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Use of a Bacterially Expressed Human Factor IX Light Chain to Develop Polyclonal Antibody Anti-hFIX

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Abstract Hemophilia B is an X-linked recessive bleeding disorder caused by deficiency or malfunctioning of human coagulation factor IX (hFIX). Hemophilia B patients are treated at present by infusion of plasma derived hFIX which is not always efficient, because development of anti-hFIX antibodies (alloantibodies) in some cases inhibits the activity of the infused hFIX. The hFIX alloantibodies are directed against γ-carboxyglutamic acid residues (Gla-domain) or protease domain in hFIX light chain. An epitope-containing fragment of hFIX light-chain was expressed in a T7-based *Escherichia coli* expression system and after purification, it was used for the immunization of rabbit to develop specific antibodies anti-hFIX. The plasma, derived from the immunized rabbit, was shown to be able to detect the normal hFIX, which indicates for the presence of a specific anti-hFIX antibody and supporting that a bacterially expressed hFIX subfragment might be able to neutralize the alloantibodies. Considering the importance of hFIX and its related investigations, both the produced hFIX antigen and its corresponding antibody will play important roles for experiments dealing with the production of hFIX and studies involved in the neutralization of the hFIX inhibitors in hFIX-related disorders and other clinical applications.

Keywords Human coagulation factor IX (hFIX) · hFIX light chain Gla-domain · *Escherichia coli* · T7 expression system · Polyclonal antibody anti-hFIX

Introduction

Human blood coagulation factor IX (hFIX) is a plasma glycoprotein with a molecular weight of about 57 kDa and approximately 17% carbohydrate, consists of 415 amino acids

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[1, 2]. It is an essential vitamin K-dependent cofactor that participates in the intrinsic pathway of blood coagulation [3, 4]. Activation of hFIX involves two cleavage steps. In the first step, an Arg^{145}–Ala^{146} bond and in the second step, an Arg^{180}-Val^{181} bond are cleaved, giving rise to activated hFIX (hFIXa) and release of an activation peptide (146Ala–Arg180) [5, 6]. This reaction occurs on the surface of an appropriate membrane, principally provided by activated platelets [7]. The hFIXa is a serine protease, consisting of the amino-terminal 145 residues "light chain", connected by a disulfide bridge to a 236 residues heavy chain. The heavy chain contains the residues involved in the process of catalysis and demonstrates a high degree of homology with the heavy chains of the other vitamin K-dependent coagulation factors and the chymotrypsin family of proteases [8, 9]. Factor IXa in turn activates factor X in a reaction requiring the presence of calcium ions, phospholipid, and factor VIII [3]. The hFIX gene is located on the long arm of X-chromosome and contains eight exons (a–h) encoding for six major domains [10].

The absence or a significant deficiency of hFIX causes hemophilia B, an X-linked recessive disorder of blood coagulation, also known as Christmas disease, affecting about one in every 30,000 males [7, 11]. Patients affected with the severe form of the disease suffer from bleeding in joints, muscles, and soft tissues. Replacement therapy is a current treatment for this disease done by infusion of hFIX. In the past, plasma-derived hFIX was used to treat hemophiliacs. However, in recent years, concerns over potential risks for the transmission of human blood born infectious agents have motivated the use of recombinant forms of therapeutics [12, 13]. Considering its key role in coagulation and its corresponding medical applications, several researches have been focused on the molecular aspects of hFIX and its production in eukaryotic systems [1, 2, 11–20].

A serious complication of the FIX replacement therapy in multitransfused patients is development of antibodies that inhibit FIX coagulant activity [21]. It has been established that such inhibitory antibodies (inhibitors) arise in 1% to 4% of the treated patients with severe hemophilia B [22]. In some patients, alloantibodies are directed against both the domain containing the γ-carboxyglutamic acid residues (Gla domain) and the protease domain of factor IX [7]. It has been shown that the bacterially produced hFIX fragments containing the major epitopes are capable of neutralizing antibodies that inhibit hFIX activity [23]. Indeed, both hFIX antigens and their corresponding antibodies have various applications in hFIX-related studies [7].

