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Journal of Chromatography B, 735 (1999) 207–217

JOURNAL OF CHROMATOGRAPHY B

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Immunopurification of the blood group RhD protein from human erythrocyte membranes

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Received 31 May 1999; received in revised form 2 September 1999; accepted 16 September 1999

Abstract

Rh proteins are membrane proteins encoded by genes at the blood group RH locus. They are of paramount importance in transfusion medicine, but their function is still unknown. Biochemical and biophysical studies of these proteins are scarce since only minute amounts of the very hydrophobic Rh proteins, can be purified from human erythrocytes. Recently, a human monoclonal antibody (LOR-15C9) was described as having the unique property to recognize the Rh30 protein carrying the major blood group D specificity (RhD protein), either in a membrane detergent extract or when blotted on a membrane. In this report, we describe one-step purification of the RhD protein from detergent extracts of red cell membranes, based on immunoaffinity chromatography carried out with immobilized LOR-15C9 IgG. The technique yielded RhD protein with high purity which was devoid of other associated proteins (RhAG, CD47, LW and GPB) that comprise the Rh complex in the erythrocyte membrane. By contrast immunoprecipitation performed with the same antibody led to co-isolation of both RhD and RhAG.

Keywords: Blood group proteins; Detergents; Red cell membrane proteins; RhD

1. Introduction

The Rh blood group antigens are carried by a family of nonglycosylated hydrophobic transmembrane proteins of molecular mass (M_r) 30 000 apparent molecular mass which are missing from the red cells of rare Rh_{null} individuals. The Rh proteins (D and Cc/Ee, respectively) are encoded by two homologous genes *RHD* and *RHCE* (with 96% sequence identity) organized in tandem on chromosome 1p34-p36 which most likely derived by duplication of a common ancestral gene. The D and non-D proteins (417 amino acids) differ by 35 substitutions and exhibit a similar membrane topology with short hydrophilic loops connecting the twelve putative transmembrane helices (reviews in Refs. [1–3]).

Analysis of many common and rare variants allowed to understand the molecular basis of the polymorphisms of the *RH* genes and Rh proteins (reviews in Refs. [4–6]). The presence or the absence of the *RHD* gene and of its product determined

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00424-7

the basis of the Rh-positive and Rh-negative phenotypes, respectively, whereas amino acid polymorphisms at positions 103 and 226 of the *RHCE* gene product determined the molecular basis for the C/c (Ser→Pro) and E/e (Pro→Ala) specificities [7]. Other polymorphisms occur mostly by gene conversion between the two *RH* genes and occasionally by single point mutations.

Rh_{null} individuals lack all Rh antigens and suffer a clinical syndrome of varying severity. Their red cells are characterized by morphological and some biochemical abnormalities. Cells present abnormal permeability to cations and a defect of the membrane phospholipid asymmetry. Moreover several other membrane glycosylated proteins (Rh associated glycoprotein of M_r 50 000 apparent molecular mass, called thereafter RhAG, CD47, LW, glycophorin B called thereafter GPB) are absent or decreased on Rh_{null} cells. These findings suggest that these glycoproteins are assembled to Rh proteins (probably both D and non-D) by non covalent linkages to form a complex. The complex is thought to contain in associated state both D and non-D Rh proteins. When RhAG or Rh proteins are missing through a genetic defect, all other members of the putative complex are present in a severely lowered amount in the erythrocyte membrane (reviews in Refs. [4–6,8]).

Biochemical and biophysical studies of purified Rh proteins, reconstitution experiments in liposomes, all techniques which might give insight into their function(s), were severely hampered by the fact that only minute amounts of the very hydrophobic Rh proteins can be purified [9–11]. Particularly, a large-scale immunopurification procedure could not be developed since all human monoclonals against the Rh antigens reacted with the native antigens but were unreactive with Rh proteins in a membrane detergent extract or blotted on a membrane. This suggested that the Rh antigens are conformation-dependent and that antigen three-dimensional structures are lost after detergent extraction. Recently, however, a unique human monoclonal anti-RhD called LOR-15C9 that reacted with a conformation-independent epitope on the RhD protein blotted on membrane filters has been described [12]. This antibody was used to devise an easy high yield purification procedure of the RhD protein by immunoaffinity chromatography as detailed in this report.

2. Experimental

2.1. Chemicals and blood samples

Detergents and other chemicals were bought from Sigma (St. Louis, MO, USA), Calbiochem (La Jolla, CA, USA) or Carlo Erba (Milan, Italy). Blood of the ccDEE phenotype was obtained from Etablissement de Transfusion Sanguine Sud-Est Francilien (Rungis, France).

2.2. Purification of human monoclonal antibody and immobilization on N-hydroxy succinimide activated-Superose

LOR-15C9 was purified on protein A agarose according to established methods [13]. In short, NaCl was added to a 3 *M* final concentration to the clarified culture supernatant and 1500 ml was pumped at 80 ml/h to a column (15×2.5 cm I.D.) packed with protein A Sepharose (Pharmacia, Uppsala, Sweden), equilibrated in 0.5 *M* glycine buffer, pH 8.9 containing 3 *M* NaCl. After sample loading, the column was rinsed with equilibration buffer and then with 0.1 *M* sodium citrate, pH 5.0 which eluted a minimum amount of material. The retained antibody was eluted with 0.1 *M* citrate, pH 2.8 buffer and the collected fractions were quickly readjusted to pH 7.4 with 1.2 *M* Tris base solution. The immunopurified antibody was concentrated to about 3 mg/ml and diafiltered using a PM30 Amicon membrane fitted in a pressure cell. Buffer used for diafiltration was 0.2 *M* sodium carbonate, pH 8.3 containing 0.5 *M* NaCl (coupling buffer). Immobilization on a *N*-hydroxysuccinimide-activated 5-ml Hitrap column was performed adhering to manufacturer's (Pharmacia) recommendations. A 15-ml volume of purified IgG was used for coupling.

