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The Blood Group Rhc Protein from Human Erythrocyte Membranes as an Immunogen for Producing Antibodies in Mice

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Abstract

Background: Various methods have been used to isolate red blood cell (RBC) membrane antigens. In this regard, obtaining the antigen and preserving its structure is of special importance. However, limited studies have been conducted to purify cellular membrane antigens such as Rh proteins.

Materials and Methods: In this experimental study, Rhc antigens of the RBC membrane was purified. Here, the RBC membrane was solubilized through the lysis buffer. Next, dialysis and affinity chromatography were performed using polyclonal anti-human RhCcEe antibody to isolate Rhc/e antigens from the RBCs with the following blood group characteristics: Rhc+, RhC-, Rhe+, and RhE-. The purified proteins were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and dot blot methods. The immunization process was performed in Balb/c mice using the Rhc antigen as an immunogen. After the last injection, the mouse serum was used to titrate antibodies.

Results: Protein bands of the purified antigen were observed in the silver-stained SDS-PAGE gel (region of 25-35 kDa). The OD405nm = 0.56 ± 0.05 results showed the reactivity with Rhc antibody. The specificity of the purified protein was evaluated using the dot blot assay. The anti-sera titration was greater than 1/10,000 against Rhc-coated microwells. Rh antigens can be isolated from the RBC membrane using the non-ionic NP-40 detergent and affinity chromatography.

Conclusion: The Rh antigen can be isolated from the RBC membrane with proper purity by solubilization with the non-ionic NP-40 detergent and purification by affinity chromatography. It seems that the membrane antigen maintains its antigenicity and structure. As a result, it can be detected by blood group-specific antibodies used in the hemagglutination method. Purified antigens may be used to generate antibodies or to study the protein structure.

Keywords: Antibodies; Erythrocyte, Immunization; Purification; Rh Antigen

Introduction

Rh blood group is the most immunogenic blood group system after ABO. To date, 49 antigens associated with this blood group have been identified, of which D, C, E, c, and e antigens are more common and immunogenic (1). These antigens are coded by the RHD and RHCE genes. Rh antigen is a protein complex passed 12 times through the red blood cell (RBC) membrane (2). Although the function of Rh antigens has not been accurately determined, they play an important role in the integrity of the membrane. Patients who need blood transfusion should receive compatible blood group products.

Therefore, it is necessary to use monoclonal antibodies (mAbs) to detect RBC surface antigens in blood donors and recipients (3, 4). In recent decades, the importance of studying the RBC membrane has increased because of the blood group antigens, including Rh. Typing of these antigens should be done to determine the blood group for blood transfusion. Antigens are purified for different purposes, such as studying their structure or producing antibodies. However, separating antigens from the RBC membrane may alter their structure such that specific antibodies do not recognize them (5-7).

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Besides, most of the available data and information about RBC membrane proteins has been obtained by RBCs hemolysis in a hypotonic buffer with different ionic strengths. In this respect, purifying antigens from RBC membranes with the lowest structural modifications is a major challenge (5). A series of detergents-containing buffers are used to solubilize the membrane (8). Additionally, free detergent-based methods have been expanded to isolate proteins. The Styrene Maleic Acid Lipid Particles (SMALP) is a method to isolate proteins and their surrounding lipids in nanodiscs. Although this method purifies the protein complex from the membrane, it has some limitations. In this method, binding the purified protein complex interferes with Ni-NTA resin and causes disturbances in the structural and functional evaluation of proteins (9). The Rhc antigen has a high prevalence and immunogenicity after RhD. However, limited studies have been done on purifying this antigen from the RBC membrane. Purified antigens can be used in animal immunization to produce antibodies. Moreover, there is a strong correlation between the nature and purity of the injected antigen and the spleen cell immunization. The increase in the immunization rate is accompanied by the rise in antibody titters (5, 10). This study was conducted concerning the importance of membrane protein purity in the immunization process.

