Alloantibodies to therapeutic factor VIII in hemophilia A: the role of von Willebrand factor in regulating factor VIII immunogenicity

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ABSTRACT

The rising incidence of neutralizing antibodies (inhibitors) against therapeutic factor VIII prompted the conduct of studies to answer the question as to whether this rise is related to the introduction of recombinant factor VIII products. The present article summarizes current opinions and results of non-clinical and clinical studies on the immunogenic potential of recombinant compared to plasma-derived factor VIII concentrates. Numerous studies provided circumstantial evidence that von Willebrand factor, the natural chaperone protein present in plasma-derived factor VIII products, plays an important role in protecting exogenous factor VIII from uptake by antigen presenting cells and from recognition by immune effectors. However, the definite contribution of von Willebrand factor in reducing the inhibitor risk and in the achievement of immune tolerance is still under debate.

Introduction

During the last six decades, the therapeutic management of hemophilia A has evolved into a multidisciplinary clinical challenge aiming at improving the quality of life and enabling a near-normal life expectancy of affected patients.[1-3] In the 1950s and early 1960s, whole blood and fresh plasma were the only available treatment options for the replacement of clotting factor VIII (FVIII). Nowadays, clinicians can choose between virus-inactivated plasma-derived FVIII (pdFVIII) concentrates [which contain FVIII in a natural complex with von Willebrand factor (VWF)], monoclonal antibody-purified pdFVIII products (which contain practically no VWF) and VWF-free recombinant FVIII proteins (rFVIII) produced by genetically engineered rodent cell lines.[1,4] In addition, primary prophylaxis, i.e. the continuous substitution of FVIII ideally starting before the age of two years, has become the standard of care in severe hemophilia A.[1,2,4]

However, one of the most serious treatment complications in hemophilia A is still the development of an anti-FVIII immune response after repeated administration of FVIII products. Currently, inhibitory antibodies ('inhibitors') are estimated to occur in 20%-35% and 3%-13% of patients presenting with severe and mild-to-moderate manifestations of hemophilia A, respectively.[5-10] As these patients usually become resistant to conventional FVIII replacement therapy, the condition is frequently associated with recurrent spontaneous bleeding into joints, muscles or vital organs leading to permanent joint deformation and represents a considerable burden to healthcare systems including the cost of alternative treatments.[11]

Patients with underlying mutations of the gene encoding FVIII (F8) that lead to complete absence or severe truncation of the gene product are at the greatest risk for inhibitor development.[12,13] Their immune system recognizes the normal FVIII protein as foreign.[14] However, among patients with similar high-risk mutations, the inhibitor plasma titer and the prognosis may vary substantially.[15] A number of additional genetic and treatment-related factors have been proposed to confer a risk for inhibitor formation (non-Caucasian ethnicity, family history, genetic variations of cytokines and cellular receptors, conditions at the time of first exposure to exogenous FVIII, upregulation of co-stimulatory molecules in answer to "danger signals") (Table 1).[14] The debate on the role of the source of therapeutic FVIII (donor plasma or DNA technology) started at the turn of the millennium. This discussion was mainly triggered by clinicians expressing their concern that rFVIII has a higher immunogenic potential than pdFVIII in treatment-naïve patients.[17]

Is the product type a determinant of inhibitor development?

In an attempt to assess the impact of types of FVIII products on the development of inhibitors, the research community compared data on inhibitor incidence following the administration of rFVIII and pdFVIII products (Figure 1).[7-10,15,18-22] These studies provided different results, which have been interpreted in different ways by experts of both interest groups. The best common consensus found in recent years is that patient populations and treatment modalities in studies are too heterogeneous to allow for a direct and unbiased comparison of clinical outcomes.[23-26]

In addition, controversy remains concerning the treatment status of patients who are to be enrolled in FVIII immunogenicity studies.[27,28] Current relevant guidelines for pivotal studies on full-length FVIII products state that safety and immunogenicity data are required from previously treated patients (PTPs) aged over 12 years.[29,30] The incidence of

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inhibitors in stable PTPs after switching treatment is consistently lower than in previously untreated patients (PUPs),[31,32] probably because many of them have developed some kind of cross-tolerance. Thus, the immunogenicity of a given FVIII concentrate in PTPs is relevant for PTPs only. However, in daily clinical practice, more situations that demand a decision between treatment options are related to PUPs, i.e. patients in their early years of life who are newly diagnosed with hemophilia A or who sustain their first bleeding episode.[27,33]

Are inhibitor testing methods a critical confounder?

