

Characteristics, Mechanisms of Action, and Epitope Mapping of Anti-factor VIII Antibodies

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Abstract The development of anti-factor VIII (FVIII) antibodies (Abs), also called inhibitors, is currently one of the most serious complications arising during the treatment of hemophilia A patients. Improved prevention and eradication of these Abs remain a challenge both for clinicians and scientists. Numerous studies in the literature have reported on their epitope specificity, on their mechanism of FVIII inactivation, as well as on the methods used for their detection. In this review, we summarize the current knowledge on the nature (isotypes, kinetic properties), epitope properties, and mechanisms of action of anti-FVIII Abs. Furthermore, we present methods for detection and epitope characterization of anti-FVIII Abs with emphasis on the Luminex technique susceptible to facilitate the monitoring of changes in the epitope specificity of these Abs.

Keywords Hemophilia A · Inhibitors · Epitope mapping · FVIII

Abbreviations

FVIII	Factor VIII
FVIIIa	Activated FVIII
pdFVIII	Plasma-derived FVIII
rFVIII	Recombinant FVIII
Ila	Thrombin
vWF	Von Willebrand factor
PLs	Phospholipids
aa	Amino acids
ap	Activation peptide
ITI	Immune tolerance induction
HC	Heavy chain
LC	Light chain

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Introduction

Hemophilia A is an X-linked bleeding disorder caused by deficiency or functional abnormality of factor VIII (FVIII) that occurs with a frequency of about one in 5,000. Substitution of defective FVIII by recombinant (rFVIII) or plasma-derived FVIII (pdFVIII) represents the current basis of the treatment for hemophilia A patients. One of the major threats facing patients treated with FVIII concentrates is the development of an immune response against FVIII that inhibits FVIII procoagulant activity. These inhibitors are observed in 10% to 40% of patients [1, 2]. According to their immunological features, they are defined as allo-antibodies (alloAbs), when they appear in transfused hemophiliac patients, and as auto-antibodies (autoAb) in patients with acquired hemophilia A. The frequency of

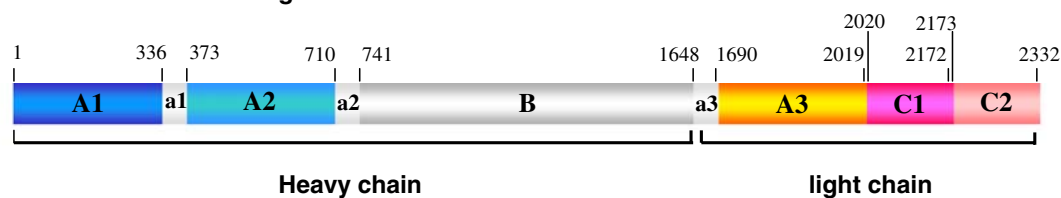
autoAb appearance is estimated to be one case per million each year [3, 4].

FVIII is synthesized in the liver as a 330-kDa precursor protein with an A1a1A2a2-B-a3A3C1C2 domain structure, which subsequently undergoes proteolytic processing. Upon its release in the circulation, FVIII circulates in association with von Willebrand Factor (vWF) as a divalent, cation-linked heterodimer formed by a heavy chain (HC) composed of the A1, A2, and B domains and a light chain (LC) composed of the A3, C1, and C2 domains [5] (Fig. 1a). To generate activated FVIII (FVIIIa) at the site of bleeding, bonds between the A2 and A1 domains within the HC are specifically cleaved at Arg372, Arg740, and within the LC, at Arg1689 [6] (Fig. 1b).

Consequently, inhibitors can alter the coagulation cascade by interfering with the activation of FVIII or with the dissociation of FVIII from vWF by inhibiting FVIIIa function or assembly of the intrinsic FXase complex. It has been established at the molecular level that inhibitors recognize epitopes, which are mainly localized in the A2,

C2, and A3 domains of FVIII, and that inhibition effects might be cumulative [7]. Factors influencing the development of inhibitors in a hemophilic patient can be intrinsic, i.e., endogenous to the patient (a positive family history of inhibitors, FVIII gene mutation type, ethnicity, and polymorphisms in the *IL10*, *CTLA4*, and *TNFA* genes), or extrinsic, i.e., intensity of initial treatment, age of first exposure to factor VIII, and rationale for first treatment (prophylaxis, bleeding, or surgery) [8]. Indeed, the nature of therapeutic FVIII (i.e., rFVIII versus high- or low-purity vWF-FVIII concentrates) appears to be an important parameter (Fig. 2). It is not yet clear whether the risk of developing inhibitors is higher with rFVIII than with pdFVIII [9]. In a recent retrospective study, the influence of the presence of vWF in therapeutic FVIII products has further been brought to light by Goudemand et al. indicating that pdFVIII (which contains vWF) is linked to fewer cases of inhibitor formation than rFVIII [10]. This observation has, however, not been confirmed in another

