REVIEW ARTICLE

Pathogenic antibodies to coagulation factors. Part one: Factor VIII and Factor IX

P. LOLLAR

Winship Cancer Institute, Emory University, Atlanta, GA, USA

Introduction

Pathogenic antibodies targeting coagulation factors usually are clinically significant because they inhibit function. 'Circulating anticoagulants' were recognized as early as 1906 ([1], cited in [2]). They interfere with the coagulation of normal blood as well as that of the patient, which is the basis of the classical mixing study. Circulating anticoagulants inhibiting factor (F)VIII and factor (F)IX were identified in the early 1940s and 1950s, nearly as soon as these factors were discovered and differentiated from one another [2], and subsequently were shown to be antibodies [3]. Another circulating anticoagulant was identified in this era that did not appear specific for a single coagulation factor and frequently was associated with systemic lupus erythematosus. Although some of these patients had abnormal bleeding, a paradoxical association of the lupus anticoagulant with thrombosis was subsequently discovered [4].

Both the hemostatic and adaptive immune systems developed rapidly during early vertebrate evolution and then produced no major changes in their principle features over the next 450 million years. Fibrinogen, factor XIII, the vitamin K-dependent proenzymes and their procofactors, factor (F)V and FVIII, are present in fish, which were the first vertebrates [5,6]. The adaptive immune system, characterized by antigen-specific lymphocyte receptors, major histocompatibility class (MHC) molecules, and antibody production, is present in jawed, but not jawless, fishes, indicating that it evolved during the 50 million years between the divergence of these two vertebrate classes [7].

The hemostatic challenge to the early vertebrates was to plug hemorrhagic leaks while maintaining overall patency of the vascular system. This includes allowing effector cells of the immune system to cross the endothelial barrier. The immunological challenge was to kill invading microorganisms

Correspondence: P. Lollar, 1365-B Clifton Road, Suite B5100, Atlanta, GA 30322., USA.
Tel.: +1 404 727 5569; fax: +1 404 727 4859; e-mail: jlollar@emory.edu

and neutralize their toxic products using a defense system that did not produce self-inflicted injury. Vertebrate immune systems possess elaborate mechanisms to tolerate self-molecules and cells. It is interesting to consider hemostasis from the point of view of the immune system. A major hemostatic event is associated with deposition of products derived from fibrinogen and other coagulation factors at potentially immunizing levels. Specific and non-specific proteolytic fragmentation exposes possible neoepitopes, often in the setting of an associated inflammatory process with its attendant adjuvant effects. Thus, it seems surprising that autoantibody formation is not a common sequela to hemorrhage. In fact, autoantibodies to coagulation factors are rare: the incidence of clinically significant autoantibodies to FVIII, which is the most commonly targeted coagulation factor in autoimmunity, is only 0.2–1 per million people per year [8] and the incidence of autoantibodies to the other coagulation factors is considerably lower. Although tolerance to the coagulation proteins has not been studied, experimental models using complement component C5 as a model plasma protein have been developed and may be relevant to the development of inhibitors [9, 10]. Conversely, the recent emergence of methods to study the immunogenicity of FVIII and FIX that are described in this review may contribute to the general understanding of immunological tolerance.

Inhibitory antibodies to coagulation factors occur more commonly as an immune response to non-self protein, e.g. during factor replacement therapy for hemophilia A or B or during the use of bovine thrombin products in surgery. Here one may wonder why the incidence is not higher. For example, most patients with hemophilia A and apparent null mutations do not develop a detectable immune response after repeated exposure to FVIII. However, the usual peaceful coexistence of the coagulation and immune systems provides little consolation to the practicing hematologist in a tertiary care center, who sees inhibitor patients often enough. Patients with autoantibodies to FVIII or other coagulation factors often present with severe bleeding that is difficult and expensive to control. The development of inhibitory antibodies in hemophilia A and B frequently transforms these disorders from treatable to refractory and now is considered the most significant complication of treatment.

In part I of this review, immune mechanisms of antibody formation will be described and pathogenic antibodies to FVIII and FIX will be discussed in this context. In part II, pathogenic antibodies to other circulating components of the hemostatic system, including prothrombin, thrombin, FV, and von Willebrand factor (VWF) will be reviewed. The lupus anticoagulant antibodies associated with thrombosis, fetal loss and other morbid complications that make up a clinical constellation known as the antiphospholipid syndrome have been recently reviewed in this journal [11]. They typically interfere with phospholipid assembly in coagulation assays, but occasionally are associated with bleeding due to inhibition of specific coagulation factors, especially prothrombin. They will be discussed only in this latter context.

Antibody formation

An operative paradigm in immunology is that the vertebrate immune system evolved to discriminate infectious non-self from non-infectious self [12]. This included the development of a specific, adaptive response to infection, which produced two problems. One is how to prevent death from an invading organism during the time it takes for the adaptive response to develop. The second results from the mechanism used to produce the vast repertoire of the adaptive immune system. There are two arms in this system: cell-mediated immunity, which utilizes MHC class I-dependent cytotoxic CD8+ T cells as the effector arm to eliminate intracellular pathogens, and antibody-mediated (humoral) immunity, which primarily deals with extracellular pathogens and toxins. Because this repertoire intrinsically cannot distinguish self from non-self antigens, the problem of potential autoimmunity arises.

Both of these problems are addressed by the innate immune system, which is mediated partly by dendritic cells (DCs), macrophages, natural killer (NK) cells, natural killer T (NKT) cells, and neutrophils bearing Toll-like and other receptors for generic microbial products (e.g. lipopolysaccharide, mannans, glycans and CpG DNA motifs). Ligation of these receptors leads to a variety of effector mechanisms to kill invading microorganisms or to hold the infection at bay until the adaptive response can develop. It also has been proposed that the prevention of autoimmunity depends on the innate system. For example, it is usually difficult to raise antibodies to a non-self protein in the absence of adjuvants that contain microbial products. This has been termed the 'immunologist's dirty little secret' [13]. In addition to the antigen, antigen-independent products of infection and cellular damage of the host help promote the immune response. These include the microbial products involved in innate immunity and host proteins, such as heat shock proteins [13,14]. In the absence of such a 'danger' signal, there is no immune response to non-self antigens or to self-antigens, and autoimmunity is avoided.

The B-cell-mediated antibody response to so-called thymus-dependent antigens, which include soluble proteins like most of the coagulation proteins, requires MHC class II-dependent CD4+ T-cell help. This occurs in secondary lymphoid organs,

primarily the spleen and lymph nodes in the case of blood-born and extravascular antigens, respectively. Naive T cells are activated to proliferate and differentiate into T helper cells (Th cells) when their T-cell receptors (TCRs) recognize antigen on the surface of MHC class II molecules of antigen-presenting cells (APCs). DCs are considered the only effective APCs early in the immune response. DCs are a phenotypically diverse group of cells that reside in immature form in all peripheral tissues, including the spleen [15]. Immature DCs undergo a constitutive macropinocytosis that allows uptake of up to half their cell volume in extracellular fluid per hour, providing an efficient mechanism for non-specific internalization of antigen. Additionally, DCs can internalize some antigens by endocytosis using Fc receptors or mannose receptors. The antigen is proteolytically degraded to peptides for presentation on MHC class II molecules. Immature DCs are activated through engagement of Toll-like receptors, by inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor-α or by interaction with activated T cells. The resulting mature DCs migrate to secondary lymphoid organs, where they reside as interdigitating DCs and encounter naive T cells in extrafollicular areas.

DC activation leads to translocation of peptide–MHC class II complexes to the cell surface for presentation to the TCR on Th cells (Fig. 1). CD4 on Th cells is a coreceptor in this complex and binds to invariant residues on the MHC class II molecule. The length of peptides bound to MHC II molecules typically is 13–17 residues. MHC-restricted T-cell responses exhibit a high level of cross-reactivity, which occurs at two levels [16]. First, many T cells within the host repertoire are capable of responding to any given MHC II-associated peptide. Second, any given T cell can bind many MHC II-associated peptides. This allows the immune system to respond to any possible foreign peptide without requiring an unacceptably large number of naive T cells. However, it also creates the
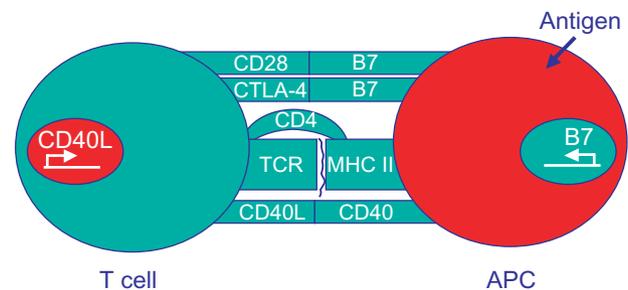

Fig. 1. Costimulation and T-cell help. Antigen-presenting cells (APCs) [dendritic cells (DCs), B cells or macrophages] present antigenic peptide-complexed MHC class II molecules to the T-cell receptor (TCR) on naive T cells (signal 1). Antigen is taken up by pinocytosis or endocytosis in DCs and by phagocytosis in macrophages. In contrast, B-cell uptake is mediated by membrane immunoglobulin and is antigen-specific. Engagement of the TCR leads to expression of CD40L on T cells, which upregulates CD80 (B7-1) and CD86 (B7-2) expression on the APC. Binding of B7 molecules to CD28 on T cells (signal 2) leads to activation, proliferation and differentiation of naive T cells into Th cells.

potential for cross-reactivity to self-molecules and autoimmunity. The mechanisms that prevent autoimmunity due to T-cell cross-reactivity are a matter of speculation.

By itself, TCR engagement by peptide–MHC II complexes is not sufficient for activation of naive T cells. In addition, a 'second signal' involving costimulatory molecules on T cells and APCs is required (Fig. 1). The costimulatory molecules include CD40, CD80 (B7-1), and CD86 (B7-2) on APCs and CD40L, CD28 and CTLA-4 on T cells. DC maturation leads to upregulation of CD40, CD80 (B7-1), and CD86 (B7-2). Engagement of the TCR leads to expression of CD40L on the T cell, which binds CD40 and further drives the expression of B7, CD40 and MHC class II molecules [17]. Binding of B7 molecules to CD28 leads to T-cell activation and differentiation into Th cells. B7 molecules also bind to CTLA-4 on T cells. The role of CTLA-4 has been controversial with both upregulatory and downregulatory functions being identified in different experimental systems. In addition to the activation of T cells by DCs, costimulation also results in the activation of DCs by T cells through a CD40L–CD40 mediated pathway [18].

