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Factor VIII Inhibitor Assays: Methodology, Shortcomings, and Challenges

M van Geffen[1], *M Dardikh*[1] *and B Verbruggen*

Affiliation: *Laboratory of Hematology, Department of Laboratory Medicine, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands*

[1] *The first two authors contributed equally to this review.*

ABSTRACT

Development of inhibitory antibodies against factor VIII is a serious complication in the treatment of hemophilia patients, as these antibodies inactivate factor VIII, resulting in an increased bleeding tendency and morbidity. The clinical manifestation of inhibitors has to be analyzed by objective laboratory tests to specify the exact nature of the inhibitor. Here, the inhibitor assays, especially the Bethesda assay and the Nijmegen assay, are discussed including their methodological shortcomings. This paper also discusses the epitope specificity of the inhibitor assays and the methods to investigate the specificity of inhibitors against factor VIII concentrates that may contribute to improve the treatment of hemophilia A patients with inhibitors. Finally, the use and limitations of overall clotting assays, such as clot wave form analysis, thromboelastography, and thrombin generation assay, in the management of hemophilia A patients with inhibitors are discussed.

Keywords: hemophilia A, factor VIII inhibitors, Nijmegen assay, Bethesda assay, epitope specificity

Correspondence: B Verbruggen, Laboratory of Hematology, Department of Laboratory Medicine, Radboud University Nijmegen Medical Center, PO Box 9101, 6500 HB Nijmegen, The Netherlands. Tel: (31)-243614796; e-mail: H.Verbruggen@chl.umcn.nl

INTRODUCTION

Factor (F)VIII is a 265-kDa protein essential for coagulation. It consists of a heavy chain with the A1, A2, and B domains, and a light chain that contains the A3, C1, and C2 domains held together by copper ions [1]. In plasma, FVIII is non-covalently bound to von Willebrand factor (VWF) [2, 3] and, upon activation by thrombin, it is cleaved into a heterotrimer consisting of A1, A2, and A3–C1–C2 domains [4]. The B domain is not essential for FVIII activity and is lost after activation [5]. Activated FVIII binds to factor IX (FIX) on negatively charged phospholipid membrane surfaces in the presence of calcium, and this complex accelerates the generation of factor Xa [6, 7]. Non-functional FVIII or absence of FVIII in blood results in a bleeding disorder. Therefore, treatment of hemophilia A patients relies on administration of exogenous FVIII. Plasma-derived FVIII (pdFVIII) concentrates used for supplementation therapy in the 1980s were frequently contaminated with human immunodeficiency virus (HIV), hepatitis B and C viruses causing mortality in a large hemophilic population. All these problems can be prevented by the use of recombinant FVIII (rFVIII).

In contrast, the development of immunoglobulin (Ig)G antibodies against the FVIII protein still constitutes a major problem in the treatment of hemophilia A [8]. Besides IgG against exogenous FVIII (alloantibodies), endogenous FVIII can also be the subject of antibody formation (autoantibodies) resulting in acquired hemophilia A. Moreover, in healthy individuals, natural autoantibodies to FVIII with and without neutralizing activity have been demonstrated [9].

Neutralizing antibodies (inhibitors) are mostly directed against epitopes located at the A2 domain (R484 to I508), C2 domain (E2181 to V2243), and the A3 and C1 domains [10–12]. The epitope at the A2 domain represents the FIXa binding site, and the epitope at the C2 domain represents, at least partially, the VWF and phospholipid binding region. Binding of antibodies to these sites prevents FVIII from exerting its function, resulting in a higher bleeding tendency with increased morbidity and mortality [13, 14].

Inhibitor assays are performed in the blood of hemophilia A patients during routine surveillance screening or when the presence of inhibitors is suspected in the case of abnormal bleeding episodes or poor response to FVIII replacement therapy. Early detection of inhibitory antibodies against FVIII allows intervention at an early stage and offers the opportunity to tailor the most effective hemostatic treatment for bleeding episodes and surgery [15, 16]. Furthermore, inhibitor levels have to be measured in patients who are being treated according to the immune tolerance strategy in order to monitor efficacy of treatment. Assays of FVIII inhibitors are also performed to test the immunogenicity of new FVIII products [17].