A/ Primary structure and domain organization of FVIII



B/ FVIII activation

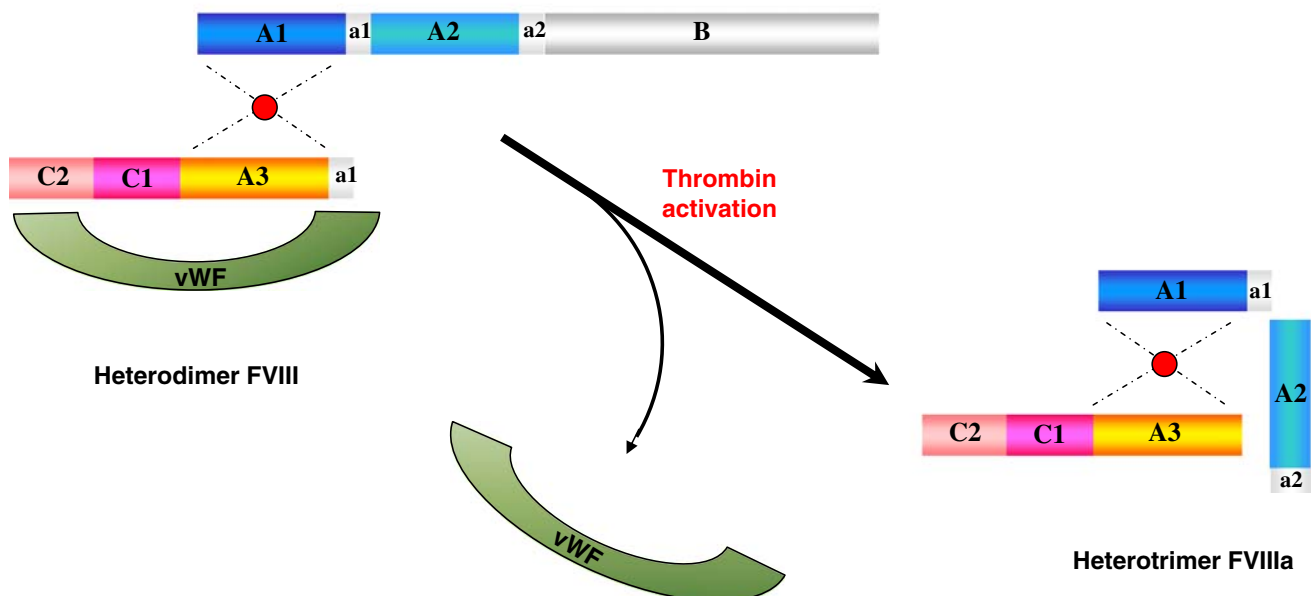


Fig. 1 Structure of the FVIII molecule. **a** FVIII is secreted by hepatocytes as a 2,332-aa polypeptide having the domain structure A1a1A2a2-B-a3A3C1C2. **b** FVIII is released into circulation as a heterodimer of a HC (domains A1a1A2a2-B) and a LC (domains a3A3C1C2) associated through a noncovalent divalent metal ion linkage between the A1 and A3 domain. Upon proteolytic activation

by thrombin, FVIII is activated to a heterotrimer of two HC fragments (the 50-kDa A1 and 43-kDa A2 domains) and the LC (the 73-kDa A3C1C2 fragments). The active form of FVIII (FVIIIa) thus consists of an A1 subunit associated through the divalent metal ion linkage to a thrombin-cleaved A3C1C2 light chain and a free A2 subunit associated with the A1 domain through an ion association

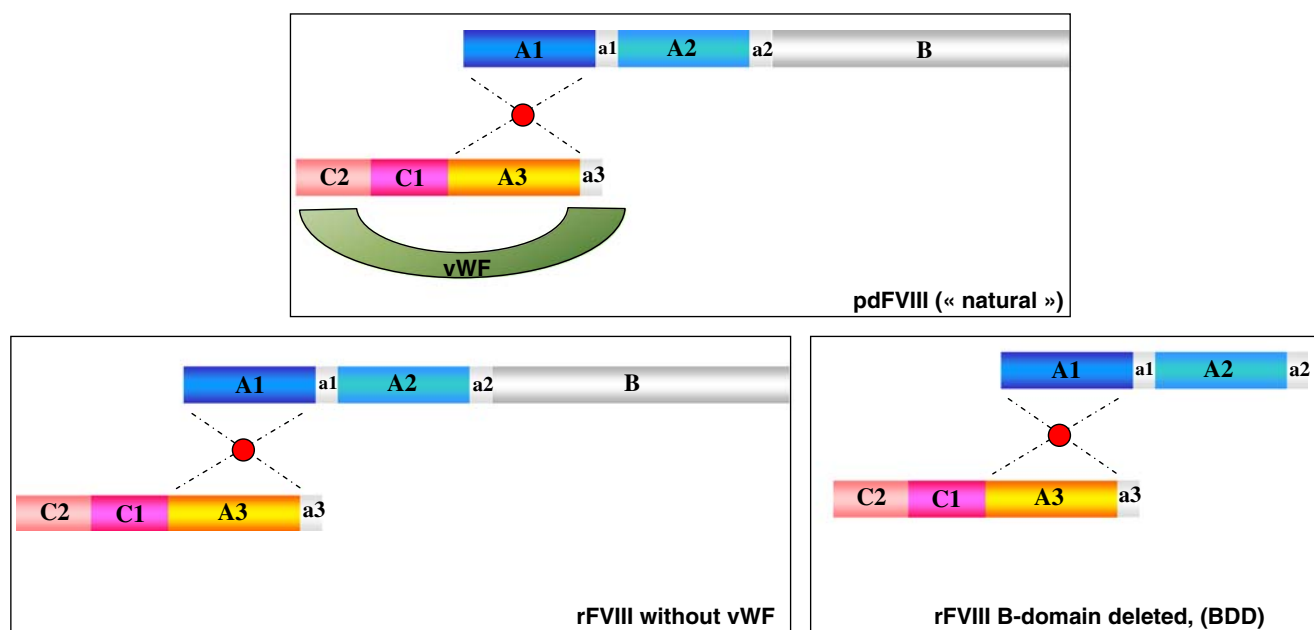


Fig. 2 Different therapeutic FVIII. The three main kinds of FVIII currently used in the clinic. plasma-derived FVIII (pdFVIII) purified from donor plasma and containing vWF, recombinant FVIII (rFVIII)

without vWF, and B domain-deleted FVIII (BDD-FVIII), a recombinant FVIII lacking B domain

recent report [11]. Anyhow, the lower risk of inhibitor development with pdFVIII could be explained by the presence of immunomodulators, which are co-purified with FVIII, and/or by the presence of vWF. vWF could also mask the C2 epitopes and therefore block some anti-C2 Abs. The impact of the product quality is an important issue, as vWF is one of the rare putative cofactors that may be controlled in the therapeutic management of patients with inhibitors. Further investigations with prospective controlled studies are needed to confirm the eventual protective effect of vWF on FVIII immunogenicity and to dissect the molecular mechanisms that could be at play.

According to the current international recommendations, the only efficient therapy against FVIII inhibitors occurring in hemophiliacs is immune tolerance induction (ITI). This approach aimed to eradicate inhibitors by regular infusions of large amounts of FVIII. Immune tolerance is effective in 70% to 80% of the cases, yet the mechanisms that allow achieving FVIII tolerance as well as possible genetic, biological, and therapeutic factors that might affect its efficacy remain unclear. Among the confirmed predictive factors for the efficacy of ITI, the titer of the inhibitor before ITI <10 Bethesda units/mL (BU/mL; i.e., inhibitor titer measured by the Bethesda method after the first massive infusions of FVIII at the beginning of ITI), its “historic peak” <20 BU/mL, the age at onset the ITI, and interval between development of inhibitor and inflammatory state of the patient appear to be important [12]. Another suggesting variable in the response to ITI is the choice of

the product used in ITI. So far, no superiority of rFVIII or pdFVIII has been found for the efficacy of ITI.