B cells develop in the bone marrow and move to the secondary lymphoid organs. These naive, mature B cells bear IgM or IgD membrane immunoglobulin as part of the B cell receptor (BCR) complex. Naive B cells die in the periphery within a few days unless they find antigen using the BCR. B cells encounter antigen-specific T cells in extrafollicular areas of secondary lymphoid organs and a B cell–T cell–DC triad forms. During the primary humoral response, recognition of antigen by the BCR leads to a signaling pathway that turns the B cell into an APC (Fig. 1). The B-cell epitope recognized by membrane immunoglobulin is the native antigen and is the region that will ultimately be recognized by the mature antibody. The BCR–antigen complex is internalized, proteolytically degraded and the antigenic peptides are presented on the surface of MHC class II molecules.

Some B cells immediately differentiate into antibody-secreting plasma cells (ASCs), which provide early defense against infection. The remaining majority of the activated B cells migrate to primary follicles, where they proliferate and produce germinal centers (GCs), forming secondary follicles. Somatic hypermutation and affinity maturation occur in the GC, where BCRs are continuously exposed to antigen. High-affinity B-cell mutants capture antigen, process it and present it to GC T cells. Low-affinity mutants are deleted. As the immune response progresses, B cells become increasingly potent APCs because of the development of high-affinity BCRs that capture antigen [19]. The Ag-specific T cells that interact with high-affinity mutants upregulate CD40L and secrete IL-4. This results in the expansion and isotype switching of high-affinity mutants, which either differentiate into memory B cells or ASCs. Memory B cells and a subpopulation of ASCs stay resident in the secondary lymphoid organs. Other ASCs migrate to the bone marrow where they can remain for years and provide long-lasting immunity. During the secondary humoral response memory B cells are activated in extrafollicular areas, producing plasma cells and more GC founder cells.

Helper T cells have been classified as Th1 and Th2 cells following the original observation that CD4 + T-cell clones could be distinguished into two distinct sets based on cytokine secretion patterns [20]. Th1 cells are characterized by secretion of interferon (IFN)-γ and IL-2, whereas Th2 cells secrete IL-4 and IL-5. IFN-γ activates macrophages and stimulates B-cell production of complement-fixing IgG antibodies (IgG1 and IgG3 in humans, IgG2a and IgG3 in mice). The principal function of Th1 cells is to elicit phagocyte-mediated defense against infection. IL-4 induces immunoglobulin class switching to IgE and IL-5 is the principal eosinophil-activating cytokine. Thus, Th2-mediated immune responses occur in allergy and in helminthic infections. Th2 cells provide B-cell help and stimulate production of high levels of IgM and non-complement-fixing IgG subclasses (IgG4 in humans, IgG1 in mice). In the murine system, IgG1 also can be supported by Th1 cells. The Th1/Th2 paradigm has evolved to include other cytokines that are not secreted by CD4 + T cells, but that promote the development of Th cells. Thus, IL-12 and IL-13 have been assigned to the Th1 and Th2 group of cytokines, respectively. IL-10 was originally assigned to the Th2 group. However, it has several functions, which makes it difficult to classify IL-10-producing cells.

In some naturally occurring infections and in model experimental systems, there is an increasing polarization toward the Th1 or Th2 phenotype as the immune response progresses. For example, in tuberculoid leprosy there is strong delayed hypersensitivity, a weak antibody response and a Th1-like phenotype. In contrast, lepromatous leprosy is characterized by a strong antibody response, weak delayed hypersensitivity and a Th2-like phenotype [21]. Polarization involves inhibition of Th1 responses by the Th2 cytokines IL-4, IL-5 and IL-10 and inhibition of Th2 responses by Th1 cytokines, most notably IFN-γ. However, extremely polarized Th1/Th2 responses are the exception to the rule in naturally occurring infections [22]. Additionally, Th1-type and Th2-type cells can coexist in immune animals, which is not explained by a model in which Th1 and Th2 cells mutually downregulate each other.

The binding energy of antigen–antibody complexes is dominated by amino acid side chain–side chain interactions. Typically, 20–25 amino acid side chains of both the antigen and antibody are in contact [23]. The antigenic determinant (B-cell epitope) corresponds to a footprint that the antibody produces. However, usually only a few of the antigen–antibody side chain interactions underneath the footprint contribute significantly to the binding energy [24]. Studies using model antigens have led to the conclusion that the entire surface of a protein is potentially a target for antibodies [25]. This is based on the finding that murine monoclonal antibodies covering essentially the entire surface topography can be produced to small model protein antigens such as ovalbumin and myoglobin, at least after immunization with large doses of antigen in strong adjuvants. However, the antibody response in nature is generally directed against a limited number of immunodominant epitopes. For example, the immune response to the 76-kDa influenza A hemagglutinin heterodimer is directed

against four immunodominant B-cell epitopes that cover only a fraction of the protein surface [26]. Analysis of the polyclonal response to single B-cell epitopes reveals considerable heterogeneity when the component monoclonal antibodies are studied. An exhaustive analysis of the number of antihemagglutinin murine B-cell hybridomas produced in response to infection with influenza A strain produced an estimate of 1500 different antibodies in the repertoire directed against the four immunodominant epitopes [27]. Thus, an immunodominant epitope could be viewed as an area underneath an antibody footprint, or set of overlapping footprints, in which there is considerable variation at the clonal level in the atomic contacts that determine the strength of the interaction.

The response of T cells and APCs, including B cells, to the same antigen is called linked recognition. However, protein epitopes recognized by the TCR generally do not contain sequences that are part of the B-cell epitope. The B-cell epitope of some antigens is protected from proteolytic degradation and MHC class II presentation by being bound to the BCR, leading to a bias against T–B-cell epitope overlap [28]. However, there are examples in which B- and T-cell epitopes do overlap [29]. Additionally, in some cases antibody binding to antigen can enhance the presentation of one T-cell determinant while simultaneously suppressing the presentation of a different T-cell determinant within the same antibody footprint [30].

MHC class I or II-restricted T-cell epitopes that develop in response to immunization with model proteins such as cytochrome c, lysozyme, and insulin or microbial proteins, such as influenza hemagglutinin, staphylococcal nuclease and malarial CS protein, tend to be limited to one or a few dominant sites [31]. In fact, immunodominance of T-cell epitopes is more generally recognized than immunodominance of B-cell epitopes. Antigen processing plays an important role in the development of T-cell epitopes, but it is not possible to predict T-cell epitopes based on sequence or structure of the native protein.

The study of immunogenicity is intimately related to that of tolerance. Tolerance is a physiological state in which the immune system does not react destructively either against self-components or against non-self antigens to which it is exposed. For developing T cells with high affinity for self-antigens, tolerance takes place in the thymus. Induction of tolerance in the thymus is referred to as 'central', as distinguished from the peripheral tolerance that may develop among already mature T cells when they encounter antigen with medium affinity in the peripheral tissues. B cells can also be rendered tolerant in a process that also occurs outside the thymus.

Central T-cell tolerance is accomplished by clonal deletion of autoreactive T cells. Deletion, anergy, receptor downregulation, and ignorance have been shown to be responsible for T- and B-cell peripheral tolerance, depending on the particular model system studied. Increasing evidence has accumulated that regulatory CD4+ T cells play a role in peripheral T-cell tolerance [32]. A revised version of the Bretscher–Cohn theory of immunogenicity and tolerance proposed in 1970 has been proposed based on the costimulation model and 'danger' theory of immunogenicity described above [33,34]. In this model, antigen-specific signaling through the BCR or the TCR and a non-specific second signal are required for B-cell and T-cell activation, respectively. In the absence of both signals, a state of tolerance develops due to cell anergy.

FVIII inhibitors

Clinical features

FVIII inhibitors are the most common pathogenic antibodies directed against the blood coagulation factors. They develop in approximately 30% of patients with severe and moderately severe hemophilia A in response to infusions of FVIII [35]. Patients who develop inhibitors usually do so within the first year of treatment [36]. The mechanisms underlying the state of apparent immune tolerance in the remaining non-inhibitor patients are unknown. The greatest risk of inhibitor development is associated with nonsense mutations, large deletions and intrachromsomal recombinations (inversions) in the FVIII gene that are predicted to cause a complete lack of endogenous FVIII. It is not possible to predict who will develop an inhibitor within these high-risk groups. Because these patients are treated more frequently due to increased bleeding and associated inflammation and tissue damage, inhibitor development may occur in conjunction with danger signals presented to the immune system. The risk of inhibitor development in patients with mild hemophilia A increases with the amount of exposure to FVIII [37].

The antibodies that arise in hemophilia A patients are usually called alloantibodies. However, in most cases they are technically isoantibodies, which are defined as antibodies that react with antigens of another member of the same species. In contrast, alloantibodies are defined as antibodies to polymorphic determinants present on proteins from another member of the same species. This distinction is commonly made with respect to antiplatelet antibodies [38]. True alloantibodies have rarely been described in patients with hemophilia A and are due to missense mutations that produce circulating, dysfunctional forms of so-called cross-reactive material positive (CRM+) FVIII [39,40]. When infused with normal FVIII, these patients can make alloantibodies that react with normal FVIII but not self-FVIII.

FVIII inhibitors that occur as autoantibodies in non-hemophiliacs produce a condition sometimes called acquired hemophilia A. As noted above, it is the most common auto-immune bleeding disorder involving the coagulation system. For unknown reasons, acquired hemophilia A patients are more likely to have a more severe bleeding diathesis than hemophilia A inhibitor patients [41]. Additionally, in contrast to patients with hemophilia A, hemarthrosis in these patients is rare. Approximately 50% of acquired hemophilia A patients have underlying conditions, including autoimmune disorders, malignancy, and pregnancy [8]. The remaining, idiopathic cases most commonly occur in elderly patients of either sex.

FVIII inhibitors also have been identified in approximately 20% of normal healthy donors [42]. These inhibitors inhibit FVIII activity in pooled normal plasma, but not autologous plasma, indicating that they are not autoantibodies, but rather alloantibodies directed against an unidentified polymorphism. Anti-FVIII IgG has also been identified in all normal plasmas tested by affinity chromatography on immobilized FVIII [43]. The increased sensitivity of the method is due to its ability to resolve anti-FVIII antibodies from anti-anti-FVIII idiotypic antibodies that are also present. Idiotypic regulation has been proposed as a mechanism for controlling autoantibody activity *in vivo* [44].

Diagnosis

In patients with hemophilia A, clinically significant FVIII inhibitors usually present as a lack of response to replacement therapy. Recovery and half-life studies, performed by measuring FVIII levels after infusion of FVIII, are the most critical aspect of the diagnostic evaluation. Acquired hemophilia A patients usually present with spontaneous bleeding, which is often severe. In either case, the activated partial thromboplastin time (APTT) of normal plasma is prolonged by the addition of patient plasma in a mixing study. This provides the basis for the Bethesda assay, which is the most common quantitative assay for FVIII inhibitors. The Bethesda titer is defined as the dilution of patient plasma that produces 50% inhibition of the FVIII activity in normal plasma [45]. Inhibitors are classified informally as low titer or high titer when the Bethesda titers are < 5 or > 5–10, respectively. Inhibitors frequently inhibit FVIII in the APTT-based assay over minutes to hours. Therefore, dilutions of patient plasma are preincubated with normal plasma for 2 h at 37 °C in the Bethesda assay. Variation in the assay is increased by poor control of the pH and/or possible loss of FVIII due to adsorption to the vessel wall during the 2-h incubation. This is particularly a problem in deciding whether a low-titer FVIII inhibitor is present. The assay has been modified by the addition of 0.1 M imidazole pH 7.4, and by diluting test plasma into FVIII-deficient plasma during the preincubation phase [46]. In a large multicenter study, this 'Nijmegen' modification of the Bethesda assay was shown to decrease spuriously positive assay results [47].