When inhibitors are suspected, an activated partial thromboplastin time (aPTT) mixing test with normal plasma may be used for quick screening. A prolonged clotting time of the mixture may indicate the presence of inhibitors, but the presence of heparin and lupus anticoagulant has to be excluded.

This paper reviews the different tests used for quantification of FVIII inhibitors and the present methodological shortcomings of these assays. Furthermore, epitope specificity in inhibitor assays is discussed and, finally, global clotting assays are discussed as experimental alternative assays in hemophilia A patients with inhibitors.

INHIBITOR ASSAYS

The first occurrence of inhibitor development after infusion of FVIII in a hemophilia A patient was described in 1941 [18]. The anticoagulant activity was detected by its ability to prolong the clotting time in a mixture with blood from a normal person. The first quantitative method for evaluating inhibitor activity in a mixing assay, the Oxford method, was reported much later by Biggs and Bidwell [19]. This initial method was based on the rate of FVIII inactivation by an excess amount of inhibitory antibody. In the New Oxford method, described by Rizza and Biggs [20], FVIII was added in surplus amount compared with antibody level with an extended incubation period up to 4 h.

Later, Kasper *et al* [21] described the Bethesda assay, a more standardized assay using normal pooled plasma as the FVIII source in a 1:1 (v/v) mixture with patient plasma and imidazole buffer as a control. One Bethesda unit (BU) is defined as that amount of inhibitor that results in 50% residual FVIII activity in this mixture. The cutoff value used in the quantification of inhibitors is usually around 0.6 BU/mL, indicating that the assay has a low sensitivity and specificity.

The Malmö assay represents a modification of the classic Bethesda assay. In this assay, normal pooled plasma is replaced by factor concentrate as a substrate for patient plasma [22]. One Malmö unit of antibody corresponds to the inactivation of 1 IU of FVIII by 1 mL of patient plasma and is approximately equivalent to 3.3 BU. The Malmö assay shows high variability in the inhibitory capacity of patient plasma when tested against different plasma-derived and recombinant FVIII concentrates [23].

NIJMEGEN ASSAY

The Bethesda assay was further improved by the Nijmegen modification [24] by buffering the normal pooled plasma with 0.1 M imidazole to pH 7.4 to prevent pH shift during incubation and replacing the buffer as a control with hemophilic or immune-depleted, FVIII-deficient plasma, which prevents dilution of the protein content in the mixture with normal plasma. The increased specificity without affecting the sensitivity was confirmed in a Canadian study that showed a reduced number of falsely positive assay results with the Nijmegen assay [25]. Hence, the Nijmegen assay is recommended as the standard assay for FVIII inhibitor testing by the International Society of Thrombosis and Hemostasis Factor VIII/IX Scientific Subcommittee. A schematic representation of the Nijmegen assay is shown in **Figure 1**. Equal amounts of patient plasma and imidazole-buffered normal pooled plasma, pH 7.4, are incubated for 2 h together with a control mixture of identical normal pooled plasma and FVIII-deficient plasma. The residual FVIII in the test mixture, defined as the percentage of FVIII remaining in the test mixture relative to the control mixture, is converted to Nijmegen Bethesda units (NBU) by reading from a calibration curve representing a linear correlation between residual FVIII activity (logarithmic) and inhibitor units (linear). The curve is fully described by 0 and 1 NBU/mL, defined as the amount of inhibitor that results in 100% and 50% residual activity respectively. The method is only reliable between 0 and 2 NBU/mL and, consequently, samples with elevated titers have to be prediluted, preferably with factor VIII-deficient plasma.