It is therefore crucial to gather in-depth information about the immune response against FVIII and to compare the available technologies to identify the different inhibitors and their epitopes. This review will focus on the characteristics and properties of anti-FVIII Abs, their mechanisms of action, and the methods to detect and to analyze their specificity. Finally, the usefulness of a new qualitative immunoassay that allows simultaneously the detection and the mapping of epitopes of all anti-FVIII Abs will be discussed.

Characteristics of anti-FVIII antibodies

Isotypes and subclasses

Most inhibitory Abs belong to the IgG isotype. The study by Gilles et al. [13] on polyclonal anti-FVIII preparations, purified from plasma by affinity chromatography, suggests that the isotypic distribution of anti-FVIII Abs follows the physiological profile of the IgG subclasses, with a preference for the IgG1 and IgG4 subclasses; results from other groups support the theory of predominance of the IgG4 subclass (although this subclass only represents 4% of the IgG in the serum), often in association with IgG1 subclass [13–16]. The predominance of IgG4 molecules in the repertoire of anti-FVIII Abs is probably a consequence of the repeated administrations of FVIII required to

maintain adequate FVIII levels for a normal hemostasis [17]. Nevertheless, it is not known why the IgG4 subclass is predominant among the anti-FVIII Abs. Noteworthy, the two human monoclonal Abs, Bo2C11 and Le2E9, which have been isolated from human memory B cells transformed with the Epstein Barr virus, are both IgG4 [18, 19]. This isotype was also observed in the case of autoAbs. On the other hand, inhibitors belonging to the IgM and IgA isotypes have also been reported in patients with acquired hemophilia [20]. Recently, using a murine model, Healey et al. have demonstrated that Abs directed against the A2 domain belong preferentially to the IgG1 subclass, whereas anti-C2 domain Abs are preferentially IgG2a, a murine isotype having the same pro-inflammatory properties than human IgG4 [21]. Van Helden et al. [22] identified in a longitudinal study the IgG subclasses of the inhibitors present in 14 patients undergoing ITI. They could show that (1) the relative contribution of IgG subclasses was similar in the majority of the patients analyzed and that (2) the levels of anti-FVIII Abs of the IgG1 and IgG4 subclasses correlated well with the inhibitor titers measured with the Bethesda assay. In low-titer inhibitor patients, anti-FVIII Abs consisted primarily of IgG1, whereas anti-FVIII Abs of the IgG4 subclass were more prominent in patients with high-titer inhibitors [22]. Although this pilot study does not provide an explanation for the observed differences in subclass distribution among patients, the results suggest that the immunological context and/or the intensity of treatment may enhance class switching of anti-FVIII Abs to IgG4. Future studies will need to address whether monitoring the relative contribution of anti-FVIII Abs of the IgG1 and IgG4 isotypes could be useful for the identification of patients who are at risk of failure after ITI.

Inhibitor kinetics

FVIII inhibitors displayed type I or II kinetics. Type I Abs completely inhibits FVIII activity in a time-dependent manner, whereas type II Abs mediate incomplete inactivation [23]. These different behaviors are related to their different epitope specificities. Type I inhibitors recognize antigenic regions that are essential for the procoagulant activity, while type II Abs recognize epitopes that are distant from these regions. The mechanism responsible for the kinetics profile of type II Abs is thought to involve competition between vWF and the inhibitor, while in the absence of vWF, most type II inhibitors seem to behave like type I inhibitors [23]. However, some type II inhibitors partially inhibit FVIII even in the absence of vWF [19], while others inhibit FVIII only when it is in interaction with vWF [24]. To summarize, the population of type II inhibitors appears to be highly heterogeneous, and the boundary between type I and II kinetics of FVIII inactivation is not always clear [25].

Mechanisms involved in the inhibition and degradation of FVIII by antibodies

Depending on the location of the target epitope, different mechanisms of action for the anti-FVIII Abs have been reported (Fig. 3): (a) impairment of the binding of FVIII to one of its partners by steric hindrance and (b) other mechanisms such as increased FVIII clearance or catalytic degradation of FVIII. Of note, inhibitors are polyclonal and might, therefore, exhibit a range of inhibitory mechanisms in the same patient. Scandella et al. also suggested the existence of an “epitope spreading” phenomenon in association with anti-FVIII Abs, characterized by modification over time of the epitopic profile [26–28], which might be associated with a concomitant change in inhibition mechanisms.

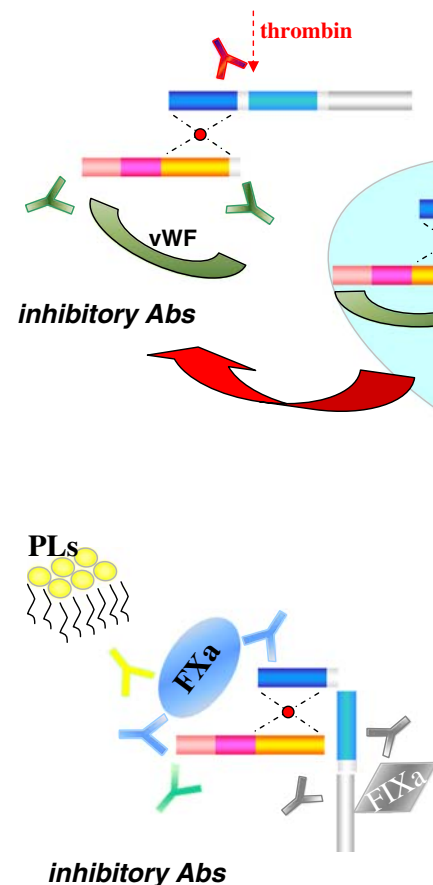
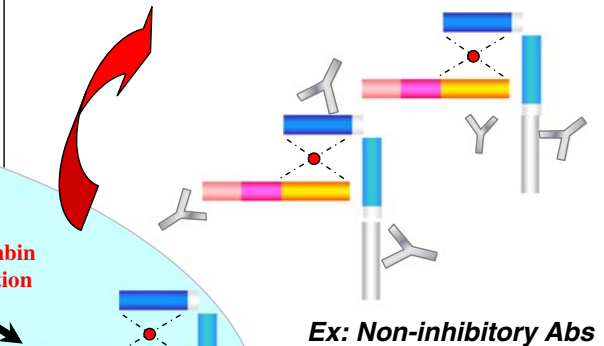
Mechanisms (a) of inhibitors

Steric hindrance

Inhibitors might block the procoagulant function of FVIII by disrupting its binding to one of its partners (PLs, vWF, FIX, or FX). Sixty-eight percent of inhibitory Abs are directed against the A2 and/or C2 domain [25], 3% against the A1 domain, and 46% against the a3 acidic region [29]. In this section, we describe the main known mechanisms of inactivation by inhibitors according to their specificity for domains and acidic regions of FVIII (Fig. 3a).

