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Mild/moderate haemophilia A: new insights into molecular mechanisms and inhibitor development

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Summary. In mild/moderate haemophilia A (MHA) patients, many factor VIII (FVIII) gene defects, mainly missense mutations, have been identified and greatly improved the understanding of the structure and function of FVIII molecule. Characterization of the molecular mechanisms involved in MHA has helped to identify regions critical for proper FVIII biosynthesis, thrombin activation, intramolecular stability as well as binding regions for important intermolecular interactions with von Willebrand factor, factor IXa and the phospholipid surface. Some missense mutations were also recognized as contributing factors to inhibitor development in MHA, in parallel to acquired factors such as inflammatory state or intensity of treatment. Treatment of MHA with inhibitor patients raises questions on how best to stop or prevent bleeding episodes and eradicate the inhibitor. Longitudinal data collection is currently being conducted in France and Belgium to enhance our knowledge in this field and to further help make treatment decision. The description of mutations in MHA finally contributed to the identification of epitopes involved in the immune response to FVIII. In some patients, the epitope specificity of inhibitor antibodies recognizing normal exogenous FVIII alone and not patient ('self') FVIII was described. This distinguished epitope specificity could also be demonstrated at the T-cell clonal level. One might expect that these molecular studies will have a major impact on development of new FVIII products in the future.

Keywords: factor VIII, genotype, inhibitor, mild/moderate haemophilia A, T-cell epitope

Introduction

Patients with mild/moderate haemophilia A (MHA) have deficient factor VIII clotting activity (FVIII:C) from >0.05–0.40 and 0.01–0.05 IU mL^{-1} respectively. Compared with severe patients, bleeding episodes are less frequent, mostly provoked by trauma or invasive procedures, and more rarely lead to recurrent haemarthroses and subsequent arthropathy. Bleeding episodes may be successfully treated or prevented either by desmopressin (DDAVP) in good responder patients or with FVIII concentrates [1]. However, delayed diagnosis, incomplete haemostasis characterization, inadequate response or tachyphylaxis to DDAVP, and loss of tolerance to either exogenous and/or endogenous FVIII remain challenges.

To address these challenges, combined analysis of genetic, clinical and immunological issues gave major insights into MHA during the last two decades. Identification of FVIII gene mutations and the corresponding variants provided pertinent tools for biochemical analysis and prediction of three-dimensional changes in the FVIII molecule structure, improving understanding of genotype/phenotype relationships and suggesting different causal mechanisms leading to MHA [2–4].

A few missense mutations potentially contributing to a higher risk of inhibitor development have also been described [5–10]. Treatment decision making in patients with MHA and inhibitors (MHAI) raises

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questions with regard to optimal treatment for bleeding episodes and how to eradicate the inhibitor effectively. This is especially important when the inhibitor is directed to the patient's own endogenous FVIII, changing the clinical phenotype to severe [11,12]. Based on the preliminary results from longitudinal data collection ongoing in France and Belgium, a few suggestions can be made. The description of mutations in MHA allowed identification of epitopes involved in the immune response to FVIII [13–15]. In parallel, antibodies recognizing either wild-type FVIII and/or self-mutant FVIII and the T and B-cell response at the clonal level were characterized [12,16,17]. The amino acid numbering given below is for the mature processed protein, as used in the HAMSTeRS database [2].

Molecular mechanisms of MHA

FVIII structure and function

Insights into molecular mechanisms of haemophilia require an understanding of the structure and function of the FVIII molecule (see Fay and Jenkins for a detailed review [3]). FVIII is a large plasma glycoprotein functioning as an essential cofactor for the proteolytic activation of factor X by activated factor IX (FIXa) within the intrinsic pathway of coagulation. The genetic sequence predicts a domain structure of A1-a1-A2-a2-B-a3-A3-C1-C2. Interdomain segments a1, a2 and a3 represent acidic amino acid rich regions. FVIII undergoes complex protein folding and disulfide bond formation followed by numerous post-translational modifications critical for efficient secretion and macromolecular interactions. It is secreted as an inactive heterodimer of a variably processed heavy chain (HC, subunits A1-a1-A2-a2-B, ~90–200 kDa) and a light chain (LC, subunits a3-A3-C1-C2, ~80 kDa) associated via a copper (Cu+)-dependent interaction between the A1 and A3 subunits. This inactive form of FVIII is stabilized in plasma by non-covalent interaction with von Willebrand factor (vWF) mediated by epitopes within the FVIII LC. Upon activation with thrombin, proteolysis removes the B domain and bisects the HC resulting in an activated FVIII heterotrimer (FVIIIa, subunits A1/A2/A3-C1-C2). Cleavage of the amino terminus of the LC (a3) results in dissociation from vWF and allows FVIII to exert its cofactor function by interacting with FIXa and FX on a phospholipid (PL) surface (e.g. activated platelet). FVIIIa can be inactivated through proteolysis by activated protein C that cleaves off the carboxy terminus of the A1 subunit and bisects the A2 subunit. However, the FVIIIa heterotrimer is unstable and also subject to spontaneous decay of its procoagulant activity attributable to first-order dissociation of its free A2 subunit that occurs at physiological pH.