A more sensitive method is warranted in samples with titers below 0.6 NBU/mL and with a suspicion of the presence of an inhibitor because of an abnormal bleeding tendency, low FVIII recovery, or a shortened half-life of infused FVIII. Therefore, our laboratory has put in much effort to develop a more sensitive method to measure very low titer inhibitors based on concentrating the putative inhibitor sample. At the moment, this test is still in validation, but the method showed at least a 10-fold increased sensitivity, resulting in a detection limit of at least 0.05 NBU/mL.

None of the currently available inhibitor activity assays detects non-neutralizing antibodies, present in 8–10% of hemophilia A patients with or without inhibitors [26, 27]. The influence of non-inhibiting antibodies on hemostasis has not been identified yet, although the formation of such immune complexes may result in increased clearance of these complexes by cells in the innate immune system [28, 29]. Non-neutralizing antibodies can be measured by immunologic methods such as enzyme-linked immunosorbent assay (ELISA), but these methods cannot discriminate between neutralizing and non-neutralizing antibodies.

METHODOLOGICAL SHORTCOMINGS IN INHIBITOR ASSAYS

The working mechanism of FVIII inhibitors is time and temperature dependent, which is in contrast to the other individual factor inhibitors. At 37 °C, an optimal inhibitor titer is reached after 120–150 min of incubation, whereas at room temperature, the FVIII inhibitor activity does not reach a steady-state condition. A number of other factors have been identified that interfere with the detection of inhibitors.

The presence of anti-phospholipid antibodies and lupus anticoagulants (LA) may influence FVIII inhibitor assays because of interference by LA in the one-stage assay. LA are antibodies directed against epitopes of phospholipid-bound coagulation factors [30–32]. Some groups have reported the simultaneous occurrence of inhibitors toward FVIII and LA in hemophiliac [33] and non-hemophilic patients [34–36]. It is difficult to discriminate between the two antibodies, although it is important because of the different implications for therapy. FVIII inhibitors are associated with hemorrhage, whereas LA is associated with thrombosis, obstetric complications, neurologic, and/or cutaneous symptoms [37, 38]. At the moment, no tests are available that discriminate fully between coagulation inhibitors and LA. The diluted Russell viper venom test may discriminate the best as it is not