We have been working on the expression of hFIX in mammalian expression systems and specific anti-hFIX antibody would be required in various steps of the work [20]. In this report, the overexpression of a hFIX subfragment and its application to develop polyclonal anti-hFIX antibody in rabbit serum is presented. The specificity of the generated antigen and its related antibody were examined and their further applications are discussed.

Materials and Methods

Bacterial Strains, Plasmids, and Primers

DH5α and BL21 (DE3) strains of *Escherichia coli* were used as hosts for subcloning and expression steps, respectively. Plasmid pET26b+ (Novagen) was used for the construction of hFIX-F1F9 expression plasmid. Primers F1F9-IXF (5′CATGCCATGGATAATTCAGG TAAATTGGAAG3′) with *Nco*I restriction site (underlined) and F1F9-IXR (5′ CGCGGATCCCCGAGTGAAGTCATTAAATG3′) with *BamH*I restriction site (underlined; synthesized by MWG, Germany) were designed respectively in forward and reverse

directions from the light chain of hFIX cDNA for amplification of the coding region of a hFIX subfragment.

Media, Enzymes, Antibodies, and Chemicals

A polymerase chain reaction (PCR) product-cloning kit (InsT/Aclon Fermentas) was used for the cloning of the hFIX-derived PCR product (hFIX-F1F9). Luria–Bertain (LB), containing 10 g/L Bacto-tryptone (Biotech. lab.), 5 g/L bactoyeast extract (Merk, Germany), and 10 g/L NaCl adjusted to pH 7.0 with NaOH, was used as culture medium. Where necessary, 100 μg/mL ampicillin (during cloning steps) or 60 μg/mL kanamycin (during expression analysis), for providing selective pressure, were added to the culture media. Enzymes including *Nco*I, *Bam*HI, taq DNA polymerase, and T4 DNA ligase were purchased from Roche, Germany. Commercially prepared columns (Roche, Germany) were applied for the purification of plasmids and DNA fragments from agarose gel as well as PCR product. The hFIX-cDNA isolated from a human liver cDNA library (purchased from MRC Clinical Science Center at the Imperial College School of Medicine, London, UK) was used as hFIX coding source. A polyclonal antibody against hFIX (ICN, USA) and a monoclonal anti-(His)6-tag peroxidase antibody (Roach, Germany) were used for immunoblotting experiment.

DNA Manipulation

Plasmid DNA isolation, DNA digestion, and subcloning steps were performed according to standard methods [24]. PCR reaction for the amplification of the sequence of hFIX-F1F9 fragment was done according to a standard protocol in thermal cycler (Touchgene Gradient, Techne) based on the following temperature profile: an initial denaturation step at 94 °C for 2 min which was followed with 30 cycles of denaturation at 94 °C (1 min), annealing at 60 °C (1 min), and extension at 72 °C. The PCR was completed with a final extension at 72 °C for 5 min. Using the InsT/Aclone kit, PCR cloning was performed based on the protocol provided by the manufacture (Fermentas). In addition to restriction analysis, PCR was also employed to verify the recombinant plasmids, followed by nucleotide sequence analysis (MWG, Germany). Comparison of the obtained sequences against those in the GenBank was performed using BLAST program [25].

Bacterial Growth and Expression Analysis

Isolated colonies were used to inoculate LB medium containing kanamycin. Cells grown at 37 °C until optical density $(OD)_{600}$=0.5–0.7 were induced by adding 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and incubated at 37 °C for various postinduction times. The cells were harvested and the protein content of samples was prepared for further analysis.