Materials and Methods

Samples collection

In this study, two packed RBC bags with the following Rh phenotype were used: O negative and D^-, C^-, E^-, e^+, and c^+. The RBC bags were obtained from healthy donors referred to the Iranian Blood Transfusion Organization (IBTO). This study was approved by the ethics committee of IBTO with the following code: IR.TMI.REC.1400.010.

RBC membrane solubilization

In the present study, RBCs were frozen at -70℃. After 24 h, RBCs were melted at room temperature. This process was repeated three times. The broken RBCs were centrifuged at 10,000 g for 10 min, followed by washing and collecting the pellet. Subsequently, a buffer was added to RBCs to solubilize the membrane antigens. The buffer consists of 10 mM EDTA, 25 mM Tris-HCL, 1% Nonidet P-40, 150 mM NaCL (pH=7.5), and 0.2 mM of phenylmethylsulfonyl fluoride (PMSF) (Sigma, USA) as protease inhibitor. This buffer breaks down the RBC membrane and releases the proteins. After 3 h of mixing, it was centrifuged at 14,000 g for 15 min. Then, the supernatant was separated, and the precipitate was removed. The obtained solution was transferred into the dialysis bags (3.5K MWCO, Thermo Scientific, USA) and dialyzed in the phosphate buffer for 24 h. Finally, the solubilized membrane proteins were stored at -30℃.

Affinity chromatography

CNBr-Activated Sepharose-4B (Sigma, USA) was used to attach the anti-RhCcEe antibody as an affinity ligand. For this purpose, 0.5 g of Sepharose gel powder (Sigma, USA) was suspended in 6 ml of 0.2 M Carbonate-Bicarbonate Buffer (pH 9.2) and homogenized with slow movements. After swelling of the gel, 60 µg of rabbit anti-human RhCcEe polyclonal antibody (Invitrogen, USA) was added to the gel. The gel tube was placed on the rotator for 3 h. During this time, the antibody could bind to the active sites of the gel. After the preparation of the column (by passing Ethanol amine and then washing), the antigen was transferred and slowly passed through the column and bound to the immobilized antibody.

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The washing step was done with phosphate-buffered saline (PBS) until the liquid coming out of the chromatographic column did not absorb light at a wavelength of 280 nm. During the elution step using glycine buffer (0.1M gly, pH=2.8), the antigens (including both Rhc and Rhe) were collected and concentrated. In the second step, instead of RhCcEe polyclonal antibody, anti-Rhe antibody (mAb, Immunodiagnostika, Germany) was used as an immobilized ligand to prepare the second chromatography column for removing the Rhe antigen from the purified antigen of the first step. The antigen was purified and concentrated using a Spin-X® UF20 concentrator tube (Corning, UK) with a 5 KD MWCO. The concentration of the purified protein was determined by the Bradford method. In this step, the protein's purity and specificity were also studied.

SDS-PAGE gel electrophoresis

The protein purity of the antigen was determined by performing the SDS-PAGE. First, 32 µL of the purified antigen was mixed with 8 µL of the sample buffer. Next, it was kept at 450 rpm and 96°C for 5 min and subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was performed using a Mini-PROTEAN® TGX™ (BIO-RAD, USA) with a current of 90 volts for 1 h using a protein size marker (6.5-116 KD, Cytomatingene, Iran). Finally, the gel was stained with silver.

Silver staining of the gel

Silver staining is used to stain proteins after a high-resolution polyacrylamide gel electrophoresis (11). For this purpose, the gel was placed in a solution of 40% ethanol, 10% acetic acid, and 50% distilled water for 1 h. After 1 h, the gel was washed with distilled water for 30 min and then incubated for 1 min in 0.02% $Na_2S_2O_3$ solution. It was washed again with distilled water for 20 s. Next, the gel was placed in a 0.1% Na_2NO_3 solution in a refrigerator at 4°C for 20 min. Then, the gel was washed 3 times with distilled water for 20 s each time. In the last step, the gel was incubated with 3% Na_2CO_3 solution. Incubation was continued until the density of the bands was suitable. After washing with distilled water, the gel was placed in 5% acetic acid for 5 min to finish the staining process, and stored in 1% acetic acid solution.

