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Original Article

Isolation, Cloning, Expression and Purification of Recombinant RhD Antigen from Cord Blood

**M Habibi Roudkenar[1], A Oodi[1], R Halabian[1], M Mohammadipour[2], N Amirizadeh[1], N Massrori[1], P Mozafari[1], E Kamali[1], A Mohammadi Roushandeh[3], H Rezvan[1]*

[1]*Research Center, Iranian Blood Transfusion Organization, Tehran Iran*
[2] *Science and Research Center, Department of Biology, Azad University, Tehran, Iran*
[3] *Dept. of Anatomy, Faculty of Medicine, Medical University of Tabriz, Iran*

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Abstract

Background: Rh (Rhesus) is a highly complex blood group system in man deeply rooted in transfusion medicine. Isolation of RhD from cord blod, cloning and expression of recombinant RhD antigen in bacterial expression system was the aim of this study.

Methods: Total RNAs were extracted from cord blood (O^+). The quality of RNA was determined by electrophoresis. In order to obtain coding sequence of RhD antigen cDNA was synthesized and Rh D gene was amplified by RT-PCR. The isolated RhD gene was cloned to pUC18 vector and transformed to *DH5α*. The confirmed construct was sub cloned into expression vector, pBADgIII/A, and expressed in *Top10 E.coli*. The expressed protein was characterized by SDS-PAGE and western blot analysis. Antigenicity of the expressed protein was assessed by ELISA using commercially available human anti-RhD polyclonal antibody with peroxidase conjugated goat anti-human IgG, IgM, IgA as secondary antibody.

Results: RhD gene was successfully cloned and expressed. The expected size of recombinant RhD protein was detected in SDS-PAGE, and confirmed by dot and western blot analysis. RhD antibody reacted with recombinant RhD antigen as well as with RhD polypeptide extracted from RBCs membrane.

Conclusion: The recombinant RhD may be helpful to further investigate the molecular basis of RhD protein and could be applicable for production anti- D antibody in an animal model

Keywords: *Recombinant RhD antigen, Cloning, Cord blood*

Introduction

Rh (Rhesus) is a highly complex blood group system in man deeply rooted in transfusion medicine, through implications in alloimmune transfusion reactions, hemolytic disease of the newborn, auto-immune hemolytic anemia and through the non-immune hemolytic condition associated with Rh-deficiency syndrome (1). Of the antigens of the Rh system, D is the most clinically significant, because it is highly immunogenic. This high degree of immunogenicity stems from the fact that the entire RhD protein is absent from the erythrocyte membranes of persons expressing D-negative phenotypes (2, 3). The RhD and CcEe proteins arise from the *RHD* and *RHCE* genes, respectively, whereas the RhAG component arises from the *RHAG* gene (4, 5). Between 30 and 35 amino acid substitutions define the Rh CcEe and D proteins, dependent on the CcEe phenotype of the individual (6,7). Rh antigen expression occurs early in hematopoietic differentiation, being detected in colony-forming unit-erythroid (CFU-E) cells, but not burst-forming unit-erythroid (BFU-E) cells (1, 8, 9).

It was hypothesized that cord blood might be a source for most hematopoetic progenitors including cells expressing RhD gene. Cloning and Expression of RhD gene has been reported in mammalian cell line previously but not in bacterial expression system (10- 13).

In this study RhD gene was isolated from cord blood, cloned and expressed in bacterial expres-

***Corresponding author:** Tel: +98 21 82052165, Fax: +98 21 88601599, E-mail: roudkenar@ibto.ir

sion system. The recombinant RhD may be helpful to further investigate the molecular basis of RhD protein.

Materials and Methods

Plasmids and bacterial strains

Plasmids *pUC* 18 (Cinagen, Iran) and *p*BAD/gIII A (Invitrogen, USA) were used for construction and expression. *p*UC18 was used for initial cloning and sequencing. Bacterial strains *E. coli DH5α* (Cinagen, Iran) used for cloning and Top10 (Invitrogen, USA) used for expression of the constructs. *p*BAD/gIII expression vector provides the opportunity to clone the desired insert as a fusion protein with two tags: C-terminal *myc* epitope and polyhistidine region. These tags facilitate detection of the expressed protein with anti-*myc* and anti-his antibodies and also purification of the protein using the metal-binding site for affinity purification of the recombinant protein.

