UDC 577.112:612.151-083:616.151-056.7

POLYCLONAL ANTIBODIES TO DOMAIN B-FREE RECOMBINANT ANTI-HEMOPHILIC FACTOR VIII: PRODUCTION, CHARACTERIZATION AND POSSIBLE BIOMEDICAL APPLICATIONS

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Introduction. Factor VIII (FVIII, anti-hemophilic factor) is an essential blood-clotting protein. FVIII gene mutations result in hemophilia A. The mature FVIII protein is a single polypeptide with M_m of 320 kDa containing three different domains (A1, A2, A3, B, C1, and C2) (Fig. 1, A). B domain does not affect FVIII activity in the process of blood coagulation, and B domain-deleted natural or recombinant FVIII heterodimers show significant activity.

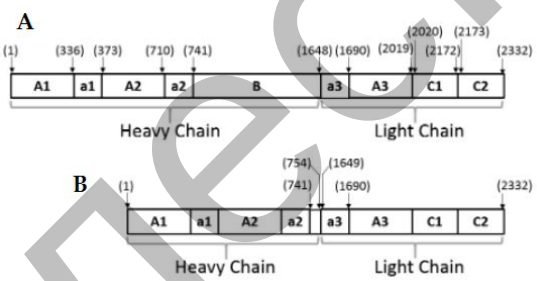

Fig. 1. Schematic structure of the full-length FVIII molecule (A) and its B-domain truncated form (moroctocog alpha) (B) [1].

Both plasma-derived FVIII concentrates and recombinant FVIII can be given to hemophiliac patients to restore hemostasis. Although various techniques for further concentrating and purifying the coagulation factors from donor plasma have been introduced, the problem of control of the quantity and quality of plasma-derived FVIII products has not been fully resolved. In screening tests during the manufacturing process immunoassays are used to quantify or detect the target antigen related to the disease in patient's blood. Quantitative analysis based on the use of highly specific antibodies to FVIII is required in order to distinguish factor deficiency from factor inhibition or defects. The use of recombinant immunogenic proteins that are maximally structurally and immunologically analogous to native target molecules is considered to be state-of-the-art industrial antigen/antibody manufacturing. Moroctocog alpha, which is also referred to as BDDrFVIII (B domain deleted rFVIII), represents a family of third-generation FVIII products, which are free of vWF and other protein impurities. Moroctocog alpha is a glycoprotein with an approximate M_m of 170 kDa consisting of 1438 amino acid residues and has functional characteristics comparable to those of endogenous FVIII [2]. In the present study, we used for the first time BDDrFVIII (moroctocog alpha) to produce polyclonal antibodies

specifically recognizing human native FVIII. Also, we describe main immunochemical characteristics of these antibodies and highlight their potential biomedical applications.

Materials and methods. ReFacto® AF (Wyeth Farma S.A., Madrid, Spain) containing 250 IU moroctocog alpha was used as immunogen. Commercially-available plasma-derived FVIII concentrates were used in this study as sources of FVIII-related antigens for immunochemical assays. Male New Zealand rabbits were immunized intracutaneously with antigen in two regimes: 1) BDDrFVIII (100 μg) as a suspension with complete Freund's adjuvant followed by repeating boost with the half-dose of antigen emulgated with incomplete Freund's adjuvant; 2) BDDrFVIII (50 μg) entrapped in 10% polyacrilamide gel (PAAG) after electrophoretic separation with the repeating boost with the half-dose of antigen as a PAAG suspension. Blood from the rabbits of the both groups was taken on 5-th, 7-th, 9-th, and 11-th days after the last antigen boost. At each time point, equal volumes of sera from each of the rabbits were analyzed by ELISA for antigen titers, using non-immune sera as a negative control. Electrophoretically homogenous IgG fraction was purified by affine chromatography with the use of Protein A-sepharose, and titers were measured by ELISA. Samples of moroctocog alpha, plasma-derived FVIII concentrates, human plasma from healthy donors and hemophilic volunteers, and human platelet lysates were used as sources of the FVIII-related antigens in immunoblotting. FVIII-specific IgG was used for FVIII-producing human umbilical vein endothelial cells (HUVECs) immunostaining followed by visualization with the use of LSM510 confocal laser scanning microscope (Zeiss, Jena, Germany).

Results and discussion. Serological analysis (ELISA) of immune sera indicates that rabbits injected with PAAG-immobilized BDDrFVIII had 4-fold higher antibody titers than did the rabbits immunized in accordance with conventional procedure (1/12000 and 1/3000, respectively). As seen in Fig. 2, ELISA data indicates that rabbits injected with PAAG-immobilized BDDrFVIII had higher antibody titers compared to the rabbits immunized in accordance with conventional procedure. Evaluation of the purified IgG titer showed the significant difference between the signal from immune IgG and non-specific control threshold baseline at the minimal IgG concentration of ~ 50 ng per well.