One of the most cited arguments used to confute a higher incidence of inhibitors associated with rFVIII treatment is that the testing frequencies and methods used for the detection of inhibitors have increased and improved over time. These improvements coincide with the introduction of rFVIII, which might have favored the detection of borderline and transient inhibitors in rFVIII-treated patient groups.[23,31,34] In fact, in 1995, the so-called "Nijmegen" method, a modification of the till then most widely used "Bethesda assay", was launched.[35] However, despite the improvements made over recent years, the methods used to detect inhibitors have not yet been standardized,[28,36] and the classical Bethesda assay originally published in 1975 is still the most frequently used assay.[34,36] Furthermore, the major advantage of the Nijmegen modification of the Bethesda assay lies in the improvement in the test's specificity near the cut-off value.[28,35,37] Hence, the advent and use of the Nijmegen assay should have caused a decrease in the rate of false positive test results and, consequently, a decrease rather than an increase in the inhibitor incidence in studies dating back to the 1990s. On the other hand, it is very possible that the increased testing frequency in the last two decades had an impact on the reported incidence of inhibitors. Studies reporting on the incidence of high-responding inhibitors [> 5 Bethesda units (BU) per milliliter] (Figure 1) avoided potential bias from assay performance and testing frequencies, because high-responding inhibitors are mostly permanently detectable as long as they are not eradicated by immune tolerance induction therapies.[27,34,36,37]

Evidence from fundamental research: possible role for FVIII phenotypes

One explanation for an increased likelihood of inhibitor development in patients treated with rFVIII is related to the fact that single nucleotide polymorphisms (SNPs) exist in the *F8* gene. Six FVIII haplotypes are known (denoted H1 through H6) which show a different distribution among ethnicities.[38,39] Of the three SNPs that distinguish H3 and H4 from H1 and H2, two are located in the domains A2 and C2 within sequences encoding target epitopes for neutralizing anti-FVIII antibodies.[39-42] The phenotypes of the currently marketed rFVIII products are H1 or H2.[39] Conversely, batches of pdFVIII are derived from thousands of donors. In view of the demographic developments in Europe and the US, it can be assumed that existing pdFVIII products contain all six wild-type forms of FVIII in highly varying proportions, albeit H1 and H2 are the most abundant forms.[38,43] The presence of different human FVIII variants in pdFVIII might reduce the likelihood of a formation of high anti-FVIII titers simply due to "antigenic competition".[44,45] In fact, it is widely accepted that fluctuations in the abundance of antigenic variants effectively reduces the immunodominance of a particular variant.[46,47]

Non-human posttranslational modifications

It was proposed that the posttranslational modifications of the therapeutic FVIII molecule play a crucial role.[48,49] All rFVIII products marketed so far are produced in Chinese hamster ovary (CHO) or baby hamster kidney (BHK) cells. These cells generate rodent-type glycosylation and sulfation patterns, which might enhance FVIII immunogenicity in several ways. Oligosaccharide motifs typical for rodents and other mammals, e.g. Gal-alpha 1-3-Gal (alpha-Gal) and N-glycolylneuraminic acid (Neu5Gc) were detected in great abundance on rFVIII, which, in other contexts than hemophilia A, are known to elicit a marked immune response in all humans.[48-52] In addition, certain rFVIII products may appear with poor sulfation at tyrosine 1680 (Tyr1680).[48] Interestingly, Tyr1680 sulfation is indispensable to the interaction between FVIII and its natural chaperone protein, VWF.[48,53,54] However, at present, there is no scientific evidence that differences in glycosylation and sulfation patterns between pdFVIII and rFVIII are of relevance to FVIII immunogenicity.

von Willebrand factor shields FVIII epitopes from recognition by the immune system

Several groups performed fundamental research to give scientific merit to the lower immunogenicity of pdFVIII products observed in distinct trials (Figure 1). Many of them focused on the most obvious difference between pdFVIII and rFVIII, namely that the former contains variable amounts of VWF in addition to FVIII.

