Review Article

Anti-factor VIII antibodies

A 2005 update

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Summary

The development of anti-factor VIII (FVIII) antibodies is currently one of the most serious complications in the treatment of haemophilia A patients. Numerous studies in literature report on their epitope specificity, their mechanism of FVIII inactivation, and their relationship with FVIII genetic alterations. During the last two years, however, a particular effort has been made to better understand their generation, with particular emphasis on the interplay of T cells and B cells specific for FVIII and the generation of anti-FVIII antibodies. Moreover, novel strategies to improve the management or treatment of patients with anti-FVIII antibodies have been recently proposed: the use of less immunogenic engineered recombinant FVIII molecules, neutralization of inhibitors by blocking their deleterious activity either by low molecular weight peptide decoys or by anti-idiotypic antibodies, and attempts to suppress the T-cell response involved in the antibody formation. All of these represent promising therapeutic approaches. This review attempts to sum up current knowledge of the nature and properties of anti-FVIII antibodies, their mechanism of action, their neutralization by anti-idiotypic antibodies, and the role of T cells in FVIII inhibitor formation. In the final part, some of the new strategies susceptible to improve the management or the eradication of anti-FVIII antibodies are presented.

Keywords

Coagulation inhibitors, Factor VIII, haemophilia A

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Introduction

Factor VIII (FVIII) is a cofactor in the generation of thrombin by the tenase complex at the surface of activated platelets. It is synthesized in the liver as a 330-kDa precursor protein with an A_1-a_1-A_2-a_2-B-a_3-A_3-C_1-C_2 domain structure, which subsequently undergoes proteolytic processing. FVIII circulates in association with von Willebrand factor (vWF) as a series of heterodimers, consisting of a heavy chain (domains A_1-a_1-A_2-a_2 and variable lengths of the B domain), and a light chain (domains a_3-A_3-C_1-C_2) associated via metal ion interaction.

Haemophilia A is an X-linked bleeding disorder caused by deficiency or functional abnormality of FVIII, at a frequency of about one in 5,000 men. Substitution of defective FVIII by human FVIII concentrates or by recombinant or plasma-derived FVIII represents the basis of the treatment for haemophilic patients. One of the major threats facing patients treated with FVIII concentrates is the development of an immune response to FVIII. Antibodies inhibiting FVIII activity, called inhibitors, are observed in 10% to 40% of patients (1). According to their immunological setting, these inhibitors are qualified as alloantibodies; when they appear in transfused haemophilic patients, and as autoantibodies or acquired inhibitors in patients with autoimmune disease. The frequency of the latter is estimated to be one case per million each year. These patients with anti-FVIII antibodies (Abs) then become resistant to substitution therapy, and their management becomes a major therapeutic challenge. Anti-FVIII Abs can be diagnosed either during routine scheduled surveillance or by a sudden lack of responsiveness to FVIII replacement therapy. The presence of anti-FVIII Abs, as well as their quantification, can be confirmed using the Bethesda assay, where one Bethesda Unit (BU) is defined as the inverse of the plasma dilution inhibiting 50% of FVIII activity in normal plasma. Indeed, preventing or suppressing inhibitors to FVIII re-

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mains a challenge for both clinicians and scientists. A better understanding of the molecular and cellular bases of this complication should lead to the development of prophylatic means to prevent the onset of FVIII inhibitors or therapeutic tools to limit their action. The aim of this article is to review current knowledge on the nature and properties of the different anti-FVIII antibodies, their mechanism of action, their potential neutralization by anti-idiotypic antibodies, and to examine the important role of T cells in anti-FVIII antibody formation. In the last paragraph, we discuss new strategies that could represent promising therapeutic approaches for the management or the eradication of anti-FVIII antibodies.

Genetic properties of FVIII inhibitors

In an attempt to identify some common features between these inhibitors, the question of their eventual isotype or sequence particularities has been addressed in several studies. Most of the anti-FVIII Abs are of the IgG isotype. Some IgM and IgA inhibitors have, however, been observed in patients with acquired haemophilia (2). Gilles et al. (3), who purified alloantibodies from plasma by affinity chromatography on FVIII, observed that the isotypic distribution of anti-FVIII Abs follows the physiological profile of IgG subclasses. However, several other studies report a prevalence of the IgG_4 subclass (although IgG_4 constitutes only 4% of serum IgG) often combined with another subclass (primarily IgG_1) (4–6). It is interesting to note that the two human monoclonal Abs (MAbs), Bo2C11 and Le2E9, isolated from human B cells, belong to the IgG_4 subclass (7, 8). Some inhibitors of the IgG_3 isotype were reported, but this was never the case for the IgG_1 or IgG_2 type. The light chains are either κ or λ chain or both (9). The prevalent contribution of the IgG_4 molecules in the anti-FVIII Ab repertoire might be explained by repeated FVIII infusions, although the underlying mechanism to explain this IgG_4 prevalence remains to be elucidated.

