, when hufVIII-R593C/E-16 KO mice were subcutaneously injected with factor VIII in the presence of an adjuvant, loss of tolerance to factor VIII was observed. The results of *chapter 7* demonstrate that hufVIII-R593C/E-16 KO mice provide a valuable model for studies directed at the mechanisms underlying inhibitor development in haemophilia A.

Finally, *chapter 8* provides a general overview that discusses the implications of our findings.

Samenvatting

Hemofilie A is een bloederziekte die wordt veroorzaakt door een tekort aan stollingsfactor VIII. Bloedingen bij patiënten met hemofilie A worden behandeld met het toedienen van factor VIII preparaten. Een ernstige en soms levensbedreigende complicatie bij deze behandeling is het ontstaan van antistoffen die de activiteit van factor VIII remmen. De afgelopen jaren hebben onderzoekers zich gericht op het ontdekken van de bindingsplaatsen van deze remmende antistoffen op factor VIII en ook de manier waarop antistoffen factor VIII kunnen remmen. De ontwikkeling van een factor VIII-deficiënte muis heeft het tevens mogelijk gemaakt om de immunologische aspecten van het ontstaan van remmende antistoffen tegen factor VIII te onderzoeken. Desondanks is onze kennis over het ontstaan van remmende antistoffen nog beperkt. De meeste studies zijn gericht op het ontstaan van remmende antistoffen bij patiënten met de ernstige vorm van hemofilie A maar remmende antistoffen komen soms ook bij milde hemofilie A patiënten voor. Remmende antistoffen kunnen bij milde hemofilie patiënten ontstaan na een periode van intensieve behandeling met factor VIII. Milde hemofilie A patiënten hebben een laag gehalte factor VIII in hun bloed (5-25 % ten opzichte van gezonde personen). De kleine hoeveelheid factor VIII in het bloed verklaart waarschijnlijk waarom het ontstaan van remmende antistoffen bij milde hemofilie patiënten minder vaak voorkomt vergeleken met ernstige hemofilie patiënten. Het onderzoek beschreven in dit proefschrift richt zich op de immunologische achtergrond van het ontstaan van remmende antistoffen tegen factor VIII bij milde hemofilie A patiënten.

In de inleiding *(hoofdstuk 1)* worden recente studies over het ontstaan van remmende antistoffen bij hemofilie A patiënten beschreven. Factor VIII is een groot eiwit en is opgebouwd uit verschillende domeinen (A1-A2-B-A3-C1-C2). Het is echter gebleken dat remmende antistoffen maar op een beperkt aantal plaatsen aan factor VIII binden (A2, A3 en C2 domein) en de manier waarop antistoffen de functie van factor VIII remmen is aangetoond. Andere onderzoekers hebben in een diermodel voor hemofilie A aangetoond dat de remming van de anti-factor VIII T cel response wellicht een mogelijkheid biedt om antistof ontwikkeling te voorkomen.

Met behulp van de faag display techniek worden remmende antistoffen tegen factor VIII geïsoleerd uit het gehele antistof repertoire van patiënten. In *hoofdstuk 2* wordt de isolatie van remmende anti-C2 antistoffen van een patiënt met milde hemofilie A beschreven. De resultaten wijzen erop dat er twee groepen van anti-C2 antistoffen zijn die op verschillende plaatsen aan factor VIII binden. De eigenschappen van de anti-C2 antistoffen worden verder geanalyseerd en vergeleken met monoklonale muizen antistoffen in *hoofdstuk 3*. Antistoffen in de eerste groep hebben dezelfde bindingsplaats als de monoklonale antistof ESH4 en de V_H domeinen van deze antistoffen zijn afkomstig van kiemlijn gen segment DP-5 (1-24). Antistoffen in de tweede groep hebben dezelfde bindingsplaats als de monoklonale antistof CLB-CAg 117. Antistoffen behorende bij deze tweede groep werden ook geïsoleerd uit het antistof repertoire van een andere milde hemofilie A patiënt *(hoofdstuk 4)*. Het V_H domein van deze groep antistoffen is niet afkomstig van één specifiek kiemlijn gen segment maar van verschillende gen segmenten. Een opvallende eigenschap van deze antistoffen is dat ze een uitzonderlijk lang CDR3 gebied bevatten. Deze laatste eigenschap doet vermoeden dat anti-C2 antistoffen ontstaan uit poly-reactieve antistoffen.

