IMMUNOCHEMICAL DETECTION OF ANTIBODIES TO COAGULATION FACTOR VIII IN HEMOPHILIA A PATIENTS

A.A. Tykhomyrov[1], V.V. Krasivska[2], O.V. Stasyshyn[2], Y.P. Kovalchuk[3], S.A. Shemet[4]

[1]Palladin Institute of Biochemistry of NAS of Ukraine, Kyiv, Ukraine; artem_tykhomyrov@ukr.net
[2]SI "Institute of Blood Pathology and Transfusion Medicine of the Academy of Medical Sciences of Ukraine", Lviv, Ukraine
[3]Oles' Honchar Dnipro National University, Dnipro, Ukraine
[4]Ukrainian Association for Haemophilia and Haemostasis 'Factor D', Dnipro, Ukraine

Introduction. Hemophilia A is an X-linked genetic hemorrhagic disorder resulting from a deficiency of blood coagulation factor VIII (FVIII). The main treatment for hemophilia is a replacement therapy, when purified concentrates of plasma-derived FVIII (pdFVIII) or recombinant FVIII (rFVIII) are infused [1]. The development of inhibitors, which are alloantibodies that inhibit the procoagulant function of FVIII, is currently the most challenging complication of treatment in persons with hemophilia A. Inhibitors significantly increase the costs of care, intensify the financial and psychosocial stressors on patients and their families, and have a negative effect on disease morbidity and mortality by making bleeding episodes more difficult to treat [2].

Among hemophilia A patients with severe FVIII deficiency, about 30 % of them respond to infusion therapy immunologically, producing anti-FVIII antibodies. An important issue related to the choice of pd- versus rFVIII is that of the comparative efficacy of the two sources of replacement therapy in achieving immune tolerance, the best method to eradicate inhibitors through the long-term treatment of patients with replacement therapy with coagulation factors. Not all antibodies to FVIII act as inhibitors. Non-neutralizing antibodies directed against coagulation factors may be seen in individuals with hemophilia and in the general population [3]. Unfortunately, no randomized clinical trials are currently available in Ukraine to provide definite evidence on whether or not a difference in immunogenicity exists between plasma-derived and recombinant FVIII concentrates.

Conventionally, inhibitor titers are measured with the Bethesda inhibitor assay (BIA). Dilutions of the patient's plasma are incubated with normal pooled plasma for 2 hours at 37°C, and the residual FVIII is measured. One Bethesda unit (BU) is defined as the quantity of inhibitor that neutralizes 50% of the FVIII in normal plasma after 2 hours' incubation at 37 °C [4]. In 1995, the BIA was modified to stabilize the protein and pH during incubation. As a result, the Nijmegen-Bethesda assay was adopted by the International Society on Thrombosis and Hemostasis. Titers of 0.6 BU/mL or greater are considered to be inhibitor positive [5]. However, there are several limitations to such laboratory measurements of inhibitors, including a

limited sensitivity for low titer inhibitors [<0.4 BU/mL]. BIA detects only inhibitors that reduce FVIII activity (inhibitory), while immunochemical assays can detect both inhibitory and non-inhibitory antibodies and may have improved detection capacity for low-titer inhibitors, but they need further clinical validation to support their widespread use [6]. Thus, due to high incidence of the development of antibodies to clotting factors (both neutralizing and non-neutralizing) in patients receiving exogenous factor concentrates, and higher sensitivity of immunological tests, there is an urgent need for affordable immunological diagnostic kits for routine screening of large populations of patients with bleeding disorders. The purpose of the present study was to compare sensitivity of distinct immunochemical assays in detecting antibodies to FVIII in patients with hemophilia A, using pdFVIII or rFVIII as target antigens.

Materials and methods. The study was performed on citrated plasma samples obtained from healthy persons (n = 3), which were used as controls, and individuals with clinically confirmed hemophilia A (n = 8), which obtained FVIII concentrates' infusions as a replacement therapy. The anti-FVIII inhibitor assay was performed as described by Kasper et al. [4]. According to BIA results, patients were divided into two groups: four inhibitor-negative patients (< 0.6 BU/mL) and four inhibitor-positive samples (> 0.6 BU/mL), which had 1.0, 1.78, 2.6, and 4.36 BU/mL. Human studies were approved by the Institutional Review Boards at the State Institution "Institute of Blood Pathology and Transfusion Medicine of AMSU". Donors' informed consents were obtained in accordance with the Declaration of Helsinki.