Periplasmic Fluid Preparation

Periplasmic–osmotic shock fluid from hFIX-F1F9 producing *E. coli* strains was obtained by a modified method described by Libby and coworkers [26]. Briefly, 50 mL of fermentation medium was centrifuged at 1,500×*g* for 5 min and the pellet was placed on ice. All the subsequent steps were carried out at 4 °C. The pellet was resuspended in 15 μL of ice-cold TES buffer (Tris-HCl 0.2 M, ethylenediaminetetraacetic acid 0.5 M, sucrose

0.5 mM) pH 8.0. Ice-cold dd-H2O (22.5 μL) was added and the samples were left on ice for 30 min before centrifugation at 15,800×*g* for 20 min. The obtained pellet was used as cytoplasmic fraction. Trichloroacetic acid was added to the supernatant up to 12% of the final volume. The mixture was centrifuged at 15,800×*g* for 20 min. The pellet was dissolved in sample buffer and saved as the periplasmic fraction for further protein analysis.

SDS-PAGE and Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by a method described by Laemmli [27], with slight modifications and gels were stained with coomassie brilliant blue. The specificity of the expressed recombinant hFIX-F1F9 was examined by Western blotting. Electroblotting of proteins onto nitrocellulose polyvinylidene fluoride membrane (Roche, Germany) was performed using a wet-blotting procedure in a transfer buffer (Tris 25 mM, glycin 192 mM, methanol 20%, pH=8.3) at 86 mA for 16 h. The blot was then probed with either polyclonal antiserum prepared against hFIX-F1F9 or monoclonal anti-(His)6. The anti-hFIX complex was then treated with horseradish peroxidase-conjugated antirabbit (in the case of polyclonal anti-hFIX) or antimouse immunoglobulin (in the case of anti-His) using a solution of 4-chloronaphtol with hydrogen peroxidase as enzyme substrate.

Estimation of the Expressed hFIX-F1F9

Total protein pattern of the recombinant bacteria, visualized on comassie brilliant blue stained gels, was scanned by a Beckmann model R-112 densitometric gel scanner for estimation (%) of the expressed hFIX-F1F9 among total bacterial proteins.

Protein Isolation

Separation of the Nonsoluble Fraction

Briefly, after reaching an OD_{600}=1.0, 50 mL of fermentation medium were centrifuged at 1,500×*g* for 5 min and the pellet was placed on ice. All the subsequent steps were carried out at 4 °C. The pellet was dissolved in sample buffer and used as the bacterial proteins for further protein analysis. After sonication, the lysed cells were centrifuged at 1,500×*g* for 15 min and the resulting supernatant and pellet were taken as soluble and nonsoluble fractions, respectively. The nonsoluble fraction was dissolved in sample buffer for electrophoresis on a preparative SDS-PAGE gel.

Protein Extraction from Polyacrylamide Gel

For the extraction of the recombinant protein from polyacrylamide gel, a preparative SDS-PAGE was performed based on a discontinuous buffer system [27]. The protein samples were prepared by mixing the nonsoluble fraction of the bacterial lysis with 4× SDS sample buffer at a 1:1 ratio (*v*/*v*) and heating at 95 °C for 2 min. A total sample volume of 2.5 mL was applied to a 13% polyacrylamide separating gel. The protein bands were separated on a constant current at 150 V for 4 h. After electrophoresis, the corresponding band to the recombinant protein was eluted from the acrylamide gel in phosphate buffered saline buffer and used for immunization of rabbit. The concentration of the purified sample was determined by a protein assay method, described by Bradford [28].

Rabbit Immunization

For antibody production, a mature female rabbit was immunized with a solution composed of 1 mL (about 250 μg) of antigen (the purified bacterially derived hFIX F1F9 peptide), mixed vigorously by a vortexing with 1 mL of complete Freund's adjuvant, at four sites (on shoulder and lumber regions) subcutaneously. First booster injection was performed similarly, using incomplete Freund's adjuvant on the 4th week. Finally, the 2nd booster was taken place on the 6th week with crude antigen (half dose, used in previous times). Blood samples were collected for antibody titration, before the 3rd immunization. After 2 months, the animal was bled and serum was collected for further application. In order to determine the specificity of the produced antibody, an immunoblotting experiment was carried out to demonstrate its recognition of the native hFIX.

