Isolation and Purification of Rh₀(D) Antigen of Human Erythrocyte Membrane and Its Serological Property

TSUYOSHI YOKOI, MINEO IWASA and KAORU SAGISAKA

Department of Legal Medicine, Gifu University School of Medicine, Gifu 500

YOKOI, T., IWASA, M. and SAGISAKA, K. *Isolation and Purification of Rh₀(D) Antigen of Human Erythrocyte Membrane and Its Serological Property.* Tohoku J. exp. Med., 1983, **141** (2), 143-154 — To elucidate immunochemical properties of Rh-Hr antigens, influence of various solubilizers on the activity, and isolation of the antigens were investigated. As a very sensitive method to detect Rh-Hr antigens, the agglutination inhibition test was recommended in which diluted antisera (1:400-1:1,600), papainized red cells and microtiter plates were used. Extraction of D antigen with Brij 35 and Triton X-100 was effective, but SDS was not successful. However, D activity of SDS solubilized stroma was recovered by adding reductants. The activity was slightly demonstrated at the preparation from D negative red cells. The influence of the reductants was discussed. SDS solubilized stroma was fractionated with gel filtrations and activated thiol Sepharose 4B. The final preparation with D activity was proved to correspond with Band 3 which was not stained with PAS reagent. Deoxycholic acid solubilized stroma was fractionated with gel filtrations and affinity chromatography, resulting in the preparation corresponding to Band 3 with potent Rh-Hr blood type activities. The activities were detected without reductants and the preparation from D negative red cells had no D activity. Rh-Hr blood type activities other than D were observed in the same fraction. ——— Rh-Hr antigens; red cell solubilization; Band 3

Rh₀(D) antigen was first recognized by Landsteiner and Wiener (1940) in the immunization of rabbit with red cells of rhesus monkey. Subsequently, the Rh-Hr blood type system was established with discovery of anti-C, -c, -E, and -e from mainly fetomaternal incompatibility. These Rh antigens are known to be located on the membrane of human red cells but not on membranes of other tissues of human body. Although details of ABH, MN, I and P antigens have been explored, attempts to characterize Rh-Hr antigens have been frustrated for many years. The red cell membrane is composed of about 20 components with different molecules as shown by SDS disc gel electrophoresis, but the relation of the components with Rh-Hr blood type is not yet known. In this paper, we describe the effects of many detergents on Rh-Hr antigen activities and the isolation and purification of Rh₀(D) antigen from red cell membranes.

MATERIALS AND METHODS

Materials. Activated thiol Sepharose 4B (Lot No. EX13978), Sepharose 6B and 4B, Sephadex G-25, G-50 and G-200 were purchased from Pharmacia Fine Chemicals, DEAE-cellulose (DE-32) from Whatman, Hydroxyapatite from Seikagaku Kogyo, AG11A8 from BioRad, Amberlite XAD-2 from Organo, Tween 80 from Kishida Kagaku Co., papain (12,000E/g) from Merck, dipyrindyl disulfide (DPDS) from Tokyo Kasei Co., lithium diiodosalicylate (LIS), deoxycholic acid (DOC), dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA), 2-mercaptoethanol (2-ME), sodium dodecyl sulfate (SDS), dimethyl suberimidate (DMS), Brij 35 and Triton X-100 (TX-100) from Nakarai Chemicals Co. Bee venom phospholipase A2 was obtained from Sigma Chemical Co. Anti-D, -C, -c and -E were purchased from Ortho Diagnostics and anti-e from Tokyo Standard Serum Co. These antisera were of human origin and albumin antibodies.