Under physiological conditions, approximately 94% of

Table 1. Factors considered as conferring an increased risk for inhibitor development.[16]

Risk factor	Evidence
Type of F8 mutation	Proven correlation with risk[a]
Family history of inhibitors	Enhanced risk reported by several authors
African or Hispanic ethnicity	Enhanced risk reported by several authors
Phenotypic mismatch between the patient's and the exogenous FVIII	A single report could not be confirmed by other authors
Polymorphisms of immunological factors: MHC class II, TNF-α, IL-10, HO-1	Inconsistent study results
On-demand therapy *versus* prophylaxis	Inconsistent study results
Age < 6 months at first treatment of a bleeding complication	Inconsistent study results
Intensity of first exposure	Inconsistent study results
First treatment associated with "danger signals"[b]	More research needed
Recombinant *versus* plasma-derived FVIII product	Ongoing debate; prospective randomized studies not completed so far

MHC: major histocompatibility complex; TNF: tumor necrosis factor; IL: interleukin. [a]Large deletions, nonsense mutations and intron 22 inversions are associated with the highest risk for inhibitor formation. [b]Conditions that stimulate antigen-presenting cells, such as bleeding, surgical procedure, injury, infection, etc.

Table 2. von Willebrand factor is protective against the neutralizing activity of anti-FVIII antibodies (inhibitors). Summary of the experiments by Shi *et al*. in a murine model of hemophilia A.[64]

First infusion	Second infusion	Survival after tail clipping (n = 4 or 5)
	FVIII^{null} mice	
–	–	0%
rFVIII[a]	Inhibitors[b] 0	100%
rFVIII	Inhibitors 2.5	100%
rFVIII	Inhibitors 25	50%
rFVIII	Inhibitors 250	50%
Inhibitors 2.5 BU/mL	rFVIII	30%
Inhibitors 25 BU/mL	rFVIII	0%
Inhibitors 250 BU/mL	rFVIII	0%
	FVIII^{null}VWF^{null} mice	
–	–	0%
rFVIII	Inhibitors 0	75%
rFVIII	Inhibitors 2.5	0%
rFVIII	Inhibitors 25	0%
rFVIII	Inhibitors 250	0%
rFVIII + rVWF[c]	Inhibitors 2.5	60%
rFVIII + rVWF	Inhibitors 25	20%
rFVIII + rVWF	Inhibitors 250	0%

[a]*Human rFVIII infused to achieve a plasma concentration of 0.02 IU/mL (concordant results were obtained with 0.015 IU/mL).* [b]*Pooled murine polyclonal anti-human rFVIII antibodies infused to achieve a plasma concentration as indicated (in BU/mL).* [c]*Recombinant human VWF infused to achieve a plasma concentration of 1 IU/mL.*