Antibodies to FVIII in plasma samples were detected by enzyme-linked immunosorbent analysis (ELISA) and immunoblotting. For evaluation of anti-FVIII antibodies, highly purified albumin-free B domain-free rFVIII (ReFacto® AF, Wyeth Farma S.A., Madrid, Spain) or pdFVIII (Bioclot A, Biopharma, Ukraine) were used as target antigens (2 μg per well). After coating and blocking procedures, plates were incubated with the tested human plasmas in various dilutions, followed by washing steps, incubation with the appropriate secondary sheep anti-human HRP-conjugated IgG, and incubation with the chromogenic substrate (3,3′,5,5′-tetramethylbenzidine, TMB). Finally, substrate reaction was stopped by adding 50 μL of 2.5 N H_2SO_4 per well. The absorbances of colored reaction products were measured at 450 nm (primary test filter) and 620 nm (reference filter) in a microELISA reader. Optical density values at 1:1,000 dilution were taken for comparison analysis. All samples were tested at least in duplicate. The final results were expressed as the mean A(450-620)nm.

In order to detect anti-FVIII antibodies by immunoblotting, two target FVIII-related antigens (pdFVIII or rFVIII, 5 μg per track) were electrophoretically separated and transferred onto nitrocellulose membranes by electroblot in 0.025 M tris-HCl solution, containing 0.195 M glycine and 25 % (v/v) methanol. After blocking in 5 % skim milk solution, membranes were stripped and probed with plasmas of healthy donors or individuals with hemophilia A (1:1,000 diluted) overnight at 4 ^{o}C. For immunoblot analysis of FVIII-related proteins in several batches of pdFVIII concentrates, the membrane was incubated with rabbit anti-FVIII antibodies generated against rFVIII B domain-free rFVIII, or moroctocog alpha [7]. After washing, bound antibodies were identified by incubating membranes with the appropriate secondary HRP-conjugated antibodies. Non-specifically bound antibodies were removed by five-times washing. FVIII specific immunostaining was detected with the use of enhanced chemiluminescence method. The membranes were treated with luminol (0.25 M luminol diluted in DMSO, 0.09 coumaric acid diluted in DMSO, 0.1 M tris pH 8.5, and 0.0035% H_2O_2) for 1 min and exposed on X-ray film (Konica Minolta, Japan). Signals were visualized, digitized, and analyzed using TL120 software (TotalLab Ltd., USA). Molecular weights were determined using standard prestained molecular weight protein ladder. The results were expressed as units of density × band area and referred to as arbitrary units (a.u.).

Statistical differences between mean values of immunostaining intensities were evaluated by one-way ANOVA followed by Tuckey's post-hoc test using OriginPro 8.6 version (OriginLab Corp., USA). The confidence interval (P) less than 0.05 was considered statistically

significant. The quantitative results of ELISA and immunoblot analysis were illustrated as 'box-and-whiskers' plots.

Results and discussion. Immunoblotting of plasma-derived concentrate of natural full-length FVIII molecules (Fig. 1) revealed two distinct immunoreactive polypeptides: major polypeptide of ~ 250 kDa and minor band of ~ 315 kDa. The identical bands were revealed by immunoblotting with the use of plasma samples as a source of primary antibodies and a pdFVIII concentrate as a target antigen (Fig. 2). Notably, one of three healthy donor samples was found to contain antibodies that bind to FVIII-related polypeptides. This observation can be explained by the fact that anti-FVIII antibodies may be present in 20 % of healthy individuals with normal levels of FVIII in plasma, as shown by Algiman et al. [8] and recently confirmed by Miller [9]. The biological significance of natural autoantibodies is not yet understood, however, there is evidence that these antibodies may have immunoregulatory functions, playing a role in protein and cell catabolism [10]. Wootla et al. [11] have reported that autoimmune FVIII inhibitors may neutralize the procoagulant activity of endogenous FVIII by proteolysis, thus acting as natural regulators of blood coagulation cascade.

However, immunostaining of the FVIII-related antigens appeared to be significantly stronger in the samples of hemophiliac individuals (Fig. 2). Compared to healthy subjects, the densities of 315 kDa band in hemophilia patients without clinically detectable inhibitor (< 0.6 BU/mL) and especially in those having clinically significant inhibitor (> 0.6 BU/mL) were 5,14- and 7.23-fold higher, respectively. Non-significant increase in immunostaining (1,81-fold, $P > 0.05$) was observed for 250 kDa band in plasma of patients without inhibitor compared to healthy individuals, while plasmas of inhibitor patients produced significantly stronger signal than control healthy individuals (4,57-fold, $P < 0.01$).