Results

Construction of the hFIX-F1F9 Expression Plasmid

After a first round of cloning in a T-vector, a 540-bp DNA fragment coding for a section of the hFIX, named F1F9 (Gla domain), was subcloned between the *Nco*I and *Bam*HI sites of a T7-based periplasmic expression vector (Fig. 1). The T7-based expression vector is equipped with a pelB signal sequence and a (Hia)6-tag coding sequences located in-frame respectively on the 5′ and 3′ ends of the hFIX-derived coding fragment. The use of the (His)

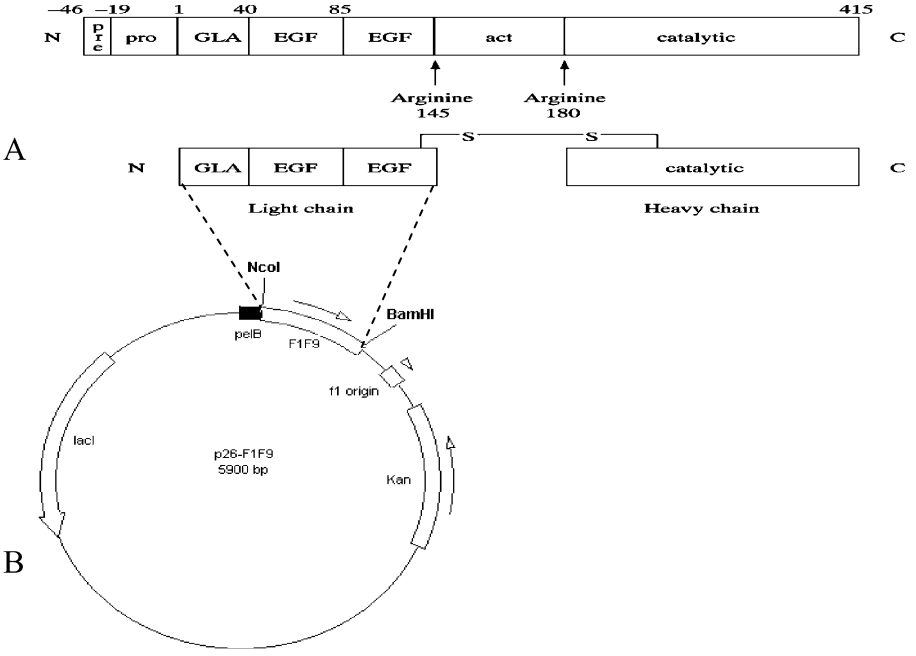

Fig. 1 **a** Structure of hFIX gene product before (*upper line*) and after (*lower line*) processing [41]. **b** Physical map of the recombinant (p26-F1F9) plasmid

6-tagged tail was considered for detection as well as the purification of the expressed recombinant protein. Therefore, periplasmic expression of a (His)6-tagged hFIX-F1F9 was expected. After verification of the cloned fragment by restriction analysis (not shown here) and nucleotide sequencing, the 5,900-bp recombinant expression plasmid (p26-F1F9) was used for the transformation of suitable *E. coli* cells.

Expression Analysis

The recombinant bacteria, carrying the p26-F1F9 plasmid, were induced with IPTG for various postinduction durations and their proteins were subjected for protein analysis. The results showed that a regulated expression of the hFIX-F1F9 takes place. Due to presence of the pelB signal peptide in the expression plasmid, the overexpression of the recombinant protein in the periplasmic space was expected. However, as the data indicates, an overexpression of a protein of approximately 25 kDa, compatible with the size of hFIXF1F9 peptide, was observable among the cytoplasmic proteins (Fig. 2). The cytoplasmic localization of the overexpressed protein can be explained by a possible formation of nonsoluble particles (inclusion bodies) of the overexpressed F1F9-related peptide. The specificity of the overexpressed protein was further confirmed by immunoblotting of the cytoplasmic proteins of the induced bacteria using rabbit serum directed against native hFIX (Fig. 3). The expression level of the hFIX-F1F9 among total bacterial proteins was estimated at 4, 6, and 8 h of postinduction time which indicated that a relatively higher level of expression with about 14% of total bacterial protein occurs after 6 h of induction. Therefore, in the subsequent steps, total proteins obtained from the recombinant bacteria, induced with 1 mM IPTG for 6 h, was subjected for purification of hFIX-F1F9.