Preparation and solubilization of stroma. Outdated red cells of D positive and negative were washed with normal saline and hemolyzed with 5 mM phosphate buffer pH 7.4. Stroma was washed with the buffer by centrifugation at 13,000 rpm for 30 min, and stroma free of hemoglobin was obtained. To the stroma were added SDS, TX-100, DOC, Brij 35, Tween 80 and saline to give final concentrations of 2%, 5%, 2%, 5%, 5% and 0.14 M, respectively. After incubation at 37°C for 20 min, the mixture was centrifuged at 13,000 rpm for 30 min and the supernatant was separated. SDS of the extract was removed by AG11A8 chromatography. TX-100 and DOC by Amberlite XAD-2 chromatography, and Brij 35 and Tween 80 by Sephadex G-25 gel filtration, respectively. For saline extraction, mixture of one volume of stroma (1 mg/ml) and the same volume of saline was stirred at 4°C for 12 hr and the supernatant was separated by centrifugation at 13,000 rpm for 30 min. Each of the extracts (protein concentration 50 µg/ml) was used for agglutination inhibition test.

Agglutination inhibition test. To each fraction (0.025 ml) of solubilized or isolated stroma was added the same volume of anti-D of two-fold serial dilution in a hole of microtitre plate (V type). After standing for 30 min, one drop of 2% indicator cell suspension was added, followed by stirring for 2 min. After standing for 2 hr, agglutination was read.

Indicator cells. Red cells of CDe/cDE type were washed with saline and treated with ten volumes of 0.1% papain in 1/15 M phosphate buffer pH 7.3. After incubation at 37°C for 30 min, the red cells were washed twice with saline.

Passive agglutination test. D negative red cells were washed with saline and treated with tannic acid by the method of Stavisky (1954). D negative red cells were coated with various concentrations of Band 3 prepared from D positive and negative red cells. Microtitre plates described above were used for agglutination.

Analytical procedures. Protein concentration was determined by the method of Bradford (1976) with bovine albumin as a standard. Sialic acid was determined by the method of Jourdain et al. (1971), in which N-acetylneuraminic acid was used as a standard and carbohydrate was stained with Schiff's reagent. SDS-PGE was performed by the method of Fairbanks et al. (1971).

Treatment of D active fractions with phospholipase A2. Equal volumes of phospholipase A2 (1865U/ml) and Band 3 (Fr. 3-2-1 or 4-2-2-1, 0.5 mg/ml) were mixed and incubated at 37°C for 10 hr. D activity was examined by the agglutination inhibition test.

RESULTS

Detection of Rh-Hr blood type antigens

Very weak activities of Rh-Hr antigens could be detected by the agglutination inhibition test, in which anti-D, -C and -e diluted 1:1, 600, 1:500, and 1:400 respectively, papainized red cells (CDe/CDe) and microtitre plates (V type) were used (Table 1).

TABLE 1. *Agglutinin titers of anti-D, -C and -e to which was added stroma (CDe/CDe) solubilized with various reagents*

Solubilizer	Anti-D	Anti-C	Anti-e
Saline extract	1: 100	1:128	1: 64
Brij 35	100	16	16
DOC	200	64	64
SDS	1, 600	512	256
TX-100	100	128	64
Tween 80	400	32	16
Stroma dilution	100	64	64
Control	1, 600	512	256

Papain treated red cells (CDe/CDe) were used as test cells.
Control was carried out using saline.

Influence of various solubilizers on Rh-Hr antigen activities

Extractions with Brij 35, TX-100 and saline were effective, but no activity was noted at the stroma treated with SDS. Rh-Hr antigens other than D showed the same behavior in these extractions (Table 1). No activity was also observed in the extracts which were prepared with the methods for isolation of ABH, MN, P or I antigen; phenol (Nagai and Springer 1962), LIS (Marchesi and Andrews 1971), acetone, ethanol, n-butanol (Green 1967), cyanogen bromide (Drickamer 1977), guanidine HCl (Jacobson et al. 1973) or choroform-methanol (Wells and Hanahan 1969).

Isolation of D active fraction from stroma

An outline of procedures was shown in Table 2. TX-100 solubilized stroma was applied on a column of Sephadex G-200, Sepharose 4B or Bio Gel P-300 and eluted with 40 mM phosphate buffer pH 7.4 containing 0.2% TX-100, resulting in a broad eluting peak. Column chromatographies with DEAE-Sephadex, CM-cellulose, CM-Sephadex or hydroxyapatite resulted in unsatisfactory fractionation. Column chromatographies of DOC or SDS solubilized stroma gave the results similar to those mentioned above.