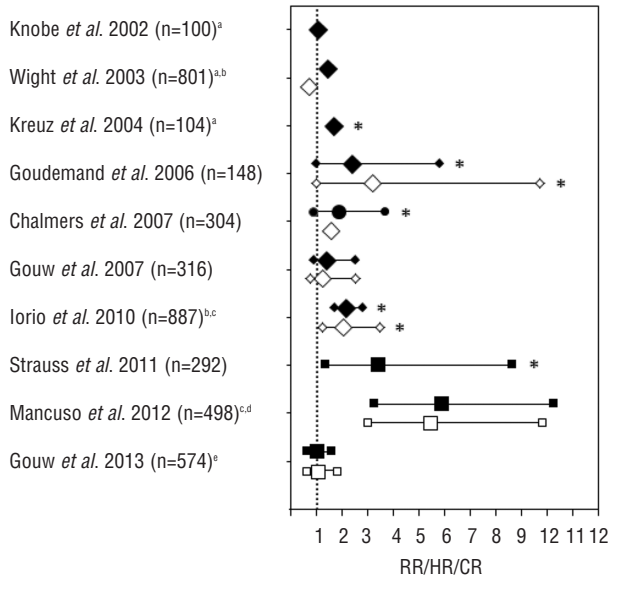

Figure 1. Risk of inhibitor development in dependence of treatment product (rFVIII products *vs.* pdFVIII products). Results from comparative studies in treatment-naïve patients with severe hemophilia A (sample sizes > 100) are shown. Multivariate analysis or adjustment for cofounders was performed unless otherwise indicated. Trapezoids: relative risk (RR); squares: Hazard ratio (HR); circles: odds ratio (OR); filled symbols: overall inhibitors; empty symbols: high-responding inhibitors (≥ 5 BU/mL); small symbols and lines: 95% CI.[7-10,15,18-22] [a]Crude data (CI not available). [b]Systematic review and meta-analysis. [c]Subpopulation of treatment-naive patients with severe hemophilia A. [d]*P* values not given. [e]Prospective study **P*<0.05.

the circulating FVIII molecules are non-covalently bound to VWF (Figure 2).[55] VWF acts as a stabilizer for FVIII in protecting it from premature degradation and plays a central role in primary hemostasis as a multi-directional bridge between FVIII, platelets and exposed sub-endothelial connective tissue.[56,57]

The light chain of FVIII, especially the C2 domain, bears epitopes that may elicit a strong antibody response.[14,41,42] In the VWF-FVIII complex, the light chain of FVIII is partly covered by VWF (Figure 2).[14,56,57] This led to the assumption that pdFVIII products which contain FVIII in its natural complex with VWF are less immunogenic simply because of "epitope masking".[57,58] In theory, steric hindrance of the binding of pre-existing C2-specific antibodies (which can be detected even in healthy individuals[59]) prevents the upregulation of phagocytic activity and the increase of antibody production to clinically relevant levels. Support for this concept comes from studies in hemophilic mice demonstrating that the administration of human rFVIII induced significantly higher levels of inhibitory anti-FVIII IgG than the co-administration of rFVIII and VWF.[60-64] Moreover, hemophilic mice showed considerably higher titers of inhibitors specifically directed at light chain epitopes when treated with rFVIII than when treated with FVIII/VWF.[60]

Franchini raised the objection that upon infusion exogenous free rFVIII rapidly (within seconds) binds to VWF already present in the patient's plasma in a 50-fold molar excess.[23,55,56] In fact, the affinity of FVIII to VWF is high.[55] However, several authors reported that, unlike pdFVIII, rFVIII concentrates contain a fraction of around 20% of the antigenic rFVIII material (rFVIII:Ag) which has no demonstrable FVIII:C activity *in vitro* and, most notably, is not capable of associating with VWF (possibly due to PTM abnormalities). It was suggested that this portion of rFVIII:Ag may trigger the specific immune response in patients.[54,65-68] The exposed VWF-binding site on FVIII, incidentally, may be occupied by anionic phospholipids which were suggested to have additional implications for the immune response.[69,70]

VWF inhibits uptake of FVIII in antigen presenting cells

Qadura and colleagues were not able to confirm a protective effect of VWF in hemophilic mice, but found variant patterns of inflammatory mediators and of immune gene expression profiles in activated (CD11c^{+}) splenic dendritic cells (DCs) between pdFVIII- and rFVIII-treated hemophilic mice (type 1 *vs.* type 2 inflammatory response).[44] As the pdFVIII-treated mice in these experiments also developed a strong immune response to human VWF, these findings may have little relevance for the situation in humans.