The characterization of the molecular mechanisms causing MHA has contributed to confirm and advance our understanding of the structure and function of this complex protein. These are presented in Fig. 1 and reviewed in some additional detail as follows.

Defects in protein folding and intracellular trafficking

Missense mutations leading to reduced secretion are a common mechanism resulting in haemophilia A. FVIII requires a scaffold of protein chaperones within the endoplasmic reticulum (ER) to help it achieve its complex tertiary structure. These intracellular interactions also serve as a quality control mechanism to retain misfolded FVIII protein until it can achieve its properly folded structure or target it to ER degradation pathways. Properly folded FVIII then relies on key post-translational modifications to assist with intracellular trafficking and ultimately secretion. Predictably, any missense mutations that significantly disrupt the tertiary structure will be mostly retained and degraded resulting in severe haemophilia A. However, some missense mutations have been described, which probably result in less severe perturbations in FVIII folding and a reduced but significant residual amount of functional material is secreted into the plasma leading to a less severe clinical phenotype. These have been described throughout the A and C domains of FVIII. Proposed structural perturbations for these amino acid substitutions include position effects (torsion angle changes of protein chain segments, 'bulky' substitutions leading to steric hindrance, altered intramolecular distances that disrupt stabilizing hydrogen bond formation), distant effects (disturbance of bonding patterns some distance from the site of the molecular substitution) and direct bonding effects (loss or creation of structural bonds such as hydrogen bonds, salt and disulfide bridges) [18]. There is a relative absence of such missense mutations within the B domain of FVIII suggesting that the primary amino acid sequence of the B domain may not be critical to FVIII folding efficiency. The role of post-translational modifications within the B domain in efficient FVIII secretion has been reviewed elsewhere [19].