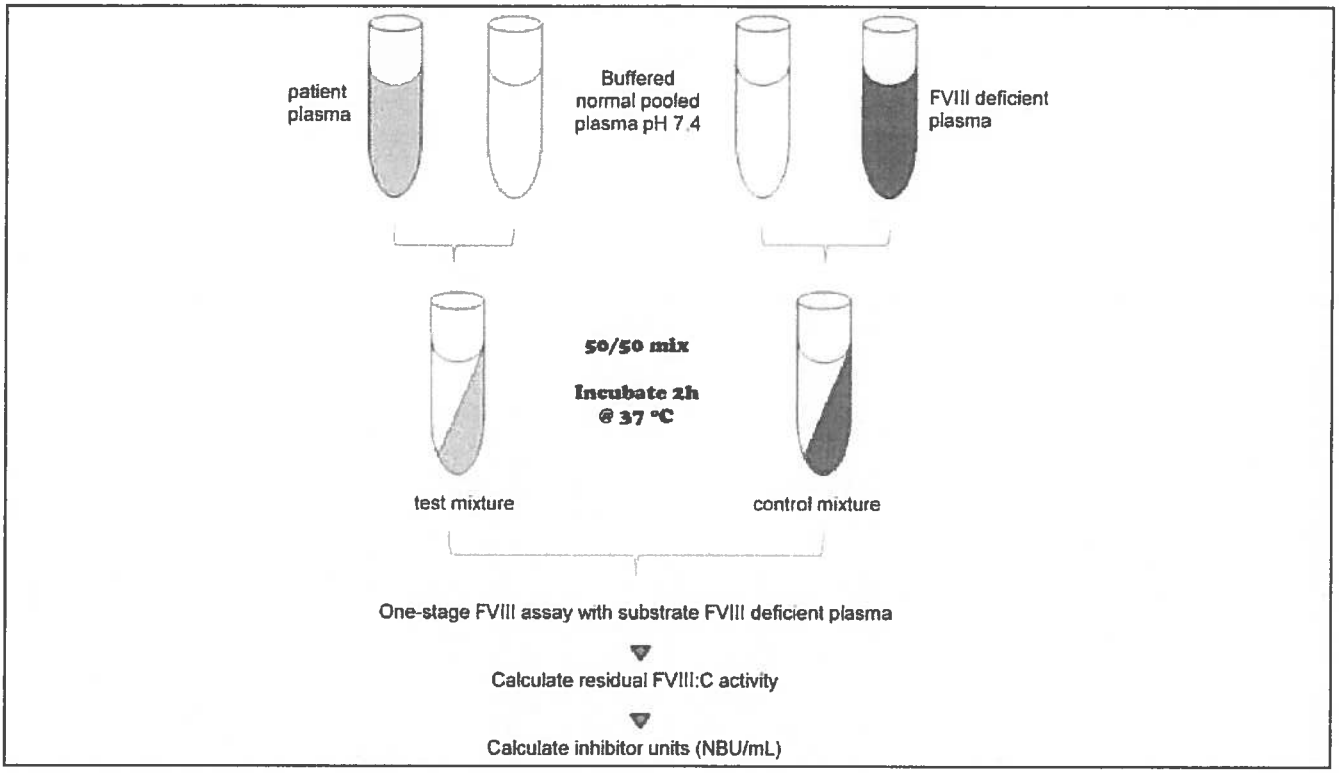

Figure 1. A schematic representation of the Nijmegen assay

influenced by factors of the intrinsic pathway including FVIII and FIX [39]. The influence of LA on inhibitor assays can be reduced by measuring the residual activity of FVIII using chromogenic substrate-based assays. These tests are not influenced by LA because of the high rate of sample dilution, and therefore are more specific than aPTT-based assays [40].

FVIII inhibitors can be characterized as either type I or type II based on their kinetic behavior. Type I inhibitors (mostly alloantibodies) have first-order inactivation kinetics that result in complete inhibition of FVIII activity at high inhibitor levels. In contrast, type II inhibitors (mostly autoantibodies) have a second-order inactivation rate leading to residual FVIII activity even at high titers. This lack of linearity between FVIII residual activity and inhibitor concentration with type II in the inhibitor test will result in dilution-dependent inhibitor data. Determination of exact titers is difficult in these cases, so typical dilutions of the patient plasma have to be used that give a residual activity of approximately 50%.

The type of deficient plasma may influence the results [41, 42]. The type of deficient plasma used as a control sample can be either congenital or immune-depleted plasma. It is highly recommended to use a VWF containing FVIII-deficient plasma. Immune-depleted plasmas containing VWF can be used, but proper control for the presence of FVIII inhibitors is strongly recommended as this type of plasma may contain FVIII antibodies that are co-eluted from the column during purification. Moreover, using chemically depleted plasma (CDP) as a control sample may also lead to aberrant results [42]. The production process of the CDP may generate minute amounts of activated factor Va causing shortening of the clotting times in the control mixture, leading to over-estimation of the inhibitor titer in a heterogeneous system. Finally, in order to confine the costs of the assay, a control sample with albumin is recommended [43].

The content of FVIII in normal pooled plasma may influence the results of the inhibitor assay. An increased FVIII content in normal pooled plasma will need more inhibitor to inactivate a certain percentage resulting in lower inhibitor titers. A pool of at least 50 healthy donors is required to guarantee a level as close as possible to 1 IU FVIII/mL. It is necessary to calibrate the normal pooled plasma against an international standard of FVIII [44].

Residual FVIII activity in the patient plasma can interfere with the inhibitor assay by increasing the remaining factor activity after incubation with normal pooled plasma, leading to falsely low inhibitor titers. Heating the test and control plasma at 58°C for 90 min overcomes this problem. This will destroy all clotting factors, but the immunoglobulins are heat resistant leaving the amount of inhibitor unchanged.