2.3. Evaluation of extraction conditions for Rh proteins solubilisation

Red cell membranes were prepared from phenotyped red cells by hypotonic lysis and extensive washings performed in a hollow fiber cartridge filtration system [14]. Red cells of the ccDEE phenotype known to contain a higher amount of RhD protein than other common erythrocytes were used

preferentially. White membranes were stored frozen in 5 m*M* sodium phosphate, pH 8.0 containing 0.5 m*M* phenylmethylsulfonylfluoride (PMSF). To evaluate the efficiency of different detergents and detergent mixtures to extract the Rh proteins, ghosts were washed three times in phosphate-buffered saline (PBS) (5 m*M* sodium phosphate, pH 7.6 containing 150 m*M* NaCl and 0.5 m*M* PMSF) and the washed pellets were suspended in nine volumes of a detergent containing extraction buffer. The extraction buffer was made of PBS containing a cocktail of protease inhibitors (final concentration, 0.5 m*M* PMSF, 1 m*M* pefablock, 0.04 m*M* leupeptin, 0.03 m*M* pepstatin, 0.01 m*M* E64, 0.02 m*M* chymostatin) and detergents at final concentrations given below in the results section (all detergent concentrations were well above the critical micellar concentration [15,16]). The suspension was shaken for 1 h at 4°C and centrifuged at 19 600 *g* for 30 min. The supernatants were collected and pellets taken in 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 6.8 buffer containing 5% sodium dodecyl sulfate (SDS) (when no pellet was seen, the centrifuged tube was treated likewise in order not to be misled by a translucent, poorly visible, pellet). Aliquots of supernatant and redissolved pellet were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting.

2.4. Preparative affinity chromatography of the RhD protein

All chromatographic steps were performed at 4°C. A 5-ml column of immobilized LOR-15C9 was equilibrated in 5 m*M* sodium phosphate, pH 7.6 containing 150 m*M* lauryldimethyl betaine (Empigen BB) at 40 ml/h flow-rate (Empigen BB is available as a 1.1 *M* solution in 1.2 *M* NaCl, hence final NaCl concentration brought by dilution of Empigen was 163 m*M*). Extract was prepared as above with Empigen so that buffer composition matched column equilibration buffer. Loaded volume corresponded to the extract obtained from about 17 ml of packed red cells. The column was then rinsed with the equilibration buffer for 60 min and for a further 60 min with intermediate buffer, i.e., PBS containing 1.6 m*M* (0.1%, w/v) Triton X-100 or other detergent as indicated in Results. The column was thereafter developed with the elution buffer, either 0.1 *M* glycine, pH 2.8 or 0.1 *M* sodium citrate, pH 2.8 without detergent or 0.1 *M* sodium citrate, pH 2.8 containing same detergent as intermediate buffer. Acidic glycine buffer eluted fractions were immediately adjusted to pH 6.5 with a pH meter, using 1.2 *M* Tris base. Concentrated Na_2HPO_4 was added to the sodium citrate eluted fractions so as to get 50 m*M* final concentration and pH was readjusted to 6.5 with 10 *M* NaOH.

2.5. Immunoprecipitation experiments

RhD-positive and RhD-negative erythrocytes were washed in PBS and 1 ml of packed red cells were suspended in 10 ml of culture supernatant containing 10% fetal bovine serum and 35 μg/ml LOR-15C9. The suspension was incubated overnight at 4°C with gentle shaking. The red cells were then washed twice with PBS and the membrane ghosts were prepared by hypotonic lysis in 5 m*M* sodium phosphate, pH 8 buffer (5P8) containing the inhibitors cocktail described above. The membranes were washed in 5P8 containing 2 m*M* PMSF until white and a detergent extract was prepared as above using 2% Triton X-100 final concentration. The detergent extracts were then incubated with protein A Sepharose (100 μl gel for 400 μl extract) at 4°C under gentle stirring overnight and the gel suspension was loaded into a centrifugal separator (Micropure device, Amicon, Danvers, MA, USA) fitted with a 0.45 μm membrane. This allowed one to easily wash the protein A agarose pellet by either four or eight aliquots of 200 μl PBS containing 2% Triton X-100. The immune complexes were finally eluted by incubating the gel for 90 min at room temperature in 100 μl 5% SDS, 10 m*M* Tris, 1 m*M* EDTA, pH 6.8 buffer and eluate was recovered by centrifugation.