Isolation of peripheral mononuclear cells from cord blood

A single cord blood with a positive blood group was obtained. The Ficoll-Hypaque method was used to isolate mononuclear cells from the cord blood. Briefly, the blood was first diluted 1:1 with phosphate-buffered saline, and 10 ml diluted blood were carefully layered onto a 3 ml Ficoll-Haque plus cushion (Pharmacia Biotech, Uppsala, Sweden) in a 15 ml centrifuge tube (Falcon 3033 Becton Dickinson, Franklin Lakes, NJ). The tube was centrifuged at 400 x *g* for 30 min at 18-20º C. The interface (containing mononuclear cells) was carefully collected and washed twice with phosphate-buffered saline. The cells were used for RNA extraction.

RNA extraction

Total RNA of mononuclear cells isolated from cord blood was extracted by Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The quantity and quality of RNA was determined by spectrophotometry (Nanodrop, USA) and electrophoresis, respectively.

cDNA synthesis

Reverse transcription was performed by Super-Script III reverse transcriptase (Invitrogen, USA) with 1µg of total RNA followed by DNaseI (Invitrogen, USA) treatment and heat inactivation.

Amplification and cloning of RhD

The RhD cDNA was amplified using specific primers containing *E coRI* and *HindIII* sites. The sequences of forward primer were, 5-TTGGAATTC ATGAGCTCTAAGTACCCGCGGTC-3 and reverse were, 5- GCAAGCTT TTAAAATCCAA-CAGCCAAATGAGG-3. The sequences of primers were designed by primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

All PCR reactions in aliquots of 50 µl contained 5 µl of 10X PCR buffer, with MgSO4, 200 µM (each) deoxynucleoside triphosphates (dNTP), 15 pmol of each primer and 200-500 ng of template DNA. PCR was carried out in Eppendorf Thermo Cycler using *Pfu* (Fermentas, Lithounia) with initial denaturation at 95º C for 5 min followed by 1 min at 94º C, 45 s at 60º C and 45 s at 72º C for 30 cycles. PCR product was visualized by UV The fragment of expected size was cleaned up by High pure PCR purification kit (Roche Diagnostic, Germany) and digested with respective restriction enzymes. The fragment was ligated to pUC18 vector. The ligation product was transformed into competent *E. coli* DH5 α and transformed cells were selected on Lauria Bertani plates containing 50 µg/ml ampicillin. The selected clones was further analyzed by Restriction enzymes and PCR and finally sequenced by a commercial facility using universal forward and reverse M13 primer (TAG Copenhage, Denmark).

Expression of Rh D gene

The RhD gene was subcloned to expression vector, *p*BAD/gIIIA, and transformed into competent *E. coli* Top10. The induction was carried out when culture had reached the OD of 0.5 at 600 nm and a further incubation at 37º C for different times and concentrations of arabinose was as instructed by the manufacturers.

Detection of the expressed protein

Total protein was extracted by complete lysis B (Roche, Germany) according to manufacture protocol. The protein concentration was quantified by BioRad protein assay kit according to the instructions of the supplier (BioRad, USA).