Purification of the hFIX-F1F9

As the previous experiments showed, the recombinant hFIX-related protein was collected as nonsoluble inclusion bodies trapped in cells. Therefore, to purify the recombinant

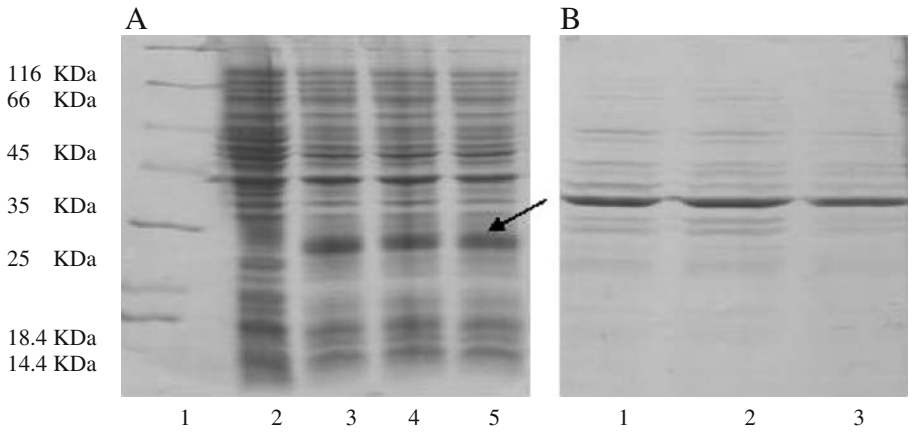

Fig. 2 SDS-PAGE (13%) analysis of the cytoplasmic (**a**) and periplasmic (**b**) proteins of the recombinant hFIX-F1F9 expressing *E. coli.* **a** *Lane 1* protein size marker, *lane 2* negative control, *lanes 3–5* cytoplasmic proteins obtained after 4, 6, and 8 h of postinduction, respectively. The overexpressed protein band of approximately 25 kDa is indicated with an *arrowhead*. **b** *Lanes 1–3* periplasmic proteins obtained after 4, 6, and 8 h of postinduction, respectively

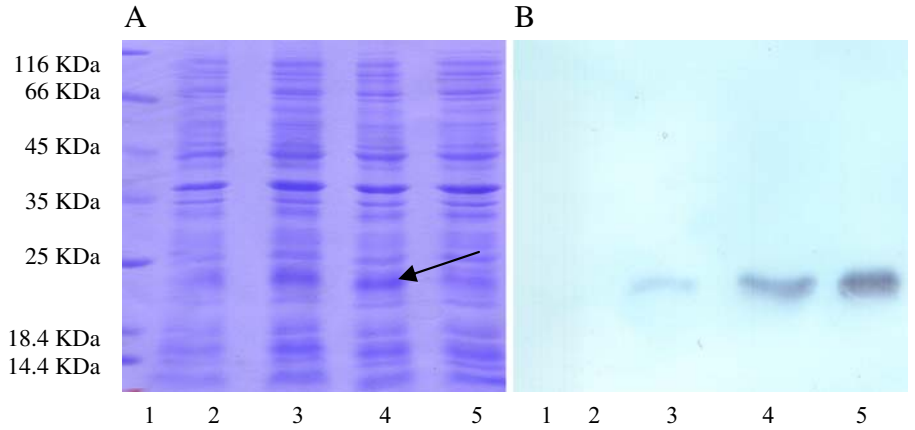

Fig. 3 Analysis of the cytoplasmic proteins of the FIX-F1F9 expressing bacteria by SDS-PAGE (13%; **a**) and Western blotting using polyclonal anti-hFIX antibody (**b**). *Lanes 1* protein size marker, *lanes 2* negative control, *lanes 3–5* 8, 6, and 4 h of postinduction with IPTG, respectively. The hFIX-F1F9 corresponding band is indicated by *arrowheads*