Incubation of monocyte-derived DCs obtained from healthy humans with FVIII in the study by Pfistershammer

et al. did not result in significant changes in cytokine expression profiles, cellular maturation markers and T-cell activation, regardless of whether rFVIII or rFVIII-VWF complexes were used for stimulation.[71] In parallel, other independent research groups showed with DCs from healthy donors or FVIII-deficient mice, endocytotic internalization of rFVIII, subsequent presentation of rFVIII fragments and upregulation of domain-specific CD4+ T cells.[61,62,72-74] Further experiments showed that VWF dose-dependently blocks endocytosis of rFVIII by DCs and leads to a significantly decreased interferon (IFN)-gamma production in co-cultured CD4+ T cells compared to controls without VWF (Figure 3).[72] In the range of the physiological molar ratio of FVIII to VWF, 1:25 to 1:80[14,56] (which corresponds to that in commercially available pdFVIII concentrates[72]) the rFVIII uptake *in vitro* was reduced by approximately 40%-70% (Figure 3A).[62,72] Moreover, pre-incubation of rFVIII with monoclonal Fab fragments that block the interaction with VWF restored the endocytosis by DCs.[72] The inhibitory effect of VWF on endocytosis could not be demonstrated for an irrelevant antigen (α-2-macroglobulin). Furthermore, VWF could not inhibit the specific T-cell activation and IFN-gamma production in response to a FVIII-derived synthetic peptide (Ile2144-Thr2161) not bearing the VWF binding site (Figure 3B). Interestingly, even an over 100-fold molar excess of VWF did not completely abrogate FVIII uptake by DCs.[61,62] This indicates the existence of an accessory VWF-independent uptake mechanism, e.g. macropinocytosis,[75] but is also in agreement with findings that a significant portion of FVIII:Ag present in rFVIII preparations is not able to associate with VWF.[54,65-68]

Delignat and co-workers reassessed these findings in a model of FVIII-deficient mice. The animals were treated by intravenous injection with rFVIII, which was either pre-incubated or not with a 50-fold molar excess of purified VWF.[62] In this experiment rFVIII/VWF resulted in a significantly (on average 8-fold) lower production of anti-FVIII IgG compared to free rFVIII. Interestingly, the presence of VWF prolonged the residence of FVIII in the splenic marginal zone, which is the main microenvironment of specialized subsets of myeloid (CD11b+/CD11c+) DCs and B cells with immune-regulatory (tolerance inducing) characteristics.[76-79] In contrast, in the absence of VWF (parallel experiment with VWF-deficient mice), only trace amounts of exogenous rFVIII were transiently detected in this important splenic zone. Taken together, the authors proposed that the inhibition of FVIII uptake by antigen-presenting cells, and possibly the facilitation of its contact with tolerogenic splenic DCs, are mechanisms by which VWF may reduce the immunogenicity of rFVIII in hemophilic mice.

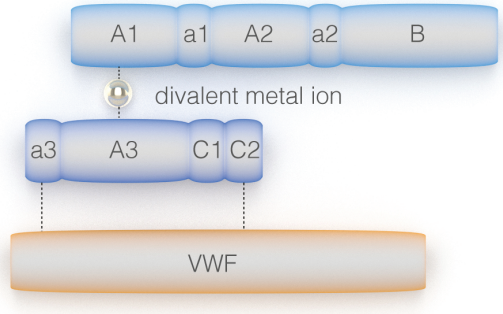

Figure 2. Scheme of the FVIII-VWF complex (FVIII heavy chain: domains A1-B; FVIII light chain: domains a3-C2).

VWF protects FVIII from being attacked by pre-existing inhibitors and may be beneficial in tolerance induction

Another intensely debated question is whether rFVIII or pdFVIII is more appropriate in treating hemophiliacs who already have developed inhibitors. The long-term goal in these patients is to induce immune tolerance to exogenous FVIII. Immune tolerance induction (ITI) is usually accomplished by repeated FVIII administration at unusually high doses or unusually short dosing intervals (with or without supporting medical measures). The resulting chronic exposure to relatively high plasma concentration of FVIII concentrations over many months is thought to down-regulate the immune response. The