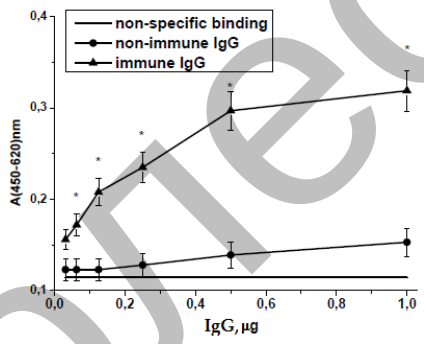

Fig. 2. ELISA absorbance profiles of specific IgG purified from serum of rabbits injected with PAAG-encapsulated antigen or BDDrFVIII in the form of emulsion with Freund's adjuvant. * - P < 0.05

Antisera taken from both groups of rabbits reacted with the moroctocog alpha, which they produced against (170 kDa), FVIII-related polypeptides in both plasma-derived concentrates and human serum (Fig. 3, A). With that, antisera of rabbits immunized with PAAG-immobilized BDDrFVIII appeared to recognize intact native FVIII molecule (320 kDa) in the samples of commercial FVIII concentrates as well as whole human serum. However, antisera obtained from conventionally immunized rabbits were not capable of such detection. Immunoblotting with the use of purified IgG as the primary antibodies showed the densities of immunostaining of the major polypeptide 320 kDa as well as FVIII-related polypeptides with the lower molecular masses to be much lesser abundant in the samples of hemophilia A persons than in healthy controls (Fig. 3, B). IgG purified from PAAG-BDDrFVIII-injected rabbits were able to recognize FVIII-related antigens in lysates of human platelets, which bear antihemophilic factor in the tenase complex or α-granules [1].

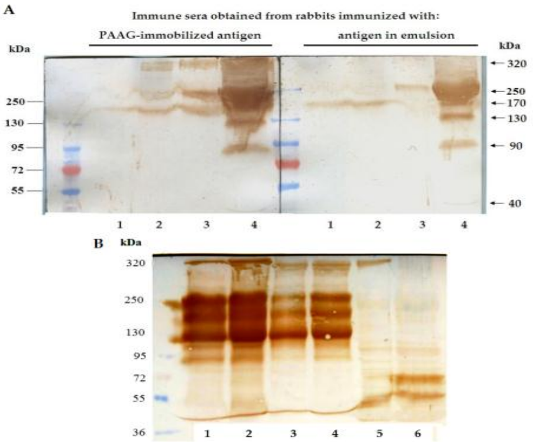

Fig. 3. Immunoblotting of FVIII-related antigens with the use of immune sera (A) or specific IgG (B) as primary antibodies (A: 1 – moroctocog alpha – 15 µg protein; 2 – BioClot A – 25 µg; 3 – Octanate – 25 µg; 4 – serum of a healthy donor – 100 µg; B: 1– healthy volunteer 1; 2 – healthy volunteer 2; 3 – person with hemophilia A 1; 4 – person with hemophilia A 2; 5 – platelet lysate 1; 6 – platelet lysate 2).

There is uncertainty about the site of FVIII synthesis, however it has been postulated that endothelial cells are generally responsible for FVIII production [3]. According to this, we performed immunofluorescence assay of HUVECs as potential FVIII producers. Fluorescence microscopy confirmed the specific binding of anti-FVIII antibodies with HUVECs, but not glioblastoma cells, which were used as a negative control (Fig. 4).

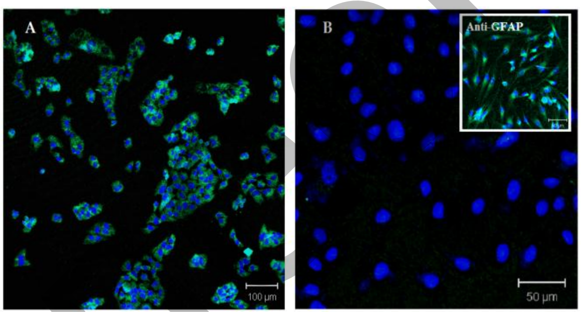

Fig. 4. FVIII immunofluorescence assay of human umbilical vein endothelial cells (HUVECs) (A) (green – FVIII; blue – nuclear staining with Hoechst-33342). Content of immune Ig G – 20 µg/mL. B – glioblastoma U373 cell line (negative control), inset: immunostaining for GFAP, the specific marker of glial cells).

Conclusions. An optimized procedure for the production of polyclonal antibodies against FVIII with the use of PAAG-encapsulated BDDrFVIII (moroctocog alpha) was proposed and successfully validated. Human recombinant B-domainless FVIII (moroctocog alpha) appeared to be suitable for the production of highly specific and affine polyclonal antibodies, capable of recognizing of native whole FVIII molecule as well as FVIII-related truncated polypeptides. The antibodies produced can be applied for the development of novel protocols of highly sensitive and specific immunoassay approaches for FVIII detection in blood and serum-derived concentrates and further clarifying of FVIII intracellular expression and localization.

References

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