To date, no comprehensive information on the gene repertoire used to encode anti-FVIII Abs is available. The only accessible data are based on the characterization of the two human MAbs, Bo2C11 and Le2E9 (7, 8). In an attempt to obtain more information, Vooberg et al. (10) used the antibody phage display technology, where the patient's immunoglobulin VH gene repertoires were cloned in a phage genome and then expressed in the form of scFvs (single chain antibody variable region fragments) at the surface of the phages. From their studies (11,12), it appeared that the anti-C_2 Abs seem to use exclusively VH genes belonging to the VH_1 family. However, depending on their epitope specificity, the VH gene of some anti-C_2 Abs is extensively modified by somatic hypermutations, and their CDR_3 loop is between 20 to 23 amino acids, whereas in general the average length is in the range of 13 amino acids (13). Other anti-C_2 Abs have a VH gene characterized by a CDR_1 and a CDR_2 being highly negatively charged (five negative charges), a characteristic not affected by VDJ rearrangement nor somatic hypermutations. It should be noted that the human anti-C_2 MAb Bo2C11 (8), whose VH gene belongs to the VH_1 family, exhibits these additional negative charges precisely in the CDR_1 and CDR_2 loops. These charges might mimic the negative surface of phospholipids (PLs) and interact with the positively charged ring of the C_2 domain, explaining how this Ab inhibits the FVIII procoagulant activity by preventing the C_2 domain from binding to PLs. The 3D structure of the C_2 domain in complex with Fab Bo2C11 (14) confirms this assumption. Concerning the anti-A_2 and the anti-A_3 Abs, they showed a broader gene repertoire, distributed among VH_1, VH_3, VH_5, and VH_6 families and VH_1 and VH_3 families, respectively (11, 12). It is interesting to note that in spite of the fact that some anti-A_2 and anti-A_3 Abs use a VH gene belonging to the VH_1 family, they show differences in their somatic mutation profile as compared with the anti-C_2 scFvs, and they do not present any cross-reactivity with the C_2 domain. Analysis of other anti-FVIII Abs appears crucial to establish precisely the rules concerning the VH gene repertoire use of these Abs.

Origin of FVIII inhibitors

Between 20% and 40% of patients with haemophilia A develop anti-FVIII alloantibodies after FVIII infusion. These inhibitors appear during the first months of treatment, generally between the first and the fifth injection (1). In the moderate and mild forms of the disease, the presence of endogenous FVIII reduces the inhibitor incidence; however the risk of inhibitor formation might increase with the growing exposure to FVIII. The reason why only some patients develop an immune response to FVIII still remains elusive. Indeed, several parameters have been identified which seem to influence the appearance of anti-FVIII inhibitors.

Numerous studies have tried to correlate genetic defects in the FVIII gene with inhibitor generation (15). Patients with severe genetic lesions (16), i.e., nonsense mutations as well as important rearrangements (in particular, intron 1 or intron 22 inversions), have a strong risk of developing inhibitors. These mutations lead, in the majority of the cases, either to a lack of FVIII synthesis or to a truncated protein unable to be secreted (15). The immune system of such patients, never having been exposed to any peptide of the FVIII sequence, might be unable to induce antigen-specific tolerance, thus leading to the occurrence of FVIII alloreactive T/B cells. However, it is not yet possible to predict which patient will develop an inhibitor within this high risk group. In patients with small deletions, missense and splice site mutations, which result in loss of function but not complete absence of FVIII protein, the inhibitor prevalence is less than 10% (15, 17). In this group, the patients are partially tolerant to the injected FVIII because they are exposed to their own endogenous FVIII. Consequently, the inhibitors in these subjects will inhibit the injected exogenous FVIII but not the self FVIII, which is seen as a self-antigen (18). It should be noted, however, that patients with certain missense mutations also have a tendency to develop an inhibitor. These inhibitors are primarily targeted to the A_2 domain but also to the C_1-C_2 junction (19). These mutations most probably give rise to a conformationally altered protein predisposing to inhibitor development after exogenous FVIII administration. Certain associations between specific mutations and the presence of Abs have been proposed (20). Similarly, a relationship between Ab occurrence and the localization of a stop codon has been highlighted (20). Nevertheless, no definitive proof has been reported even if some mechanisms in

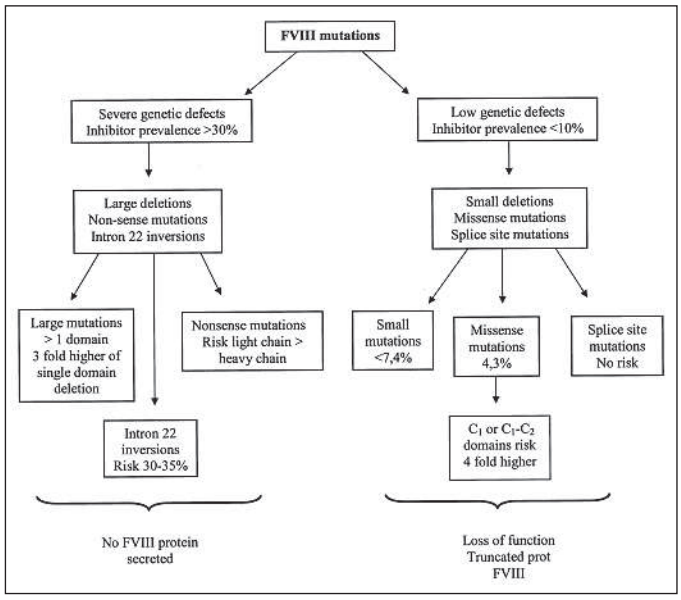

Figure 1: Inhibitor development in correlation with FVIII genotypes.

