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Reconstitution of Rh (D) Antigen Activity from Human Erythrocyte Membranes Solubilized by Deoxycholate

Abstract. *Proteins were selectively solubilized from human erythrocyte membranes with deoxycholate. After ultracentrifugation and preliminary fractionation procedures, the detergent was removed from the soluble membranes by Bio-Beads SM-2 and dialysis against 5 millimolar magnesium chloride. Reaggregation took place with the apparent formation of vesicles which showed serologically specific Rh antigen activity.*

Since their initial description (*1*), the Rh antigens have been found on the surface of erythrocytes of man and other primates. The Rh antigenic sites are molecularly dispersed in a random two-dimensional array on the surface of the erythrocyte (*2*) and appear related to the problem of erythrocyte membrane integrity (*3*). Attempts to completely solubilize membrane Rh antigens, by techniques known to solubilize other membrane proteins and carbohydrate antigens, have been unsuccessful (*4*). The loss of Rh activity caused by some of these procedures (*5*) is due in part to loss of phospholipid which appeared bound to a Rh antigen-related membrane protein and required for the antigen activity to be manifested.

We now report the selective solubilization of the human erythrocyte membrane proteins with recovery of Rh antigenic activity by the following solubilization procedure. Erythrocyte membranes (*6*) were solubilized in a 20 m*M* sodium phosphate buffer (*p*H 7.5) containing 0.8 percent (weight to volume) NaCl, 10 m*M* dithiothreitol, 1 m*M* ethylenediaminetetraacetate, 20 percent (weight to volume) glycerol (*7*), and 0.9 percent (weight to volume) deoxycholate at a concentration of 4.5 mg of dry membranes per milliliter. The mixture was stirred for 30 minutes at 4°C and then centrifuged at 151,000*g* (average) for 90 minutes to sediment the unsolubilized material. Protein determinations of the mixture and of the supernatant fraction were used to calculate the percentage of membrane protein solubilized (*8*). The supernatant fraction was treated with Bio-Beads SM-2 (*9*) to remove most of the deoxycholate and then dialyzed for 2 to 3 days against water buffered to *p*H 7.5 with tris base (1 mg/liter), or buffered water containing 5 m*M* $MgCl_2$ or $CaCl_2$. The dialyzed supernatants were then centrifuged at 151,000*g* (average) for 60 minutes. The pellets were either suspended in phosphate-buffered saline, *p*H 7.4, or lyophilized; the Rh antigenic activity was then determined (*10*).

Solubilization of erythrocyte membranes by this procedure produced supernatant fractions which contained 55 to 70 percent of the membrane protein, while the pellets contained less than 1 percent of the original Rh antigenic activity on a dry weight basis. The solubilization exhibited some degree of specificity, since sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis indicated that spectrin and some polypeptides contained in bands 3, 4, and 5 (*11*) were not solubilized. More protein was pelleted when the samples were dialyzed against magnesium or calcium than when they were dialyzed against buffered water (Table 1). Each type of dialysis showed recovery of Rh antigenic activity, but maximum reactivation per milligram of protein occurred with dialysis against $MgCl_2$.

Table 1. Distribution of protein and Rh antigen activity after membrane solubilization and reconstitution with dialysis. After treatment with Bio-Beads SM-2, the soluble preparations were dialyzed against water, 5 m*M* $MgCl_2$ or 5 m*M* $CaCl_2$. The samples were then centrifuged, and the reconstituted pellets were assayed for Rh antigenic activity (*10*) and protein. Samples retained activity for at least 1 week when stored at 4°C, either as pellets or suspensions in phosphate-buffered saline, but lost activity within 1 day when lyophilized. The numbers in parentheses are the number of experiments.

Cation	Pellet	
	Protein* (%)	Rh antigen† (%)
None	45–60 (4)	30–60 (2)
Magnesium	70–90 (4)	50–240 (6)
Calcium	81–86 (4)	25–75 (6)

* Percent of total protein after dialysis. † Percent of original membrane protein.

To rule out any possibility of nonselective absorption of antiserum (anti-D) to the D antigen during the titer assays, membranes that were Rh(D)–negative, c-positive were run as controls. The Rh(D)–negative membranes do not have D antigen specificity, but do exhibit c antigen specificity, among others. The amount of protein solubilized, and the distribution of protein after dialysis and centrifugation were the same as with D-positive membranes. Assay of preparations from D-negative membranes with anti-D showed no binding of anti-D to the samples, as would be expected. These same samples, however, showed c antigen activity. Therefore, the absorption of anti-D by the D-positive samples was completely specific. In addition, the conditions used to reconstitute the D antigen were also favorable for the reconstitution of the c antigen.