Properties of FVIII inhibitors

FVIII inhibitors almost always consist of a polyclonal IgG population. IgG4 is usually a major component of the antibody population [48–51], even though IgG4 accounts for only 5% of the total IgG in normal plasma. However, IgG1 and IgG2, but not IgG3, subclasses also are usually present in populations of anti-FVIII antibodies. There is no correlation between anti-FVIII IgG subclasses and whether the patient has congenital or acquired hemophilia A. These findings suggest that FVIII inhibitors are associated with a mixed Th1/Th2 response in both patient populations. IgG4 antibodies do not fix complement, which has been cited as a reason that immunopathology

due to antigen–antibody complex is not observed in FVIII inhibitor patients. However, it is more likely that FVIII simply is not present in sufficient quantity to mediate tissue damage when complexed to antibody. This also would account for the lack of immune complex-mediated effects due to IgG1, which does fix complement.

FVIII inhibitors are classified based on the kinetics and extent of inactivation of FVIII [52]. Type I inhibitors follow second-order kinetics and inactivate FVIII completely, which would be expected for a simple bimolecular antigen–antibody reaction. Type II inhibitors inactivate FVIII incompletely and display more complex kinetics of inhibition. Hemophilia A inhibitor patients and acquired hemophilia A patients tend to have type I and type II inhibitors, respectively [53]. However, the borderline between type I and type II inhibitors is not always clear and the distinction is not useful clinically.

Epitopes recognized by FVIII inhibitors

FVIII is an approximately 300-kDa glycoprotein that circulates bound non-covalently to VWF. In the absence of an interaction with VWF (e.g. in severe von Willebrand disease or in some naturally occurring VWF missense mutations), FVIII is cleared rapidly, resulting in low circulating levels. VWF is a multimeric protein that is multivalent for FVIII. It has an average molecular mass of several million Daltons and a hydrodynamic radius in the submicron range [54]. Thus, the FVIII–VWF complex may look something like a microorganism to the immune system and present a danger signal under certain circumstances. Additionally, BCR cross-linking by a multivalent antigen can produce receptor clustering and an increase in immunogenicity [55], which also suggests that the immunogenicity of FVIII may depend on being bound to VWF.

FVIII contains a sequence of domains designated A1-A2-B-*ap*-A3-C1-C2 (Fig. 2), where *ap* is an acidic 41-residue activation peptide. During biosynthesis, FVIII is cleaved intracellularly at several sites in the B domain. This produces a series of heterodimers that contain a common light chain consisting of the *ap*-A3-C1-C2 domains. During the activation of FVIII by thrombin, cleavages occur between the A1 and A2 domains, the A2 and B domains, and the *ap* and A3 domains. The B-domain fragments and the acidic 41-residue *ap* peptide are released, producing an A1/A2/A3-C1-C2 activated FVIII heterotrimer [56].
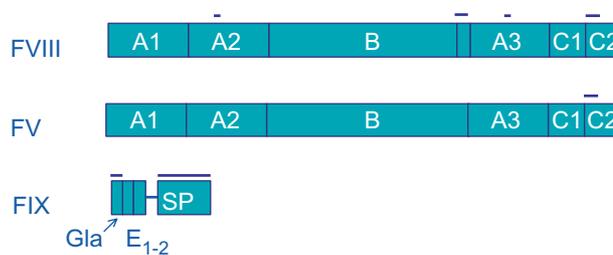

Fig. 2. B-cell epitopes in factor VIII, factor V and factor IX.

Western blotting analysis and antibody neutralization studies of large series of FVIII inhibitor patients have shown that inhibitory activity is usually restricted to the A2 domain and the FVIII light chain (*ap*-A3-C1-C2 polypeptide) in both hereditary and acquired hemophilia A [57–59]. The epitope specificity can fluctuate with time in individual patients [60]. The C2 domain contains the dominant epitope within the light chain [59,61]. Homolog scanning mutagenesis using hybrid human/non-human FVIII molecules has mapped anti-A2 antibodies to a segment bounded by Arg484–Ile508 and anti-C2 antibodies to the NH$_2$-terminal half of the C2 domain (Fig. 2) [62,63]. Interestingly, inhibitory antibodies to FV, which is homologous to FVIII (Fig. 2), also map to the N-terminal half of the C2 domain [64]. Inhibitory activity directed against the *ap*-A3-C1 region of FVIII has been identified in some patients by the observation that the FVIII light chain neutralizes more antibody than the isolated C2 domain [58,65]. Inhibitor IgGs have been identified that are neutralized by a synthetic peptide corresponding to A3 domain residues Lys1804–Val1819 [65,66], which contains the FIXa binding site [67] and A3 domain residues His2009–Val2018, which contains an activated protein C binding site [65]. Additionally, experiments using hybrid FVIII molecules have identified the *ap* segment as an antigenic site in some patients [68].

The unusual inhibitory alloantibodies that have been described in CRM+ patients occurred in association with an Arg593Cys mutation in the A2 domain outside the immunodominant Arg484–Ile508 epitope [39] and an Arg2150His substitution in the C1 domain [40]. The epitopes recognized by antibodies from these patients are at the site of the mutation because they do not recognize self FVIII, which presumably only differs from wild-type FVIII at the mutation site. Conceivably, other CRM+ patients develop alloantibodies that escape detection because they are not inhibitory.

Despite the different immunological settings in which they arise, antibodies in hereditary and acquired hemophilia A inhibitor plasmas are both primarily directed to the A2 and C2 domains. This suggests that intrinsic structural features in the FVIII molecule are an important determinant driving the immune response. Consistent with this, the inhibitory antibody response to human FVIII in hemophilia A mice can be reduced by mutagenesis of the A2 epitope [69]. However, although the A2 and C2 epitopes are immunodominant in both populations, antibody neutralization experiments have shown that the distribution of antibodies differs significantly [58]. Most hemophilia A inhibitor plasmas recognize both the A2 and C2 domains. In contrast, most autoantibody plasmas recognize either the A2 or C2 domain, but not both, with the C2 domain being more frequently targeted. Epitope spreading between domains does not appear to be a property of FVIII inhibitors because anti-C2 antibodies can occur in the absence of anti-A2 antibodies and *vice versa*.

An X-ray structure of the human FVIII C2 domain has been solved, revealing a putative hydrophobic three-prong phospholipid membrane-binding site consisting of Met2199/Phe2200, Val2223, and Leu2251/Leu2252 [70]. Additionally,
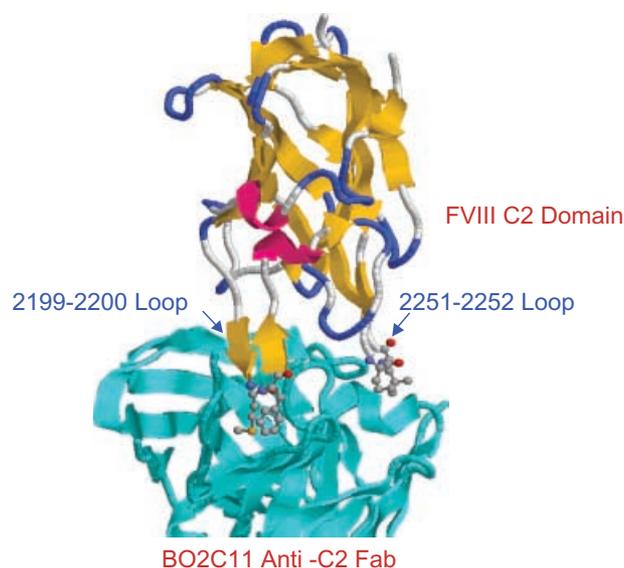

Fig. 3. Structure of the BO2C11 Fab–factor C2 complex. X-ray coordinates were obtained from Protein Data Bank accession number 1IQD [72].

several basic residues contribute to a ring of positively charged residues that may contribute to electrostatic interaction of FVIII with negatively charged phosphatidylserine. Mutagenesis of several of these residues using conservative replacements with non-human amino acids indicates that C2 inhibitors frequently target the Met2199/Phe2200 and Leu2251/Leu2252 β-hairpins [71]. The X-ray structure of an Fab fragment of a human hemophilia A inhibitor patient-derived monoclonal IgG4κ anti-C2 antibody, BO2C11, in complex with the FVIII C2 domain, has been solved [72]. The antibody-combining site was in contact with the Met2199/Phe2200 and Leu2251/Leu2252 loops (Fig. 3). Site-directed mutagenesis studies have indicated that, although the antibody footprints on the A2 and C2 domains may be similar from patient to patient, the antibodies vary with respect to the individual amino acids that they recognize [71,73].

Phage display technology has been used to characterize human anti-FVIII antibodies from inhibitor patients [74–77]. In this method, a patient-derived, subclass-specific immunoglobulin heavy-chain gene repertoire obtained from peripheral blood mononuclear cells is combined with a non-specific light-chain gene repertoire and displayed as single-chain variable domain antibody fragments (scFv) on filamentous phage. Phages are selected by binding to antigen or antigenic fragments. An underlying assumption in this method is that most of the binding energy for antigen–antibody interactions is derived from the heavy chain. Several IgG4-specific, hypermutated clones have been identified and the corresponding scFv molecules have been characterized. Three anti-C2 domain scFvs have been isolated that have different epitope and V$_H$ germline specificities. None of the scFvs inhibited FVIII function but one overlapped with an inhibitory epitope. These results indicate that the immune response to the FVIII C2 domain is complex. However, an alternative possibility is that

at least some of the scFvs are derived from inhibitory antibodies, but have low affinity due to the non-native immunoglobulin light chain. Five anti-A3 domain scFvs recognized an epitope that included residues Gln1778–Asp1840. Only two of the five scFvs inhibited FVIII activity, which also may be due to low binding affinity. Two anti-A2 domain scFvs have been isolated. One was directed against the Arg484–Ile508 epitope and had inhibitory activity against FVIII. The second recognized an epitope that included residues Asp712–Ala736 and was non-inhibitory.