INTRA- AND INTERLABORATORY QUALITY ASSESSMENT

Interlaboratory surveys of FVIII inhibitor assays have been organized by the ECAT (European Concerted Action on Thrombosis) Foundation on a regular basis (twice a year) since 2006. Occasionally, this kind of survey is also organized by UKNEQAS (United Kingdom National External Quality Assessment Scheme) and by the RCPA (Royal College of Pathologists of Australasia) Haematology QAP (Quality

Assurance Programme). The results of the ECAT surveys show a rather high interlaboratory coefficient of variation (average ~40%) for the Nijmegen assay and slightly more (~45%) for the original Bethesda assay. Unfortunately, no real improvement in interlaboratory variation has been shown in the 3 years of ECAT surveys. Therefore, our laboratory, together with the ECAT Foundation, planned to organize a workshop on this subject. The aim of this workshop is to investigate components that contribute to the high between-laboratory variability of the results of the factor VIII inhibitor assays (observed in ECAT surveys) and to come up with suggestions to reduce the variability of the assay. Meanwhile, it is very important that more laboratories participate in external quality survey programs, and thus will improve their assays if needed. Intralaboratory day-to-day quality assessment has to be performed by assaying negative and positive inhibitor samples that are stored at minus 80°C.

The use of a calibrator may result in more uniform inhibitor data and lower interlaboratory variation. Unfortunately, FVIII inhibitor standards or calibration materials are not yet available. The FVIII and FIX Scientific Subcommittee of the International Society on Thrombosis and Haemostasis (ISTH) together with the National Institute for Biological Standards and Controls (NIBSC, Potters Bar, UK) are currently working on the production of suitable standards. The materials include a mouse monoclonal anti-human A2 and anti-human C2 antibody, a rabbit polyclonal antibody and, finally, a human high-titer polyclonal inhibitor plasma diluted to reference values of 1.8 and 10 BU [45].

EPITOPE SPECIFICITY OF THE INHIBITOR ASSAY

Inhibitor binding to immune dominant epitopes inhibits different functions of the FVIII molecule (e.g., A2 and C2), although the intensity [46] and specificity [47] of immune responses may fluctuate over time. Epitope specificity may possibly affect FVIII activity in a distinctive way, but no data are available on this topic. A mechanism that may cause heterogeneity in reactivity is the binding competition of inhibitors and VWF or anionic phospholipids for the C2 domain. However, mapping of FVIII inhibitors to its exact epitope still remains a challenge. Various techniques have been used for epitope mapping: immunoblotting assays with polypeptide fragments [48–50], phage display [51–53], inhibitor neutralization assays and competitive inhibition assays using overlapping synthetic peptides [54, 55], in silico prediction [56], and mass spectrometry [57]. For instance, the epitope of the monoclonal antibody ESH8 has been determined using three different techniques resulting in three distinctive epitopes. First, the region 2248–2285 was detected using deletion mutants [58]; second, peptide array analysis indicated that amino acids 2265–2280 [59] were involved; and last, it was determined by peptide array analysis that ESH8 may have a discontinuous epitope containing two sites at 2234–2238 and 2267–2270 [60] in the FVIII sequence. These different results demonstrate that the identification of the exact epitope remains a challenge.

