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The Role of Membrane Phospholipid in Expression of Erythrocyte $Rh_o(D)$ Antigen Activity (40917)

FRED V. PLAPP, MARK M. KOWALSKI, JAMES P. EVANS, LOWELL L. TILZER, AND MASAHIRO CHIGA

Department of Pathology and Oncology, University of Kansas Medical Center, Kansas City, Kansas 66103

Abstract. Previous investigators have reported that the expression of $Rh_o(D)$ antigen activity by human erythrocytes and their membranes depended on the presence of phospholipid. In order to further elucidate the role of phospholipids in expression of $Rh_o(D)$ antigen activity, erythrocyte membranes and partially purified $Rh_o(D)$ antigens were incubated with bee venom phospholipase A_2. Treatment of erythrocyte membranes with phospholipase A_2 resulted in loss of $Rh_o(D)$ antigen activity as detected by hemagglutination inhibition assays. However, subsequent solubilization of these treated membranes with deoxycholate allowed recovery of $Rh_o(D)$ antigen activity. Phospholipase treatment of solubilized $Rh_o(D)$ antigens, which had been partially purified by affinity chromatography on anti-$Rh_o(D)$ IgG agarose columns, did not destroy $Rh_o(D)$ antigen activity. These results suggested that phospholipids did not affect the antigenic determinants of the $Rh_o(D)$ antigen since solubilized, partially purified $Rh_o(D)$ antigens retained their antigenicity following exposure to phospholipase. Phospholipids were presumably required for proper orientation of $Rh_o(D)$ antigens within erythrocyte membranes since $Rh_o(D)$ membranes lost their antigenicity following phospholipase treatment.

Previous investigators have suggested that the $Rh_o(D)$ antigen present in human erythrocyte membranes is a lipoprotein. This suggestion was based on the observations that removal of phospholipids from erythrocyte membranes by butanol extraction (1, 2) or phospholipases (3) resulted in loss of membrane $Rh_o(D)$ antigen activity. In the former case, $Rh_o(D)$ antigen activity was partially restored by addition of phosphatidylcholine or phosphatidylethanolamine. Thus, these results suggested that phospholipids were necessary for the antigenic activity of membrane-bound $Rh_o(D)$ antigen. More recently, however, our laboratory (4) and others (5) demonstrated that partially purified $Rh_o(D)$ antigen was predominantly protein and was not inactivated by phospholipase (6, 7). These results seemed to indicate that phospholipids were not required for the activity of solubilized $Rh_o(D)$ antigen. In order to investigate this apparent discrepancy between the phospholipid requirements of membrane-bound and solubilized $Rh_o(D)$ antigen, we studied the effect of phospholipase A_2 on the antigenic activity of erythrocyte membranes and solubilized, partially purified $Rh_o(D)$ antigen fractions.

Methods. Erythrocyte membranes were prepared from outdated group O, Rh-positive erythrocytes as previously described (4). Packed membranes were suspended in an equal volume of 0.02 *M* Tris–HCl (pH 8.0) to give a protein concentration of approximately 4 mg/ml.

Membranes were treated with phospholipase A_2 by adding 200 μl of membranes, containing 80 μg of protein, to 1.80 ml of buffer containing 10 m*M* Hepes (pH 7.4), 148 m*M* NaCl, and 10 m*M* $CaCl_2$. Three units (2 μg of protein) of bee venom phospholipase A_2 (Sigma) were then added and the suspensions were incubated at 37° for 1 hr. One unit of phospholipase A_2 hydrolyzed 1.0 μmole of phosphatidylcholine to lysophosphatidylcholine and a fatty acid per minute at pH 8.5 and at 37°. Control membranes were treated identically but without phospholipase. Following incubation, the suspensions were centrifuged at 20,000*g* for 15 min and the pellets were suspended in 200 μl of 20 m*M* Tris–HCl (pH 8.0). In some cases control and phospholipase-treated membranes were solubilized by addition of sodium deoxycholate (Sigma) to a final concentration of 1% (w/v) and incubation for 30 min at room tem-

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perature. Solubilized membranes were then centrifuged at 54,000*g* for 30 min at 4° in a Spinco Type 50 rotor. The supernatants were mixed with 0.2 ml of packed Bio-Beads SM-2 (Bio–Rad) for 1 hr at 25° and the supernatants, which were free of deoxycholate, were recovered following centrifugation at 900*g* for 2 min.