Mechanism of action of FVIII inhibitors

The only biological function of FVIII is to become proteolytically activated and participate as a cofactor for FIXa during intrinsic pathway factor (F)X activation on phospholipid membranes. Theoretically, antibodies could inhibit FVIII procoagulant function in several ways, including blocking the binding of FVIIIa to FIXa, FX or phospholipid, or by interfering with the proteolytic activation of FVIII. Additionally, antibodies that interfere with binding to VWF could displace FVIII from VWF *in vivo* and increase the clearance of FVIII. These antibodies would not affect the procoagulant activity of FVIII *in vitro* in the Bethesda assay unless they also interfered with intrinsic pathway FX activation or the activation of FVIII.

Many, if not all, anti-C2 antibodies inhibit the binding of FVIII to phospholipid [78], which was the basis for the hypothesis that anti-C2 antibodies bind the proposed phospholipid-binding site of FVIII. However, VWF also binds the C2 domain [79,80] and VWF competes for the binding of factor to phospholipid [81,82]. In most cases, type II inhibitors behave like type I inhibitors when they are tested against FVIII in the absence of VWF, indicating that inhibition by the type II antibodies is blocked due to competition for binding by VWF [83]. Thus, VWF could interfere with the binding of anti-C2 antibodies, producing a type II antibody pattern. This is consistent with the observation that polyclonal patient antibodies that recognize only the C2 domain are much more common in autoantibody patients [58], in which most type II antibodies are found [52,83]. Alternatively, the difference between type I and type II inhibitors may be a function of binding affinity for FVIII.

The analysis of FVIII inhibitors has been confounded by the polyclonal nature of the antibodies. The BO2C11 antibody described above is one of only two human monoclonal anti-FVIII antibodies that have been isolated [84]. It is a high-affinity antibody that was isolated by immortalization of the peripheral blood memory cell pool from a hemophilia A patient with a high-titer inhibitor. BO2C11 inhibits the binding of FVIII to phosphatidylserine and VWF. However, contrary to expectations, it is a type I inhibitor. Thus, type II inhibitors may represent another population of inhibitors with different epitope specificity from BO2C11, yet still inhibit the binding of FVIII to the C2 domain and to VWF. The other human monoclonal anti-FVIII antibody was cloned from the patient

with the C1 domain Arg2150His missense mutation described above [85]. Polyclonal IgG from the patient competed with the monoclonal antibody for binding to FVIII, indicating that the immune response was directed entirely to a C1 epitope that included Arg2150. Surprisingly, the monoclonal antibody was a type II inhibitor and blocked the binding of FVIII to VWF, implying a possible interaction of VWF with the FVIII C1 domain. Three unusual acquired hemophilia A patients with type II inhibitors have been identified with normal levels of FVIII antigen but decreased FVIII activity [65]. Analysis of the autoantibodies in these patients revealed that they formed immune complexes with FVIII that were protected from degradation by activated protein C. The autoantibodies inhibited the binding of FVIII to VWF. This indicates that the normal antigen levels are due to displacement of FVIII from VWF to immune complexes, which are protected from proteolysis by activated protein C and subsequent rapid clearance. The epitopes recognized by the autoantibodies were mapped using synthetic peptides to the previously recognized Lys1804–Val1819 FIXa binding site and also to the His2009–Val2018 activated protein C binding site. The properties of anti-FVIII light-chain antibodies remain poorly understood with respect to their relative pathophysiological effects as anticoagulants compared with their inhibition of VWF binding.

Outbreaks of C2-specific inhibitors have been described in previously treated hemophilia A patients with no history of inhibitor development receiving specific lots of plasma-derived FVIII [86,87]. The C2-specificity is remarkable because it is unusual in the hemophilia A population. The results suggest that exposure to denatured forms of FVIII may produce an immunogenic neoepitope or that antigens other than FVIII may have provided a 'second signal' for the immune response.

Anti-A2 inhibitors inhibit intrinsic FXase noncompetitively [88]. Noncompetitive inhibitors do not prevent binding of substrate (FX) to enzyme (FIXa/FVIIIa/phospholipid), but the resulting complex is inactive. Anti-A2 inhibitors do not block the binding of FVIIIa to fluorescent dye-labeled FIXa, but prevent a secondary change that occurs when FX binds the dye-labeled FIXa–FVIIIa complex. Studies with prothrombinase, which is homologous to the intrinsic FX activation complex, indicate that substrate binding to an enzyme exosite determines substrate affinity. This is followed by an intramolecular binding step that docks the scissile bond region of the substrate to the active site of the enzyme [89]. Anti-A2 inhibitors may bind FVIIIa and block the intramolecular binding event without disrupting the initial encounter of FX with the enzymatic complex.

Lacroix-Desmazes *et al.* have described antibodies in polyclonal IgG preparations from hemophilia A inhibitor patients that catalyze the proteolytic degradation of FVIII [90]. Multiple, unidentified cleavage sites in the FVIII molecule appear to be involved. They have proposed that proteolysis of FVIII may increase clearance *in vivo*. The clinical significance of catalytic anti-FVIII antibodies is unknown.

Non-inhibitory anti-FVIII antibodies

Anti-FVIII antibodies can be measured by ELISA, which does not discriminate between inhibitory and non-inhibitory antibodies. Hemophilia A patients with a positive ELISA but undetectable inhibitor levels by Bethesda assay have rarely been identified, indicating the presence of non-inhibitory antibodies [91]. It has been proposed that antibodies in this subset of hemophilia A patients could interfere with the binding of FVIII to VWF and decrease the circulatory lifetime of FVIII. However, in another study, only four of 26 hemophilia A patients with no detectable inhibitor by Bethesda assay showed a non-inhibitory antibody by ELISA [92]. In three of these patients the ELISA assay was only weakly positive. Overall, the results indicate that a significant antibody response to FVIII usually includes a clinically significant inhibitor response. Whether there is a significant subpopulation of non-inhibitory antibodies in FVIII inhibitor plasmas is not known.

T-cell help in FVIII inhibitor development

As discussed above, antibody response to most soluble proteins requires T-cell help in which antigen-specific peptides are presented on the surface of MHC class II molecules to the TCR. MHC loci are highly polymorphic, which is a major factor in the nature of the antigen that is presented. However, inhibitor formation in patients with hemophilia A is influenced only weakly by MHC loci [93]. Several observations suggest that T-cell help is required for the production of anti-FVIII antibodies in congenital and acquired hemophilia A. High-affinity antibodies, which characterize FVIII inhibitors, require affinity maturation, which is a T-cell-dependent process. Additionally, the identification of IgG4 antibodies as an important subclass for production of anti-FVIII antibodies indicates the presence of Th2-cell help.

T-cell help clearly has been identified in the murine FVIII inhibitor model (*vide infra*). However, it has been difficult to demonstrate unequivocally FVIII-specific T-cell responses in human hemophilia A. The analysis of FVIII inhibitors is limited by the inaccessibility of lymphoid tissue. Peripheral blood T-cell proliferation in response to *in vitro* stimulation with FVIII has been observed in some hemophilia A inhibitor patients [94]. However, major differences were not observed between hemophilia A patients with or without inhibitors, acquired hemophilia A patients and in normal controls in T-cell proliferation assays using FVIII-specific synthetic peptides [95]. Peripheral blood T-cell proliferation in response to synthetic peptides spanning the entire sequence of full-length human FVIII was also measured in this study. The peptides were divided into pools based on their corresponding FVIII domains (A1, A2, A3, B, C1, and C2). T-cell proliferation occurred in response to native FVIII and equally well to pools from all domains. Subsequently, T-cell proliferation in response to 16 overlapping 20-residue peptides spanning the human C2 domain was studied in patients with congenital or acquired hemophilia A and in normal controls [96]. Significant responses were obtained in all patient groups. Several peptides were recognized in all groups, except hemophilia A patients with inhibitors, in whom there was a more limited response. Thus, CD4+ recognition of FVIII appears to be complex with a surprisingly robust response in the normal population. The more restricted response in hemophilia A inhibitor patients compared with hemophilia A patients without inhibitors or normal subjects, which seems counterintuitive, possibly results in the inability of inhibitor patients to activate a regulatory CD4+ T cell that suppresses the immune response to FVIII [96].

FVIII-specific T-cell clones were produced from the patient with the Arg2150 His missense mutation described above whose inhibitor recognizes Arg2150 [97]. These clones proliferated in response to antigen presentation by FVIII-specific B cells of a peptide corresponding to the normal FVIII sequence that spans Arg2150. However, they did not respond to a peptide representing the corresponding mutant sequence. Thus, Arg2150 also is part of a T-cell epitope. As discussed above, examples of overlapping B- and T-cell epitopes exist, such as this case, but are not necessarily the rule. This case may be exceptional because the single amino acid difference between mutant and wild-type FVIII may have driven the selection of overlapping B- and T-cell epitopes.

Treatment

Treatment of bleeding episodes for patients with acquired hemophilia A or congenital hemophilia A with inhibitors depends on the inhibitor titer. Low-titer inhibitors can be overwhelmed with FVIII. FVIII bypassing agents [prothrombin complex concentrates, activated prothrombin complex concentrates, or recombinant factor (F)VIIa] or porcine FVIII concentrates can be used to treat patients with high-titer inhibitors [8,98]. Recombinant FVIIa (NovoSeven™) is effective in controlling most bleeding episodes [99–101]. There have been no reports of inhibitory antibodies developing to the product. Prospective trials have not been performed to compare these agents, although a trial comparing FEIBA VH™, an activated prothrombin complex concentrate, with NovoSeven, is under way (the FENOC study). The availability of porcine FVIII is limited because of problems associated with its manufacture. Immunosuppressive agents, intravenous gammaglobulin and plasmapheresis have been used in acquired hemophilia in conjunction with FVIII bypassing agents and porcine FVIII [100]. Immune tolerance induction protocols are discussed below.

The immunogenicity of FVIII in hemophilia A mice

Hemophilia A mice provide an important model system for studying the immunogenicity of FVIII. There are two strains of FVIII knockout mice [102], which were produced by targeted disruption of the FVIII gene at exons 16 and 17, respectively, both of which encode A3 domain sequences. E16 and E17 mice

have a bleeding diathesis and undetectable functional FVIII. They secrete detectable FVIII antigen because the gene 5′ to the encoded A3 domain is intact [103]. Thus, both strains may be partially tolerant to murine FVIII. Consistent with this, human FVIII is more immunogenic than murine FVIII in E16 mice [104]. In contrast to the E16 and E17 mice, the genetics of human hemophilia A is very heterogeneous. As a model, E16 and E17 mice correspond to a subset of CRM+ patients that have nonsense mutations in the FVIII light chain.