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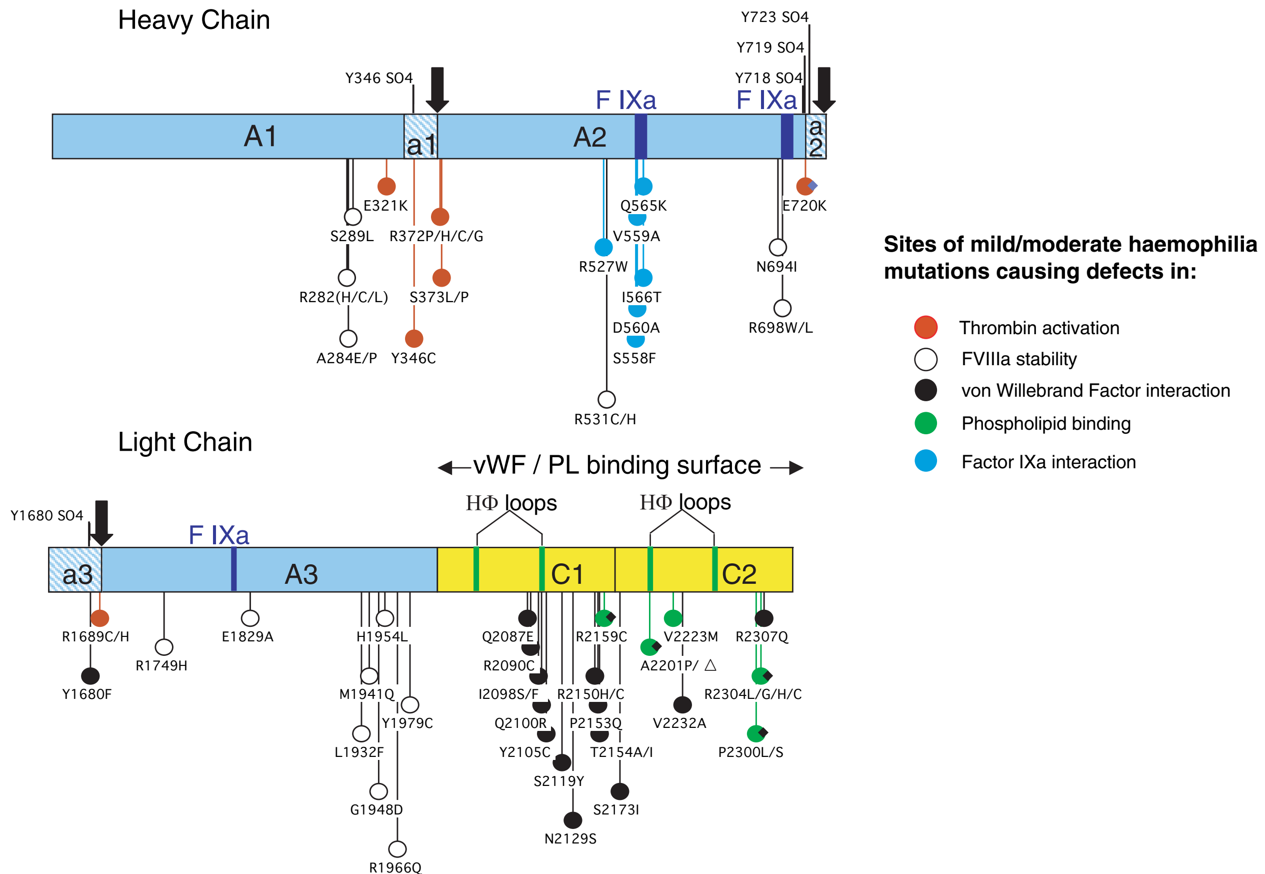

Fig. 1. Sites of mutations and related molecular mechanisms causing mild and moderate haemophilia (excluding mutations associated with defects in protein folding and intracellular trafficking). Black arrows correspond to thrombin activation sites. SO4, sulfation; vWF, von Willebrand factor; PL, phospholipid; FIXa, factor IXa; HΦ, hydrophobic.

Defects in vWF interaction

The role of the FVIII–vWF interaction in stabilizing FVIII in plasma, extending its half-life and reducing clearance is well described. Accordingly, missense mutations that disrupt this interaction can lead to haemophilia. These mutations have been identified primarily within the C1 and C2 domains and may alter the core structure of these domains or disrupt surface site interactions [20]. In particular, distinct clusters of residues have been identified as haemophilia-yielding sites that may disrupt vWF interaction: Gln2100, Tyr2105, Ser2119, Arg2150 and Thr2154 within the C1 domain and Pro2300, Arg2304, and Arg2307 within the C2 domain. Additional mutations have been described near the C1 cluster at Glu2087, Arg2090, Ile2098, Asn2129, and Pro2153 [21]. Outside the C domains, a single residue within the A3 domain, Tyr1680, has been associated with mild haemophilia through elimination of a site for tyrosine sulphation that is critical for vWF affinity [22].

Defects in thrombin activation

Thrombin cleaves FVIII after Arg372, Arg740 and Arg1689. Acidic regions in the interdomainal connecting regions that precede these cleavage sites probably provide important contacts for thrombin. Sulphated tyrosines have also been demonstrated to be important for facilitating efficient thrombin cleavage. FVIII has sulphated tyrosine residues at 346, 664, 719, 721, 723 and 1680. The role of Tyr1680 for vWF binding was described above. Tyr346Cys mutation has been described and patients with this mutation exhibit higher FVIII activity as measured by a two-stage assay than by a one-stage assay [23]. Preincubation with thrombin in the two-stage assay may explain this observation suggesting a role for this sulphated tyrosine in coordinating efficient thrombin cleavage. However, this particular mutation has been observed in patients without a clinical bleeding phenotype such that this may be an *in vitro* phenomenon rather than a clinically relevant defect in thrombin activation [24]. A similar FVIII

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activity assay discrepancy has been described with Glu321Lys and Glu720Lys [3]. Glu321 is predicted to lie in close proximity to Arg372 based on three-dimensional modelling of the A domains of FVIII, whereas Glu720 probably contributes to the acidic nature of the a2 interdomain segment.