connection with mRNA splicing are suspected to explain this observation (20). On the basis of the various mutations indexed in HAMSTeRS database (http://europium.csc.mrc.ac.uk) (20), it appears that: (i) patients with large gene deletions affecting more than one domain show a higher risk of developing an inhibitor than those where only one domain is affected (74% of inhibitors for multi-domain deletions versus 21% for single domain deletions), (ii) the nonsense mutations affecting the FVIII light chain (29 inhibitors associated with 9 different codons) are more frequently associated with inhibitor development than those affecting the heavy chain (3 inhibitors for 3 different codons). The different genetic defects are summarized in figure 1. Nevertheless, in patients from the same family showing a similar FVIII mutation, not all develop an inhibitor, suggesting that the occurrence of a particular mutation in itself is not sufficient for inhibitor development and that other factors are important too.

Some accompanying factors are closely linked to treatment: age at the beginning of FVIII treatment, FVIII level, interval between injected doses, and the nature of injected FVIII. Indeed, the composition and purity of the injected FVIII have been suspected to have an influence on inhibitor development as the different steps of purification and virus-inactivation of the FVIII preparations may modify the physico-chemical properties of the molecule and make it more immunogenic. Several cases of inhibitor appearance that could be directly linked to the method of FVIII preparation have been reported. The first case occurred in Belgium and Holland, where multitransfused patients usually treated with plasma-derived FVIII concentrates suddenly developed inhibitors when a new FVIII preparation, including a supplementary pasteurization step (CPS-P product), was injected (21, 22). These inhibitors recognized specifically the C_2 domain, suggesting that the pasteurization step may have modified the conformation of the C_2 domain, rendering it more immunogenic (23, 24). The second series of cases was observed in Germany and Belgium in patients treated with a FVIII for which an additional step of viral inactivation by pasteurization was added (25). It thus seems that the application of some viral inactivation methods may result in an important alteration of FVIII conformation, which might lead to inhibitor generation. Concerning the recombinant FVIII preparations (rFVIII), post-translational modifications specific for each expression system used to prepare the therapeutic molecule somewhat modify FVIII as compared with the native FVIII. Also, the absence of vWF during the manufacturing process may alter the spatial conformation of FVIII making it more immunogenic. Indeed, in a comparative study, whose goal was to follow the immune response in FVIII knockout mice after injections of different FVIII concentrates (recombinant as well as plasmatic FVIII), it appeared that the absence or the presence of reduced levels of vWF correlated significantly with an increase in inhibitor generation (26). This result, however, was not confirmed in humans, and several studies seem to show that rFVIII presents an immunogenicity similar to that of plasma-derived FVIII (27). Consequently, no clear association concerning the increase in the incidence of the inhibitors with specific FVIII concentrates has been reported (28).

Other parameters pertain to the uniqueness of each patient. Inhibitor production is under the control of auxiliary T cells, namely FVIII-specific CD4+ T cells. These auxiliary T cells are subdivided into Th_1 and Th_2 cells on the basis of their secreted cytokine profile (29). The Th_2 cells are implicated in humoral immunity, and several observations tend to highlight their participation in the immune response directed against FVIII. Firstly, as mentioned, IgG_4 is the most commonly encountered subclass among anti-FVIII Abs (5), and Th_2 cells are necessary for subclass isotype switching, generating IgG unable to bind complement, that is, IgE and IgG_4 (30). Secondly, analysis of the primary sequences of the two human MAbs obtained by immortalization with the Epstein-Barr virus (7, 8) as well as those of the human scFvs obtained by phage display (10), indicates that they present several mutations as compared with the germline genes. This suggests that the B cells producing anti-FVIII Abs undergo affinity maturation, a step which requires T-cell help (31). Thirdly, a more direct proof of T-cell involvement was the observation of an inhibitor in haemophilia A patients infected by HIV that disappeared when the T-cell number fell (32). T-cell involvement has intensively been studied in animal models of haemophilia A. Two strains of FVIII-deficient mice generated by insertion of a neo gene into the 3' end of exon 16 and exon 17 of the FVIII gene (probably blocking FVIII production after the A_3 domain), respectively were used (33, 34). In these animals, human FVIII injections were followed by the generation of anti-FVIII Abs. Before their detection, a significant proliferation of specific FVIII T lymphocytes was recorded three days after the first FVIII injection (35). Specific antibodies were observed in 100% of the animals after two or three injections. This suggests that the antibody response to FVIII is T-cell dependent and that the blockade of T-cell activation might prevent inhibitor antibody formation in haemophilia A mice. However, more difficulties were met to demonstrate unequivocally FVIII-specific T-cell responses in human haemophilia A patients. Several investigators (36–43) have tried to isolate FVIII-specific CD4+ T cells from