Qian *et al.* developed an immunogenicity model using E16 and E17 hemophilia A mice in a C57BL/6 (H2^b) background [105]. The mice are infused intravenously with human FVIII in the absence of adjuvants at a frequency and dosage on a body weight basis (10 µg kg^{-1}, approximately 80 U kg^{-1}) that mimics treatment of human hemophilia A. Under these or similar conditions, the mice develop detectable FVIII inhibitors after two or three doses that increase from 10 and 1000 Bethesda units (BU) mL^{-1} with further injections [105–107]. Measurement of anti-FVIII antibodies by ELISA indicates that 1 BU of anti-FVIII activity corresponds to approximately 1 µg of anti-FVIII antibody [105], similar to inhibitors that develop in humans. The relative contribution of inhibitory antibodies to the total antibody population is not known. Normal C57BL/6 mice also make antibodies to human FVIII, but the response is slower than in hemophilia A mice [105,107]. This indicates that endogenous murine FVIII partially tolerizes normal mice to human FVIII.

The delivery of the immunogen in human and murine hemophilia A differs considerably from most model systems used by immunologists in which antigens typically are given in adjuvants at a dose of about 5000 µg kg^{-1}. The strong immune response at 100-fold lower doses of FVIII in the absence of adjuvants is striking. Hemophilia A mice do not have spontaneous bleeding and are healthy when handled with care. Specifically, they do not have chronic inflammatory joint disease, or other evidence of 'danger' signals that would act as natural adjuvants. As noted above, the immunogenicity of FVIII conceivably depends on the carrier effect of VWF. On the other hand, anti-C2 antibodies, presumably including membrane immunoglobulin in the cognate BCR, compete with VWF for binding to FVIII. This makes it difficult to see how the immunodominant anti-C2 response is initiated unless FVIII and VWF dissociate in the spleen and other secondary lymphoid organs. Furthermore, results by Reipert *et al.* in the hemophilia A mouse model may have relevance to the possible role of VWF in the immunogenicity of FVIII [107]. Anti-FVIII antibody levels are comparable when FVIII is delivered intravenously or subcutaneously. FVIII is not absorbed into the circulation when given subcutaneously and VWF is confined to the intravascular space. Thus, FVIII is probably delivered to draining lymph nodes in an unbound state, suggesting that the immunogenicity of FVIII is independent of VWF.

The production of antihuman FVIII antibodies in hemophilia A mice is T-cell-dependent, as shown by a T-cell proliferative response to exposure to FVIII *in vitro* [105]. The T-cell response is detectable in most mice before anti-FVIII antibodies are measurable. IgG1, IgG2a and IgG2b anti-FVIII antibodies are produced, which is consistent with a combined Th1 and Th2 response [106,108–110]. FVIII-specific CD4+ IFN-γ-positive and CD4+ IL-2-positive T cells have been identified by flow cytometry, consistent with a strong Th1 response [108]. FVIII-specific CD4+ IL-10-positive T cells also are present. As discussed above, the role of IL-10 in Th1 and Th2 responses is complex and incompletely understood. CD4+ IL-4-positive Th2 cells have not been definitively identified, but IL-4 is secreted in spleen cells from immunized hemophilia A mice in response to FVIII [106], which, however, may not be due to CD4+ cells.

The proliferation of CD4+ T cells prepared from spleens of immunized mice in this model in response to intact FVIII and synthetic peptides spanning the entire sequence of full-length human FVIII was measured [110]. The peptides were divided into pools based on the corresponding FVIII domains (A1, A2, A3, B, C1, and C2). T-cell proliferation occurred in response to native FVIII and equally well to pools from all domains, similar to the results obtained using human peripheral blood T cells described above [95]. Thus, specific immunodominant T-cell epitopes have not been identified. This may be due to the large size of the molecule and the presence of numerous immunodominant epitopes.

FVIII-specific ASCs can be identified in the spleen after two doses of FVIII and in the bone marrow after three doses by ELISPOT analysis [111]. The number of ASCs decreases in the spleen over a period of 5 months but persists unchanged in the bone marrow. Memory B cells have been isolated from spleens of immunized mice, which differentiate into ASCs upon exposure to FVIII *in vitro* and in adoptive transfer experiments *in vivo* [112]. The differentiation of memory B cells into ASCs is T-cell-dependent. However, in mice presensitized with ovalbumin, ovalbumin-specific T cells can provide T-cell help and FVIII-specific T cells are not necessary. In this setting, ovalbumin behaves as a third-party antigen that activates T cells to stimulate memory B cells in a phenomenon called bystander help [113].

The role of the costimulatory molecules in hemophilia A mice immunized with FVIII has been addressed in several studies. These were motivated partly by observations in other experimental systems that exposure of T cells to antigens in the absence of costimulation can render them anergic due to lack of signal 2. B7-1$^{-/-}$ and B7-2$^{-/-}$ mice were crossed with hemophilia A mice and evaluated in the immunogenicity model [114]. Hemophilia A/B7-2$^{-/-}$ mice did not develop anti-FVIII antibodies, but the A/B7-1$^{-/-}$ mice produced high-titer antibodies. The differential response of hemophilia A/B7-2$^{-/-}$ and hemophilia A/B7-1$^{-/-}$ was not predicted from previous studies on the function of B7-1 and B7-2 molecules. CTLA4-Ig, which blocks B7/CD28 and CTLA4/CD28 interactions, blocked the anti-FVIII antibody response when given concomitantly with FVIII [114]. However, further injections of FVIII to these mice in the absence of CTLA4-Ig resulted in the production of anti-FVIII antibodies. The effect of CTLA4-Ig is potentially complex

because it can block both an upregulatory response (B7-CD28) and a downregulatory response (B7-CTLA4).

Anti-CD40L antibody prevents a primary immune response when given concomitantly with FVIII over a series of injections [106,108,115]. Additionally, anti-CD40L antibody given to hemophilia A mice after they have been immunized with FVIII decreases anti-FVIII antibody titers, FVIII-specific T-cell proliferation and proliferating germinal centers in the spleen [115]. However, additional repeated exposure to FVIII in the absence of anti-CD40L in both settings results in a high-titer anti-FVIII response. The levels of FVIII-specific CD4 + IFN-γ-positive and CD4 + IL-2-positive T cells are decreased in CD40L-treated mice, but increase with additional immunizations of FVIII in the absence of anti-CD40L [108]. The interruption of the B7-1/CD28, B7-2/CD28, and CD40/CD40L pathways blocks differentiation of memory B cells into ASCs, but interruption of the ICOS/ICOS ligand pathway is not inhibitory [112]. In this system, interference with the B7/CD28 pathways blocks restimulation of memory T cells. In contrast, work in other systems has suggested that B7/CD28 interactions are necessary for activation of naive T cells but not for memory T-cell responses [116]. Costimulatory interactions may depend on the nature and concentration of the antigen. The studies by Hausl *et al.* were done using physiological concentrations of FVIII, whereas studies in other systems have used higher concentrations of antigen. Interruption of the B7-1/CD28, B7-2/CD28, and CD40/CD40L pathways does not decrease the production of antibodies by mature ASCs. This suggests that blocking costimulation pathways in existing FVIII inhibitor patients could possibly blunt the anamnestic response to additional infusion of FVIII, but would not reduce the inhibitor levels due to existing ASCs [112].

FIX inhibitors

The incidence of hemophilia B is approximately one-fifth that of hemophilia A. Hemophilia B patients are more likely than hemophilia A patients to have missense mutations and dysfunctional circulating FIX. These patients are designated CRM + based on detectable FIX antigen and are less likely to develop FIX inhibitors than CRM– hemophilia B patients [117]. This is presumably due to the development of immune tolerance to the self-FIX polypeptide that extends to the wild-type protein used in replacement therapy. The incidence of inhibitor development in hemophilia B patients is less than in hemophilia A patients and is approximately 1–3% [118]. However, the lower incidence cannot be completely accounted for by the higher fraction of CRM + hemophilia B patients. Additionally, the development of autoantibodies to FIX is much less common than to FVIII [119]. This indicates that FVIII generally may be more immunogenic than FIX.

Hemophilia A and hemophilia B are indistinguishable clinically in the absence of specific clotting factor assays, which is consistent with the fact that the only known biological functions of FVIII and FIX are to participate in the intrinsic pathway FX activation complex. Likewise, the clinical presentation, diagnostic evaluation and management strategies of FIX and FVIII inhibitor patients are similar. However, in contrast to hemophilia A patients, hemophilia B inhibitor patients are at significant risk of developing anaphylactoid reactions or frank anaphylaxis in response to FIX replacement therapy [120]. This usually occurs at the time the inhibitor first appears, at a median age of 1.3 years and a median of 11 exposure days to product. Additionally, some of these patients develop the nephrotic syndrome.

As in FVIII inhibitor patients, inhibitor titers are characterized using the Bethesda assay, modified by using FIX-deficient plasma. The Bethesda assay is predictive of poor response to replacement therapy in hemophilia B, indicating that non-inhibitory antigen–antibody complexes that promote rapid clearance do not occur. FIX inhibitors from several patients have been characterized [121]. They are polyclonal IgG antibodies, predominantly in the IgG1 and IgG4 subclasses. FIX epitopes recognized by the IgG preparations were localized using chimeric human FIX/FX molecules. FIX contains a Gla-EGF1-EGF2-AP-SP domain structure (Fig. 2), where Gla, EGF, AP and SP refer to γ-carboxyglutamic acid, epidermal growth factor-like, activation peptide, and serine protease domains. Most of the IgG preparations recognized both the Gla and SP domains, but none recognized the EGF or AP domains. The anti-FIX antibodies inhibited the FIXa/FVIIIa intrinsic FX activation complex. The antibodies from most of the patients that were studied inhibited the binding of FIX to phospholipid, which could account for the inhibitory activity. However, the antibodies blocked the binding of FIX to the FVIII light chain in a phospholipid-independent assay, indicating that more than one inhibitory mechanism may be involved.

In patients with high-titer FIX inhibitors that cannot be overwhelmed by infusion of FIX, bleeding episodes can be treated with prothrombin complex concentrates, activated prothrombin complex concentrates, or recombinant FVIIa [118,122]. Immune tolerance induction protocols are discussed below. Because of the risk of anaphylaxis associated with the formation of a FIX inhibitor, it is recommended that the first 10–20 infusions of FIX be given in a treatment center equipped for the treatment of shock. In the absence of controlled clinical trials, treatment is based on the clinical situation, physician preference, product availability and cost.

Hemophilia B mice have been created by targeted disruption of the FIX gene in the promoter region, which results in no detectable FIX expression [123]. T-cell responses in hemophilia B mice in C57BL/6 (H2^b) or BALB/c (H2^d) backgrounds after immunization with 3000 μg kg^{-1} human FIX subcutaneously in complete Freund's adjuvant have been studied [124]. CD4 + T cells from the mice in both strain backgrounds proliferated in response to human FIX. In both strains of mice, single dominant T-cell epitopes were identified using proliferation assays in response to synthetic peptides. The peptides were different, which was expected because H2^b and H2^d MHC class II molecules have different peptide-binding specificities. Surprisingly, CD4 + T cells from normal C57BL/6 mice also

recognized the immunodominant peptide recognized by hemophilia B C57BL/6 mice. This indicates that normal C57BL/6 mice have autoreactive T cells that do not produce an immune response to murine FIX.