Anti-A2 inhibitors

These inhibitors affect the interactions between the A2 domain and FIXa in a noncompetitive manner, resulting in the formation of an inactive FVIIIa/FIXa/PLs and FX complex [30, 31]. The epitope that is most often recognized by these anti-A2 domain Abs is located in the 484–508 region, which is recognized by the monoclonal Ab 413 (Fig. 4) [32]. In 2006, Ansong et al. characterized the epitope of the R8B12 monoclonal Ab as comprising residues 497–510 and 584–593 and thus differing from the previously identified 484–508 continuous epitope [33] (Fig. 4). More recently, Healey et al. analyzed the antibody response to human FVIII in hemophilia A mice subjected to a dosage schedule that mimics the human therapy [21]. Using an enzyme-linked immunosorbent assay (ELISA) method to assign domain specificity and a high-throughput FVIII inhibitor assay to assess their functional properties, they characterized Abs from 506 hybridomas. In addition to the classical anti-A2 and anti-C2 inhibitory responses, they described, for the first time, the relative contribution of noninhibitory Abs to the immune response. They also discovered a novel inhibitory A2 epitope and identified a class of anti-FVIII Abs with

A/ Inhibition by steric hindrance**B/ Others mechanisms****Immune complex formation and FVIII clearance****Active hydrolysis of FVIII by catalytic Abs**

Other inhibitors specific for the C2 domain or the acidic region a3 (green) block protection by vWF, rendering FVIII less susceptible to be proteolytically activated by thrombin. **b** Others mechanisms: one is based on an increase of the FVIII clearance. Some inhibitors (grey) generate immune complexes with FVIII, increasing its clearance from the circulation. Another mechanism is due to catalytic Abs against FVIII. Some FVIII inhibitors present proteolytic activity and degrade the FVIII molecule

Fig. 3 Mechanisms of action of anti-FVIII Abs. **a** Mechanisms based on the inhibition of FVIII activation by blocking functional epitopes through steric hindrance. Inhibitors with specificity for the C2 domain (green) prevent FVIII interaction with vWF or with phospholipids. Some anti-C2 Abs, in a manner similar to anti-a1 Abs (blue), prevent FVIII interaction with FXa. Anti-A2 and anti-A3 Abs (grey) disrupt FVIII interaction with FIXa. Some Abs directed against the acidic region a1 (red) interfere with the activation of FVIII by thrombin.

dual specificity for the A1 and A3 domains. Although obtained in a mouse model, these results represent a very significant step forward towards a better understanding of the complex immune response to FVIII.

Anti-C2 domain inhibitors

Depending on the location of the target epitope of the anti-C2 Abs, different mechanisms of inactivation are possible (Fig. 4):

1. Some anti-C2 inhibitors act by inhibiting the *FVIIIa*–*vWF* interaction [34] and by inhibiting *FVIIIa* binding to phospholipids (PLs) [35]. Study of the 3D structure of the human monoclonal antibody Bo2C11 in complex
2. Some anti-C2 Abs are thought to alter *exclusively the FVIIIa*–*vWF* interaction. A prototype of this class is the

with the C2 domain [36] has provided a good understanding of its mechanism of inactivation. Indeed, Bo2C11 recognizes a conformational epitope composed of residues located in the N-terminal (2170–2216) and C-terminal region (2303–2332) of the C2 domain. Bo2C11 has an extremely high affinity for FVIII due to a very low dissociation constant [18] and can compete with vWF to form an inactive Bo2C11–FVIII complex that is extremely stable over time. Furthermore, the Bo2C11 epitope in the C2 domain overlaps with the binding site for PLs [37]. It seems that inhibitors from patients directed towards the C2 domain behave like Bo2C11 [38].