Effect of reductants on D activity of SDS solubilized preparation

As shown in Table 1, D activity was inactivated with SDS. In this examination, reductants (2-ME, DTT, ascorbic acid) or polymerizing reagent (DMS) was added to reacting medium. Using 2-ME or DTT of appropriate concentrations, D activity of SDS solubilized preparation was obviously recovered (Table 3). This recovering was not observed in the preparations prepared with the phenol, LIS, or n-butanol extraction.

Fractionation of SDS solubilized stroma

After SDS in stroma was removed by AG11A8 column, the solubilized stroma was applied onto a Sephadex G-50 column which was equilibrated beforehand with

TABLE 2. *Procedures of purification of D antigen*

Exp. 1. Stroma solubilized with TX-100 or SDS			
DEAE-cellulose	Fr. 1-1	D-active (non-absorbed)	
	Fr. 1-2	D-active (absorbed)	
Hydroxyapatite	Fr. 1-3	D-inactive (0.30 M phosphate buffer elution)	
	Fr. 1-4	D-active (0.35 M phosphate buffer elution)	
	Fr. 1-5	D-active (0.40 M phosphate buffer elution)	
Exp. 2. Stroma solubilized with SDS			
Sephadex G-50	Fr. 2-1	D-active	
	Fr. 2-2	D-inactive	
	Fr. 2-3	D-active — Sepharose 6B Fr. 2-3-1 D-active	
Exp. 3. Fr. 2-1			
Activated thiol Sephacrose 4B	Fr. 3-1	D-weak activ (non-absorbed)	
	Fr. 3-2	D-active — Sepharose 6B Fr. 3-2-1 D-active (absorbed) (single band corresponding with Band 3)	
Exp. 4. Stroma solubilized with DOC			
Sephacrose 4B	Fr. 4-1	D-active	
	Fr. 4-2	D-active — Activated thiol Fr. 4-2-1	
	Fr. 4-3	D-inactive Sepharose 4B (non-absorbed)	
		Fr. 4-2-2 — Sepharose 6B Fr. 4-2-2-1 (absorbed) (single band corresponding with Band 3)	

All fraction numbers correspond with those in the figures and text.

TABLE 3. *Agglutination inhibition test of anti-D with SDS solubilized stroma containing reductants*

Reductants	Dilution of anti-D						
	1: 50	1: 100	1: 200	1: 400	1: 800	1: 1,600	1: 3,200
0.3% 2-ME	+	+	—	—	—	—	—
0.3% 2-ME*	+	+	+	+	+	+	—
0.15% DTT	+	+	—	—	—	—	—
0.15% DTT*	+	+	+	+	+	+	—

+, not inhibited; —, inhibited.

* Control experiment was carried out without stroma.

saline. After washing with saline, absorbed fraction (Fr. 2-3) was eluted by saline containing 0.2% SDS (Fig. 1). After concentration with ultrafiltration, Fr. 2-3 was applied onto a Sepharose 4B column and eluted with 40 mM phosphate buffer pH 7.4 containing 0.2% SDS. Rechromatography of the eluate with Sepharose 4B gave a D active fraction (Fr. 2-3-1) which corresponded with Band 3 in SDS-PGE. But Fr. 2-3-1 prepared from D negative red cells showed slight D activity.

Separation of sialoglycoproteins (SGP) from Fr. 2-1-1.

To ascertain Rh-Hr blood type activities in Band 3, D active fraction (Fr. 2-1) in 40 mM phosphate buffer pH 7.4 containing 0.2% SDS, 1 mM EDTA and 0.14M

Fig. 1. Chromatography of SDS solubilized stroma on Sephadex G-50. Twenty ml of stroma (5 mg/ml) was applied onto the column and eluted with saline. The arrow indicates the addition of 0.1% SDS.

Fig. 2. An affinity chromatography of SDS solubilized stroma on activated thiol Sepharose 4B.