Examining the reactivity of the purified antigens with specific antibodies by ELISA method

The enzyme-linked immunosorbent assay (ELISA) method was used to study the reactivity potential of the purified antigen with the Rhc-specific human mAbs. For this purpose, the purified antigen was coated at 10 µg/mL concentration in the wells of a microplate during overnight incubation. Then, the blocking step was done using 2% BSA for 24 h at 4°C. About 50 µL of anti-Rhc antibodies (Immunodiagnostika, Germany) was added to the wells and incubated for 40 min at 37°C. In the next step, the plate was washed with PBS-tween in triplicate. Afterward, 50 µL of HRP-conjugated anti-human globulin was added to each well and incubated for 40 min at 37°C. The wells were washed again, and added with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution. After 20 min, the stop solution (H2SO4) was added, and the absorbance of the wells was read at 450 nm using an ELISA reader (Lab Systems Multiskan, Thermo Scientific, USA).

Dot blot procedure

Dot blots, including western blot, are among the methods for detecting proteins using antibodies. In this method, the presence of proteins is determined by the appearance of a spot on the membrane (12). Dot blotting was performed by immersing the nitrocellulose membrane in methanol for 1 min. Then, it was washed with distilled water. It was immersed again

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in the transfer buffer for 15 min. After 15 min, the buffer was removed and the membrane was placed at room temperature to dry. Afterward, 10 µL of the purified antigen and 10 µL of the BSA solution (as the negative control) were loaded on the membrane. It was placed at room temperature to dry the blots. In the next step, the nitrocellulose membrane was blocked with BSA. After BSA depletion, the membrane was incubated with primary antibodies (anti-Rhc or Rhe) for 2 h. In the next step, the secondary antibody (HRP-conjugated anti-human globulin) was added and incubated for 2 h. The membrane was washed with PBS-tween, and the antibody binding was detected using enhanced chemiluminescence (ECL) (Cytomatin gen, Iran) and quantified by Bio-Rad molecular Imager (Biorad ChemiDoc XRS system, USA).

The immunization procedure

The appropriate amount of purified Rhc antigen (i.e., ImmunoGen) was injected into 4-week-old male Balb/c mice. This solution was completely mixed with an equal volume of adjuvant inside the syringe. After creating a milky emulsion, the injection was performed according to Table I. The first booster injection was given 12 days after the first injection. The last injection was done without any adjuvants on day 25 of the immunization.

Results

Evaluation of the reactivity of the purified antigen by ELISA method

After antigen purification, an indirect ELISA method was used, as mentioned in the "methods" section. The OD_{405nm} = 0.56 ± 0.05 results showed the reactivity with Rhc antibody.

Purity evaluation of the antigen by SDS-PAGE

The purified antigen was electrophoresed on 8% polyacrylamide gel. After silver staining, two bands were observed. These bands are related to the Rhc and Rhe antigens in the size range of 30-35 kDa (Fig. 1A). The electrophoresis was carried out again after absorption of the Rhe antigen by passing the antigen through the anti-Rhe-immobilized sepharose-4B column. The remaining 35 kDa-band is related to the presence of Rhc antigen (Fig. 1B).

Evaluation of the specificity of the purified antigen using dot blot assay

The dot blot assay was performed to check the specificity of the purified antigen. The dark spots marked in Fig. 2 show the positive reaction points of ECL substrate with HRP-conjugated antibody. Observing these dots confirms the existence and antibody reactivity of Rhc or Rhe in the purified antigen (Fig. 3). After removing the Rhe antigen, dot blotting was performed again to confirm the presence of Rhc antigen alone.

Serum antibody titration of the Rhc-immunized mice

Serum titration was done using the ELISA method using purified Rhc antigen-coated plates. According to Table II, mice produced good titers of antibodies against the antigen after receiving the third dose of antigen (Table II).

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Table I. The injection times and days, the immunogen quantity, the type of adjuvant, and the route of antigen administration for the immunization of Balb/c mice are presented.