One mg of total protein was used for Dot, western blot and ELISA. Expressed protein was detected by running the samples heated in 1x SDS-PAGE sample buffer at 95º C for 5 min on 12% gel and stained with Coomassie blue. The proteins were also blotted onto PVDF paper (Hi-bond Amersham Biosciences, USA) and blocked with a solution containing 5% skimmed milk and 0.1% Tween 20. Blocked membranes were washed with PBS-0.05% Tween 20 and incubated with horseradish peroxidase-conjugated *myc* antibody according to manufacture protocol (Invitrogen, USA) at room temperature for 1 h. Membranes were then washed 3 times with PBS containing 0.1% Tween 20 and developed using ECL kit according to manufacture protocol (Amersham Biosciences, USA). Antigenicity of the expressed protein was assessed by ELISA.1 mg of total protein was mixed with 100µl of coating solution (R & D, USA) and coated to ELISA micro plate's wells and were incubated over night in 4º C. Subsequent steps were performed according to R & D manufacture protocol. Human anti-Rh (D) polyclonal antibody (Immunodiagnostika, USA) in different concentrations as a first antibody and goat anti-human IgG, IgM, IgA antibody conjugated with peroxidase (Abcam, UK) as secondary antibody were used. RhD polypeptide extracted from RBCs membrane (14) used a positive control in ELISA. Ghost extracted, RBC membrane, from RhD negative RBC membrane also used as negative control.

Purification of polyhistidine-tagged RhD fusion protein

The RhD encoded by pBAD carries six histidine residues at its C terminus. Polyhistidine has a high affinity for a nickel-nitrilotriacetate acid resin (Invitrogen, USA), permitting single-step purification of the fusion protein. Bacterial pellets were collected by centrifugation at 10,000 x g for 10 min and periplasmic extract were prepared by osmotic shock. The nickel-nitrilotriacetate acid resin was washed and periplasmic extract was added to the column and bound protein eluted according to manufacturer's instruction.

Results

Isolation of RhD

Specific primers were designed to amplify RhD. The expected size of the PCR product, 1254 bp, was obtained (Fig. 1). The isolated fragment was cloned in pUC18 vector (Fig 2) and the nucleotide sequence of the gene was confirmed by sequencing. The sequence was searched for homology by BLAST analysis (Ref; NM_016124.3).

Construction and expression of RhD

Schematic representations of constructs are summarized in Fig. 2. The expression vector containing RhD was constructed as described. The *p*BAD/gIII can be used to add a C-terminal *myc* epitope and a polyhistidine tag, facilitating detection and purification of the expressed protein. In this study, RhD fragment was used without a stop codon, therefore, the hybrid gene was expressed containing both C-terminal *myc* epitope and polyhistidine region. The molecular mass of RhD was approximately 35 kDa. Top10 cells transformed with *p*BAD/RhD were treated with different concentration of arabinose as instructed by the manufacturer. The induction was carried out for 6 h, and the cells were collected and processed as instructed. Aliquots were dissolved in SDS-PAGE sample buffer (whole-cell extract) and analyzed by electrophoresis. A protein of approximately 35 kDa was induced (Fig. 3). The optimization of protein expression was attempted in different range of arabinose (0.002-2%) concentration and induction period. The best result was obtained at 0.2% arabinose and 6 h incubation. Next, expression was performed in 100 ml bacterial culture and Bacterial extract containing RhD was purified by passage through a nickel-Sepharose column and eluted protein was analysed by SDS-PAGE (Fig 4). Purity was measured visually and spectrophotometrically. This purified protein was used to determine the bioactivity of RhD.

Detection of expressed protein

Dot and Western-blot analysis were performed for the protein using anti-*myc* antibody. Dot blot was used for optimization of concentration of an-

tibody. The RhD protein was detected using *myc* antibody both in Dot blot and western blot (Fig. 5). A single protein band was detected after developing by ECL system. To prove the recombinant protein contains RhD epitope, ELISA was performed with human anti- RhD antibody. The reacted with recombinant RhD antigen as well as with RhD polypeptide extracted from RBCs membrane while no reaction was detected with Ghost extracted from Rh D negative RBC membrane. Threshold of detection for human anti- RhD antibody in ELISA was 10 fold dilutions. These results indicate the recombinant expressed protein carries RhD epitope (Table 1).