peripheral lymphocytes to identify immunodominant T-cell epitopes. To achieve this, they examined CD4+ T-cell repertoires from haemophilia A patients with and without inhibitors, from acquired haemophilia A patients and from healthy subjects. Some studies reported the use of whole recombinant FVIII and pools of peptides spanning whole domains of FVIII to stimulate proliferation of FVIII-specific T cells (39, 40), whereas other relied on overlapping peptides spanning only one precise domain (C_2, A_2, or A_3) (36, 41, 42). These studies revealed that all individuals possess CD4+ T cells that are able to proliferate in response to FVIII or FVIII peptides. Some T-cell epitopes are common between groups, regardless of their inhibitor status, but the occurrence of some other T-cell epitopes can be correlated with the presence of an inhibitor. Similarly to the restricted use of defined VH genes in inhibitor, some Vβ genes such as Vβ2, Vβ5, and Vβ9 used by T-cell receptors appeared to be more often expanded among inhibitor positive patients (40). T-cell epitopes were identified in all domains of the protein. More surprisingly, several T-cell epitopes included residues that contribute to inhibitor binding sites, as shown by the work of Jacquemin et al. (37), who isolated and characterized T-cell clones specific for FVIII derived from a patient with a moderate form of haemophilia A (Arg^{2150}His substitution in the C_1 domain) and showed that both B cells and T cells recognized epitopes within the mutated region.

In an attempt to identify individual factors that might affect inhibitor development, other investigators have focused on the study of the MHC class II molecules involved in antibody generation, as these molecules present antigen-derived peptides (T-cell epitopes) to T-cell receptors (17). This approach was privileged as several pieces of evidence tend to indicate a possible genetic predisposition for developing inhibitors. Indeed, inhibitor development is observed in approximately 30% of patients with haemophilia A, and ethnic variations are well documented (44). Thus, depending on the inherited HLA haplotypes, only certain individuals are able to generate FVIII peptide-HLA molecule complexes susceptible to induce Abs directed against biologically relevant epitopes. However, studies aimed at proving an association between defined HLA haplotypes and the capacity to elicit anti-FVIII antibodies were not conclusive. Oldenburg et al. (45) as well as Hay et al. (46) were, nevertheless, able to demonstrate that in a group of patients with intron 22 inversion, the HLA haplotypes A3, B7, C7, DQA0102, DQBO602, and DR15 could be classed as risk alleles and haplotypes C2, DQA0103, DQB0603, and DR3, considered in contrast as protective alleles.

Finally, another explanation for the fact that inhibitors are present only in certain patients could be a difference in stimulation or suppression mechanisms that regulate the immune response. For example, it has been demonstrated that anti-idiotypic Abs (Ab_2) play a role in this regulation. In several cases of anti-FVIII autoimmune disease, a spontaneous remission was associated with an increased production of anti-idiotypic Abs able to neutralize the inhibitory activity of the anti-FVIII Abs (47). Similarly, Gilles et al. (48), and more recently Sakurai et al. (49), suggested that the success of immune tolerance induction was associated with the development of anti-idiotypic antibodies, as the follow-up of the level of anti-FVIII Abs in five unrelated haemophilia A patients remained unchanged with time, whereas Ab_2 were elicited. In a simplified model using the 20F2 monoclonal anti-idiotypic Ab directed against anti-FVIII autoantibodies from a patient, Dietrich et al. (50) established proof of the concept that idiotype/anti-idiotype interactions represent a physiological mechanism modulating the activity of anti-FVIII Abs, an observation which was later confirmed by Gilles et al. with two MAbs (51). Finally, several groups (47, 52, 53) have shown that the inhibitory activity of natural anti-FVIII Abs in healthy individuals can be neutralized by anti-idiotypic Abs. Thus, anti-idiotypic antibodies may shift the immune system toward a steady-state equilibrium that prevents immune response. Nevertheless, the current data do not allow us to generalize the conclusion that the *de novo* development of inhibitors results from a deregulation or a deficiency of Ab_2 production.

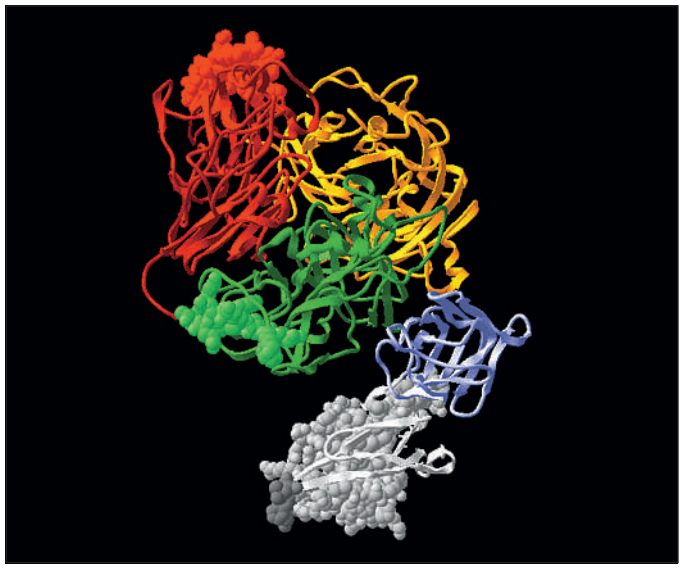

Figure 2: Localization of inhibitor epitopes. The five domains of FVIII are drawn in ribbon format following the model proposed by Stoilova et al. (109). The main epitopes recognized by inhibitors are illustrated in a space-filling format: red for the A_2 epitope (484–508), green for the A_3 epitope (1778–1840) and grey for the C_2 epitope.