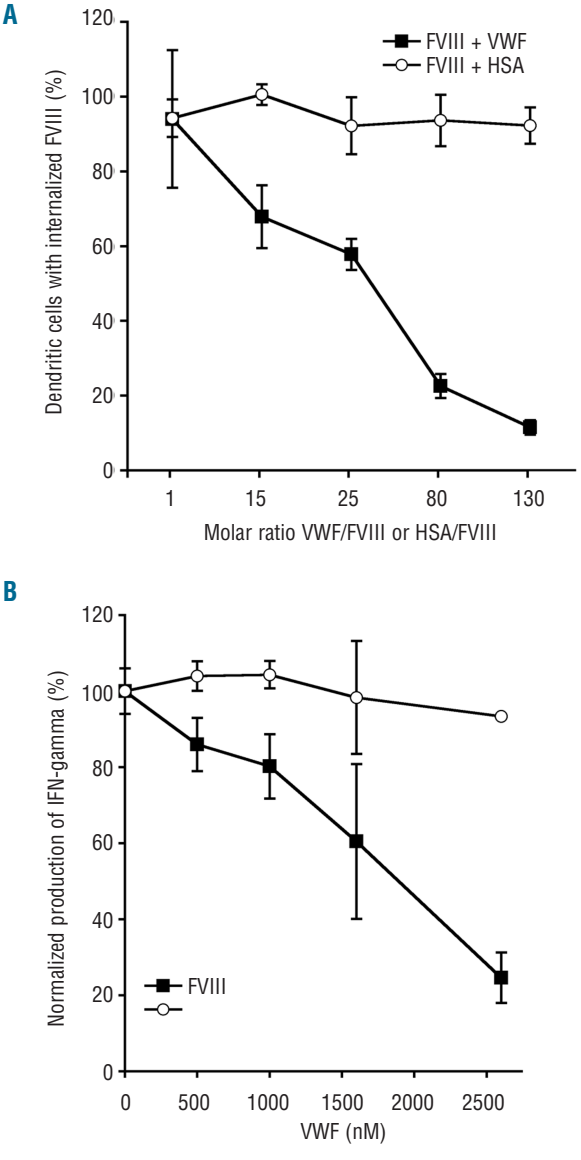

Figure 3. Effect of VWF on uptake of FVIII into antigen presenting cells (A) and IFN-gamma production (B). Data from Dasgupta *et al.*[72] HSA: human serum albumin; synthetic FVIII peptide: spans amino acids Ile^{2144}-Thr^{2161} (immunogenic, but no VWF binding site).

exact mechanism is still not fully elucidated.[54,80] Overall, in 60-80% of the affected patients ITI is successful, with peak inhibitor titers being the strongest adverse prognostic factor.[81-84] In a consensus meeting of European stakeholders of hemophilia care, pooled outcome data for ITI according to the Bonn protocol (100-150 IU FVIII per kg bodyweight twice daily) from two German hemophilia centers were presented. Prior to 1990, the success rate of ITI with pdFVIII products was 87% (44 of 51 cases), whereas between January 1990 and July 2001 the success rate was 82% with high-purity pdFVIII products (23 of 28 cases) and 43% with rFVIII products (6 of 14 cases). In 10 of 13 patients treated with low-VWF pdFVIII or rFVIII who initially did not respond, immune tolerance was later achieved after switching to a high-VWF pdFVIII product. In 2 of these successfully switched patients, the recurrence of inhibitors was noted after switching back to a non-VWF product (*EMA, unpublished data, 2007*). Several other authors provided similar evidence that pdFVIII concentrates rich in VWF may be especially valuable in high-risk patients, in rescue ITI, and in cases where the dominating inhibitors are specific for the C2 epitope.[84-88]