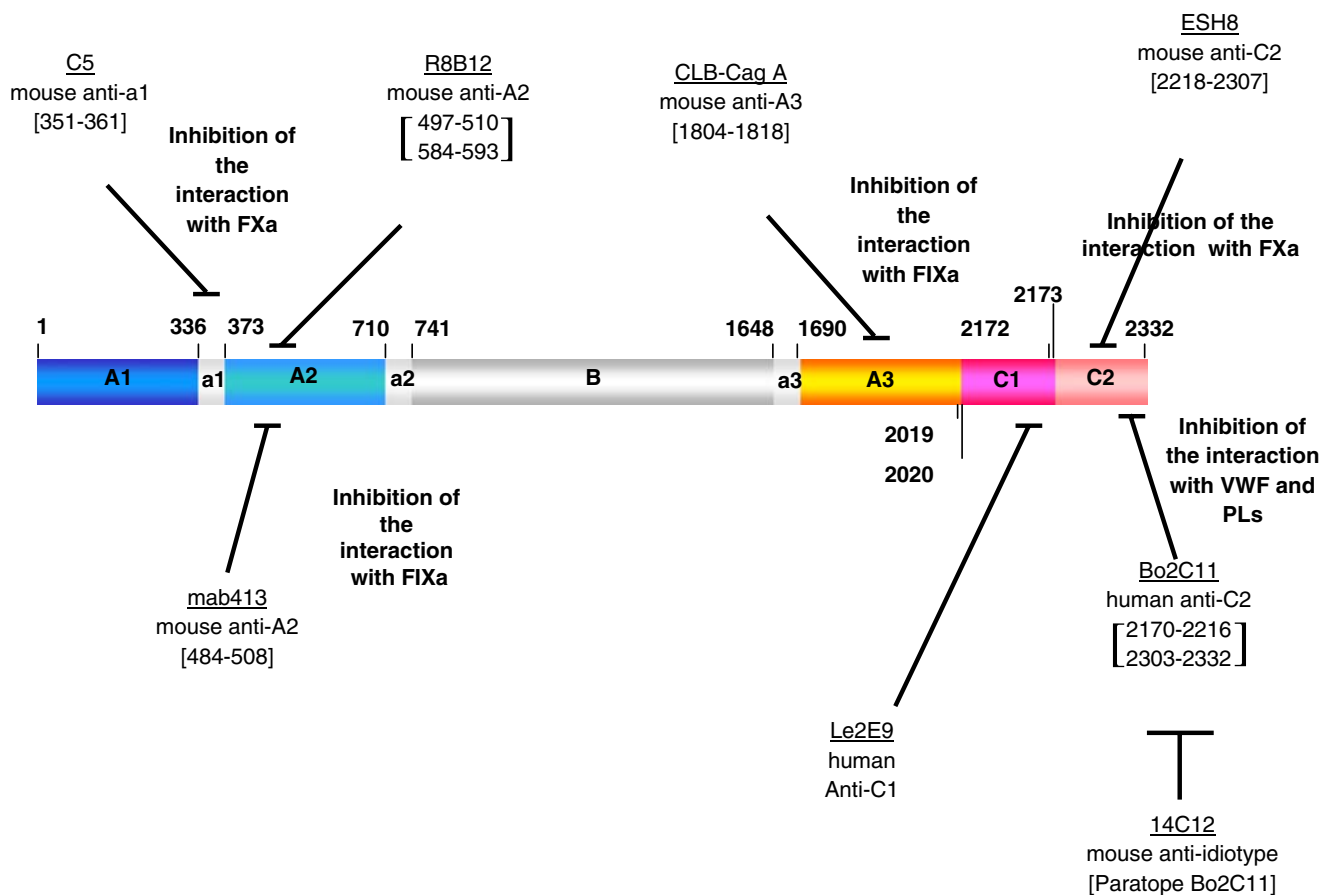


Fig. 4 The main representatives of monoclonal anti-FVIII Abs and their mechanisms of inhibition. The anti-FVIII Abs could block the procoagulant function of FVIII by inhibiting the interaction with FXa (C5, ESH8), FIXa (413, CLB-Cag A), or with vWF and/or PLs (Le2E9, Bo2C11). These monoclonal anti-FVIII Abs recognized

either continuous epitopes (C5) or conformational epitopes (Bo2C11, Le2E9). Moreover, these Abs could be blocked by anti-idiotypic Abs (14C12). *Numbers in brackets* refer to epitope position in the FVIII sequence

mouse monoclonal antibody ESH8 [26], which recognizes an epitope located between residues 2218 and 2307 of the C2 domain [39]. This Ab slows down the dissociation of FVIIIa from vWF, thus indirectly preventing the binding of FVIIIa to PLs [40]. During activation by thrombin, elimination of the a3 acidic region [40, 41] induces a conformational change in the C2 domain that disrupts the association with vWF [42]. ESH8 binding, prior to FVIII activation by thrombin, prevents this conformational change during the elimination of a3. As a consequence, FVIII retains its affinity for vWF [42]. The release of FVIII from vWF is delayed, and FVIIIa inactivation (by dissociation from the A2a2 subunit of the dimer) begins before it participates in the tenase complex.

3. In a mouse model of hemophilia A, some “nonclassical” *anti-C2 domain* Abs (i.e., not inhibiting FVIII binding to PLs or vWF) were described [43] that alter the mechanisms of FVIII activation by thrombin or FXa. The diversity of the anti-C2 immune response was

clearly demonstrated in the mouse model, where five groups of structural epitopes and two groups of functional epitopes were described. Three of them (A, AB, and B) consist of classical Abs that inhibit the binding of FVIII to PLs and vWF (see (1) and (2)). The other two groups (BC and C) contain nonclassical anti-C2 Abs that block the activation of FVIII by thrombin or FXa. Abs from the BC group display high inhibitory activity and have type II kinetics. Moreover, these “nonclassical” anti-C2 Abs seem to be present in hemophilia A patients with inhibitors [43]. These results reveal the complexity of the anti-C2 Abs response. Further investigations are needed to better understand the molecular mechanisms of action and the in vivo pathogenicity of nonclassical anti-C2 Abs.

Anti-A3 domain inhibitors

Several observations suggest that anti-A3 domain Abs neutralize the procoagulant activity of FVIII by preventing

its interaction with FIXa (Figs. 3a and 4). Fijnvandraat et al. demonstrated that a human inhibiting alloantibody targeted to the 1778–1823 region interfered with FIXa binding to FVIII LC [44]. Indeed, one epitope has been mapped to residues Lys1804–Lys1818 and Glu1811–Gln1820 of the FVIII LC. The overlapping sequence Glu1811–Lys1818 comprises the minimal requirements for binding of FVIII to FIXa [45, 46].

Anti-a1 and anti-a3 inhibitors

Inhibitors recognizing the acidic region a1 may act through two mechanisms: (1) by altering FVIII activation or inactivation since a1 is flanked by two cleavage sites (the first after Arg372 is recognized by thrombin or FXa to activate FVIII, whereas the second, after Arg336 is recognized by thrombin, activated protein C (APC) and FXa to inactivate FVIII) [47]; (2) by modifying the binding of the A2a2 subunit to the dimer and thus the formation of the active trimer [48]. Anti-a3 Abs inhibit the FVIII–vWF interaction as the acidic region a3 contains the second vWF binding site [39].