Localization of the epitopes

Many studies have dealt with epitope mapping of inhibitors in an attempt to understand their antigenic specificities. The first studies were initiated approximately 15 years ago by Fulcher et al. (54). By immunoblotting of thrombin cleaved FVIII, they showed that most of the epitopes recognized by the inhibitors were localized within the FVIII light chain as well as in the 44-kDa fragment, corresponding to the A_2 domain, and more rarely within the 54-kDa fragment, which corresponds to the A_1 domain (5). Indeed, most of the FVIII mutations concern residues localized after the A_1 domain (55), so that patients may synthesize a certain percentage of protein showing a cross-reactivity with the N-terminal part of FVIII. More detailed epitope mapping was obtained by using recombinant FVIII fragments expressed in *E. coli* (56). From this analysis it appeared that the light chain epitopes are multiple with, however, a predominance

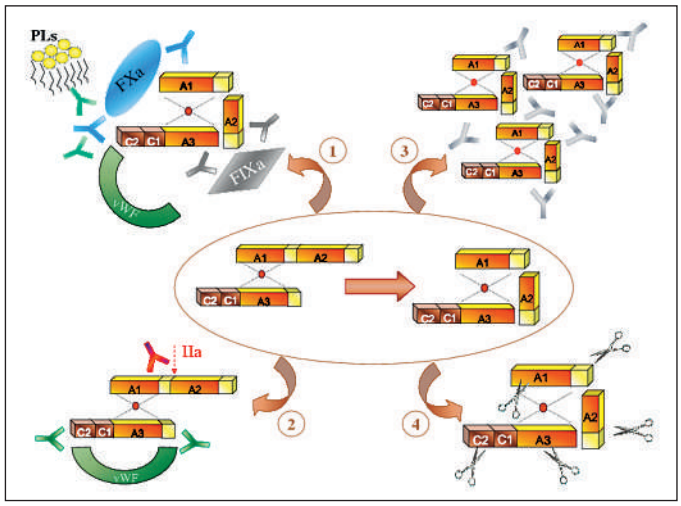

Figure 3: Inactivation mechanisms of anti-FVIII antibodies. (1) Mechanisms based on the binding inhibition of FVIII with one of its partners. Inhibitors with specificity for the C_2 domain (cyan) prevent FVIII interaction with vWF as well as with phospholipids. Some anti-C_2 antibodies, in a similar manner to anti-a_1 antibodies (blue) prevent FVIII interaction with FXa, and anti-A_2 and anti-A_3 antibodies (grey) disrupt FVIII interaction with FIXa. (2) Inhibition of FVIII activation by blocking functional epitopes through steric hindrance. Some antibodies directed against the acidic region a_1 (red) interfere with the activation of FVIII by thrombin. Other inhibitors, specific for the C_2 domain or the acidic region a_3 (green), block protection by vWF, rendering FVIII less susceptible to be proteolytically activated by thrombin. (3) Mechanism based on the increase in FVIII clearance. Some inhibitors (grey) generate immune complexes with FVIII, increasing its clearance from the circulation. (4) Catalytic antibodies against FVIII. Some FVIII inhibitors present proteolytic activity and degrade the FVIII molecule.

in the C_2 domain. The main epitopes recognized by the anti-FVIII Abs are represented in figure 2.

Epitopes on the A_2 domain are essentially localized in the N-terminal part of the domain, between residues 379–538. Based on the observation that clinically significant inhibitors usually cross-react poorly with porcine FVIII, Lollar et al. (57) elegantly mapped the A_2 and C_2 domain epitopes. Indeed, by using recombinant hybrid human/porcine FVIII fragments, they identified amino acid substitutions that resulted in a decrease in Ab cross-reactivity. By taking advantage of exceptional patient's plasma monospecific for the A_2 or C_2 domains, they were able to identify the human FVIII amino acids responsible for the noted antigenic differences. Thus, a major anti-A_2 domain was narrowed to residues Arg^{484}-Ile^{508} (58). This antigenic region contains either a unique epitope recognized by the anti-A_2 Abs or several overlapping epitopes. Alanine scanning of all amino acids in the 484–508 sequence that differ between human and porcine FVIII showed that Tyr^{487} plays a crucial role in the A_2 epitope (59). However, a 26-mer synthetic peptide corresponding to the human FVIII sequence Val^{483}-Glu^{507} neutralized only weakly FVIII inhibition by anti-A_2 Abs, suggesting that other amino acids outside this region may contribute to the epitope (58).

Epitope localization in the C_2 domain is more complex. The use of human/porcine hybrids in the study of five human plasma samples and a monoclonal murine Ab suggested that part of the functional epitope recognized by the inhibitor was located near the N-terminal part of the C_2 domain, within residues 2181 to 2243 (60). On the other hand, an approach based on a series of overlapping synthetic peptides covering the C_2 domain underlined the importance of residues 2303 to 2332 in the C_2 epitope (61, 62). These results are consistent with earlier ones and suggest rather that amino acids present both in the N- and C-terminal parts of the C_2 domain take part in formation of the epitope. It is possible since the crystallographic model of the C_2 domain shows that both ends are in close proximity, being linked by a disulfide bridge between Cys^{2174} and Cys^{2236} (63).