2.6. Gel electrophoresis and Western blotting procedures

SDS–PAGE was performed using a Novex apparatus (San Francisco, CA, USA) on 10% (w/v) acrylamide Tris–glycine gel [17]. Unstained molecular mass standards were from New England Biolabs. (Beverly, MA, USA), while prestained molecular mass standards used for blotting were from the same

supplier and from Novex. Samples were loaded after β-mercaptoethanol reduction except those used to analyze immunoprecipitation experiments which were run without reduction. Silver staining of gels was performed using a recipe derived from [18]. Western blot analysis on nitrocellulose was performed with the following antibodies: (i) LOR-15C9, (ii) MPC8, a rabbit antiserum which was obtained by immunization against a synthetic peptide spanning the 408–416 sequence of the Rh protein [19], and which recognizes all Rh proteins (RhD and RhCcEe) from all human red cells, (iii) a murine anti-band 3 monoclonal antibody purchased from Sigma (clone BIII-136), (iv) a murine anti-Kell protein monoclonal antibody (clone 5A11, [20]), (v) a murine anti-CD47 monoclonal antibody (gift from Dominique Blanchard, ETS, Nantes, France, clone F463E12), (vi) a murine anti-glycophorins A and B monoclonal antibody (clone 3F4, [21]), (vii), a murine anti-LW monoclonal antibody (gift from Dr. H.H. Sonneborn, Biotest, Dreiech, Germany, clone BS56) and (viii) a murine anti-RhAG monoclonal antibody (clone 2D10 [22]).

Conditions for immunoblotting of LOR-15C9 to nitrocellulose membranes have been described elsewhere [12], whereas other primary antibodies were used as in [23]. Secondary antibodies were as required, anti-human, anti-rabbit or anti-mouse IgG peroxidase-tagged antibodies (Biosys, Compiègne, France). Immunoblots were finally stained with the ECL chemiluminescent system from Amersham (Little Chalfont, UK) and exposed to X-ray film (Biomax MR Kodak, Rochester, NY, USA).

2.7. Protein assay

Protein assays were performed using 3-(4-carboxybenzoyl)quinoline-2-carboxyaldehyde (CBQCA) purchased from Molecular Probes (Eugene, OR, USA) by mixing unknowns, 0.1 *M* borate buffer, pH 9.3 and reagents, then incubating reaction mixtures for 90 min at room temperature in the dark [24]. The calibration curve with points ranging from 10 to 1500 ng per assay was made with bovine serum albumin (BSA) dissolved in buffer used for elution readjusted to pH 6.5 as was done for collected fractions. Obviously, since assay is based on reaction with primary amines, it was performed only with citrate and not glycine based elution buffers. The fluorescence of reacted samples was evaluated by injecting aliquots into a steady stream of 0.1 *M* sodium borate buffer, pH 9.3 flowing through the continuous flow cuvette of a Shimadzu S 356 fluorometer with wavelengths for excitation and emission set at, respectively, 460 and 560 nm.

2.8. Ultracentrifugation

Ultracentrifugation was performed in a Beckman (Fullerton, CA, USA) airfuge at 140 000 *g* for 45 min. The supernatants were collected and pellets taken in 10 m*M* Tris, pH 6.8 buffer containing 5% SDS (when no pellet was seen, the centrifuged tube was treated likewise in order not to be misled by a translucent, poorly visible, pellet), both supernatant and redissolved pellet were analyzed by SDS–PAGE.

3. Results

3.1. Evaluation of extraction conditions for Rh proteins solubilisation

Fig. 1 shows that 80 m*M* Triton X-100 or, 16 m*M* Triton X-100 plus 4.8 m*M* deoxycholate (DOC) do not lead to the complete extraction of Rh proteins from the red cell membrane: the pellet still contained significant amounts of Rh proteins as demonstrated by western blots probed with MPC8 (bottom, right panel, lanes 3 and 4). Positions of colored molecular mass standards which were used to evaluate apparent molecular masses were not reported on autoradiographs presented in the figures. MPC8 revealed Rh proteins as bands with apparent M_r 30 000. MPC8 which is a rabbit antibody does also recognize bands which are not Rh related since there are also recognized in membranes of Rh null patients, (not shown) these unidentified high molecular mass bands are marked by stars right to the blots on Fig. 1. A band which most probably corresponds to some dimer of RhD protein is indicated by the arrow marked di.

A large proportion of Rh proteins was also found in pellets obtained after extraction with 150 m*M* octyl glucoside and 150 m*M* dodecyl maltoside (bottom lanes 7 and 8).