Anti-C1 domain inhibitors

The human monoclonal antibody Le2E9 was obtained from an immortalized memory B-lymphocyte cell line isolated from a patient with moderate hemophilia A who had developed anti-FVIII IgG specific for exogenous FVIII [19, 49]. The characteristics of this antibody are very specific: (1) it partially inhibits the procoagulant activity of wild type FVIII; (2) it completely inhibits FVIII–vWF binding; (3) it does not inhibit the endogenous FVIII of the patient (which carries an Arg2150His mutation); and (4) it binds to the C1 domain of exogenous FVIII. Inhibition of the FVIII–vWF interaction is caused by the high dissociation rate of the FVIII^{His2150}–vWF complex [49, 50]. Gilbert et al. studied the catalytic activity of the tenase complex to evaluate the effect of phospholipidic membranes on the binding of FIXa to FVIIIa and on the enzymatic cleavage of the product. The apparent affinity of FIXa for FVIIIa was tenfold lower in the absence of phospholipidic membranes [51]. Thus, Le2E9 seems to block a PL binding site on the C1 domain and then to compete with FIXa for binding to FVIIIa in the tenase complex. However, the role of the C1 domain has not really been elucidated. To address the potential role of the C1 domain in FVIIIa attachment to platelets, Hsu et al. analyzed the binding of the C1C2 or C2 fragments to platelets using flow cytometry and proposed a new role of this domain in the procoagulant activity of the FVIII. Binding of the C1C2 fragment to the platelet surface was lower than that of the C2 fragment both before and after

platelet activation. This study suggests that the C1 domain, together with FVIII LC, could contribute to platelet-binding affinity. Further studies are needed to investigate the special orientation of this domain with respect to the platelet membrane surface and the role of specific residues in binding to phospholipidic membranes [52] (Fig. 4).

Mechanisms (b): other mechanisms

Formation of immune complexes and FVIII clearance

Some authors have previously described anti-FVIII Abs that do not interfere with the functional properties of FVIII [53, 54]. These Abs are directed towards so-called “nonfunctional” epitopes and can be detected using immunoassays such as ELISA techniques. They are present in 8% to 10% of hemophilia A patients with or without inhibitors depending on the study [50, 55, 56]. Although the consequences of their presence on the efficacy of the coagulation cascade are not yet fully understood, some authors have suggested that the formation of immune complexes (Abs + FVIII) might lead to an accelerated clearance of transfused FVIII by cells of the innate immunity (macrophages and neutrophils) [56] (Fig. 3B). However, in 2001, Mondorf et al. failed to establish a correlation between recovery and the presence of “non-inhibitory” Abs detected by immunoprecipitation in 16 patients with severe hemophilia [57]. These results led to question the importance of this type of Abs. To date, their real clinical significance remains unclear, and international recommendations do not as yet require detecting those Abs using ELISA or immunoprecipitation techniques. Efforts to understand the role of the “noninhibitory” Abs as well as to develop new tools to detect easily all types of anti-FVIII Abs and to study their epitope specificity need to be pursued.

Catalytic antibodies

Some anti-FVIII Abs can also degrade FVIII through an intrinsic proteolytic activity. Lacroix-Desmazes et al. described the presence of IgG that hydrolyze FVIII in patients with severe hemophilia A who developed inhibitors [58] (Fig. 3b). These catalytic Abs are present in over 50% of patients with severe hemophilia and FVIII inhibitors. The same group further demonstrated that the kinetics of FVIII degradation by IgG Abs is compatible with a pathogenic role for catalytic IgG. In addition, they reported that each patient’s FVIII-hydrolyzing IgG exhibits multiple cleavage sites on FVIII, while the specificity of cleavages varies from one patient to another [59].

Techniques and tools for epitope mapping of anti-FVIII antibodies

The epitopes of FVIII inhibitors have been studied with different techniques. A rough localization of epitopes was first obtained, followed by a more precise knowledge thanks to technical breakthroughs). Most of the techniques are adapted to the environment of a research lab but present numerous drawbacks for studying the epitopes targeted by inhibitors in routine clinical practice. We briefly describe here the techniques used to map the FVIII epitopes, as well as their limitations (summarized in Table 1).

Immunoblotting/immunoprecipitation techniques

In immunoprecipitation (IP) techniques, an immune complex (antigen–antibody) is captured on a solid support to which either protein A or protein G has been immobilized. The immune complex is eluted from the support and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblotting consists of probing the antigenic reactivity of proteins or fragments that have been previously separated by gel electrophoresis. The first studies on the specificity of epitopes recognized by inhibitors were initiated about 20 years ago by Fulcher et al. [60]. They evaluated by immunoblotting the reactivity of inhibitors against different FVIII fragments broken down by thrombin. They found that most of them bound to the LC of FVIII as well as to a 44-kDa fragment that corresponds to the A2 domain and more rarely to a 54-kDa fragment, which comprises the A1 domain [60]. Subsequently, a more precise mapping of epitopes was carried out by Scandella's group, using IP techniques with recombinant fragments of different FVIII domains (including deletions) expressed in *Escherichia coli* [61]. They demonstrated that most of epitopes recognized by FVIII inhibitors were localized on a 18.3-kDa fragment of the N-terminal part of A2 and/or on a 17.3-kDa fragment of the C-terminal part of the C2 domain. A more detailed analysis

of the LC showed that there was also an epitope on the A3 domain. In fact, mapping using recombinant fragments (Asp1563–Asp1840, Asp1563–Gln1778, and Asp1563–Met1823) showed that the residues Gln1778–Met1823 comprised a part of the epitope [44]. These results demonstrated that the immune response directed against FVIII was oligoclonal, with a complex pattern of epitope recognition, affecting preferentially the same antigenic regions corresponding to the A2, C2, and a3A3–C1 domains. The major limitations of these techniques are that they use denatured FVIII or FVIII fragments, and therefore, they are unlikely able to recognize conformational epitopes. In addition, these techniques are somewhat unwieldy and cannot be considered as a systematic, simple-to-use and rapid method for clinical biology laboratories.