Injections Time	The First	The Second	The third
The injection day	1	12	**25**
Immunogen quantity	20 µg (200 µL)	15 µg (150 µL)	**15 µg (150 µL)**
Type of adjuvant*	Complete (CFA)	Incomplete (IFA)	--------
The route of antigen administration	Subcutaneous	Subcutaneous	**Intravenous Tail Vein Injections**

*Complete (CFA) and incomplete (IFA) Freund's adjuvants were used. CFA contained oil emulsion and inactivated mycobacteria. IFA had the same oil emulsion without the mycobacteria.

Table II. Results of anti-Rhc-sera titration of the immunized mice using the antigen-coated ELISA plates (N=2).

Mice Sera Dilution factor	OD_{450nm}	
	Serum 1	Serum 2
$\frac{1}{100}$	2.47 ± 0.19	2.19 ±0.06
$\frac{1}{500}$	2.62 ± 0.02	1.92 ± 0.11
$\frac{1}{2000}$	2.11 ± 0.13	1.36 ± 0.15
$\frac{1}{4000}$	1.67 ± 0.25	0.86 ± 0.08
$\frac{1}{10000}$	0.660±0.28	0.463±0.31
Negative control	0.09 ± 0.01	0.22 ± 0.01

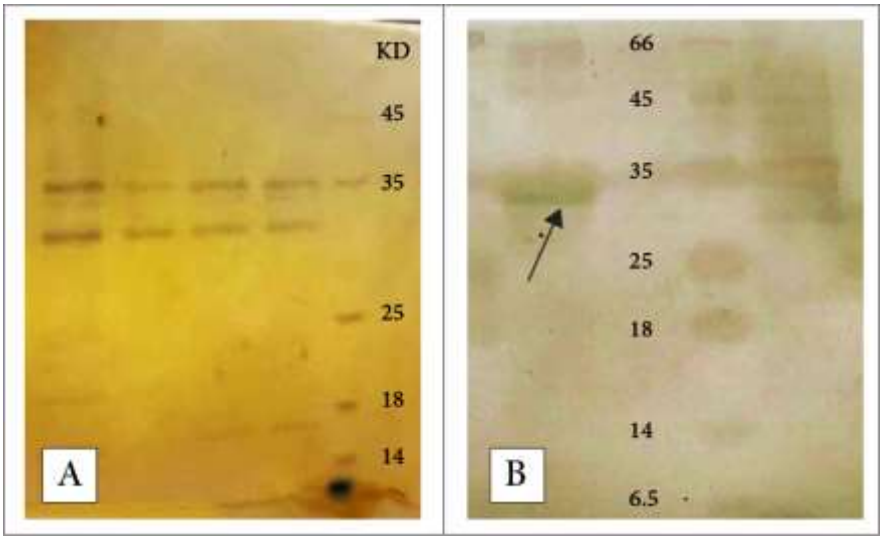

Figure 1. SDS-PAGE of the purified antigen on 8% polyacrylamide gel: A) Electrophoresis before Rhe antigen removal and B) Electrophoresis after Rhe antigen removal. After absorption, only one 35 kDa- band was observed (arrow). The range for unstained protein size marker was 6.5 to 116 kDa.