Immune tolerance induction in hemophilia inhibitor patients

In 1974, Brackmann and coworkers attempted to overwhelm an inhibitor in a hemophilia A patient with large doses of FVIII and made the serendipitous observation that the inhibitor titer declined [125]. This led to the development of the Bonn protocol, which was followed by several other protocols to eliminate FVIII and FIX inhibitors [126,127]. The number of hemophilia B patients that have been studied is small because of the low incidence of inhibitor development in this group. The mechanism underlying inhibitor elimination is unknown, but the process is commonly called immune tolerance induction (ITI). The most rigorous definition of a successful outcome is a return to normal pharmacokinetic recovery and half-life of infused product. If the pharmacokinetics are normal, inhibitors are not detected in the Bethesda assay or other *in-vitro* measurements of inhibitor formation. However, abnormal pharmacokinetics can persist after the inhibitor titer has disappeared.

All protocols have in common the use of the specific coagulation factor, but there is considerable variation in the dose, dose frequency and whether modulators such as immunosuppressives, intravenous immune globulin (IVIG) or extracorporeal inhibitor adsorption are used. ITI is clearly antigen-specific because it has been achieved using highly purified FVIII or FIX. Whether intermediate purity FVIII concentrates, which contain VWF, are superior to highly purified plasma-derived or recombinant FVIII is controversial [126].

Success rates for ITI in hemophilia A inhibitor patients in the range of 60–80% have been reported in retrospective analyses. However, the optimal dosing regimen in terms of treatment outcome and cost has not been determined and will require a prospective trial. The current Bonn protocol utilizes the most FVIII, 300 U kg^{-1} day^{-1}, which is usually given for several months. An average of approximately 2 million units of FVIII are used per patient in the Bonn protocols. FVIII use is as low as 20% of this amount in other protocols. Relapse following successful ITI is rare. The reported success rate in hemophilia B is only 30% and the treatment is associated with a high incidence of allergic reactions and the nephrotic syndrome.

Inhibitor development in gene therapy for hemophilia A and B

The threat of inhibitor development is a major concern in gene therapy of hemophilia A and hemophilia B. Inhibitors to FVIII and FIX have been observed in both murine and canine models of hemophilia, which was recently reviewed in this journal [128]. In animal models, the risk appears to depend on several factors, including the vector, expression levels of FVIII or FIX and site of transgene expression. The apparent development of immune tolerance has been noted in some studies. Chao and Walsh observed that a high-titer inhibitor that developed in normal C57BL/6 mice disappeared spontaneously and abruptly after several months [129]. Apparent immune tolerance to human FIX was produced in normal CD-1, C3H, BALB/c and C57BL/6 mice by adeno-associated viral hepatic gene transfer as judged by sustained expression of human FIX [130]. Tolerance was adoptively transferred by CD4+ cells from C57BL/mice, indicating that regulatory T cells mediated the process. Gene transfer was less successful in hemophilia B mice in a CD-1 or C3H background using the murine FIX transgene. However, sustained expression of FIX was obtained in all four BALB/c hemophilia B mice using a liver-specific promoter. These mice did not develop inhibitors when challenged with a single-dose of murine FIX subcutaneously in complete Freund's adjuvant, suggesting that immune tolerance had been induced.

References

1 Weil PE. Etude du sang chez les hemophiles. *Bull E Mem Soc Med Par* 1906; **23**: 101.

2 Margolius A Jr, Jackson DP, Ratnoff OD. Circulating anticoagulants: a study of 40 cases and a review of the literature. *Medicine* 1961; **40**: 145–202.

3 Bidwell E, Denson KWE, Dike GWR, Augustin R, Lloyd GM. Antibody nature of the inhibitor to antihemophilic globulin (Factor VIII). *Nature* 1966; **210**: 746–7.

4 Bowie EJW, Thompson JH Jr, Pacuzzi CA, Owen CA Jr. Thrombosis in systemic lupus erythematosus despite circulating anticoagulants. *J Lab Clin Med* 1963; **62**: 416–30.

5 Jiang Y, Doolittle RF. The evolution of vertebrate blood coagulation as viewed from a comparison of puffer fish and sea squirt genomes. *Proc Natl Acad Sci USA* 2003; **100**: 7527–32.

6 Davidson CJ, Hirt RP, Lal K, Snell P, Elgar G, Tuddenham EGD, McVey JH. Molecular evolution of the vertebrate blood coagulation network. *Thromb Haemost* 2003; **89**: 420–8.

7 Flajnik MF, Miller K, Du Pasquier L. Evolution of the immune system. In: Paul WE, ed. *Fundamental Immunology*. Philadelphia: Lippincott Williams & Wilkins, 2003: 519–70.

8 Cohen AJ, Kessler CM. Acquired inhibitors. *Bailliere's Clin Haematol* 1996; **9**: 331–54.

9 Harris DE, Cairns L, Rosen FS, Borel Y. A natural model of immunologic tolerance. Tolerance to murine C5 is mediated by T cells, and antigen is required to maintain unresponsiveness. *J Exp Med* 1982; **156**: 567–84.

10 Zal T, Volkmann A, Stockinger B. Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. *J Exp Med* 1994; **180**: 2089–99.

11 Arnout J, Vermylen J. Current status and implications of autoimmune antiphospholipid antibodies in relation to thrombotic disease. *J Thromb Haemost* 2004; **1**: 931–42.

12 Janeway CA Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* 1992; **13**: 11–6.

13 Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symposia Quantitative Biol* 1989; **54 Part 1**: 1–13.

14 Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* 1994; **12**: 991–1045.

15 Bell D, Young JW, Bancherau J. Dendritic cells. *Adv Immunol* 1999; **72**: 255–324.

16 Mason D. A very high level of cross-reactivity is an essential feature of the T cell receptor. *Immunol Today* 1998; **19**: 395–404.

17 Muraille E, De Trez C, Pajak B, Brait M, Urbain J, Leo O. T cell-dependent maturation of dendritic cells in response to bacterial superantigens. *J Immunol* 2002; **168**: 4352–60.

18 Bjorck P, Flores-Romo L, Liu YJ. Human interdigitating dendritic cells directly stimulate CD40-activated naive B cells. *Eur J Immunol* 1997; **27**: 1266–74.

19 Lanzavecchia A. Antigen uptake and accumulation in antigen-specific B cells. *Immunol Rev* 1987; **99**: 39–51.

20 Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986; **136**: 2348–57.

21 Abbas AK, Murphy KM, Sher A. Functional diversity of helper T-lymphocytes. *Nature* 1996; **383**: 787–93.

22 Muraille E, Leo O. Revisiting the Th1/Th2 paradigm. *Scand J Immunol* 1998; **47**: 1–9.

23 Davies DR, Padlan EA. Antibody–antigen complexes. *Ann Rev Biochem* 1990; **59**: 439–73.

24 Jin L, Fendly BM, Wells JA. High resolution functional analysis of antibody–antigen interactions. *J Mol Biol* 1992; **226**: 851–65.

25 Benjamin DC, Berzofsky JA, East IJ, Gurd FRN, Hannum C, Leach SJ, Margoliash E, Michael JG, Miller A, Prager EM, Reichlin M, Sercarz EE, Smith-Gill SJ, Todd PE, Wilson AC. The antigenic structure of proteins: a reappraisal. *Ann Rev Immunol* 1984; **2**: 67–101.

26 Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* 1982; **31**: 417–27.

27 Staudt LM, Gerhard W. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. I. Significant variation in repertoire expression between individual mice. *J Exp Med* 1983; **157**: 687–704.

28 Watts C, Lanzavecchia A. Suppressive effect of antibody on processing of T cell epitopes. *J Exp Med* 1993; **178**: 1459–63.

29 Barnett BC, Graham CM, Burt DS, Skehel JJ, Thomas DB. The immune response of BALB/c mice to influenza hemagglutinin: commonality of the B cell and T cell repertoires and their relevance to antigenic drift. *Eur J Immunol* 1989; **19**: 515–21.

30 Simitsek PD, Campbell DG, Lanzavecchia A, Fairweather N, Watts C. Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants. *J Exp Med* 1995; **181**: 1957–63.

31 Berzofsky JA, Berkower IJ. Immunogenicity and antigen structure. In: Paul WE, ed. *Fundamental Immunology*. Philadelphia: Lippincott Williams & Wilkins, 2003: 631–83.

32 Shevach EM. Regulatory T cells in autoimmunity. *Annu Rev Immunol* 2000; **18**: 423–49.

33 Schwartz RH. Immunological tolerance. In: Paul WE, ed. *Fundamental Immunology*. Philadelphia: Lippincott-Raven, 1999: 701–39.

34 Hodgkin PD, Basten A. B cell activation, tolerance and antigen-presenting function. *Curr Opin Immunol* 1995; **7**: 121–9 [Review].

35 Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T, Krackhardt B, Kornhuber B. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. *Lancet* 1992; **339**: 594–8.

36 Lusher J, Arkin S, Abildgaard CF, Hurst D, Kogenate PUP, Study Group. Kogenate treatment of previously untreated patients (PUPS) with hemophilia A. Update of safety, efficacy, and inhibitor development after five study years. *Blood* 1994; **84**: 239a.

37 Sharathkumar A, Lillicrap D, Blanchette VS, Kern M, Leggo J, Stain AM, Brooker L, Carcao MD. Intensive exposure to factor VIII is a risk factor for inhibitor development in mild hemophilia A. *J Thromb Haemost* 2004; **1**: 1228–36.

38 Yamamoto N, Ikeda H, Tandon NN, Herman J, Tomiyama Y, Mitani T, Sekiguchi S, Lipsky R, Kralisz U, Jamieson GA. A platelet membrane glycoprotein (GP) deficiency in healthy blood donors: Naka-platelets lack detectable GPIV (CD36). *Blood* 1990; **76**: 1698–703.

39 Fijnvandraat K, Turenhout EA, van den Brink EN, Ten Cate JW, van Mourik JA, Peters M, Voorberg J. The missense mutation Arg593 → Cys is related to antibody formation in a patient with mild hemophilia A. *Blood* 1997; **89**: 4371–7.

40 Peerlinck K, Jacquemin MG, Arnout J, Hoylaerts MF, Gilles JG, Lavend'homme R, Johnson KM, Freson K, Scandella D, Saint-Remy JM, Vermylen J. Antifactor VIII antibody inhibiting allogeneic but not autologous factor VIII in patients with mild hemophilia A. *Blood* 1999; **93**: 2267–73.

41 Ludlam CA, Morrison AE, Kessler C. Treatment of acquired hemophilia. *Semin Hematol* 1994; **31**: 16–9.

42 Algiman M, Dietrich G, Nydegger UE, Boieldieu D, Sultan Y, Kazatchkine MD. Natural antibodies to factor VIII (anti-hemophilic factor) in healthy individuals. *Proc Natl Acad Sci USA* 1992; **89**: 3795–9.