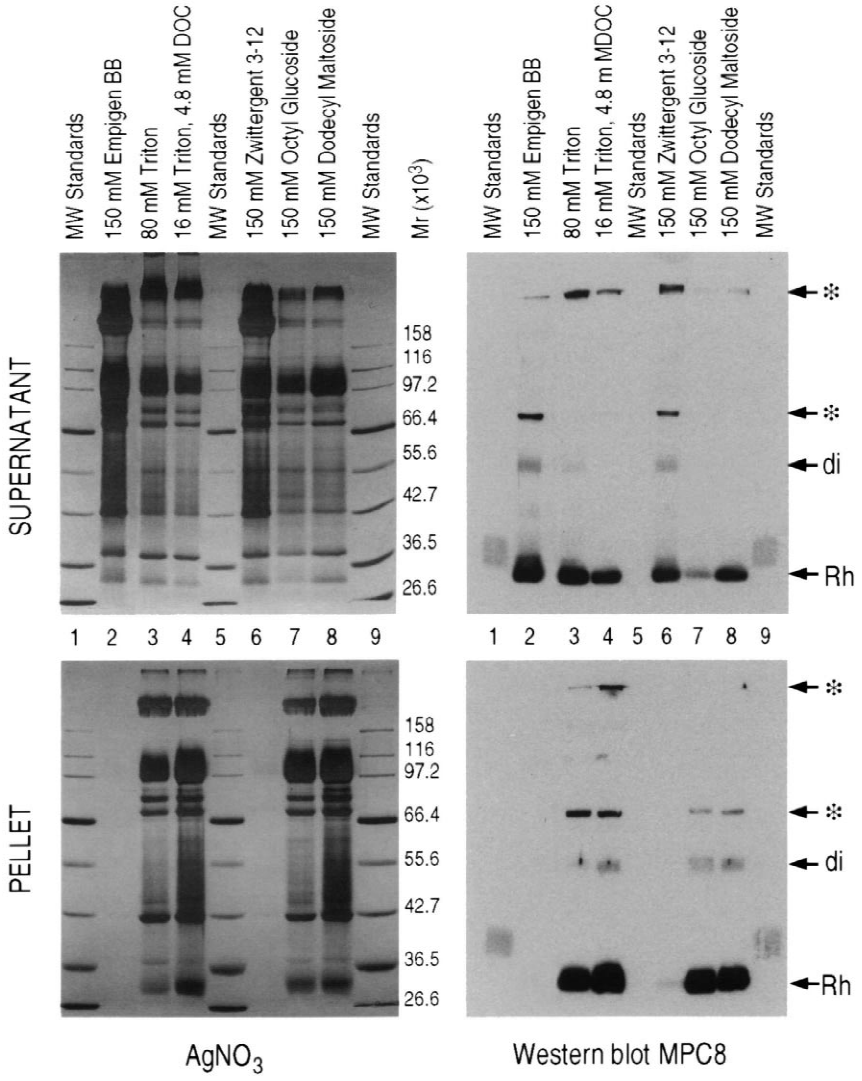

Fig. 1. Rh proteins extraction efficiency of different detergents. White erythrocyte membranes were washed three times in PBS and the washed pellets were suspended in nine volumes of extraction buffer (PBS containing a protease inhibitors cocktail and detergents to give final concentrations indicated at the top of the picture). The suspensions were then shaken for 1 h at 4°C and centrifuged. The supernatants were collected and the pellets resuspended in 10 m*M* Tris, 1 m*M* EDTA buffer, pH 6.8 containing 5% SDS. Aliquots of each supernatant and redissolved pellet were analyzed by SDS–PAGE performed in 10% acrylamide gels. The gels were either stained with silver nitrate (left panels) or immunostained by Western blotting with the MPC8 anti-Rh 30 antibody (autoradiographs used for revelation of blots by the ECL chemioluminescent reagent are shown on the right panels). Lanes 1, 5 and 9 were loaded with molecular mass markers (M_r of the standard proteins loaded onto the silver nitrate dyed gel are indicated). The position of the Rh protein is indicated on the right of the blot as well as position of its dimer (arrow di). Stars indicates bands which are not Rh related (see text for details).

Conversely, only a small amount of Rh protein was recovered in the pellet corresponding to 150 m*M* Zwittergent 3-12 extracted membranes (bottom, right panel, lane 6) and interestingly, 150 m*M* Empigen dissolved completely Rh protein (bottom, right panel, lane 2).

Staining with silver nitrate of the various fractions showed that solubilization of Rh protein paralleled

solubilization of whole proteins from ghosts and that only pellets derived from extraction mixtures containing 150 m*M* Empigen were free of un-dissolved proteins (Fig. 1, left panel).

3.2. Preparative chromatography of RhD protein

SDS–PAGE analysis of fractions from chromatography on immobilized LOR-15C9 is shown in Fig. 2 (no bands were seen on SDS gels of fractions collected during development of column with intermediate buffer, not shown).

Aliquots of breakthrough peak (lanes 3–6) and of column wash through equilibration buffer did not contain RhD proteins as demonstrated by western blot with the LOR-15 C9 antibody (bottom, left panel). However as expected, RhCcEe proteins were present in these fractions as demonstrated by western blot analysis with the MPC8 antiserum directed against Rh30 proteins (D+CcEe) (middle panel).

The purified RhD protein was recovered in fractions eluted by acidic elution buffer (lanes 8–11). Indeed, these fractions contained a band of M_r 30 000 apparent molecular mass which is detected by silver nitrate staining (upper panel, lanes 9 and 10) is reactive with LOR-15C9 antibody (bottom left panel) and with MPC8 rabbit anti Rh proteins antiserum (middle panel). The M_r 60 000 band revealed with MPC8 (arrow di) represents obviously aggregated material (dimer). On blots treated with LOR-15 C9, bands at ca. 95 000 apparent molecular mass were also revealed in acid eluted fraction as well as faint 75 000 and 70 000 bands (arrow X). These bands which are also discernible on the silver nitrate stained gel are clearly IgG derived since they might be seen when blot was incubated with the second antibody only, i.e., peroxydase tagged anti human IgG raised in goat, (Fig. 2 bottom, right panel marked control). Nature of these bands was further confirmed using a two stage western blot revelation (anti human IgG raised in rabbits followed by peroxidase tagged anti rabbit IgG raised in goat, not shown). The same technique also identified as IgG related the bands with M_r 95 000 apparent molecular mass which were revealed on LOR-15C9 probed membrane with the starting material in loaded extract (Fig. 2, SM, lane 2) and breakthrough peak (lane 3).