Defects in stability of FVIIIa

Discrepancies in FVIII activity as measured by one-stage and two-stage assays have been informative in elucidating mechanisms for many mild haemophilia presentations. As described above, an increased FVIII activity in the two-stage assay can in some cases be attributed to mechanisms that affect thrombin activation. However, a more common presentation is a reduced FVIII activity in the two-stage assay compared with the one-stage assay. Haemophilia A patients with this clinical phenotype have been recognized for decades. Once genetic testing was available, a number of missense mutations have since been described, mainly clustered within the A domains [25–31]. Biochemical analysis of recombinant forms of these mutations demonstrated that these FVIII mutants all exhibited accelerated decay in FVIII activity after thrombin activation secondary to an increased rate of A2 subunit dissociation from the FVIIIa heterotrimer [32]. Three-dimensional modelling highlighted these residues as being located at the hydrophobic A1-A2 and A2-A3 and A1-A3 interfaces. It has been hypothesized that amino acid substitutions at some of these critical residues cause subtle structural perturbations resulting in destabilization of the interdomain interactions. Whereas the majority of these mild haemophilia patients would still be identified by the commonly used one-stage FVIII assay, at least one mutation (His1954Leu) exhibits normal activity by one-stage assay and only exhibits FVIII activity in the mild haemophilia range as tested by the two-stage assay [33]. Thus, at least some patients with this clinical phenotype could be missed through routine screening evaluations.

Defects in phospholipid binding

The FVIII C1 and C2 domains are important interactive surfaces for the PL membrane. Insights into the critical residues involved in this interaction have come through the study of crystallographic structures derived for the C2 domains of FVIII [34] and FV [35], FVIII bound to PL [36], a homology model of the FVIII C1 domain [20] and a recent crystal structure of the FVIII heterodimer [4]. Because of the critical nature of this interaction for FVIII cofactor activity, it is not surprising that missense mutations leading to even mild structural perturbations in these domains could result in haemophilia A. An analysis of 57 reported mutations corresponding to substitutions at 43 separate residues within the C1 and C2 domains have been analysed [20]. Missense mutations were evenly divided between both domains with only 20% associated with severe disease. A majority of these probably interfere with protein folding by disrupting the protein core. At least one haemophilia mutation, Val2223Met, involves a surface exposed hydrophobic residue on the C2 domain that may represent a PL interactive site. In addition, Arg2159 within the C1 domain has been associated with haemophilic missense mutations and is also located on the same relative surface as Val2223. Ala2201Pro may change the orientation of a preceding Met2199/Phe2200 beta-hairpin that is one of the key proposed interactive sites with PL. Interestingly, although mutations at Pro2300 have been proposed to disrupt vWF binding, the PL binding profile is different dependent on the amino acid substitution – PL binding is reduced if Pro2300Leu but normal if Pro2300Ser [37].

Defects in interaction with FIXa

The FIXa interactive site has been localized to residues 558–565, and 698–712 within the A2 domain and residues 1811–1818 within the A3 domain. Haemophilia mutation Ile566Thr creates a new asparagine-linked glycosylation site and Ser558Phe introduces a bulky side chain, both of which could create steric hindrance impairing FIXa interaction with the 558–565 segment [38]. Other missense mutations in this region include Val559Ala, Asp560Ala, and Gln565Lys [39]. In addition, three-dimensional modelling of the A domains positions residues 511–530 in close proximity to residues 558–565 [26]. Thus, mutations in this segment, for example Arg527Trp, could also interfere with FIXa interaction at that site.

Other genetic defects associated with MHA

In addition to the multiplicity of missense mutations in this patient population, other genetic mechanisms can lead to MHA. Novel splicing errors (~9% of mild haemophilia A) and rare small rearrangements and promoter mutations have now been described [18]. Point deletions are another mechanism. Del2201 causes a structural perturbation in the C2 domain, which impairs FVIII interaction with both

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vWF and PL [40]. Interestingly, this point deletion also eliminates a major antigenic determinant of the FVIII molecule as will be discussed below.