43 Gilles JG, Saint-Remy JM. Healthy subjects produce both anti-factor VIII and specific anti-idiotypic antibodies. *J Clin Invest* 1994; **94**: 1496–505.

44 Guilbert B, Dighiero G, Avrameas S. Naturally occurring antibodies against nine common antigens in human sera. I. Detection, isolation and characterization. *J Immunol* 1982; **128**: 2779–87.

45 Kasper CK, Aledort LM, Aronson D, Counts R, Edson JR, van Eys J, Frantatoni J, Green D, Hampton J, Hilgartner M, Lazerson J, Levine PH, McMillan CW, Pool JG, Shapiro SS, Shulman NR, van Eys J. A more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh* 1975; **34**: 869–72.

46 Verbruggen B, Novakova I, Wessels H, Boezeman J, van den Berg M, Mauser-Bunschoten E. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *Thromb Haemost* 1995; **73**: 247–51.

47 Giles AR, Verbruggen B, Rivard GE, Teitel J, Walker I. A detailed comparison of the performance of the standard versus the Nijmegen modification of the Bethesda assay in detecting factor VIII:C inhibitors in the haemophilia A population of Canada. Association of Hemophilia Centre Directors of Canada. Factor VIII/IX Subcommittee of Scientific and Standardization Committee of International Society on Thrombosis and Haemostasis. *Thromb Haemost* 1998; **79**: 872–5.

48 Hoyer LW, Gawryl MS, de la Fuente B. Immunochemical characterization of factor VIII inhibitors. In: Hoyer LW, ed. *Factor VIII Inhibitors*. New York: Alan R. Liss, 1984: 73–85.

49 Fulcher CA, de Graaf MS, Zimmerman TS. FVIII inhibitor IgG subclass and FVIII polypeptide specificity determined by immunoblotting. *Blood* 1987; **69**: 1475–80.

50 Matsumoto T, Shima M, Fukuda K, Nogami K, Giddings JC, Murakami T, Tanaka I, Yoshioka A. Immunological characterization of factor VIII autoantibodies in patients with acquired hemophilia A in the presence or absence of underlying disease. *Thromb Res* 2001; **104**: 381–8.

51 Gilles JGG, Arnout J, Vermylen J, Saint-Remy JM. Anti-factor VIII antibodies of hemophiliac patients are frequently directed towards nonfunctional determinants and do not exhibit isotypic restriction. *Blood* 1993; **82**: 2452–61.

52 Biggs R, Austen DE, Denson KW, Borrett R, Rizza CR. The mode of action of antibodies which destroy factor VIII. II. Antibodies which give complex concentration graphs. *Br J Haematol* 1972; **23**: 137–55.

53 Hoyer LW, Scandella D. Factor VIII inhibitors: structure and function in autoantibody and hemophilia A patients. *Semin Hematol* 1994; **31**: 1–5.

54 Slayter H, Loscalzo J, Bockenstedt P, Handin RI. Native conformation of human von Willebrand protein. Analysis by electron microscopy and quasi-elastic light scattering. *J Biol Chem* 1985; **260**: 8559–63.

55 Jun JE, Goodnow CC. Scaffolding of antigen receptors for immunogenic versus tolerogenic signaling. *Nat Immunol* 2003; **4**: 1057–64.

56 Lollar P, Parker CG. Subunit structure of thrombin-activated porcine factor VIII. *Biochemistry* 1989; **28**: 666–74.

57 Fulcher CA, Mahoney SD, Roberts JR, Kasper CK, Zimmerman TS. Localization of human factor FVIII inhibitor epitopes to two polypeptide fragments. *Proc Natl Acad Sci USA* 1985; **82**: 7728–32.

58 Prescott R, Nakai H, Saenko EL, Scharrer I, Nilsson IM, Humphries J, Hurst D, Bray G, Scandella D, Recombinate and Kogenate Study Groups. The inhibitory antibody response is more complex in hemophilia A patients than in most nonhemophiliacs with fVIII autoantibodies. *Blood* 1997; **89**: 3663–71.

59 Scandella D, Mattingly M, de Graaf S, Fulcher CA. Localization of epitopes for human factor VIII inhibitor antibodies by immunoblotting and antibody neutralization. *Blood* 1989; **74**: 1618–26.

60 Fulcher CA, Lechner K, de Mahoney GS. Immunoblot analysis shows changes in factor VIII inhibitor chain specificity in factor VIII inhibitor patients over time. *Blood* 1988; **72**: 1348.

61 Scandella D, Mahoney SD, Mattingly M, Roeder D, Timmons L, Fulcher CA. Epitope mapping of human factor VIII inhibitor antibodies by deletion analysis of factor VIII fragments expressed in *Escherichia coli. Proc Natl Acad Sci USA* 1988; **85**: 6152–6.

62 Healey JF, Lubin IM, Nakai H, Saenko EL, Hoyer LW, Scandella D, Lollar P. Residues 484–508 contain a major determinant of the inhibitory epitope in the A2 domain of human factor VIII. *J Biol Chem* 1995; **270**: 14505–9.

63 Healey JF, Barrow RT, Tamim HM, Lubin IM, Shima M, Scandella D, Lollar P. Residues Glu2181-Val2243 contain a major determinant of the inhibitory epitope in the C2 domain of human factor VIII. *Blood* 1998; **92**: 3701–9.

64 Ortel TL, Moore KD, Quinn-Allen MA, Okamura T, Sinclair AJ, Lazarchick J, Govindan R, Carmagnol F, Kane WH. Inhibitory antifactor V antibodies bind to the factor V, C2 domain and are associated with hemorrhagic manifestations. *Blood* 1998; **91**: 4188–96.

65 Nogami K, Shima M, Giddings JC, Hosokawa K, Nagata M, Kamisue S, Suzuki H, Shibata M, Saenko EL, Tanaka I, Yoshioka A. Circulating factor VIII immune complexes in patients with type 2 acquired hemophilia A and protection from activated protein C-mediated proteolysis. *Blood* 2001; **97**: 669–77.

66 Zhong D, Saenko EL, Shima M, Felch M, Scandella D. Some human inhibitor antibodies interfere with factor VIII binding to factor IX. *Blood* 1998; **92**: 136–42.

67 Lenting PJ, van de Loo JW, Donath MJ, van Mourik JA, Mertens K. The sequence Glu1811-Lys1818 of human blood coagulation factor VIII comprises a binding site for activated factor IX. *J Biol Chem* 1996; **271**: 1935–40.

68 Barrow RT, Healey JF, Gailani D, Scandella D, Lollar P. Reduction of the antigenicity of factor VIII toward complex inhibitory plasmas using multiply-substituted hybrid human/porcine factor VIII molecules. *Blood* 2000; **95**: 557–61.

69 Parker ET, Healey JF, Barrow RT, Craddock HN, Lollar P. Reduction of the inhibitory antibody response to human factor VIII in hemophilia A mice by mutagenesis of the A2 domain B cell epitope. *Blood* 2004 in press.

70 Pratt KP, Shen BW, Takeshima K, Davie EW, Fujikawa K, Stoddard BL. Structure of the C2 domain of human factor VIII at 1.5 A resolution. *Nature* 1999; **402**: 439–42.

71 Barrow RT, Healey JF, Jacquemin MG, Saint-Remy JM, Lollar P. Antigenicity of putative phospholipid membrane binding residues in factor VIII. *Blood* 2001; **97**: 169–74.

72 Spiegel PC Jr, Jacquemin M, Saint-Remy JM, Stoddard BL, Pratt KP. Structure of the factor VIII, C2 domain–immunoglobulin G4k Fab complex: identification of an inhibitory antibody epitope on the surface of factor VIII. *Blood* 2001; **98**: 13–9.

73 Lubin IM, Healey JF, Scandella D, Lollar P. Analysis of the human factor VIII, A2 inhibitor epitope by alanine scanning mutagenesis. *J Biol Chem* 1997; **272**: 30191–5.

74 van den Brink EN, Turenhout EA, Davies J, Bovenschen N, Fijnvandraat K, Ouwehand WH, Peters M, Voorberg J. Human antibodies with specificity for the C2 domain of factor VIII are derived from VH1 germlines. *Blood* 2000; **95**: 558–63.

75 van den Brink EN, Turenhout EA, Bank CM, Fijnvandraat K, Peters M, Voorberg J. Molecular analysis of human anti-factor VIII antibodies by V gene phage display identifies a new epitope in the acidic region following the A2 domain. *Blood* 2000; **96**: 540–5.

76 van den Brink EN, Turenhout EA, Bovenschen N, Heijnen BG, Mertens K, Peters M, Voorberg J. Multiple VH genes are used to assemble human antibodies directed toward the A3–C1 domains of factor VIII. *Blood* 2001; **97**: 966–72.

77 van den Brink EN, Bril WS, Turenhout EAM, Zuurveld M, Bovenschen N, Peters M, Yee TT, Mertens K, Lewis DA, Ortel TL, Lollar P, Scandella D, Voorberg J. 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. *Blood* 2002; **99**: 2828–34.

78 Arai M, Scandella D, Hoyer LW. Molecular basis of factor-VIII inhibition by human antibodies—antibodies that bind to the factor-VIII light chain prevent the interaction of factor-VIII with phospholipid. *J Clin Invest* 1989; **83**: 1978–84.

79 Saenko EL, Scandella D. The acidic region of the factor VIII light chain and the C2 domain together form the high affinity binding site for von Willebrand factor. *J Biol Chem* 1997; **272**: 18007–14.

80 Saenko EL, Shima M, Rajalakshmi KJ, Scandella D. A role for the C2 domain of factor binding to von Willebrand factor. *J Biol Chem* 1994; **269**: 11601–5.

81 Lajmanovich A, Hudry-Clergeon G, Freyssinet JM, Marguerie G. Human factor VIII procoagulant activity and phospholipid interactions. *Biochim Biophys Acta* 1981; **678**: 132–6.

82 Andersson LO, Brown JE. Interaction of factor VIII–von Willebrand factor with phospholipid vesicles. *Biochem J* 1981; **200**: 161–7.

83 Gawryl MS, Hoyer LW. Inactivation of factor VIII coagulant activity by two different types of human antibodies. *Blood* 1982; **60**: 1103–9.

84 Jacquemin MG, Desqueper BG, Benhida A, Vander Elst L, Hoylaerts MF, Bakkus M, Thielemans K, Arnout J, Peerlinck K, Gilles JG, Vermylen J, Saint-Remy JM. Mechanism and kinetics of factor VIII inactivation: study with an IgG4 monoclonal antibody derived from a hemophilia A patient with inhibitor. *Blood* 1998; **92**: 496–506.