An additional epitope in the light chain, far apart from the C_2 domain, was assigned by two independent studies on the A_3 domain. An approach based on recombinant fragments showed that the region Gln^{1778}-Met^{1823} constituted part of this epitope (64). In parallel, Zhong et al. (65) demonstrated that the synthetic peptide Lys^{1804}-Val^{1819} was able to inhibit the binding of anti-A_3 Abs to FVIII. Moreover, recently, Barrow et al. (66) showed that an A_3 domain human/porcine hybrid molecule was much less antigenic than its human counterpart, and that the Gln^{1778}-Asp^{1840} residues belonged to the epitope recognized by anti-A_3 Abs.

In addition to these inhibitors, mainly found in patients, other anti-FVIII Abs with more uncommon epitope specificities have been identified in certain subjects. These specificities are localized within the different acidic regions (a_1, a_2, and a_3). Their mapping is more difficult as they are not frequent (67), and monospecific Abs against these regions are rare. Using murine MAbs, thought to be representative of human anti-a_1 Abs, several groups have shown that the sequence Thr^{351}-Asp^{361} appears to be the main epitope recognized in the a_1 region (67–69). The antigenicity of this region was confirmed by our group by characterizing another murine MAb (70). The anti-a_3 Abs are more frequent (71), and several studies underline their existence (66, 72, 73). The effects of these inhibitors are additive, suggesting that inhibitors to a given domain act independently of inhibitors to other domains. From one patient to another, the epitope specificity can vary to a considerable extent. This epitopic complexity is further increased as the epitope profile may change with time: inhibitors present in low concentrations at a given time may become dominant later (74). An epitope spreading phenomenon has also been noticed: some inhibitors having initially a specificity for the A_2 domain later on recognize the C_2 domain (75). Despite the different immunologic settings in which they arise, alloantibodies and autoantibodies appear to bind to the same epitopes, though autoantibodies are directed primarily against single domains (2).

Methods of FVIII inactivation

A schematic representation of the different mechanisms of inactivation of anti-FVIII antibodies is shown in figure 3. Depending on their epitope specificities, anti-FVIII Abs prevent FVIII from interacting with its various partners (PLs, vWF, FIX, or FX), thus impairing the coagulation cascade (Fig. 3–1). The most documented inactivation mechanism is the inhibition of FVIII binding to PLs and vWF by anti-C_2 Abs. Indeed by resolution of the crystallographic structure of the C_2 domain, two PL interaction

regions were identified: a positively charged belt (Arg^{2215}, Arg^{2220}, Lys^{2227}, Lys^{2249}), interacting with negatively charged groups from phosphatidyl-L-serines, and a hydrophobic interface, involving the two loops Met^{2199}/Phe^{2200} and Leu^{2251}/Leu^{2252}, with a certain participation of Val^{2223}, which interacts with the hydrophobic side chains of the lipid bilayer. By using C_2 mutants, Gilbert et al. (76) demonstrated that these two hydrophobic loops were also constituents of the vWF binding motif. In parallel, inspection of the crystallographic structure of the Fab Bo2C11/C_2 domain complex revealed that the four hydrophobic residues (Met^{2199}, Phe^{2200}, Leu^{2251}, and Leu^{2252}) are hidden in the Bo2C11 paratope and that Val^{2223} is located at the interface and is in contact with the Fab (14). Moreover, two basic residues of the C_2 domain, Arg^{2215} and Arg^{2220}, are also hidden, forming salt bridges with respectively Asp^{52} and Asp^{102} of the heavy chain of Bo2C11. Consequently, all the key residues taking part in the binding to PLs and vWF being sequestered by Bo2C11, FVIII is unable to bind to its partners and cannot fulfil its procoagulant function. In parallel, Foster et al. (72) reported the case of an anti-a_3 Ab, which by steric hindrance interferes with the interaction between FVIII and vWF, as the acidic region a_3 contains the second site of the vWF binding motif (77). As vWF plays a protective role for circulating FVIII, the inhibition of this interaction renders FVIII more susceptible to proteolytic inactivation. For FVIII interaction with FIX, two binding motifs were reported. Region 1811–1819 along the A_3 domain is responsible for the high affinity for FIX, and region 484–508 of the A_2 domain is involved in the 100-fold increase in the kcat of the FX conversion to FXa (78). Inhibition of this interaction is easily explained as the main epitopes of anti-A_3 Abs as well as anti-A_2 Abs are included or are confined within FIX binding regions (64, 65, 79). Moreover, the anti-A_2 Abs inhibit directly the interaction of the A_2 domain with the protease domain of FIX, which results in the decrease of the Vmax of the activation of FX to FXa by >95% at saturating concentrations without altering the Km (80). Finally, concerning the interaction of FVIII with FX, antibodies directed against residues 2253–2270 of the C_2 domain (81) and the acidic region a_1 (82) prevent this interaction.

Other inactivation mechanisms delay the activation of FVIII by thrombin, a crucial step for the procoagulant activity of FVIII. One mechanism is the slowing down of the dissociation of FVIII from vWF, whereas the second relies on masking the thrombin cleavage site by steric hindrance (Fig. 3–2). The vWF binding motif is composed of two sites, one in the C_2 domain and the second one in the acidic region a_3 (77). FVIII activation by thrombin involves elimination of the a_3 region, leading to a conformational change in the C_2 domain, resulting in vWF release (83). In the first case, some rare anti-C_2 Abs, exemplified by the murine MAb ESH8 (62) and by some human anti-C_2 Abs, whose epitope belongs to the region 2218–2307 (84), seem to prevent the conformational switch that follows a_3 elimination. FVIII thus preserves its strong affinity for vWF and is indirectly prevented from binding to PLs (83). This delay in FVIII release from vWF appears to be sufficient to allow FVIII inactivation by dissociation of the A_2 subunit from the dimer. In the second case, several Abs binding to the acidic regions a_1 and a_3, that are in close proximity with the thrombin cleavage site, were reported to prevent FVIII proteolysis by thrombin by steric hindrance (75, 90).