Membrane phospholipids were extracted by the method of Reed *et al.* (8). Phospholipid, 40 μg in 5 μl of chloroform, was layered onto silica gel K-5 plates (Helena) and chromatographed for 25 min at 25° in a solvent consisting of 38 ml chloroform, 14 ml methanol, and 2 ml of 7.4 *N* NH_4OH. Phospholipids were visualized by immersing the plates in phosphomolybdic acid (Whatman) for 1 min and heating at 80° for 10 min. The plates were then scanned in a Helena scanning densitometer at 525 nm. Lysophospholipids were not detected by this method.

$Rh_o(D)$ antigens were partially purified from sodium deoxycholate-solubilized Rh-positive membranes by affinity chromatography as previously described (4). Membrane proteins were labeled with fluorescamine (Fluram, Roche) according to Cross and Briggs (9). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in 5% gels at 8 mA per gel for 90 min according to Fairbanks *et al.* (10). Gels were scanned in a Gilford Model 240 spectrophotometer equipped with a Model 2515 fluorescent gel accessory (11).

The $Rh_o(D)$ antigen activity of membranes and solubilized membrane protein fractions was determined by hemagglutination inhibition assays performed at 37° in rigid microtiter plates with V-shaped wells (Linbro), using papain-treated type O, Rh-positive erythrocytes (4). Rh(c), Rh(E), and LW antigen activities were similarly assayed using papain-treated type O erythrocytes, containing the appropriate Rh or LW antigen, and the appropriate antisera.

The A,B,M, and N activities of erythrocyte membranes were also determined by hemagglutination inhibition assays, except that the assays were performed at 4° and the erythrocytes, containing the appropriate A,B,M, or N antigens, were not treated with papain. In each case 200 μl of membrane fraction were incubated with 50 μl of antisera. All antisera were obtained from Ortho Diagnostics. Complete effacement of wells by erythrocytes indicated hemagglutination, whereas the small button of erythrocytes in the bottom of wells indicated hemagglutination inhibition.

Erythrocyte membrane proteins, which had been solubilized with 1% sodium deoxycholate, were incorporated into liposomes according to Gerritsen *et al.* (12). Two milliliters of deoxycholate solubilized membrane proteins, prepared as described above, were added to 12 mg of egg phosphatidylcholine (Sigma). Following 1 hr of mixing at 25°, 1 ml of Bio-Beads SM-2 was added and the suspension was mixed for another hour. The beads were then removed by centrifugation at 900*g* for 2 min and the supernatants were centrifuged at 54,000*g* for 2 hr. The pellets were suspended in 200 μl of phosphate-buffered saline (15 m*M* Na_2HPO_4, pH 7.4, 0.9% NaCl) and assayed for $Rh_o(D)$ antigen activity.

Results. Incubation of Rh-positive membranes with bee venom phospholipase A_2 resulted in nearly total degradation of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, while sphingomyelin was preserved (Fig. 1). In order to determine the effect of this phospholipid hydrolysis on $Rh_o(D)$ antigen activity, phospholipase-treated membranes were tested by hemagglutination inhibition assay. When Rh-positive erythrocyte membranes were exposed to phospholipase A_2 they did not inhibit the hemagglutination of Rh-positive erythrocytes, indicating the absence of $Rh_o(D)$ antigen activity (Fig. 2b). In contrast, control Rh-positive membranes, which had been incubated under identical conditions, but without phospholipase A_2, inhibited hemagglutination (Fig. 2c), indicating $Rh_o(D)$ antigen activity (4). Thus, phospholipase A_2 appeared to abolish the $Rh_o(D)$ antigen activity of Rh-positive erythrocyte membranes.