IN VITRO TESTING OF IN VIVO EFFECTIVENESS OF CONCENTRATES

Inhibitor assays with FVIII concentrates as a FVIII source can be used for in vitro reactivity testing of inhibitor activity against different types of concentrates in order to select the best match for treatment. Inhibitor plasmas may vary in responsiveness against different concentrates because of variation in epitope specificity of the polyclonal inhibitors and because of variations in the FVIII structure, e.g., binding to vWF, variable glycosylation, and variation in tertiary structures. Studies have demonstrated that inhibitors directed against the light chain were less neutralizing in the presence of VWF-containing concentrates [61–64], as vWF in concentrates protects against neutralization by factor VIII antibodies [65, 66] because they mask the epitope for further binding of inhibitors [67]. For instance, Tagariello *et al* [68] demonstrated that FVIII concentrates containing a small amount (0.06 U/ml) of VWF had significantly increased responsiveness toward inhibitors than concentrates with a high amount of VWF (2.12 U/mL). A recombinant concentrate without VWF demonstrated lower inhibitor responsiveness, probably due to non-specific FVIII inactivation because of lack of stabilizing factors. Salvagno *et al* [69] described the procoagulant effect of inhibitor plasmas of various titers on four different FVIII concentrates. The results indicated that FVIII products containing VWF may have beneficial effects in patients with inhibitors against the C2 domain. However, these experiments were performed in the presence of variable amounts of VWF, leading to variable results because of possible non-specific, temperature-dependent FVIII inactivation in the samples with low or absent VWF [70]. In conclusion, it is a challenging idea to develop in vitro inhibitor assays to predict the in vivo effectiveness of FVIII replacement therapy.

ALTERNATIVE ASSAYS TO MONITOR REPLACEMENT THERAPY IN PATIENTS WITH INHIBITORS

Hemophilia A patients with inhibitors who are treated with FVIII bypassing agents balance between a sufficient hemostatic ability and a thrombogenic state. It is important to monitor the patients' inhibitor titer by the Nijmegen assay, although it may not fully correlate with the clinical risk of bleeding or thrombosis. Global clotting assays such as clot wave form analysis (CWFA) [71], thromboelastography (TEG) [72], and thrombin generation assays (TG) [73] may give additional information on the hemostatic balance. CWFA reflects the whole clotting process including the fibrinolytic activity [74] during the measurement of an aPTT and prothrombin time (PT). Algorithms have been developed to measure several parameters such as coagulation velocity and acceleration. CWFA is especially sensitive to changes in low coagulation factor levels [75, 76]. For instance, a normalization of aPTT clot waveform parameters was observed after FVIII infusions in two hemophilia A patients with high responding inhibitors (FVIII:C of 3.2 and 6.5 IU/dL respectively) [77]. In theory, this normalization should result

in a decreased bleeding tendency, which was indeed observed in these two patients.

TEG evaluates the kinetics of formation, stabilization, and subsequent lysis of the clot. In various experiments, the effect of treatment of factor VIIa or factor VIII inhibitor bypassing activity (FEIBA) was tested using TEG in hemophilia A patients [78–80] and in a hemophilia B monkey model with inhibitors [81]. Treatment of patients resulted in correction of the clotting times (time to initial clot formation and the time to reach arbitrary clot strength) and, in the monkey model, the maximum amplitude was corrected. Furthermore, Young *et al* [82] used TEG to assess the effect of bypass therapy in 16 hemophilia A patients with high inhibitor titers. TEG allowed individualization of treatment for each of the patients and resulted in more effective, convenient, and less expensive treatment regimens.

TG measures the time-dependant changes in thrombin activity using a specific fluorescence peptide substrate, which is cleaved by thrombin and releases a fluorophore. TG is proposed as a sensitive and reliable method for assessing overall clotting function in a hemophilic population with or without inhibitors [83–86]. TG assays enable the monitoring of the in vivo thrombin-generating capacity of FVIII-bypassing agents, and thus might help to optimize treatment [87].

In conclusion, a number of experimental methods are available that may contribute to a better understanding of the relation between laboratory data and clinical findings in hemophilia patients with inhibitors. However, these methods need to be further evaluated in order to explore their usefulness.

CONCLUSION

Development of accurate assays to measure inhibitor activity remains an essential requirement in the diagnosis and monitoring of treatment of patients with hemophilia and inhibitors. The Nijmegen method has led to increased specificity and reduced falsely positive results, but a more sensitive method of measuring inhibitor levels is still needed. The various methodological shortcomings remain to be fully explored, and further standardization and optimization is required for improvement of these assays. Moreover, the development of epitope-dependent inhibitor activity assays may contribute to increased specificity of the assay, and may predict the in vivo effectiveness of FVIII replacement therapy. Alternatively, global clotting assays for the study of FVIII inhibitors are available but need further evaluation.

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