The acidic buffer eluted fractions were examined with other antibodies reactive with band 3 (M_r 90 000) and Kell protein (M_r 92 000). Only minute reactivity with anti band 3 antibodies was occasionally observed (not shown) and no reactivity with anti Kell protein antibody was ever detected. Furthermore antibodies to the RhAG did not detect any RhAG in the immunopurified Rh D protein fractions (Fig. 4, lower panel, lane 1). Antibodies to CD 47, LW and GPB detected these proteins only in aliquots of breakthrough and wash and not in acid eluted fractions (not shown).

The same results with regard to SDS–PAGE of eluted fractions were consistently obtained with the different protocols used for developing the column. Protocols differed by the (i) nature of eluting buffer (citrate or glycine buffer), (ii) nature of added detergent to intermediate buffer i.e., 1.6 m*M* Triton X-100, or 2 m*M* docecylmaltoside, or 1.7 m*M* $C_{12}E_{9,}$ or 3.7 m*M* Empigen BB, or 4.4 m*M* lauryldimethylamine oxide (LDAO), or 41 m*M* octyl glucoside (all these concentrations are greater than the critical micelle concentration [15,16]), (iii) addition to the eluting buffer of the detergent present in intermediate buffer or absence of detergent in eluting buffer.

3.3. Ultracentrifugation of purified Rh protein

Ultracentrifugation experiments established that while all protocols ended with a perfectly transparent solution of RhD protein, some of them clearly yielded aggregated material sedimentable in the ultracentrifuge in the conditions given above. Fig. 3 shows that when Triton X-100 was used in elution buffer of the immunoaffinity column a significant proportion of purified RhD protein was recovered in pellet after centrifugation. Similar results were obtained with either, LDAO, octylglucoside, dodecylmaltoside or no detergent addition (not shown). Only $C_{12}E_9$ and Empigen BB allowed to recover RhD as soluble unaggregated protein which was not sedimented in the ultracentrifuge in the given conditions (Fig. 3). Faint band present in pellet of Empigen BB sample is likely to correspond to some IgG derived material leached from the column (see above).