Inhibitor development in MHA patients

Mild/moderate haemophilia A patients with inhibitors are increasingly recognized. The largest cohort reported so far included 26 such patients and gave major insights into the factors influencing their development and the characteristics of these inhibitors [5]. The global incidence of inhibitors remains low in MHA patients (2.7–13%), [5] but some particular FVIII gene missense mutations contribute to an unexpectedly high incidence, sometimes up to the level observed in severe patients [6]. These mutations are mainly located within the exons encoding for the light chain of FVIII. Indeed, patients with Tyr2105Cys [5,10,41,42], Arg2150His [5,9,43–45] and Trp2229Cys [5,46,47] mutations included in the HAMSTeRS database [2] (accessed January 2008) developed inhibitors in 44% (4/9 patients), 23.8% (15/63) and 40% (8/20), respectively. An Arg593Cys [5,42,44,48] mutation in the FVIII A2 domain has also been found to be related to inhibitor formation in 11.7% (6/51) of the patients included [2]. As in severe patients, a familial predisposition to develop inhibitors may exist for patients with MHA [49]. In the UK cohort [5], 41% of treated family members had a history of FVIII inhibitors. However, further data are required to assess fully the role of familial factors in inhibitor formation. Similarly, the impact of human leukocyte antigen class II [50], or polymorphisms of genes involved in the modulation of the immune response to FVIII needs further study specifically within MHA patients [51].

Environmental factors can also be involved in inhibitor development in MHA [52]. Increases in the dose or duration of FVIII treatment, route of administration and even the type of FVIII concentrate may affect the risk of inhibitor formation [53–56]. The context in which FVIII is given is potentially crucial. Endogenous danger signals released by tissue damage as observed after a surgery or during an inflammatory state may trigger the immune response [57]. However, appropriate case–control studies are warranted to address such associations.

A range of therapeutic options for the eradication of inhibitors in MHAI patients is available, including induction of immune tolerance (ITI), immunomodulatory drugs (such as corticosteroids, cyclophosphamide, anti-CD20 monoclonal antibody rituximab), and even no specific treatment [5,11,55,58–62]. Among these, the best approach to eradicate the inhibitor and restore durable tolerance to endogenous and/or exogenous FVIII effectively is currently unknown. Similarly, optimum treatments either to stop or to prevent haemorrhages are not well-defined in MHAI patients. A variety of treatments can be used, including DDAVP [5,9,43,58], FVIII concentrates [5], FEIBA® (Baxter Corp, Deerfield, IL, USA) and recombinant activated factor VII (Novoseven®; Novo Nordisk, Bagsvaerd, Denmark) [15,58,63]. However, such treatment choices may have an impact on inhibitor outcome [64].

To provide further data and help make treatment decision in MHAI patients, a retrospective and prospective data collection is currently underway in France and Belgium. Based on the preliminary results, a few directions can be suggested. Specific inhibitor assays should be performed on a regular basis in MHA patients to allow early diagnosis of inhibitor, particularly after intensive FVIII exposure. It must be emphasized here that baseline measurements of FVIII:C alone are not sufficient to rule out the possibility of inhibitor directed exclusively to exogenous FVIII. A prompt inhibitor discovery may avoid the inappropriate reintroduction of FVIII concentrates that would lead to unexpected FVIII inefficacy and to anamnesis. When an inhibitor is still present, treatment of bleeds with FVIII or activated prothrombin complex concentrates potentially induces an anamnestic response, and thus may delay the disappearance of inhibitor and the return to detectable endogenous baseline FVIII:C level. Moreover, the profile of inhibitor specificity may also change in parallel to anamnesis, and subsequently modify the severity of the clinical phenotype. This complication has also been observed after reintroduction of FVIII containing products in patients who already had returned to a negative inhibitor assay and had normalized FVIII:C baseline levels. In the absence of reliable criteria to predict whether or not any individual MHAI patient will respond to rechallenge with FVIII, the choice of therapy for treatment of haemorrhages should take into account this risk.

The preliminary data from the MHAI study suggest that immune tolerance induction could be more effective than no specific treatment or immunomodulating drugs in preventing further risk of anamnesis (data not shown). However, the indication of performing such a long and restrictive treatment should take into account the characteristics of the patient such as age, bleeding phenotype, FVIII:C baseline level, coexisting morbidities and the potential need for FVIII in the future. The predictive success criteria of ITI described for severe HA

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patients may not be appropriate in MHAI patients and should be further studied.