Another recently described inhibitory mechanism involves FVIII hydrolysis by anti-FVIII Abs endowed with proteolytic properties (85) (Fig. 3–4). Lacroix-Desmazes et al. reported that this antibody-mediated proteolytic activity was present in the IgG fraction of anti-FVIII Abs and was specific for FVIII since these IgG were unable to degrade irrelevant proteins such as albumin or FIX and as normal polyclonal IgG did not hydrolyse FVIII (85). The authors also reported that 13 of 24 patients with FVIII inhibitors had catalytic antibodies in their plasma, and they found a statistically significant correlation between the presence of FVIII-hydrolysing and the FVIII-inhibitory antibodies (86). The kinetic parameters of FVIII hydrolysis as well as the estimated quantity of these catalytic Abs in plasma suggest that a functional role can be allotted to this phenomenon in FVIII inactivation, even if their biological relevance remains unclear.

It is important to note that if inhibitors are identified through their blocking activity, other anti-FVIII antibodies may exist that do not interfere with the functional properties of FVIII. They could be detected by ELISA, and although their precise physiopathological role is unknown, they can be responsible for the increase in FVIII clearance, through the capture of the FVIII-immunoglobulin complexes by phagocytic cells of the reticuloendothelial system (87) (Fig. 3–3). Another possibility would be that they favour the interaction with the endocytic receptor, low density lipoprotein receptor-related protein (LRP). Indeed, the A_2, A_3, and C_2 domains, that are the main targets of the anti-FVIII Abs, were also identified as interacting with LRP (88–91). Therefore, inhibitors may induce a conformational alteration of FVIII, allowing interaction with the LRP, resulting in increased FVIII clearance. Nevertheless, this mechanism has not yet been demonstrated.

Future perspectives

Considerable advances have been made in recent years in understanding various facets of the problem of anti-FVIII Abs, although many questions still remain to be answered. In this final section, we report the most promising studies, which might eventually lead to new strategies to reduce or ideally to eradicate the anti-FVIII antibodies. Improvements could be achieved by intervening at several levels: (i) by treating haemophilia A patients with less immunogenic FVIII molecules, (ii) by reducing the Ab titre by blocking Ab binding to FVIII with peptide decoys mimicking anti-FVIII Ab epitopes, or (iii) by acting directly at the source by suppressing the B-cell and/or T-cell responses, which are involved in the Ab formation.

The concept of using less immunogenic FVIII molecule is already of actuality, as some patients are being treated with porcine FVIII (Hyate: C) (92), a molecule reported to be poorly cross-reactive with human anti-FVIII antibodies (93). To overcome drawbacks like the development of anti-porcine FVIII Abs after exposure to porcine FVIII (94), and the possible transmission of viral agents (95), Lollar et al. (96) constructed active human/porcine hybrid FVIII molecules by replacing the B-cell epitopes along the A_2, A_3, and C_2 domains with the homologous porcine sequence. Barrow et al. (66) have demonstrated that *in vitro* human anti-FVIII Abs recognized preferentially the human FVIII molecule rather hybrid FVIII molecules suggesting a re-

duced antigenicity of these hybrids. Nevertheless, reduced immunogenicity has not yet been reported. These new molecules may also be very interesting for clinical use because they retain full procoagulant activities. They can still be improved by modifying their T-cell epitopes to reduce or block the ability of T cells to recognize the molecule and to stimulate the B-cell response. Nevertheless, there is a risk of losing certain biochemical properties of FVIII or the introduction of new epitopes. This approach is very encouraging although supplementary *in vitro* and *in vivo* studies are required before using these engineered FVIII molecules as therapeutics.

Currently, any approach susceptible to significantly reduce antibody titres of the patients may improve the medical management of this disease. As this complication is largely antibody mediated, our group has proposed a strategy based on blocking the deleterious activity of these Abs by low molecular weight peptide decoys mimicking epitopes recognized by anti-FVIII Abs with the objective of restoring normal procoagulant activity (97, 98). This concept was validated in a simplified model based on the human inhibitory anti-FVIII MAb Bo2C11. Despite the fact that this MAb is an extremely potent inhibitor (7,000 BU/mg), a series of small peptides (12-mer) selected by the phage display technology was shown to be able to restore FVIII procoagulant activity in a murine model of haemophilia A after injection with MAb Bo2C11 (97). Moderate and mild haemophilia A patients with inhibitor could be more prone to benefit from peptide-based therapy, insofar as the anti-FVIII immune response of such patients appears to involve only a limited number of B-cell clones, the specificities of which are directed essentially against the epitope corresponding to the region containing the FVIII mutation. In severe haemophilia A cases, although the immune response is usually more heterogeneous, B-cell epitopes cluster to only a limited number of regions; consequently an optimal combination of peptides to obtain a "universal" decoy could inhibit a majority of anti-FVIII Abs. Another advantage of this approach is that peptides are poor immunogens due to their small size, and the immune system would probably ignore them. Nevertheless, usage of peptides in therapy is generally challenged by their short *in vivo* half-life consequent to their susceptibility to degradation. Introduction of modifications to peptides, such as the replacement of L amino acids by D amino acids or by unnatural amino acids may, however, be an alternative to obtain more stable analogues. Moreover, these epitopic peptides could be used to prepare affinity chromatography columns. Indeed, some clinicians carry out plasmaphoresis to deplete the anti-FVIII Abs, but the drawback is the low specificity of this operation. Removal of inhibitors on a FVIII column is conceivable, but such a technique is very expensive due to the requirement for FVIII. Therefore, use of immobilized antigenic peptides as affinity ligands may be a good alternative to remove inhibitors from patients' blood. Consequently, the use of peptide decoys may be a promising new approach for the neutralization of pathologic Abs.