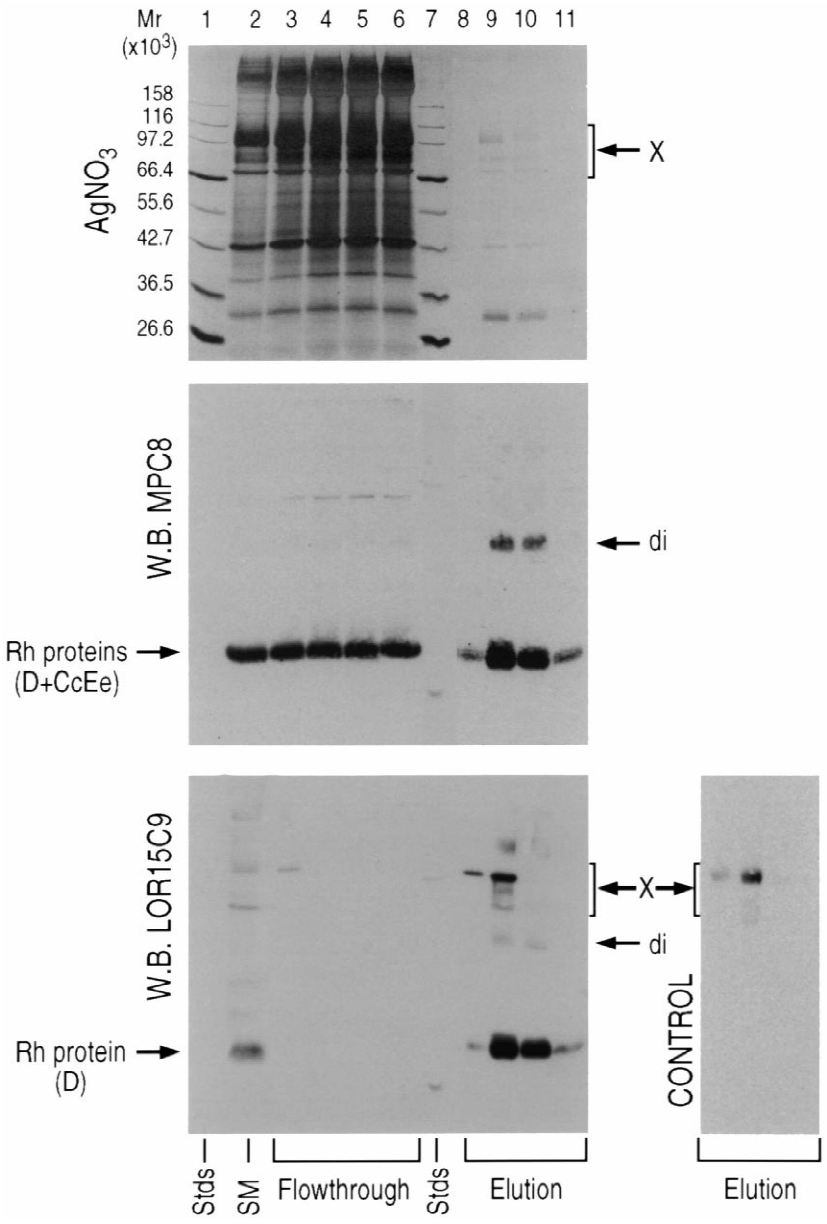

Fig. 2. Preparative affinity chromatography of RhD protein on immobilized LOR-15C9 antibody. An Empigen extract of membranes prepared from ccDEE red cells was chromatographed on the immobilized LOR-15C9 column as indicated in Methods. The chromatographic fractions were analyzed by SDS–PAGE performed in 10% acrylamide gels and Western blotting. Aliquots of starting material were loaded in lanes marked SM. Other lanes contained as indicated aliquots of breakthrough peak, of 0.1 *M* glycine, pH 2.8 buffer eluted fractions or molecular mass standards (Std; M_r of the standard proteins are indicated left to lane 1). The gels were stained with silver nitrate (top) and the nitrocellulose membranes were probed with MPC8, an anti-Rh proteins antiserum (detecting the D and CcEe proteins) (middle) and LOR-15C9, a human monoclonal antibody detecting only the D protein of apparent M_r 30 000 (bottom). The blot marked control (bottom right) was probed with a secondary anti-human IgG antiserum only. The position of the Rh protein is indicated on the right of the blot as well as position of its dimer (arrow di). The bands marked X correspond to IgG derived material (see text for details).

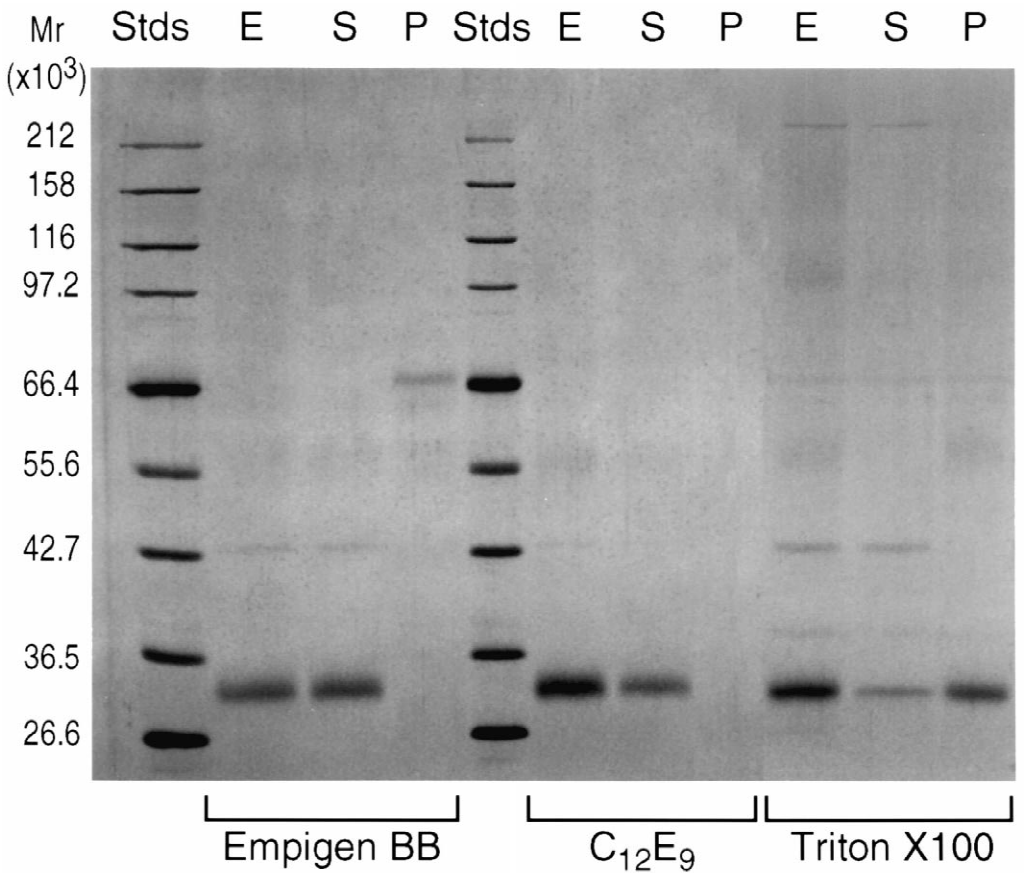

Fig. 3. High-speed centrifugation of purified RhD protein. Aliquots of purified RhD protein were centrifuged in the Airfuge apparatus at 140 000 *g* for 45 min. Detergent added to 0.1 *M* citrate, pH 2.8 eluting buffer is indicated on the bottom of the figure. Lanes marked E were loaded with purified protein eluted from the column before centrifugation, S correspond to aliquots of supernates and P to aliquots of redissolved pellets.