Thirteen ml of SDS solubilized stroma (Fr. 2-1) was applied onto the column (1.5×15 cm) and eluted with 40 mM phosphate buffer pH 7.4 containing 0.14 M NaCl, 1 mM EDTA, 0.2% SDS and 50 mM 2-ME. Fractions of 2.5 ml each were collected. Absorbance at 600 nm indicates the quantity of protein measured by the method of Bradford.

NaCl was adopted to activated thiol Sepharose 4B in which PAS components were separated by the method of Brocklehurst et al. (1974). After washing with 40 mM phosphate buffer pH 7.4 containing 0.14 M NaCl, 1 mM EDTA and 0.2% SDS, an absorbed fraction (Fr. 3-2) was eluted with 50 mM 2-ME in the same buffer (Fig. 2). Fr. 3-2 had strong D activity and Fr. 3-1 had weak one. After concentration and dialysis against 40 mM phosphate buffer pH 7.4 containing 0.2% SDS, Fr. 3-2 was applied twice onto a Sepharose 6B and eluted with the same buffer (Figs. 3a, b). The final preparation (Fr. 3-2-1) corresponded to the Band 3 free from PAS in SDS-PGE (Fig. 4). As shown in Table 4, D activity of Fr. 3-2-1 was detected by

Fig. 3. Chromatographies of Fr. 3-2 on Sepharose 6B. Five ml of the fraction (Fr. 3-2) from Fig. 2 was applied onto the column (3×10 cm) and eluted with 40 mM phosphate buffer pH 7.4 containing 0.2% SDS. Fractions of 3 ml each were collected. In Fig. 3a, the fractions indicated by the bar contained D activity. The fractions were rechromatographed under the same condition (Fig. 3b). The first peak (Fr. 3-2-1) had D activity.

Fig. 4. SDS-polyacrylamide gel disc electrophoretic patterns of fractions from Fig. 3b. Tracks 1 and 3, stroma; 2 and 4, Fr. 3-2-1. Tracks 1 and 2 were stained with Coomassie blue and 3 and 4 with PAS.

TABLE 4. *Agglutination inhibition test of anti-D with Band 3 (Fr. 3-2-1) obtained in Fig. 3b containing 0.15% 2-ME*

Band 3 from	Dilution of anti-D						
	1: 50	1: 100	1: 200	1: 400	1: 800	1: 1,600	1: 3,200
D positive	+	+	—	—	—	—	—
D negative	+	+	+	+	—	—	—
Control	+	+	+	+	+	+	—

Note slight inhibiting activity of Band 3 from D negative red cell.
+, not inhibited; —, inhibited.

addition of reductants. However, Fr. 3-2-1 of D negative stroma showed slight D activity.

Further investigation of D activity in Band 3 (Fr. 3-2-1)

Passive agglutination test was performed using red cells coated with Band 3 (Fr. 3-2-1). As shown in Table 5 D activity was observed in Band 3 without reductants and not observed in Band 3 prepared from D negative red cells. The optimal protein concentration of Band 3 used for coating red cells was approximately 0.1 mg/ml.

TABLE 5. *Passive agglutination test of tannic acid treated D negative red cells with anti-D*

Band 3 from	Dilution of anti-D					
	1:32	1:64	1:128	1:256	1:512	1:1,024
D positive	+	+	+	+	+	—
D negative	—	—	—	—	—	—
Control	—	—	—	—	—	—

The red cells were coated with Band 3 (Fr. 3-2-1) from D positive or negative red cells. Note agglutination of the red cells coated with Band 3 from only D positive red cells.