Use of recombinant hybrid porcine/human FVIII in a Bethesda assay

Porcine/human FVIII hybrids constructed by the Lollar's group had allowed precise mapping of the epitopes recognized by FVIII inhibitors in a coagulation assay (Bethesda assay). Chimeric molecules, combining human and porcine FVIII sequences [62], made it possible to identify a major determinant of the epitope of the A2 domain inhibitors (exemplified in Fig. 3 by the mAb 413) which is localized on residues Arg484–Ile508 [63]. Hybrid porcine/human FVIII also allowed the identification of a major epitope in the N-terminal part of the C2 domain (close to residues Glu2181–Val2243) [63]. These results suggested that the residues present in the N- and C-terminal part of the C2 domain participated in the formation of the epitope. Then, Barrow et al. demonstrated that an antigenic region was recognized in the A3 domain, in particular residues Gln1778–Asp1840. They also identified an epitope in the a3 region; the representativeness of this epitope in the patient's inhibitor appears to be more substantial than that of the a1 acidic region [64] (Fig. 3). The use of hybrid porcine/human FVIII despite allowing a precise epitope

Table 1 Techniques and tools for epitope mapping of anti-FVIII Abs compared to Bethesda method

Techniques and tools	Detection of				Suitability for clinical practice
	Continuous epitope	Conformational epitope	Inhibitory Abs	Noninhibitory Abs	
Bethesda method	–	–	X	–	current
Immunoblotting/immunoprecipitation techniques	X	–	X	X	–
Recombinant hybrid porcine/human FVIII	X	X	X	–	X
Spot technique	X	–	X	X	–
Phage-displayed peptides	X	X	X	X	–
ELISA technique	X	X	X	X	X
Luminex technique	X	X	X	X	X

localization of inhibitors has some limitations. Indeed, a certain homology/identity of sequences exists between human and porcine FVIII segments, and thus, hybrid constructions are not always helpful to characterize epitope specificity of human inhibitors [65]. Furthermore, a heavy investment in terms of molecular biology (cloning and expression of different mutants, purification of constructions, etc.) is required. Thus, this technique is not appropriate to study the epitope repertoire of anti-FVIII Abs in hemophilia A patients in clinical practice.

Spot technique

Developed by Merrifield in 1963, the solid phase peptide synthesis (SPPS) consists in binding the C-terminal amino acid (aa) of the target peptide to an insoluble solid support (i.e., polystyrene beads), then lengthening the peptide chain by successive additions of residues from the C terminal to the N terminal. The spot technique is a derivative of the SPPS in which the solid support is a cellulose membrane [66]. Overlapping peptides covering the amino acid sequence of domains of FVIII can easily be prepared in this way and further tested for antigenic reactivity with monoclonal antibodies or anti-FVIII patients' Abs. In 2003, Raut et al. characterized at the aa level the epitopes of two murine monoclonal Abs, directed against the a1 and a2 acidic regions of FVIII [67]. The same approach was used by Schwaab et al. for the mapping of anti-FVIII Abs developed in patients with hemophilia A [68]. Later on, Kopecky et al. studied the epitope profile of anti-FVIII Abs in six hemophilia A patients. They demonstrated that the immune response of the patients was directed against all FVIII domains, with a predominance of inhibitors against the LC and more particularly against the C1 domain. This study also confirms the presence of noninhibitory Abs preferentially directed against the A1 and B domains [69]. The spot technique only allows the characterization of continuous epitopes and requires large volumes of plasma. This approach is thus inappropriate for epitope study of inhibitors out of the research laboratory environment.

Phage-displayed peptides

Phage display is a technique in which a foreign protein or peptide is expressed at the surface of filamentous bacteriophages. This system, developed by Smith, was originally used to create large libraries of randomly expressed peptides for the purpose of selecting those that specifically bind to a particular antibody [70]. This allows selection of peptides that mimic either continuous or discontinuous epitopes (called mimotopes). Villard et al. successfully used phage display for the identification of mimotopes recognized by the monoclonal Abs ESH8 or Bo2C11 [71]. They

derived synthetic peptide decoys able to block the FVIII inhibitory activity of ESH8 and Bo2C11 and demonstrated their *in vivo* ability to restore FVIII procoagulant function in the presence of the potent Bo2C11 antibody in a hemophilia A mouse model [71]. More recently, Kessel et al. used a similar approach in order to compare humoral immune response before and after anti-CD20 treatment in a hemophilia A patient with inhibitors. The authors thus selected a peptide which is still recognized after inhibitor relapse despite complete B-cell depletion and which mimics a conformational epitope in the A2 domain. This peptide partially restored FVIII activity *in vitro* [72]. Although interesting, the phage display technique is not fully appropriate for characterizing the epitopes of polyclonal inhibitors in hemophilic patients because the use of polyclonal antibodies does not provide unambiguous mapping of epitopes.

ELISA technique

Enzyme-linked immunosorbent assay uses an immunoassay principle (detecting interactions between antigen and antibody where one of those is captured on a solid support) with an enzyme as the reporter of the interaction. It is well adapted to routine measurements of analytes in patient's samples. Martin et al. have evaluated the ELISA technique for the detection of anti-FVIII developing in hemophilia A patients. In a population of 131 patients with hemophilia A with inhibitors, these authors demonstrated that their ELISA technique was sensitive (97.7%), specific (78.8%), and with a high negative predictive value (98.6%) [73]. Shetty et al. confirmed these results in a large cohort of 312 patients and also highlighted the interest of this technique for the detection of noninhibitory Abs [74]. More recently, Towfighi et al. found a highly significant correlation between the Bethesda titer and the absorbance values measured with either a global (Ig, IgG/A/M) or specific (IgG and IgG4) ELISA for detecting anti-FVIII Abs [75]. The ELISA technique is promising since it detects all the anti-FVIII Abs with a good correlation with the Bethesda method; this method also made possible to confirm the presence of noninhibitory Abs [76]. However, this method is not adapted to a precise epitope mapping of anti-FVIII Abs which would require the immobilization in ELISA plates of the two chains and different domains of FVIII and thus necessitate using relatively large volumes of plasma.

Immunoassay based on the Luminex technique

The Luminex technique allows the simultaneous analysis of different analytes in a single microplate well [77]. It is based on the recognition by Abs of a probe (e.g., FVIII or fragments) attached to a polystyrene bead tagged by a specific fluorescent address. Several beads can be mixed,

each bearing a different antigen, and the binding signal on each bead is finally deconvoluted. This approach has many advantages: it is fast (3 to 4 h), it requires small volumes of plasma (<100 µl), and it is suitable for multiplexing by coupling of different markers of interest (e.g., chains, domains, peptides...) to different beads (theoretically, up to 100 different markers) (Fig. 5). In 2008, we reported the use of the Luminex technology to develop a multiplex (duplex) assay that allows the detection of all anti-FVIII Abs and the rough localization of their epitope and requires a small plasma sample [78]. We confirmed that all anti-FVIII Abs (inhibitory or noninhibitory) can be simultaneously detected with this technology. Indeed, the duplex assay allows establishing two distinct epitope profiles: monospecific for autoAbs or polyspecific for alloAbs. The autoAbs recognized preferentially the LC of FVIII mainly in C2 domain, as previously described [27]. Concerning alloAbs, the results were in agreement with the observation

that the immune response in patients with congenital hemophilia A is more complex than those patients with acquired hemophilia. Moreover, as most of the alloAbs arise early in the course of patient's treatment (generally after about 9–20 days exposure in children with severe hemophilia A) the multiplex assay could be advantageous for the clinical management as the low plasma requirements could preserve venous access for treatment. Further improvements in the follow-up of patient's management may be achieved with this technology since the epitope specificity and its evolution along time could be analyzed precisely and quite easily.