Maximum Rh antigenic activity was obtained with dialysis against $MgCl_2$. To study this, we performed phospholipid analysis, SDS polyacrylamide gel electrophoresis, and electron photomicroscopy on representative reconstituted samples. Total phospholipid content was the same and ranged from 16 to 17 percent on a dry weight basis compared with erythrocyte membranes which contain 32 percent phospholipid. The 50 percent reduction in phospholipid content was presumably due to removal of phospholipid-deoxycholate mixed micelles by Bio-Beads SM-2. Phospholipid distribution on thin-layer chromatography plates was unchanged. The SDS polyacrylamide gel electrophoresis of samples reconstituted in each of the three ways showed the same number of protein peaks in approximately the same ratios. This data, plus the data in Table 1, suggested that samples reconstituted with Mg and Ca ions differed from the water samples because the proteins had reaggregated

(*12*). Electron photomicroscopic studies (*13*) sustained this possibility, since only amorphous material was seen in the water samples, while membrane vesicular structures were seen in Mg-treated samples and spicules were visible in Ca-treated samples. The data suggest that some membrane structure may be necessary for Rh antigenic activity.

Two additional procedures were used to exclude the possibility that membrane fragments were responsible for all of the Rh antigenic activity in the "solubilized" membrane fraction: column chromatography, with Sepharose 4B and ultrafiltration (*14*) with a Diapor microporous filter with an average pore size of 0.2 μm. Gel filtration of the supernatant with Sepharose 4B showed little or no protein at the void volumn, and revealed two protein peaks with an apparent molecular weight of approximately 200,000 and 10,000, respectively. Ultrafiltration (Table 2) retained only 1 to 2 percent of the protein of the supernatant fraction. This small amount of protein may have been membrane fragments, or reaggregated soluble membrane protein. Rh antigenic activity was found in the ultrafiltrate (Table 2). The above data suggest that the recovered Rh antigenic activity from the supernatant fraction could not possibly be explained by residual membrane fragments, but was due predominantly, if not exclusively, to reconstituted soluble protein complexes of fairly small molecular weight.

Because deoxycholate forms a gel on prolonged standing below 16°C in the buffer system used for solubilization (*15*), the proteins were first fractionated by ultrafiltration at 18°C. Two ultrafilters were used, one with a cut-off for molecules having a molecular weight of 300,000 (XM 300) and the other of 100,000 (XM 100 A). Only 20 percent of the supernatant protein passed the XM 300 filter, and 5 percent of the protein passed through the XM 100 A filter (Table 2). Only the protein obtained from the filtrate on the XM 300 showed Rh antigen activity. Not enough pelleted material was obtained from the XM 100 A filter after removal of the deoxycholate and dialysis to perform Rh activity assays. The amount of protein obtained in the filtrate from the XM 300 and XM 100 A filters was lower than was expected from the column data. The same apparent increase in molecular weight (increased retentivity) was observed with standard soluble protein molecular weight markers. This increased retentivity was probably due to the development of a "secondary" boundary membrane containing deoxycholate and protein and having a smaller pore size, thereby retaining proteins that would have penetrated the primary membranes. Although an exact molecular weight of the D antigen in deoxycholate could not be determined from the ultrafiltration data, the D antigen must be fairly small (less than 300,000) to penetrate both the "secondary" and primary membranes of the XM 300 filter.

Table 2. Distribution of proteins and Rh antigenic activity after ultrafiltration of deoxycholate-solubilized membranes. The solubilized membrane fraction (SM) was filtered by a single pass, and the ultrafiltrate (UF) was then dialyzed against $MgCl_2$. The reconstituted pellet was assayed for Rh antigenic activity and protein.

Filter	Pore size (Å)	Protein concentration (mg/ml)		Rh* (%)
		SM	UF	
DPO-2	2000	0.95	0.94	84
XM 300	180†	0.88	0.17	57
XM 100 A	55‡	0.91	0.05	§

* Percent activity (in ultrafiltrate) of original membrane protein. † Equivalent to a molecular weight of 3×10^5. ‡ Equivalent to a molecular weight of 10^5. § Insufficient quantity.

The molecular weights on Sepharose 4B are approximate only, since the binding of detergent to the lipophilic membrane proteins and hydrophilic marker proteins occurs to an unknown extent and would tend to increase the apparent molecular weight of the proteins (*16*). The determinations of the exact molecular weight and other molecular properties of the antigen must await its isolation from the deoxycholate-bound state.

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