protein, after homogenization of the induced recombinant cells, the nonsoluble proteins of the bacterial proteins were subjected to solubilization using either 8 M urea or guanidine hydrochloride denaturants. The solubilized proteins were then subjected to the purification of (His)6-tagged protein, using a nickel nitrilotriacetic acid-based resin which appeared not to be an efficient method in this case. Therefore, the protein was subjected directly for electrophoresis on a 13% a preparative gel. After electrophoresis, the desirable fragment was isolated and eluted from the gel. The purified hFIX subfragments were confirmed by immunoblotting experiment using a polyclonal anti-hFIX antibody (Fig. 4).

Development of Polyclonal Anti-hFIX-F1F9

With the aim of producing a specific polyclonal anti-hFIX antibody, the bacterially derived hFIX-F1F9 was used to direct rabbit serum against a major epitopic region of hFIX (Gla-domain). The serum obtained from the immunized rabbit was used for a set of immunoblotting experiments to confirm its specificity (Fig. 5). As the results indicate, the

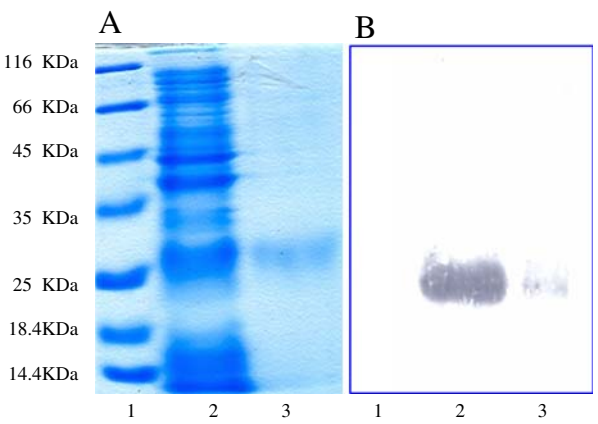

Fig. 4 Analysis of the purified rhFIX by 13% SDS-PAGE (**a**) and Western blotting using polyclonal anti-hFIX antibody (**b**). *Lanes 1* protein size marker, *lanes 2* total bacterial proteins, *lanes 3* eluted fraction

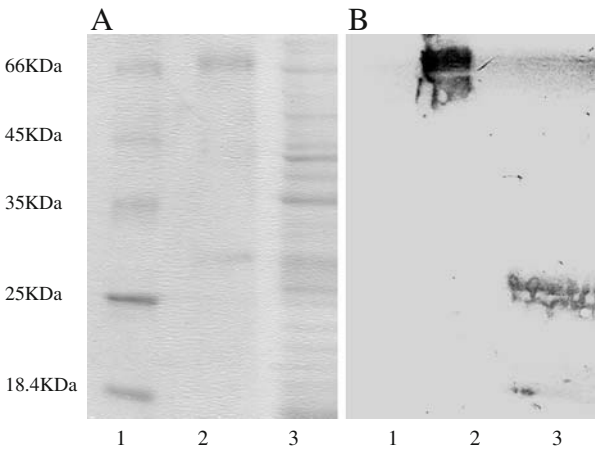

Fig. 5 Evaluation of the developed polyclonal anti-hFIX. **a** SDS-PAGE. **b** Western blotting. *Lanes 1* protein size marker, *lanes 2* plasma-derived native hFIX, *lanes 3* total protein of the recombinant F1F9-expressing bacteria

rabbit anti-hFIX-F1F9 polyclonal antibody recognizes the human plasma-derived hFIX. Appearance of multiple bands in the Western blotting experiment of the native hFIX suggests for the presence of various hFIX-derived fragments of different sizes in human plasma.