The treatment of MHAI patients remains a difficult challenge and thus emphasizes the importance of preventing this complication. In this regard, limitation of intensity (dose and duration) of FVIII treatment should first be discussed when suitable. Desmopressin may offer a viable alternative to correct haemostasis [1]. Preliminary testing is required to assess the FVIII:C level at peak and the released FVIII:C half-life to enable appropriate treatment choices for effective haemostasis.

Antifactor VIII antibodies and FVIII-specific T lymphocytes in mild/moderate haemophilia A

To understand the relation between mutation in the FVIII gene and inhibitor development, the antigenic determinants (epitopes) recognized by inhibitor antibodies were investigated in patients with a significant FVIII:C activity despite the presence of a high titre inhibitor. That study indicated that, in some cases, the inhibitor antibodies distinguish between the patient's self FVIII and normal exogenous FVIII [7,9,43].

In the latter study, a patient with mild haemophilia A carrying a mutation Arg2150His in the FVIII C1 domain developed a high titre inhibitor although FVIII levels remained about 25%. The patient's polyclonal anti-FVIII antibodies and a human monoclonal antibody derived by immortalization of the patient's B lymphocytes recognized the C1 domain of normal FVIII, but not a C1 domain carrying the mutation Arg2150His found in the patient FVIII gene [14,43]. Similarly, a deletion of Ala2201 in the C2 domain eliminates a major antigenic determinant recognized by some inhibitor antibodies [40]. So far, the patients carrying the latter mutation were treated with DDAVP and hence it is still unknown whether deletion of Ala2201 also predisposes to inhibitor development. However, there is one report of a patient with a missense mutation of Ala2201 who developed an inhibitor [17].

Similarly, the analysis at the clonal level of the T-cell response to FVIII in a MHA patient who had developed a strong antibody response to wild-type FVIII while remaining tolerant to his own FVIII indicated that the T cells recognized normal exogenous FVIII, but not the patient's FVIII [16]. All T cells derived from that patient recognized a single stretch of amino-acids, Ile2144-Tyr2161, located in the C1 domain. Peptide Ile2144-Tyr2161 also bound to other DR molecules than those expressed by the patient, such as DRB1*0101, DRB1*0401, DRB1*0701, DRB1*1101, DRB1*1301, or DRB1*1501, which are each expressed in about 2–20% of the Caucasian population. Accordingly, that peptide could be presented to T lymphocytes of at least 60% of the population [16] in agreement with the high risk of inhibitor development associated with the mutation Arg2150His [5].

Factor VIII-specific T cells were also studied in a recent report of a patient with a missense genotype Ala2201Pro in the FVIII C2 domain. Peptides containing A2201 (amino acid present in native FVIII) were recognized by $CD4^{+}$ T cells at all time points after inhibitor development, whereas a Pro2201 peptide was recognized only near the initial antibody peak response. Patient-derived T-cell clones proliferated in response to C2 protein and to peptides containing Ala2201 but not Pro2201, indicating that, as for the mutation Arg2150His, the cellular immune system is able to distinguish self mutant FVIII from exogenous FVIII [17].

Altogether, these observations have demonstrated that both B cells and T cells can distinguish between self and wild type FVIII molecules differing by a single point mutation, which provides a plausible mechanism for the frequent occurrence of inhibitor in patients carrying some mutations.

The mechanisms allowing the development of an antibody response to self FVIII in addition to exogenous FVIII are still unknown, although the enlargement of an immune response from one antigenic determinant to another is a well known phenomenon, called epitope spreading, which is frequently observed in experimental models [65]. In theory, any T lymphocyte specific for exogenous FVIII should be able to provide help to any B-cell recognizing antigens shared by the patient's FVIII and exogenous FVIII. Why such an extension of the immune response is observed in some cases [7,11] but not in others [43] is currently unknown.

Interestingly, the extension of the immune response to self FVIII is not restricted to the antibody response. Indeed, T cells derived from a patient with antibodies neutralizing both exogenous and self FVIII also recognized the latter [12]. Thus, in a patient with MHA, who develops an immune response to his own FVIII, the T-cell tolerance to self FVIII can also be broken. However, it is still unknown whether such a cellular response to self FVIII renders restoration of tolerance to FVIII more difficult.

These observations have important implications for the treatment of mild/moderate haemophilia A and the prevention of inhibitor development, such as the use of DDAVP [8].

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