3.4. Immunoprecipitation with LOR-15C9 antibody

LOR-15C9 immunoprecipitated the RhD protein of M_r 30 000 from RhD-positive cells (D+) as proven by Western blot analysis of the immunoprecipitates with the MPC8 antiserum: MPC8 revealed bands with M_r 30 000 and 60 000 apparent molecular masses (Fig. 4, top left, lanes 3 to 5). No MPC8 reactive band was observed when the starting material was RhD-negative cells (D−) (lanes 6 to 8). The RhAG was seen to coprecipitate with the RhD protein, as demonstrated by probing the nitrocellulose membrane with the 2D10 monoclonal antibody (Fig. 4, bottom left, lanes 3 to 5). The signal detected by 2D10 diminished only slightly when protein A-agarose pellet was washed twice (lane 3), four times (lane 4) or eight times (lane 5). Immunoprecipitates from RhD positive cells (run on the gel without prior reduction) contain high-molecular-mass bands marked by stars. These bands reveal the presence on the blot of the LOR-15C9 antibody used for immunoprecipitation. The antirabbit and antimouse secondary antibodies we used clearly cross react with human IgG as demonstrated in control experiments, moreover a control membrane obtained as those of Fig. 4 when incubated with peroxidase tagged anti human IgG antiserum developed a strongly positive signal at this same position (not shown).

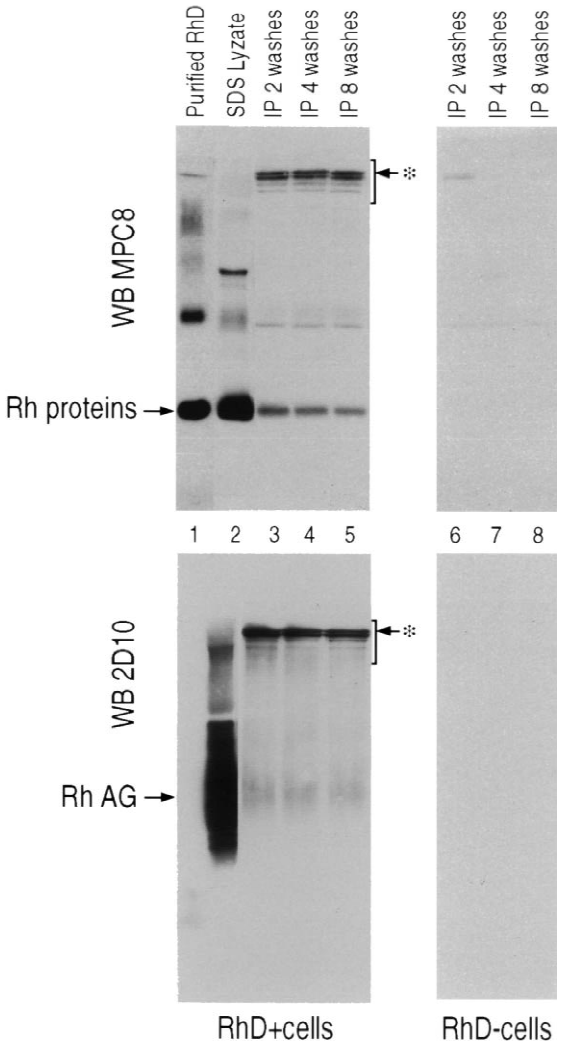

Fig. 4. Immunoprecipitation with LOR-15C9 antibody. Immunoprecipitates from RhD positive (D+, lanes 3 to 5) and RhD negative cells (D−, lanes 6 to 8) were recovered on immobilized protein A as described in Methods and analyzed by SDS–PAGE and Western blotting. Unreduced samples were loaded onto the gels. The nitrocellulose membranes were probed with the MPC8 rabbit antiserum (directed against Rh proteins of M_r 30 000 apparent molecular mass) and with the murine monoclonal antibody 2D10 directed against the RhAG, as indicated. Lanes 1 were loaded with aliquots of immunopurified RhD protein. Lanes 2 were loaded with SDS lyzates of red cell membranes. Other lanes were loaded with Protein A eluates. Stars identify bands due to the presence on the blot of LOR-15C9 antibody used for immunoprecipitation; these bands were revealed because of cross reactivity of the tagged secondary antibodies with human IgG. The number of washes performed before immunoprecipitates elution from protein A aliquots are indicated on top of the gels.

4. Discussion

The results of this study indicate that among the reagents which were tested, Empigen is the best detergent for Rh proteins solubilization. This detergent has already been claimed to be an efficient solubilizer for membrane proteins and to have no or minimal effect on enzymatic [24] or antigenic [25] properties of solubilized proteins. Immunochromatography experiments confirmed, as expected, that Empigen did not interfere with RhD protein recognition by the human monoclonal antibody LOR-15C9.

Immunochromatography is an easy way to obtain the membrane RhD protein in a highly purified form. The silver nitrate stained protein band of M_r 30 000 was identified as the RhD protein by immunoblot analysis with polyclonal antibodies directed against Rh proteins (D+CcEe) and the monoclonal antibody LOR-15C9 which is only reactive with the RhD protein [12]. The RhD protein is clearly the major component in acid eluted fractions even if other faint protein bands with higher molecular mass are detectable (Fig. 2 upper panel). Identified impurities were essentially IgG derived material, which may have leached from the immunoaffinity column. Bands with IgG reactivity identified in RhD containing fractions do not show the apparent molecular masses of heavy and light chain but IgG may be only partially dissociated on SDS gels when samples are not boiled [26]. IgG material present in Empigen extracts of ghosts probably originate from IgG adsorbed on red cells which were detected by the sensitive chemiluminescence technique but should stay undetected by Coombs techniques.