Discussion

Two major epitopes of hFIX are localized at the γ-carboxyglutamic acid residues (Gla-domain) and the protease domain of the human FIX within exons b and c, the locations where anti-hFIX alloantibodies are directed against [7]. In the present work, a 180-amino acid length peptide, derived from the light chain of hFIX containing Gla-domain of hFIX, was expressed in *E. coli*. The cytoplasmic protein pattern of the recombinant bacteria which was taken after induction with IPTG showed an overexpression of a protein, comparable to the expected size of the hFIX-derived (F1F9) peptide. In spite of the application of signal peptide, no protein of expected size appeared in the osmotic shock fluid obtained from periplasmic fractions that can be explained by a possible formation of nonsoluble particles of the overexpressed F1F9 fragment. The formation of inclusion bodies is the major cause of the processing inhibition of the protein precursor [29–32]. The nature of host strain and the growth and inducing conditions are involved in the maturation process of recombinant proteins [33]. The above-mentioned factors are very much influenced by the nature of the signal sequence and its combination with corresponding protein [34]. In such cases, a number of solutions have been suggested to decrease the extent of the metabolic load, such as the use of low copy number plasmid vector [35].

We were able to use the unprocessed protein as a source for hFIX antigen for antibody development. The data provided by immunoblotting experiments demonstrate the specificity of the generated antibody for experiments such as detection and purification of hFIX. The specific reaction of the developed polyclonal antibody against bacterially expressed hFIX subfragment and plasma-derived hFIX showed that neither the carbohydrates nor the conformation of the native hFIX play key roles in the epitopes of these domains.

Based on these results and reports from other researchers [23], bacterially generated peptides might be able to recognize the inhibitory antibodies (inhibitors) generated against

the infused human factors VIII and IX, in hemophilia A and B patients, respectively. This assumption is further supported by the fact that most inhibitors are directed against epitopes [23, 36, 37]. In a separate work, it was shown that highly antigenic hFIX-derived synthetic peptides conjugated with bovine serum albumin are able to neutralize factor IX inhibitors in plasma and there is a possibility of peptide neutralization inhibitor therapy [2]. Using a bacterially derived hFVIII-C1C2 peptide and plasma samples from hemophilia A inhibitor patients in a previous work (unpublished data), we demonstrated a specific Ab/Ag reaction between the bacterially derived hFVIII subfragment and the patient's plasma that supports the idea of probable medical application of bacterially produced peptides as neutralizing factor in FVIII inhibitor-generating patients [38]. Production of specific polyclonal antibodies antihuman factor IX using the whole hFIX protein was reported previously by Chaves and colleagues [39]. The application of that polyclonal antibody against the whole hFIX protein might be limited to experiments dealing with the entire length of hFIX protein. In this work, we produced a subfragments specific antibody antihuman FIX. To our knowledge, there has not been a report on the use of a bacterially derived fragment from hFIX light chain for the production of anti-hFIX antibody. In our experiments, we have demonstrated the ability of the generated antibody for detection of human factor IX which is an indication for its potential for other applications both with medical and economical importance. Both the recombinant hFIX subfragment and its specific polyclonal antibody, produced in this work, has provided valuable tools for further studies dealing with the production of recombinant hFIX (rhFIX) and the hFIX-related clinical and diagnostic applications on hemophilia B patients who develop inhibitors against subregion of the infused hFIX. Enzymatic immunoassays using anti-hFIX antibodies are appropriate tools to detect hFIX antigen in various steps of the hFIX production and its final product.

The antibody anti-hFIX, generated in this work, could be used to reduce hFIX-related investigational costs and providing independence in studies dealing with hFIX manufacture. This work has described a simple, economic, and high yield production of specific antibodies antihuman FIX in rabbits. Peptide neutralization inhibitor therapy has been suggested as an effective treatment to eradicate the inhibitor antibodies [40]. In this regard, the generated hFIX epitope has provided useful mean for studies concerning the therapeutic approaches to patients suffering from the factor IX inhibitor therapy.

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