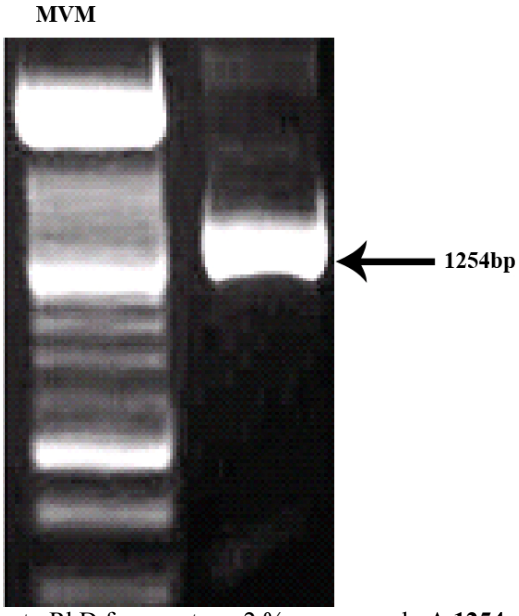

Fig. 1: Electrophoresis of PCR product , RhD fragment, on 2 % agarose gel. A **1254** pb fragment was amplified by specific primers from cord blood (lane 2). Lane 1 representing Molecular weight marker 100 bp ladder.

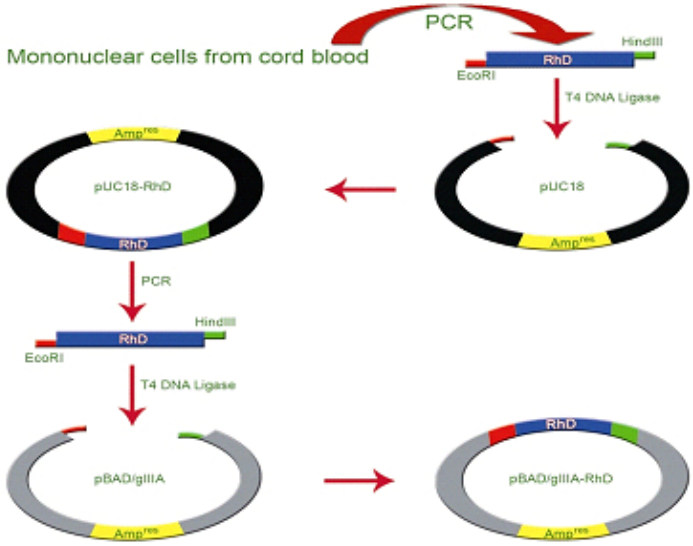

Fig. 2: RhD was isolated from cord blood by PCR. The RhD fragment was ligated to pUC18 vector that has been cut with EcorI and HindIII. The recombinant pUC18-RhD was selected on LB medium containing 50µg/ml Ampicillin and further analyzed by restriction enzymes or PCR for existence of RhD insert. Its accuracy was verified by DNA sequencing; it was then amplified and sub-cloned to pBAD/gIIIA vector at EcorI and HindIII sites. The recombinant vector, pBAD/gIIIA-RhD, was transformed to *Top10 E. coli* and induced by arabinose to express recombinant RhD protein.