Rh-Hr blood type activities of DOC solubilized stroma

DOC solubilized stroma was fractionated with Sepharose 4B gel filtration (Fig. 5). D activity was found in Fr. 4-1 and 4-2. Fr. 4-2 was conjugated with DPDS by the method of Svenson et al. (1977) and was applied onto a reduced thiol Sepharose 4B which was washed beforehand with 0.3M borate buffer pH 8.0 con-

Fig. 5. Chromatography of DOC solubilized stroma on Sepharose 4B. Five ml of stroma solubilized with 1% DOC was applied onto the column (3×70 cm) and eluted with 10 mM Tris-HCl buffer pH 7.4 containing 0.2% DOC. Fractions of 3 ml each were collected. Absorbance at 630 nm indicated the quantity of PAS positive. Inhibition titer showed the titer of anti-D being inhibited with the fractions.

taining 50 mM 2-ME and equilibrated by 10 mM Tris-HCl buffer pH 7.5 containing 0.14 M NaCl. The column was washed by the equilibrating buffer and the absorbed fraction was eluted by the same buffer containing 50 mM 2-ME. Elution pattern was the same as in Fig. 2, and its D activity was shown in Table 6. After concentration, the absorbed fraction (Fr. 4-2-2) was applied twice onto a Sepharose 6B and eluted with 10 mM Tris-HCl buffer pH 7.5 containing 0.2% DOC. The final preparation (Fr. 4-2-2-1) corresponding to Band 3 in SDS-PGE had potent D activity. The activity was detected without reductants and the preparation from D negative stroma had no D activity (Table 6). Rh-Hr blood type activities other than D were observed in the same fraction.

TABLE 6. *Agglutination inhibition test of anti-D with the fractions (Fr. 4-2-1 and 4-2-2) and Band 3 (Fr. 4-2-2-1)*

Protein conc.		Dilution of anti-D						
		1: 50	1: 100	1: 200	1: 400	1: 800	1: 1,600	1: 3,200
Fr. 4-2-1	10 μ g/ml	+	+	—	—	—	—	—
Fr. 4-2-2	10 μ g/ml	+	+	+	+	—	—	—
	50 μ g/ml	+	+	—	—	—	—	—
Band 3 (Fr. 4-2-2-1)		+	+	—	—	—	—	—
from D positive								
Band 3 (Fr. 4-2-2-1)		+	+	+	+	+	+	—
from D negative								
Control		+	+	+	+	+	+	—

+, not inhibited; —, inhibited.

Treatment of D active fractions with phospholipase A2

D activity of Band 3 (Fr. 3-2-1 and 4-2-2-1) prepared from SDS or DOC solubilized red cells was not affected with treatment of phospholipase A2.

DISCUSSION

Of all major blood types, the Rh-Hr system has been most intractable for a biochemical approach. This difficulty has been attributed to the small quantity of Rh-Hr antigens present in red cell and to the severe procedures to separate the antigens from red cell membrane. The component carrying Rh-Hr antigens has been reported to be protein (Mollison 1979a) because antigenic activities were diminished by treatment by alkylating reagents or non-ionic detergents. On the other hand, it has been claimed that lipid is required for expression of the antigen activities (Green 1972; Weicker et al. 1973; Hughes-Jones et al. 1975; Mollison 1979b). Green (1972) reported that D activity which had disappeared by n-butanol treatment was restored by addition of phosphatidylcholine or phosphatidylethanolamine. However, Plapp et al. (1979) demonstrated that partially purified D antigen prepared by anti-D IgG affinity chromatography was predominantly protein and that phospholipids were not needed for recovery of D antigen.

We first investigated detecting methods of Rh-Hr blood activities. As a very sensitive method the agglutination inhibition test was recommended in which anti-D, -C and -e diluted 1:1, 600, 1:500 and 1:400 respectively, papainized red cells and microtiter plates (V type) were used. This method was more sensitive and inexpensive than usual agglutination inhibition methods. Therefore, it was useful for detecting Rh-Hr blood types of blood stains in forensic practice (Yokoi et al. 1982).

As for solubilizing reagent, extractions with Brij 35, TX-100 and saline were effective, but extraction of Rh-Hr antigens with EDTA-NaCl treatments as reported by Abraham and Bakerman (1974, 1975, 1976, 1977) was not reproduced in this experiment. It has been admitted that Brij 35 could extract glycoproteins possessing MN and ABH antigens (Sagisaka and Hamada 1975). In general, extraction with non-ionic detergent contained proteins which was degenerated and contaminated with lipids. On the other hand, extraction with ionic detergents isolated proteins from membrane as micelles. Glycoproteins extracted with the method of phenol (Nagai and Springer 1962) or LIS (Marchesi and Andrews 1971) were known to be mainly major sialoglycoproteins corresponding with PAS-I. From results of this experiment and data reported by Lorusso et al. (1977), it is considered that Rh-Hr antigens are not involved in the sialoglycoproteins.