Instead of fighting existing anti-FVIII antibodies, blocking their production by inducing anergy or deletion of FVIII-specific B cells would be particularly interesting. One possible approach relies on immunoregulation by anti-idiotypic Abs. Indeed, the idiotopes recognized by the Ab_2 at the surface of the inhibitors are also displayed by surface immunoglobulins (sIg) of the corresponding B cells. Therefore, the Ab_2-sIg interaction may prevent the production of inhibitors or even lead to cell apoptosis (99). Several lines of evidence may simplify the feasibility of such an approach. Gilles et al. showed that the 14C12 anti-idiotypic MAb recognized idiotopes expressed by several patients having anti-C_2 domain Abs. Moreover, as the residues constituting these idiotopes belong to the Ab germline sequence (51) and the fact that Van den Brink et al. (11, 12) demonstrated that all the VH genes of the anti-C_2 Abs were from the VH_1 gene family, we can expect to find a "universal idiotope" able to generate an Ab_2 susceptible to bind to the majority of anti-C_2 Abs. Extrapolating this hypothesis to the anti-FVIII Abs with different epitope specificities may however be more difficult since their VH gene repertoires are less restricted. To improve their use, selected Ab_2 can be humanized to reduce their immunogenicity and can be engineered in such a way that their Fc part interacts with complement or with the Fcγ receptor present at the surface of natural killer cells. Nevertheless, such an approach is difficult since the B-cell repertoire is in constant renewal.

Another attractive B-cell depleting approach relies on the use of the chimeric MAb rituximab, directed against the CD20 antigen (100). Rituximab is essentially being used successfully in patients with B-cell malignancies. Nevertheless, Stasi et al. (101) reported its ability to deplete selective B cells in ten patients with acquired haemophilia A. The treatment was completely effective for patients with low inhibitor titre. For patients with high antibody titre, additional drugs such as cyclophosphamide were required to obtain a full and durable response.

In contrast with the B-cell population, the T-cell pool is relatively fixed at birth. Consequently FVIII-specific immunosupressive treatments directed against the CD4+ T cells that drive antibody formation is a promising perspective. T-cell activation requires two signals: one is based on an antigen-specific interaction between the T-cell receptor and the peptide-MHC on antigen presenting cells, and the other involves interactions between receptors and their ligands such as CD40/CD40L and members of the B7 family (B7.1, B7.2) and their ligands CD28/CTLA4. Preliminary attempts in the murine model of haemophilia A are quite encouraging. Qian et al. (102) focused on a potential link existing between Ab generation and these costimulation pathways. They showed that administration of an Ig-fused CTLA4 protein (CTLA4-Ig) prevents initiation of Ab formation, and they underlined the critical role of the B7.2 ligand, but not B7.1, in anti-FVIII Ab generation in the mouse model. Furthermore, in haemophiliac mice with an established anti-FVIII immune response, injection of CTLA4-Ig prevented a further increase in anti-FVIII Abs. However, tolerance induction was not achieved because initially immune-suppressed mice rechallenged with FVIII responded with anti-FVIII Abs. In the same time, Scandella et al. showed that blocking the CD40/CD40L pathway by a MAb specific for the murine CD40 ligand delayed anti-FVIII antibody formation and reduced antibody titres in challenged mice, reflecting suppression of the primary immune response (103). In another set of observations, Reipert et al. reported that during treatment of haemophilic mice, the development of anti-FVIII Abs was completely prevented via the blockage of CD40/CD40L interaction and that the induction of FVIII-specific T cells was suppressed (104–106). In

a clinical trial carried out with an anti-CD40L antibody, confirmatory results were obtained in humans. Anti-CD40L may effectively block anamnestic responses to FVIII. However, no long-term tolerance was induced (107).

There are thus several different ongoing research programs that are promising in the perspective of finding an efficient treatment to prevent development of anti-FVIII Abs. Nevertheless, costimulation blockade impairs all T-cell dependent immune responses, and a more selective approach, which would target T cells of defined specificity, is desirable. Encouragingly, in an autoimmune context, Appel et al. (108) demonstrated that bivalent soluble MHC/peptide complexes, which only bind to T-cell receptors with the appropriate MHC/peptide specificity, induced anergy of antigen specific T cells. This selective approach could be appropriate in the context of haemophilia A as more and more studies tend to identify "universal CD4 epitopes".

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