These observations are in accordance with the hypothesis that VWF shields FVIII light chain from attack from antibodies. In fact, the competition between VWF and antibodies for free binding sites was confirmed through binding experiments.[89,90] However, it should be noted that inhibitors are polyclonal and may thus also be directed against the uncovered heavy chain.[41,42] International registry data and a systematic literature review show no clear association between FVIII product and ITI outcome (equivalent success rates of approx. 70%).[82,83] Accordingly, the participants of the International Workshop on Immune Tolerance Induction declared by consensus that FVIII products with and without VWF may be used for ITI, but that after failure with rFVIII, switching to a VWF-containing pdFVIII product should be considered (evidence level IIB).[91] At the functional level, *in vitro* experiments have shown that VWF dose-dependently interferes with the neutralizing effect of light chain-specific inhibitors on rFVIII:C.[64,92-94] Shi and colleagues provided both *in vitro* and *in vivo* evidence that rFVIII in a preformed complex with VWF (rFVIII-VWF) is better protected from the inactivation by inhibitors than rFVIII that encounters inhibitors and VWF at the same time. In particular, when 'naked' rFVIII was mixed with murine plasma containing VWF and inhibitors, the normalized apparent Bethesda titers (as a measure of FVIII inactivation) were 5.8-fold lower compared to VWF-free control mixtures. When a pre-formed rFVIII-VWF complex was used, the titers were even 38.9-fold lower. In further experiments, the authors gave two infusions to hemophilic (FVIIInull) mice: either first FVIII, then inhibitory anti-FVIII antibodies (at defined amounts) or in a reversed order. They noted that considerably more mice survived the tail clip test when therapeutic rFVIII was allowed to associate with the endogenous VWF prior to the infusion of inhibitors compared to the reverse (clinical) setting, i.e. when rFVIII was infused into the circulation with inhibitors already present. The role of VWF was further highlighted by the observation that in FVIIInull/VWFnull mice (no endogenous VWF), therapeutic rFVIII was apparently completely inactivated by inhibitors, as the entire double-knockout mouse population died from bleeding after tail clipping. The animal experiments by Shi *et al*. are summarized in Table 2.[64]

The authors meticulously ruled out the possibility that dilution artefacts had impacted their findings.[23] Thus, the study convincingly demonstrated that VWF, the natural chaperone protein of FVIII, not only protects FVIII against degrading enzymes,[56,57] but also from inactivation by pre-existing inhibitory antibodies. The findings by Shi *et al*. speak in favor of the use of products containing pre-formed FVIII-VWF complexes for FVIII replacement and ITI in inhibitor patients. Such products appeared to have a head start in the competition of VWF and inhibitors for binding to FVIII.

Summary and outlook

The controversy about whether pdFVIII is superior over rFVIII with regard to immunological safety and tolerogenicity is far from being resolved. This becomes apparent when two authors after reviewing basically the same literature come to an opposite conclusion.[24,95] While some authors regard the apparently rising incidence of inhibitors as the consequence of altered replacement schemes and improved diagnostic tests, others expressed concerns about the rodent-type posttranslational modification of rFVIII produced in genetically engineered hamster cells. Several research groups focused on the presence of VWF in pdFVIII products compared to none in rFVIII. They provided evidence that VWF protects FVIII epitopes from recognition by the immune system, inhibits uptake of FVIII by DCs, and antagonizes the neutralizing activity of pre-existing inhibitors. The non-clinical research supports the view that VWF plays a beneficial role in the therapy of patients with hemophilia A with respect to natural tolerance at first FVIII exposures or induced tolerance by ITI.

On the other hand, the results of one prospective and several retrospective observational studies comparing the incidence of inhibitors among patients treated with VWF-containing pdFVIII products *versus* rFVIII are highly inconsistent. It is, thus, unambiguously accepted that well-planned randomized trials will throw more light on this issue. Thus, the results of two open, randomized trials, the Survey of Inhibitors in Plasma-Product Exposed Toddlers (SIPPET) and the Rescue Immune Tolerance Study (RESIST), are eagerly awaited.[96,97] In parallel, novel therapeutic strategies are being developed in order to prevent formation of FVIII alloantibodies or to restore immune tolerance. These strategies mainly focus on co-administration of immunomodulatory drugs, desensitization using modified autologous dentritic cells, and gene therapy.[98-100] In the meantime, clinicians are left to use their personal experience and insights and the treatment preferences of their patients. Undoubtedly, there will be a demand for both types of FVIII concentrates for many years to come.

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