In *hoofdstuk 5* wordt het ontstaan van remmende antistoffen beschreven bij een milde hemofilie A patiënt. Bij deze patiënt is een Arg^{593}- Cys substitutie in het A2 domein van factor VIII de oorzaak van milde hemofilie A. Met behulp van de faag display techniek

werden anti-A2 antistoffen geïsoleerd en verder werden de anti-factor VIII antistoffen die aanwezig waren in het bloed van de patiënt gekarakteriseerd. De resultaten van *hoofdstuk 5* geven een mogelijke verklaring voor de snelle ontwikkeling van remmende antistoffen bij patiënten die al eerder remmende antistoffen in hun bloed hadden.

Hoofdstuk 6 beschrijft de karakterisering van anti-factor VIII antistoffen in een andere milde patiënt met de Arg^{593}- Cys mutatie. Resultaten van deze analyse en resultaten van andere onderzoekers wijzen erop dat patiënten die deze mutatie hebben en die een hoge antistof titer ontwikkelen, remmende antistoffen ontwikkelen die binden aan het A2 domein. Deze bevinding suggereert dat de ontwikkeling van remmende antistoffen bij patiënten met de Arg^{593}- Cys substitutie op dezelfde manier gebeurd. De invloed van HLA klasse II allelen op de ontwikkeling van remmende antistoffen werd onderzocht in een grote groep milde hemofilie patiënten met de Arg^{593}- Cys mutatie. Bij drie (van de vier) patiënten met een hoge antistof titer werd haplotype HLA-DQB1*06, DRB1*13 gevonden en bij patiënten met een lage antistof titer werd een zwakke associatie gevonden met haplotype HLA-DQB1*05, DRB1*01. De gegevens van de HLA-typering suggereren een zwakke associatie van HLA genen met het ontstaan van remmende antistoffen bij deze groep van milde hemofilie patiënten.

De karakterisering van een muis transgeen voor humaan factor VIII met de Arg^{593}-Cys substitutie (hufVIII-R593C muis) wordt in *hoofdstuk 7* beschreven. Het ontstaan van anti-factor VIII antistoffen werd geanalyseerd in transgene hufVIII-R593C muizen gekruist met factor VIII-deficiënte muizen (exon 16 knock-out, of E-16 KO muizen). Intraveneuze injecties met (humaan) factor VIII geven geen antistof ontwikkeling in de hufVIII-R593C/E-16 KO muizen. Dit geeft aan dat de transgene muis tolerant is voor humaan factor VIII. Deze tolerantie wordt doorbroken wanneer de injectie subcutaan worden toegediend en in aanwezigheid van Freund's adjuvant. Deze resultaten geven aan dat de hufVIII-R593C/E-16 KO muis een waardevol instrument zal zijn in verder onderzoek naar het ontstaan van remmende antistoffen bij hemofilie A patiënten.

Hoofdstuk 8 is een algemene discussie over het onderzoek beschreven in dit proefschrift.

Curriculum Vitae

Wendy Simone Bril werd op 26 januari 1975 te Giesbeek geboren. Na het behalen van het VWO diploma aan Het Rhedens Lyceum te Rozendaal, begon zij in 1993 met de studie Medische Biologie aan de Universiteit Utrecht. Tijdens deze studie liep zij stage bij de vakgroep Besmettingsleer aan het Eijkman-Winkler instituut voor Microbiologie, Universitair Medisch Centrum Utrecht (onder leiding van Dr. J.A.G. van Strijp en Dr. A.C. Fluit) en bij de vakgroep Immunologie, Universitair Medisch Centrum Utrecht (onder leiding van Prof. dr. T. Logtenberg). Het doctoraal examen werd behaald in april 1998 en in augustus van dat jaar begon zij als onderzoeker in opleiding bij de afdeling Plasma Eiwitten van het CLB (Sanquin Research) te Amsterdam onder leiding van Dr. J. Voorberg. Vanaf 1 november 2002 is zij werkzaam als post-doc bij de vakgroep Immunologie, Universitair Medisch Centrum Utrecht.

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