No difference regarding the band pattern on SDS–PAGE of acidic buffer eluted fractions or band intensities was noted when the column was developed with either 0.1 *M* glycine or 0.1 *M* sodium citrate, pH 2.8 buffers. Only this latter buffer allowed to assay protein with the sensitive CBQCA fluorogenic reagent which reacts with primary amines. The amount of recovered RhD protein in 0.1*M* sodium citrate eluted fractions was 4.1 to 28.5 μg/ml of ccDEE red cells (mean 12.3). We are well aware that choice of protein standard for protein assays may well have an influence on absolute figures obtained for assays (bovine serum albumin

contains 59 lysine residues and RhD only 14 for a M_r 45 000 theoretical molecular mass). Differences in amounts recovered after the purification runs most likely reflects a quantitative variation of RhD protein in membranes of ccDEE individuals, this is suggested by serological evaluations of RhD antigen reactivities in different Rh phenotypes [27]. No effort has been made to correlate RhD antigen reactivity on red cells used for one given purification experiment and the actual amount recovered after immunochromatography on immobilized LOR-15C9.

However the amount of purified RhD protein recovered through one single chromatography step on a 5-ml column is such that to accumulate several milligrams of this protein seems to be feasible. In this regard, the lifetime of the immobilized LOR-15C9 column might be rather long since more than 50 preparative chromatographies have been performed using the described protocol without obvious deterioration of its performance.

It is worth noticing that immunochromatography afforded an easy way to exchange detergent hence it allowed us to quickly identify by the simple ultracentrifugation experiment described above the detergent useful to recover RhD protein as a soluble and non-aggregated protein. Indeed, that detergents are not interchangeable for use with membrane proteins is an established notion [28,29] but systematic studies performed with other proteins are of little help, e.g., dodecylmaltoside was shown optimal to stabilize sarcoplasmic reticulum Ca^{++} ATPase [28] while it is clearly inadequate for RhD protein. Among the various detergents tested, Empigen BB and $C_{12}E_9$ seems to be the detergents of choice able to maintain protein in non aggregated state.

Surprisingly, neither RhAG, which is thought to compose with the Rh30 proteins themselves the core of the Rh membrane complex, nor the accessory chains like CD47, LW and GPB were present in sufficient amounts in pools of purified RhD protein to be detected by Western blotting with specific antibodies and chemiluminescent detection. These results suggest that noncovalent associations between the postulated components of the Rh membrane complex do not survive to the conditions used here for extraction and immunoaffinity chromatography. This contrasts with published results of immunoprecipitation experiments [30–33] which support the Rh complex model, since at least both Rh proteins and RhAG were immunoprecipitated by anti-Rh proteins antibodies. In addition, anti-LW [34] and anti-RhAG antibodies [22,35] precipitate Rh proteins.

The Rh complex hypothesis is supported also by the study of Rh_{null} patients. For instance, (i) RhAG, CD47, LW and GPB are absent or severely reduced in Rh_{null} red cells which lack Rh antigens and Rh proteins (for review see Ref. [1]), (ii) the most frequent cause of Rh_{null} syndrome is not due to the defect of genes coding for Rh proteins (D or CcEe) but to a defect of the gene coding for RhAG [36].

Considering the Rh complex hypothesis one may wonder if our purified RhD protein might contain some non-D Rh protein (i.e., Rh protein coded by the RhCcEe gene): Rh complex in erythrocyte membrane is thought to contain in associated state both D and non D Rh protein but presence of non-D protein in our purified D could not be directly tested since no specific antibody able to specifically recognize non-D Rh proteins on blots was available. However this seems to be unlikely since no other partner of the Rh complex was found associated with RhD in column eluate, hence large amount of protein reactive with the MPC8 antiserum in flowthrough (Fig. 2) might well correspond to most if not all of non-D Rh proteins.

Some experiments were designed to explore why immunochromatography was unable to detect the postulated physical association between Rh protein and RhAG. This was clearly not related to a greater dissociation caused by Empigen (used for chromatography) compared to Triton X-100, (commonly used in immmunoprecipitation experiments). Indeed, when we used a Triton X-100 extract of ghosts as starting material for immunochromatography, the RhAG, CD47, LW and GPB were again recovered in the flowthrough and wash (not shown). Moreover, LOR-15C9 was proven to co-immunoprecipitate RhAG (see Fig. 4). Hence the binding of this antibody obviously does not interfere with the assembly of the Rh membrane complex. Examination of Fig. 4 also shows that the amount of RhAG immunoprecipitated was only slightly lower when the immunoprecipitate was washed more extensively, suggesting that the washing efficiency is not responsible for the discordant results between immuno-

chromatography and immunoprecipitation. Finally it may be assumed that antibody binding on its target prior to solubilization with the detergent (as is done in immunoprecipitation), in some way, protects the Rh complex from complete dissociation induced by detergent extraction.

To conclude even though, immunochromatography did not provide any insight into the nature and composition of the Rh membrane complex, it stands as an efficient preparative procedure of the RhD protein. Hence it is presently possible to consider reconstitution experiments in liposomes and even trials to generate two dimensional crystals, all experiments which might give insights into antigenicity, function and structure of the enigmatic Rh protein.

Acknowledgements

We gratefully acknowledge Dr. D. Blanchard and Dr. H.H. Sonneborn for their gifts of antibodies. A.B. and F.R are indebted to the Agence Française du Sang for financial support (contract No. 94 001 30 12 91).

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