In this investigation, a non ionic detergent, TX-100, was first adopted to solubilize membrane. As shown in Table 2, separation of D active fraction was unsatisfactory, and the similar results were obtained by solubilization with DOC or SDS. It might be due to fragmentation of the detergents by which membrane was cut randomly into pieces possessing Rh-Hr activities.

There is general agreement that SDS was effective to solubilize red cell or plasma cell membrane. In the present experiments, preparations from SDS solubilized stroma had no Rh-Hr activity. But the activity was evidently recovered by adding reductants, 2-ME or DTT. In addition, weak D activity was noted at the preparation from D negative red cells. Romans et al. (1977) and Mandy et al. (1965) treated incomplete Rh antibody of human IgG with 2-ME with the result that the antibody was converted to complete one. They explained the phenomenon that mild reduction of the antibody brought about liberalization of disulfide bonds between heavy chains in Fc fragments of IgG, resulting in an increase of agglutinating ability. Therefore, it was suggested that slight D activity of preparation from D negative red cells was caused by a chemical structure similar to D antigen which was activated by the mild reduction.

The recovery of Rh-Hr antigens indicated the possibility of fractionation with some chromatographies. By fractionations with Sephadex G-50, Sepharose 4B, activated thiol Sepharose 4B and Sepharose 6B, the final preparation with D activity was found to correspond with Band 3 in SDS-PGE which was not stained with PAS reagent. The isolation of Band 3 was performed according to the method of Fukuda et al. (1978) with some modifications to keep D activity. However, contrary to our expectation the specific activity of D antigen did not

increase by these procedures. It might be attributed to splitting or inactivation of D antigen during the procedures. The passive agglutination test using red cells coated with Band 3 revealed that D activity was detected without reductants in Band 3 from only D positive red cells. Recently, Plapp et al. (1979, 1980, 1981) have reported that D antigen was prepared by affinity chromatography with anti-D absorbed gel column. They demonstrated that D antigen was protein with low molecular weight and that the antigen was presented in not only D positive but also D negative red cells. It seemed that the proposals of Plapp et al. might be incompatible with biochemical evidence and genetics of the Rh-Hr system reported hitherto as pointed out by Victoria et al. (1981) and Mollison (1981).

Victoria et al. (1981) described that D antigen was located in Band 3. They solubilized stroma by Brij 36T and fractionated with gel filtrations and Protein A affinity chromatography. As a result, D active fraction complexed with anti-D IgG was proved to be a dimer of Band 3 in SDS-PGE. However, D activity was not confirmed by serological assay.

Further isolation using DOC, a mild anionic detergent (Lorusso and Green 1975), was performed to certify D activity in Band 3. DOC solubilized stroma was fractionated with gel filtrations and reduced thiol Sepharose 4B by the method of Svenson et al. (1977). The final preparation corresponding to Band 3 in SDS-PGE had potent D activity. Preparation of Band 3 by the activated thiol Sepharose 4B was not so efficient because only one-fifth of Band 3 had disulfide crosslinkage (Lutz et al. 1979) which could be isolated by reduced form of activated thiol Sepharose 4B. As shown in Table 6, D activity was noted in both absorbed and non-absorbed fractions. These results suggest that D activity has no relation with disulfide bonds in Band 3 structure. In this isolation, Rh-Hr activities were detected without reductants and the preparation from D negative red cells had no D activity. Treatment of Band 3 with phospholipase A2 indicated that lipid did not take part in the antigenicity of D.

From these results, it is concluded that Rh-Hr blood type activities are extracted with ionic and non-ionic detergents and that the activities are located in Band 3 with no PAS reaction.

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