Conclusions

The development of anti-FVIII inhibitors represents a major medical hurdle and a critical societal concern; indeed,

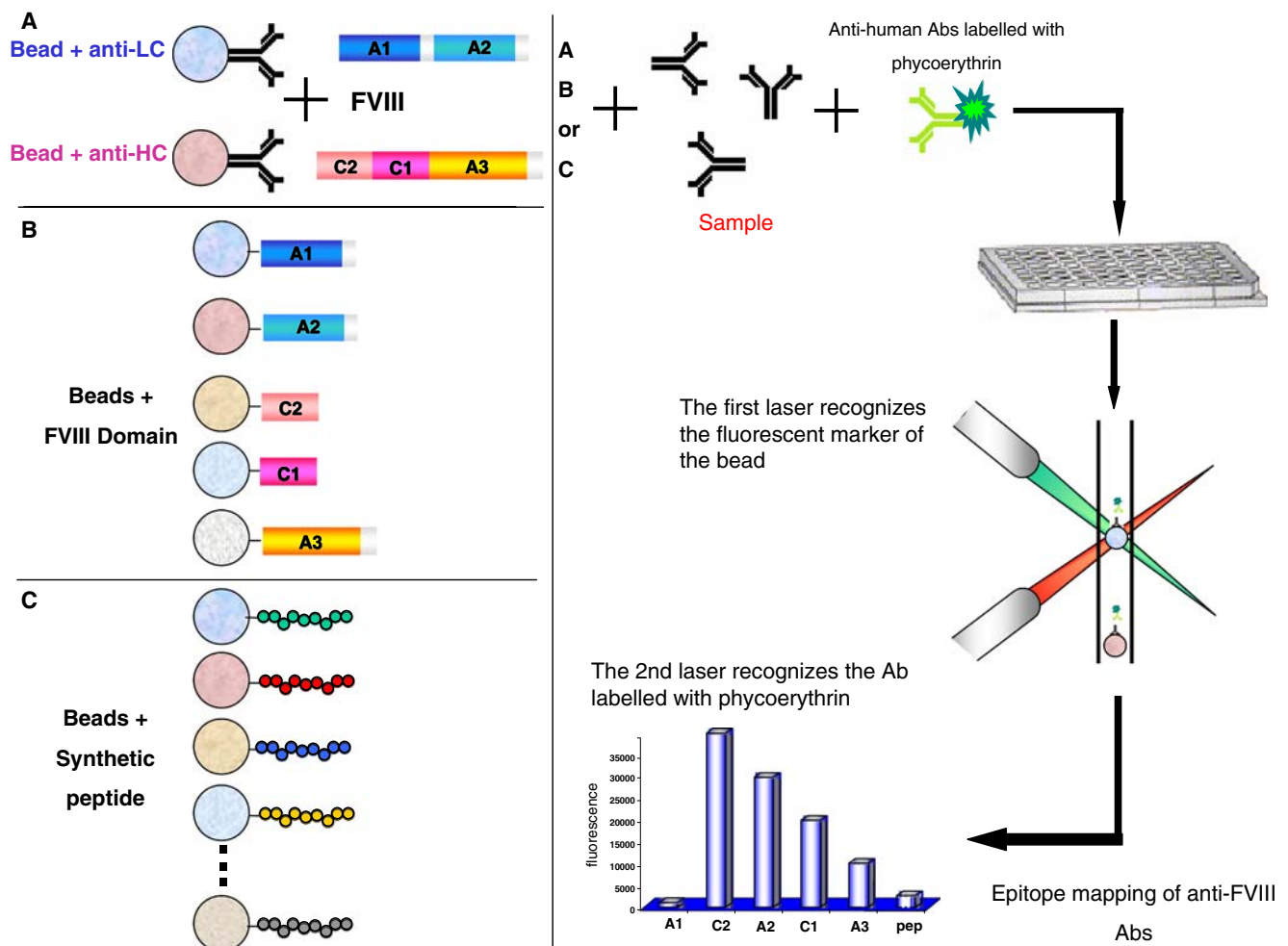


Fig. 5 Simultaneous detection and epitope specificity determination of anti-FVIII Abs using the Luminex technique. The Luminex immunoassay detects simultaneously anti-LC and anti-HC Abs (a), detection of different domain of FVIII (b), or epitopic peptides (c).

Ab, peptide, or protein can be covalently immobilized on beads, the putative ligand is incubated, and PE-labeled Ab directed against the human IgG reveals the reactions

patient with inhibitors become resistant to conventional therapy, and their quality of life is dramatically affected, while their morbidity and mortality are increased. Moreover, the development of inhibitors in patients with congenital hemophilia A increases by more than threefold the cost of their treatment. Consequently, this problem remains at the center of many scientific and medical debates. Questions concerning the pathophysiology and therapeutic management of patients with inhibitors are plentiful. However, in the clinical practice, few tools are currently available to improve our knowledge about the immune response against FVIII Abs that arises in patients with hemophilia A. The study of epitopes specificity of anti-FVIII Abs is not feasible with most of the available tools. In addition, the noninhibitory Abs that are not detected by the routine Bethesda technique are not studied. Therefore, new tools are needed that allow better detection and understanding of the mechanisms of appearance of anti-FVIII Abs in hemophilic patients (autoAbs or alloAbs). The analysis of the epitope repertoire of all types of anti-FVIII (inhibitors and noninhibitory) Abs and of IgG subclasses could help to unravel the underlying pathophysiological mechanisms and, above all, should allow to define the best therapeutic strategy for these patients. Compared to other methods, the Luminex approach seems to be a promising tool, simple, rapid, and reproducible, allowing to the possibility of analyzing many samples simultaneously on several antigens.

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