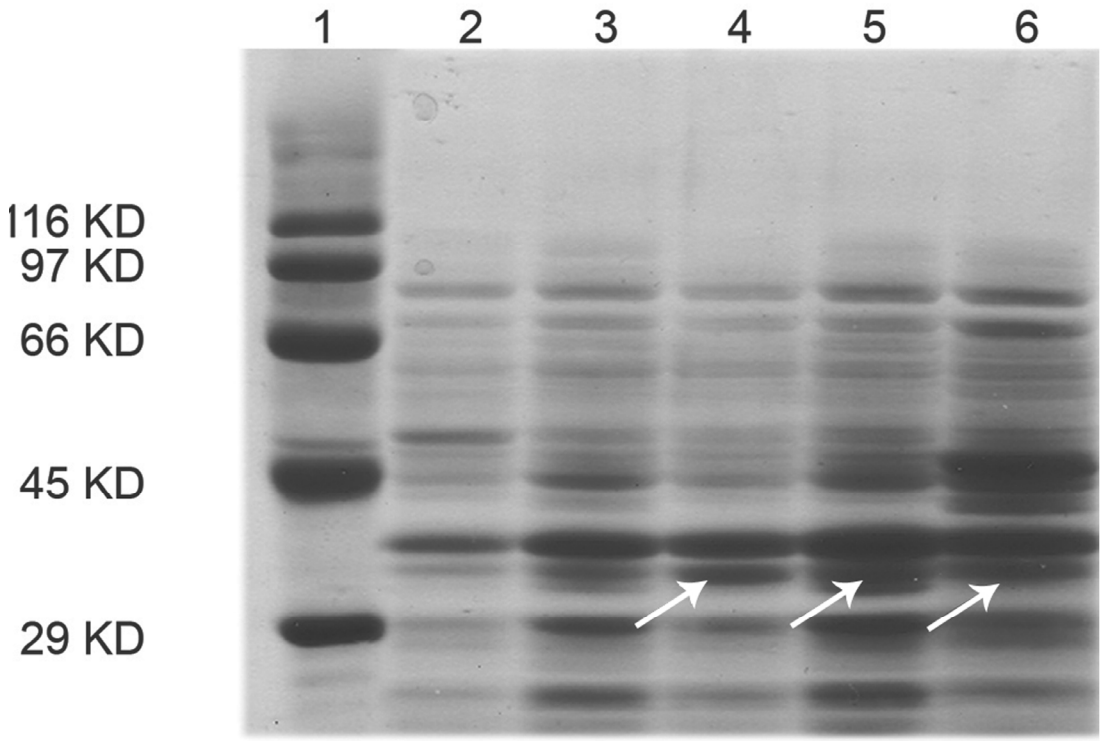

Fig. 3: SDS-PAGE analysis was performed to detect the expression of RhD protein. Total protein was extracted and the concentration was measured with Brad ford method. Same protein concentration of different extracts was loaded on 12% SDS-PAGE. Lane 1, MWM (**Fermantase, Lithuania)**, Lane 2 before induction, lane 3 uninduced, lane 4, 2% of arabinose, lane 5, 0.2 % of arabinose, lane 6, 0.02 % of arabinose. In the presence of 2, 0.2 and 0.02 % of arabinose, a protein band with approximately 35 KD can be observed **(arrows).**

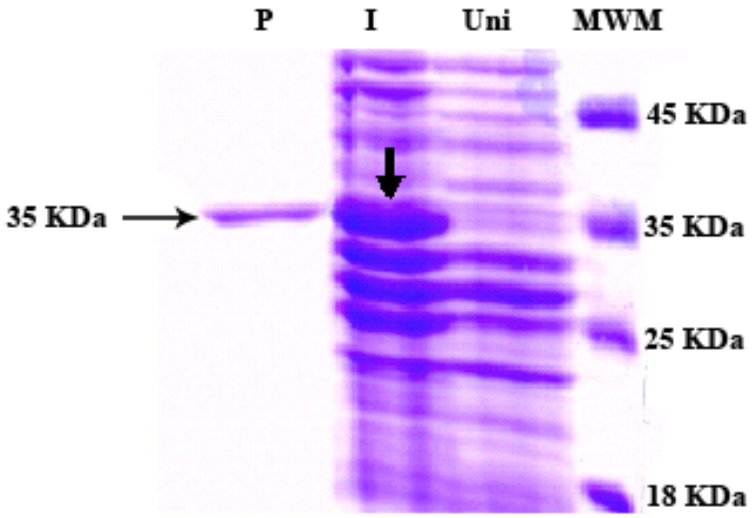

Fig. 4: large scale expression and purification of RhD. SDS-PAGE (% 12) was performed to detect the protein. Single band can be observed after elution from Ni-NTA column elution (P). Lane 2, induce, lane 3 is uninduce and lane 4 is MWM (**Fermantase, Lithuania)**.