Several possibilities exist regarding the mechanism(s) by which phospholipase A_2 abolishes $Rh_o(D)$ antigen activity. Some investigators have reported that various phospholipase preparations contained

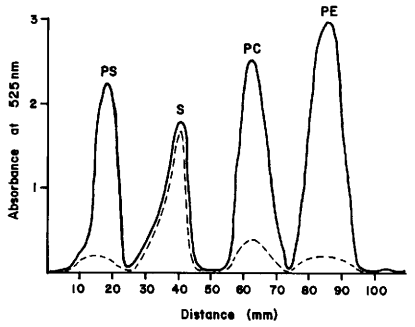

FIG. 1. Thin-layer chromatography of phospholipids extracted from untreated (control) erythrocyte membranes (——) and phospholipase-treated erythrocyte membranes (– –). PS, phosphatidylserine; S, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

contaminating proteases (3), which might have degraded the protein moiety of the Rh_o(D) antigen. On the other hand, if the Rh_o(D) antigen was a lipoprotein, as previously proposed (1–3), it might have been released from the membrane following phospholipid hydrolysis. Alternatively, if the antigenic determinants of the Rh_o(D) antigen were at least partially composed of phospholipids, phospholipase treatment might have diminished the ability of the antigen to bind anti-Rh_o(D) IgG.

In order to determine if the Rh_o(D) antigen had been degraded by contaminating proteases both control and phospholipase-treated membranes were labeled with fluorescamine, solubilized with sodium dodecyl sulfate, and electrophoresed on sodium dodecyl sulfate–polyacrylamide gels. The membrane proteins pattern obtained with control membranes (Fig. 3A) was similar to the pattern described by Fairbanks *et al.* (10). Phospholipase treatment did not affect the membrane protein pattern (Fig. 3B). If proteases had been present they probably would have degraded spectrin bands 1 and 2, since these proteins are very sensitive to proteolysis (13). More definitive evidence that the Rh_o(D) antigen had not been degraded or antigenically altered was obtained by electrophoresis of Rh_o(D) antigen extracts which had been partially purified from control and phospholipase-treated membranes by affinity chromatography on anti-Rh_o(D) IgG agarose columns (4). As seen in Figs. 3C and D, antigens from both control and treated membranes migrated the same distance, consistent with an approximate molecular weight of 7000, as previously described (4). Thus, the protein moiety of the Rh_o(D) antigen had not been significantly degraded.

In order to determine if phospholipid degradation had resulted in release of the Rh_o(D) antigen from Rh-positive membranes, the supernatants obtained after centrifugation of membrane–phospholipase mixtures were concentrated to the volume of the original membrane suspension and tested by hemagglutination inhibition assays for Rh_o(D) antigen activity. As seen in Fig. 2d no Rh_o(D) antigen activity was detected. When the membrane pellets from the same experiments were solubilized with sodium deoxycholate, which solubilizes the Rh_o(D) antigen (4), much Rh_o(D) antigen activity was recovered (Fig. 2e). The degree of hemagglutination inhibition observed was similar to that obtained with the same quantities of intact control Rh-positive membranes (Fig. 2c) and control membranes solubilized with sodium deoxycholate (Fig. 2f). These results suggested that the Rh_o(D) antigen had not been released from the membrane or degraded to an inactive form since as much antigen activity was recovered from phospholipase-treated membranes as from control membranes.