85 Jacquemin M, Benhida A, Peerlinck K, Desqueper B, Vander EL, Lavend'homme R, d'Oiron R, Schwaab R, Bakkus M, Thielemans K, Gilles JG, Vermylen J, Saint-Remy JM. A human antibody directed to the factor VIII C1 domain inhibits factor VIII cofactor activity and binding to von Willebrand factor. *Blood* 2000; **95**: 156–63.

86 Sawamoto Y, Prescott R, Zhong D, Saenko EL, Mauser-Bunschoten E, Peerlinck K, van den Berg M, Scandella D. C2 domain restricted epitope specificity of inhibitor antibodies elicited by a heat pasteurized product, factor VIII CPS-P, in previously treated hemophilia A patients without inhibitors. *Thromb Haemost* 1998; **79**: 62–8.

87 Laub R, Di Giambattista M, Fondu P, Brackmann HH, Lenk H, Saenko EL, Felch M, Scandella D. Inhibitors in German hemophilia A patients treated with a double virus inactivated factor VIII concentrate bind to the C2 domain of FVIII light chain. *Thromb Haemost* 1999; **81**: 39–44.

88 Lollar P, Parker ET, Curtis JE, Helgerson SL, Hoyer LW, Scott ME, Scandella D. Inhibition of human factor VIIIa by anti-A2 subunit antibodies. *J Clin Invest* 1994; **93**: 2497–504.

89 Krishnaswamy S, Betz A. Exosites determine macromolecular substrate recognition by prothrombinase. *Biochemistry* 1997; **36**: 12080–6.

90 Lacroix-Desmazes S, Bayry J, Misra N, Horn MP, Villard S, Pashov A, Stieltjes N, d'Oiron R, Saint-Remy JM, Hoebeke J, Kazatchkine MD, Reinbolt J, Mohanty D, Kaveri SV. The prevalence of proteolytic antibodies against factor VIII in hemophilia A. *N Engl J Med* 2002; **346**: 662–7.

91 Dazzi F, Tison T, Vianello F, Radossi P, Zerbinati P, Carraro P, Poletti A, Girolami A. High incidence of anti-FVIII antibodies against non-coagulant epitopes in haemophilia A patients: a possible role for the half-life of transfused FVIII. *Br J Haematol* 1996; **93**: 688–93.

92 Ling M, Duncan EM, Rodgers SE, Street AM, Lloyd JV. Low detection rate of antibodies to non-functional epitopes on factor VIII in patients with hemophilia A and negative for inhibitors by Bethesda assay. *J Thromb Haemost* 2003; **1**: 2548–53.

93 Hay CR, Ollier W, Pepper L, Cumming A, Keeney S, Goodeve AC, Colvin BT, Hill FG, Preston FE, Peake IR. HLA class II profile: a weak determinant of factor VIII inhibitor development in severe haemophilia A. UKHCDO Inhibitor Working Party. *Thromb Haemost* 1997; **77**: 234–7.

94 Singer ST, Addiego JE Jr, Reason DC, Lucas AH. T lymphocyte proliferative responses induced by recombinant factor VIII in hemophilia A patients with inhibitors. *Thromb Haemost* 1996; **76**: 17–22.

95 Reding MT, Wu H, Krampf M, Okita DK, Diethelm-Okita BM, Christie BA, Key NS, Conti-Fine BM. Sensitization of CD4+ T cells to coagulation factor VIII: response in congenital and acquired hemophilia patients and in healthy subjects. *Thromb Haemost* 2000; **84**: 643–52.

96 Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Human CD4+ T-cell repertoire on the C2 domain of coagulation factor VIII. *J Thromb Haemost* 2003; **1**: 1777–84.

97 Jacquemin M, Vantomme V, Buhot C, Lavend'homme R, Burny W, Demotte N, Chaux P, Peerlinck K, Vermylen J, Maillere B, van der BP, Saint-Remy JM. CD4+ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A. *Blood* 2003; **101**: 1351–8.

98 Brettler DM. Inhibitors in congenital haemophilia. *Bailliere's Clin Haematol* 1996; **9**: 319–29.

99 Roberts HR. Recombinant factor VIIa (Novoseven) and the safety of treatment. *Semin Hematol* 2001; **38**: 48–50.

100 Hay CR, Negrier C, Ludlam CA. The treatment of bleeding in acquired haemophilia with recombinant factor VIIa: a multicentre study. *Thromb Haemost* 1997; **78**: 1463–7.

101 Abshire T, Kenet G. Recombinant factor VIIa: review of efficacy, dosing regimens and safety in patients with congenital and acquired factor VIII or IX inhibitors. *J Thromb Haemost* 2004; **2**: 899–909.

102 Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet* 1995; **10**: 119–21.

103 Sarkar R, Gao GP, Chirmule N, Tazelaar J, Kazazian HH Jr. Partial correction of murine hemophilia A with neo-antigenic murine factor VIII. *Hum Gene Ther* 2000; **11**: 881–94.

104 Doering CB, Parker ET, Healey JF, Craddock HN, Barrow RT, Lollar P. Expression and characterization of recombinant murine factor VIII. *Thromb Haemost* 2002; **88**: 450–8.

105 Qian J, Borovak M, Bi L, Kazazian HH Jr, Hoyer LW. Inhibitor antibody development and T cell response to human factor VIII in murine hemophilia A. *Thromb Haemost* 1999; **81**: 240–4.

106 Rossi G, Sarkar J, Scandella D. Long-term induction of immune tolerance after blockade of CD40–CD40L interaction in a mouse model of hemophilia A. *Blood* 2001; **97**: 2750–7.

107 Reipert BM, Ahmad RU, Turecek PL, Schwarz HP. Characterization of antibodies induced by human factor VIII in a murine knockout model of hemophilia A. *Thromb Haemost* 2000; **84**: 826–32.

108 Reipert BM, Sasgary M, Ahmad RU, Auer W, Turecek PL, Schwarz HP. Blockade of CD40/CD40 ligand interactions prevents induction of factor VIII inhibitors in hemophilic mice but does not induce lasting immune tolerance. *Thromb Haemost* 2002; **86**: 1345–52.

109 Sasgary M, Ahmad RU, Schwarz HP, Turecek PL, Reipert BM. Single cell analysis of factor VIII-specific T cells in hemophilic mice after treatment with human factor VIII. *Thromb Haemost* 2002; **87**: 266–72.

110 Wu H, Reding M, Qian J, Okita DK, Parker E, Lollar P, Hoyer LW, Conti-Fine BM. Mechanism of the immune response to human factor VIII in murine hemophilia A. *Thromb Haemost* 2001; **85**: 125–33.

111 Hausl C, Maier E, Schwarz HP, Ahmad RU, Turecek PL, Dorner F, Reipert BM. Long-term persistence of anti-factor VIII antibody-secreting cells in hemophilic mice after treatment with human factor VIII. *Thromb Haemost* 2002; **87**: 840–5.

112 Hausl C, Ahmad RU, Schwarz HP, Turacek PL, Dorner F, Reipert BM. Modulating re-stimulation of memory B cells in hemophilia A with factor VIII inhibitors. *Blood* 2004 in press.

113 Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 2002; **298**: 2199–202.

114 Qian J, Collins M, Sharpe AH, Hoyer LW. Prevention and treatment of factor VIII inhibitors in murine hemophilia A. *Blood* 2000; **95**: 1324–9.

115 Qian J, Burkly LC, Smith EP, Ferrant JL, Hoyer LW, Scott DW, Haudenschild CC. Role of CD154 in the secondary immune response: the reduction of pre-existing splenic germinal centers and anti-factor VIII inhibitor titer. *Eur J Immunol* 2000; **30**: 2548–54.

116 Schwartz JC, Zhang X, Nathenson SG, Almo SC. Structural mechanisms of costimulation. *Nat Immunol* 2002; **3**: 427–34.

117 Ljung R, Petrini P, Tengborn L, Sjorin E. Haemophilia B mutations in Sweden: a population-based study of mutational heterogeneity. *Br J Haematol* 2001; **113**: 81–6.

118 Lusher JM. Inhibitor antibodies to factor VIII and factor IX. management. *Semin Thromb Hemost* 2000; **26**: 179–88.

119 Largo R, Sigg P, von Felten A, Straub PW. Acquired factor-IX inhibitor in a nonhaemophilic patient with autoimmune disease. *Br J Haematol* 1974; **26**: 129–40.

120 Warrier I, Ewenstein BM, Koerper MA, Shapiro A, Key N, DiMichele D, Miller RT, Pasi J, Rivard GE, Sommer SS, Katz J, Bergmann F, Ljung R, Petrini P, Lusher JM. Factor IX inhibitors and anaphylaxis in hemophilia B. *J Pediatr Hematol/Oncol* 1997; **19**: 23–7.

121 Christophe OD, Lenting PJ, Cherel G, Boon-Spijker M, Lavergne JM, Boertjes R, Briquel ME, Goede-Bolder A, Goudemand J, Gaillard S, d'Oiron R, Meyer D, Mertens K. Functional mapping of anti-factor IX inhibitors developed in patients with severe hemophilia B. *Blood* 2001; **98**: 1416–23.

122 Negrier C, Goudemand J, Sultan Y, Bertrand M, Rothschild C, Lauroua P. Multicenter retrospective study on the utilization of FEIBA in France in patients with factor VIII and factor IX inhibitors. French FEIBA Study Group. Factor Eight Bypassing Activity. *Thromb Haemost* 1997; **77**: 1113–9.

123 Lin HF, Maeda N, Smithies O, Straight DL, Stafford DW. A coagulation factor IX-deficient mouse model for human hemophilia B. *Blood* 1997; **90**: 3962–6.

124 Greenwood R, Wang B, Midkiff K, White GC, Lin HF, Frelinger JA. Identification of T-cell epitopes in clotting factor IX and lack of tolerance in inbred mice. *J Thromb Haemost* 2003; **1**: 95–102.

125 Brackmann HH, Oldenburg J, Schwaab R. Immune tolerance for the treatment of factor VIII inhibitors—twenty years' 'Bonn protocol'. *Vox Sang* 1996; **70** (Suppl. 1): 30–5.

126 Mariani G, Siragusa S, Kroner B. Immune tolerance induction in hemophilia A. *Biomed Prog* 2003; **16**: 52–6.

127 DiMichele DM, Kroner BL. The North American Immune Tolerance Registry: Practices, outcomes, outcome predictors. *Thromb Haemost* 2002; **87**: 52–7.

128 Vandendriessche T, Collen D, Chuah MK. Gene therapy for the hemophilias. *J Thromb Haemost* 2003; **1**: 1550–8.

129 Chao H, Walsh CE. Induction of tolerance to human factor VIII in mice. *Blood* 2001; **97**: 3311–2.

130 Mingozzi F, Liu YL, Dobrzynski E, Kaufhold A, Liu JH, Wang Y, Arruda VR, High KA, Herzog RW. Induction of immune tolerance to coagulation factor IX antigen by *in vivo* hepatic gene transfer. *J Clin Invest* 2003; **111**: 1347–56.