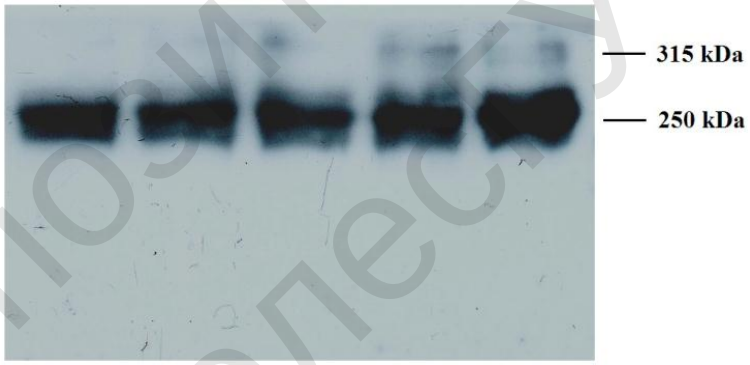

Fig. 1. – Immunoblot of various batches of pdFVIII (Bioclot A, Biopharma): primary rabbit antibodies to FVIII, secondary goat anti-rabbit IgG-HRP conjugates (1:80,000 diluted) (Sigma Aldrich, USA)

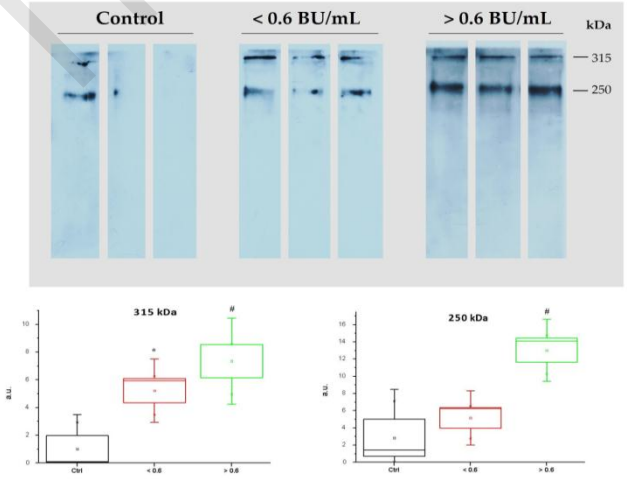

Fig. 2. – Immunoblot of anti-FVIII antibodies in healthy donor plasma and plasma of patients with hemophilia A with the use of pdFVIII (Bioclot A, Biopharma) as a target antigen. "Box-and-whiskers" represent results of densitometry analysis. * - P < 0.05 vs. Control, # - P < 0.01 vs. Control

Results of immunoblot assay demonstrate significant difference in binding capacities of circulating anti-FVIII antibodies to the target antigens of different origin. Unlike pdFVIII target antigen, B domain-free rFVIII (moroctocog alpha) was recognized by plasma IgG to much lesser extent (Fig. 3). Detectable signal levels were produced only in plasmas of patients with clinically significant inhibitor. Weak level of immunostaining in the case of rFVIII molecules could reflect the lesser amount of epitopes due to deleted B-domain, or changes in their conformation after denaturing gel-electorphoresis, as compared to pdFVIII. Therefore, the main conclusion which may be made on the basis of the described results is that immunoblotting with the native pdFVIII molecules as target antigen was capable to discriminate between normal plasmas and plasmas containing a FVIII-antibodies, including those unrecognized by the BIA (< 0.6 BU/mL).

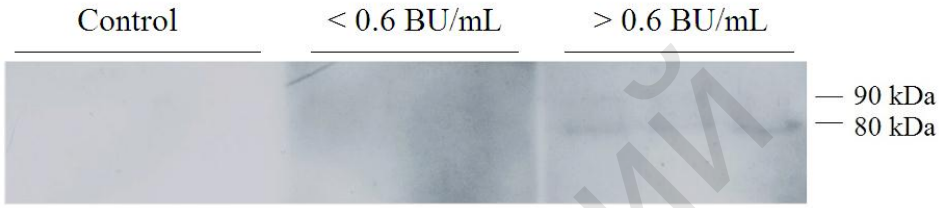

Fig. 3. – Immunoblotting of FVIII antibodies in donor plasma and plasma of patients with hemophilia A with the use of rFVIII (B domain-free rFVIII, or moroctocog alpha, ReFacto® AF, Wyeth Farma S.A.) as a target antigen

In contrast, when rFVIII was used as an coating antigen on the ELISA plates, the mean level of anti-FVIII IgG did not differ significantly between healthy donor plasmas and plasmas of patients without clinically significant inhibitor (< 0.6 BU/mL) (Fig. 4). However, pdFVIII gave the highest sensitivities for anti-FVIII antibody detection in plasmas without detectable BIA titers (at least 3-fold, $P < 0.05$), but not for plasma samples with > 0.6 BU/mL values, providing equal sensitivity with rFVIII in that BU titer range. Thus, our results obviously demonstrate that characteristics of FVIII molecules used as target antigens might affect the results of immunochemical detection.