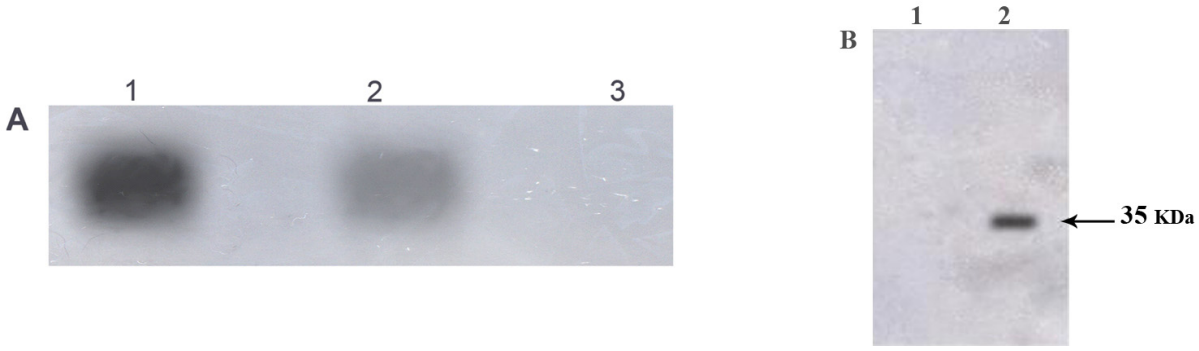

Fig. 5: A; Dot blot analysis was performed with C-myc antibody. Total protein was loaded on PVDF membrane with different concentration of C-myc antibody. The dilutions 1/500 (lane 1), 1/1000 (lane 2) and 1/2000(lane 3) of antibody were examined. B; Western blot analysis was performed with C-myc antibody. A single protein band was detected after developing by ECL kit (lane 2). There was no band on uninduced well (Lane 1).

Table 1; ELISA of human polyclonal anti– RhD toward recombinant RhD and RhD Polypeptide Extracted from RBCs membrane (OD 450)

	Recombinant RhD	**RhD Polypeptide Extracted from RBC membrane(Positive control)**	**Ghost extracted from Rh D negative RBC membrane (Negative control)**
Human Polyclonal anti- RhD			
1:2	0.451±0.122	0.316±0.152	0.091±0.023
1:10	0.285±0.091	0.186±0.162	0.015±0.021

Discussion

The Rh blood group is one of the most complex blood groups known in humans (1). From its discovery 60 yr ago where it was named (in error) after the Rhesus monkey, it has become second in importance only to the ABO blood group in the field of transfusion medicine (15, 16). It has remained of primary importance in obstetrics, being the main cause of hemolytic disease of the newborn (HDN). Since RBCs lack nucleus, it would be impossible to obtain mRNA of RhD from RBCs. Progenitors of RBCs in peripheral blood expressing RhD gene are too low to obtain RhD mRNA while in cord blood they are adequately available (1, 8, 9). In this study we isolated cDNA of RhD from cord blood. DNA sequencing showed the accuracy of the RhD gene. This indicates that cord blood cells may represent a source for obtaining RhD gene. In this study, *p*BAD/gIII expression vector, which is a tightly regulated system based on arabinose operon was employed, and it is designed to express the desired protein in preplasmic space (17). Expression of the heterologous recombinant proteins in preplasmic space abolishes the need for denaturation/renaturation cycle which is required when proteins are produced in the form of inclusion bodies (18). The RhD encoded by pBAD carries six histidine residues and C-myc at its C terminus. Polyhistidine has a high affinity for a nickel-nitrilotriacetate acid resin permitting single-step purification of recombinant RhD protein. It is noted, in bacteria cells there is not C-myc epitop. So monoclonal antibody against C-myc epitop is suitable to detect the expression of recombinant protein. Recombinant RhD protein was detected as a single bound in western blot analysis indicating the expression C-myc epitop. Next, we optimised the expression of recombinant RhD in large scale and RhD was purified by nickel-nitrilotriacetate acid resin. Finally, the antigenicity of the chimeric protein was confirmed by ELISA using anti-RhD antibody. Expression of RhD has been reported in K562 cell line by yan et al. (19). Expression of RhD in bacterial system has not been reported previ-

ously and the present report seems to be among the first ones. The recombinant RhD may be helpful to further investigate the molecular basis of RhD protein and could be applicable for production anti -D antibody in an animal model such as guinea pig (20).

Acknowledgements

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The authors declare that they have no Conflict of Interests.

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