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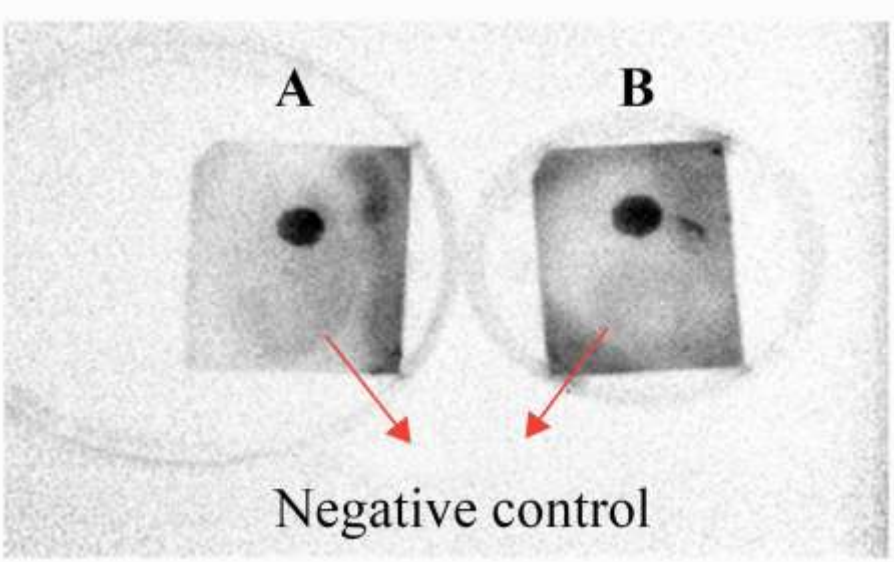

Figure 2. Dot blot test for Rhc and e antigens. Points A and B are related to the reaction of the purified antigen with anti-e and anti-c, respectively.

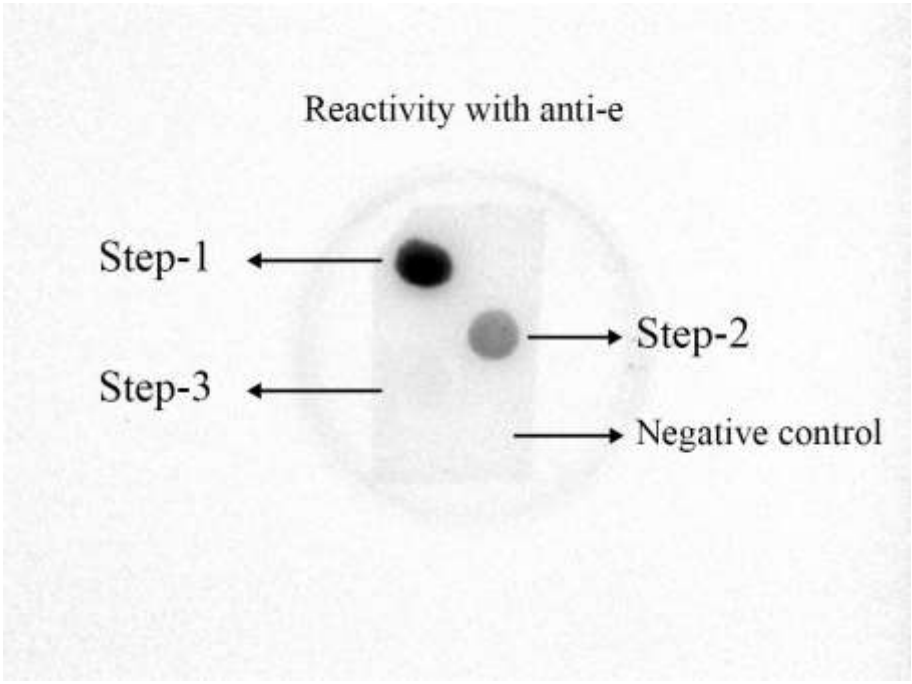

Figure 3. Dot blotting after the Rhe antigen removal. The Rhe antigen removal was performed in three steps.

Discussion

This study aimed to isolate the Rhc antigen from the cellular membrane of the circulating red blood cells. SDS-PAGE and dot blotting were used to examine the characterization of the purified antigen. The purified antigen was able to react with anti-Rhc. The purified antigen could immunize the mice with a good antibody titer.

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Isolation of cell membrane proteins is necessary to investigate their structure, antigenicity, and other biological activities (13). To this end, suitable detergents have been introduced to preserve the structure and antigenicity of membrane proteins (13, 14). The membrane of RBCs has proteins, lipids, and other macromolecules that interact with each other. Maintaining the structure and function of the blood group antigens during their separation from the surface of the RBC membrane is of specific importance (15, 16).