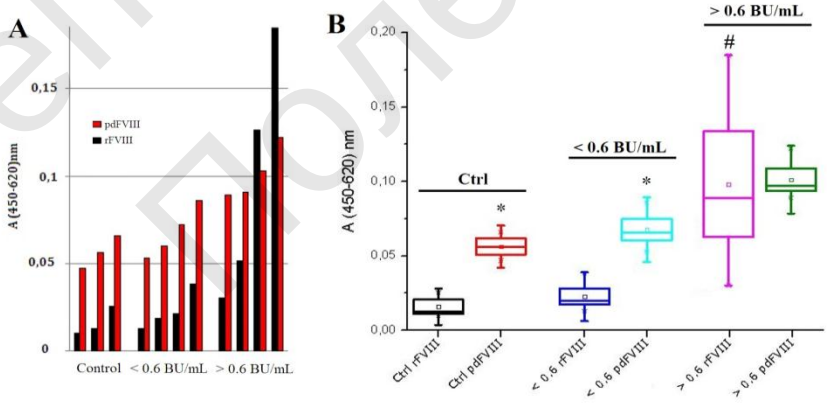

Fig. 4. – ELISA detection of anti-FVIII antibody levels in donor plasma and plasma of patients with hemophilia A with the use of pdFVIII or rFVIII as target antigens (plasma dilution 1:1,000): A – individual data, B – mean data. * - $P < 0.05$ between corresponding "pdFVIII" and "rFVIII" mean values; # - $P < 0.05$ between "> 0.6 rFVIII" and other "rFVIII" mean values

FVIII functions as a cofactor to factor IXa in the tenase complex, and a deficiency of factor VIII thus reduces the generation of thrombin on the surface of activated platelets. FVIII is synthesized as a 330-kDa precursor protein having an A1-A2-B-A3-C1-C2 domain structure with an activation peptide between the B and A3 domains. After proteolytic processing, FVIII associates with von Willebrand factor (VWF) in heterodimers of a heavy (A1-A2) and a light

(A3-C1-C2) chain associated by a metal ion interaction [12]. Most acquired FVIII inhibitors bind to the A2, A3 or C2 domains. Anti-C2 antibodies disrupt the binding of FVIII to phospholipid and VWF, while antibodies to A2 and A3 interfere with FVIII binding to factor X and factor IXa [13]. Higher IgG binding capacity to the native pdFVIII-related proteins compared to rFVIII molecules, found by blotting assay, could be a result of dissociation of FVIII molecules from VWF due to protein denaturation during gel-electrophoresis. B domain-free rFVIII (moroctocog alpha) is a genetically engineered antihemophilic factor with an approximate molecular weight of 170 kDa and consisting of two chains, 90 and 80 kDa [14]. Possibly, lack of B domain, bearing additional epitopes, in rFVIII molecule used as a target antigen may as well cause its decreased performance in both immunoblotting and ELISA. These results are in line with earlier observation of Butenas et al. [15] who demonstrated B domain to bind a significant fraction of anti-FVIII antibodies.

Immunochemical assays are much more sensitive than functional assays in detection of anti-FVIII antibodies. Because they measure both inhibitory and non-inhibitory antibodies, they are not equivalent to and cannot be substituted for functional inhibitor assays. They may be used to screen specimens for those requiring inhibitor assays or to confirm the presence of specific antibodies. The present pilot study is the first report providing a comprehensive description of immunoblot assay based on the enhanced chemiluminiscence technique for anti-FVIII antibody polypeptide profile investigations. The apparent consistency of the findings made by immunoblot analysis and results scored in plasmas by BIA supports the legitimacy and potential usefulness of the described immunochemical method as a supplementary approach to BIA.

Conclusions. We showed here that, unlike ELISA, immunoblot analysis using pdFVIII as a source of antigen and optimized by chemiluminescence technology is able to efficiently discriminate between normal plasmas and hemophiliac patients' plasmas, containing even low titers of FVIII antibodies, undetectable by BIA (< 0.6 BU/mL). Summarizing, we highlight that the use of a similar immunoblot technique for detection and screening for anti(auto)-FVIII antibodies could facilitate future researches of coagulation inhibitors development, and potentially serve as highly sensitive and informative diagnostic and/or prognostic tool.

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