In this study, the solubilization of Rh antigens from the RBC membrane was performed using the non-ionic detergent NP-40. Rh antigens were obtained from the RBC membrane (ghost). The RhCcEe polyclonal antibody was used as an affinity ligand for antigen purification. Notably, the anti-Rhc antibody was not available at the time of study achievement, except in the form of diluted antibody for blood group typing. Therefore, at the first step, both Rhc and Rhe antigens were obtained from the membrane of (c+, e+, E-, C-) packed RBCs. The Rhc antigen was purified by passing the Rhc/e antigen through another chromatography column containing anti-Rhe as an affinity ligand to remove the Rhe antigen. The results of this study showed that the removal of the Rhe antigen was done while maintaining the specificity of the Rhc antigen. An interesting and important point is that after purification, the reactivity of both Rhc and Rhe antigens was preserved with the corresponding antibodies. This study showed that the isolation of membrane antigens using NP-40 can keep its immunogenicity. As a result, it can react with specific antibodies in immunoassays such as ELISA and dot blot.

Additionally, in this study, the purified antigen was used to immunize mice. The antisera showed good titters and could agglutinate Rhc+ cells from triplicate RBC panel cells (data not shown).

The results of this study suggest that the purified antigens preserve their structure to a good extent during the isolation process. In line with this finding, Ji showed that NP-40 was the best detergent to minimize the dysfunction of the purified proteins using lysis of cultured cells for immunoprecipitation (8). Previous studies mainly used Triton X-100 and Tween 20 as other non-ionic detergents to separate proteins from the RBC membrane (9, 17, 18).

However, solubilization of the membrane antigens using detergent-based methods may involve a series of challenges. Evidence shows that Tween20 and Triton X-100 detergents have a series of polyoxyethylene derivatives that cause false positive results in protein assays, such as the Bradford assay (18). Hence, it seems that NP-40 has an advantage over other non-ionic detergents because it does not have this problem. Among other problems related to using non-ionic detergents, it can be mentioned that NP-40 and Triton X-100 have optical absorption at 280 nm and cause interference in the ultraviolet absorbance to evaluate the purity of proteins using chromatography (18). In this study, serum proteins were dialized sufficiently to remove NP-40 detergent efficiently.

After the solubilization and dialysis of the membrane proteins, it is necessary to use an appropriate method to purify the target protein from other components of the cell membrane. Many techniques have been investigated for the separation of proteins of interest. However, in the past, the separation of RBC membrane components was typically done using lectin-based chromatography (18). Although the immunoprecipitation method has been previously used for the isolation of RhD antigen after using Triton X-100 as a non-ionic detergent (19, 20), no scientific report was found for the isolation of Rhc

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antigen by an immunoaffinity method like this study.

The present study used a polyclonal antibody as a ligand for affinity chromatography for Rh antigen purification. The method was in line with the study of Dakour et al., who used monoclonal antibodies as ligands to isolate Lewis blood group antigens, Leb and Lea (21). The difference between their study and this study is in the type of the isolated antigen.

Overall, it can be stated that using antibody-based chromatography can be the most specific method to purify the desired protein. So far, we have not found any studies regarding the use of an antibody-based affinity chromatography method to separate Rh antigens from the RBC membrane. This method can be effective concerning the optimal purification of membrane antigens of RBC.

Conclusion

Using the ghost method by applying the non-ionic detergent (NP-40) can preserve the reactivity of the purified antigen with a specific antibody. Affinity chromatography may be an efficient method for separating Rh antigens from the RBC membrane. Furthermore, the purified antigen may be used to produce antibodies or to investigate the protein structure.

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Authors' contributions

F.Y. designed and supervised the study. H.R. did the research and wrote the manuscript. S.M. was the consultant for the project.

Ethical approval

All the procedures performed in this study were in accordance with the ethical standards of the local ethics committee of the Iranian Blood Transfusion Organization (IR.TMI.REC.1400.010). Written informed consent was obtained from the healthy blood donors.

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Conflict of interests

There is no conflict of interests.

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