In order to further demonstrate that the Rh_o(D) antigen, itself, was not affected by phospholipid hydrolysis, partially purified Rh_o(D) antigen was incubated with phospholipase A_2. As seen in Fig. 2j, there was no loss of Rh_o(D) antigen activity compared to partially purified antigen which had not been incubated with phospholipase (Fig. 2i). Also, further evidence that the Rh_o(D) antigen, itself, had not been degraded was established by the finding that the Rh_o(D) antigen, solubilized from phospholipase-treated membranes, could interact with phosphatidylcholine and exhibit Rh_o(D) antigen activity. As seen in Fig. 2g, liposomes containing proteins from phospholipase-treated membranes expressed Rh_o-

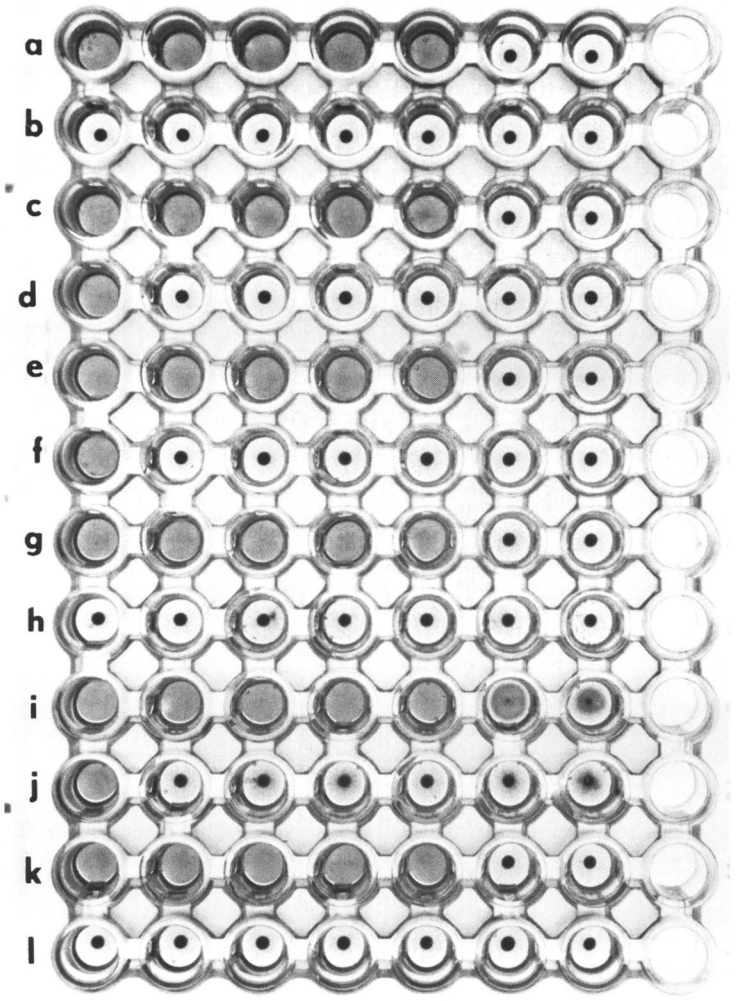

FIG. 4. Hemagglutination inhibition assay. Anti-Rh_o(D) IgG was adsorbed with: (a) nothing, (b) untreated (control) erythrocyte membranes, (c) phospholipase-treated erythrocyte membranes, (d) solubilized phospholipase-treated erythrocyte membranes. Anti-Rh(E) IgG was adsorbed with: (e) nothing, (f) untreated (control) erythrocyte membranes, (g) phospholipase-treated erythrocyte membranes, (h) solubilized phospholipase-treated erythrocyte membranes. Anti-LW IgG (Group A Bigelow) was adsorbed with: (i) nothing, (j) untreated (control) erythrocyte membranes, (k) phospholipase-treated erythrocyte membranes, (l) solubilized phospholipase-treated erythrocyte membranes.

membranes by hypotonic lysis (14). Sphingomyelin was not degraded since it is not a substrate for bee venom phospholipase A_2 (14).

Phospholipase treatment of erythrocyte membranes resulted in loss of membrane Rh_o(D) antigen activity which was not due to degradation of antigenic determinants or release of the antigen from membranes. Instead, these results suggested that intact Rh_o(D) antigen remained bound to the membrane following phospholipase treatment, but could no longer react with anti-Rh_o(D) IgG, until the membrane was solubilized.

These findings could be explained if it is

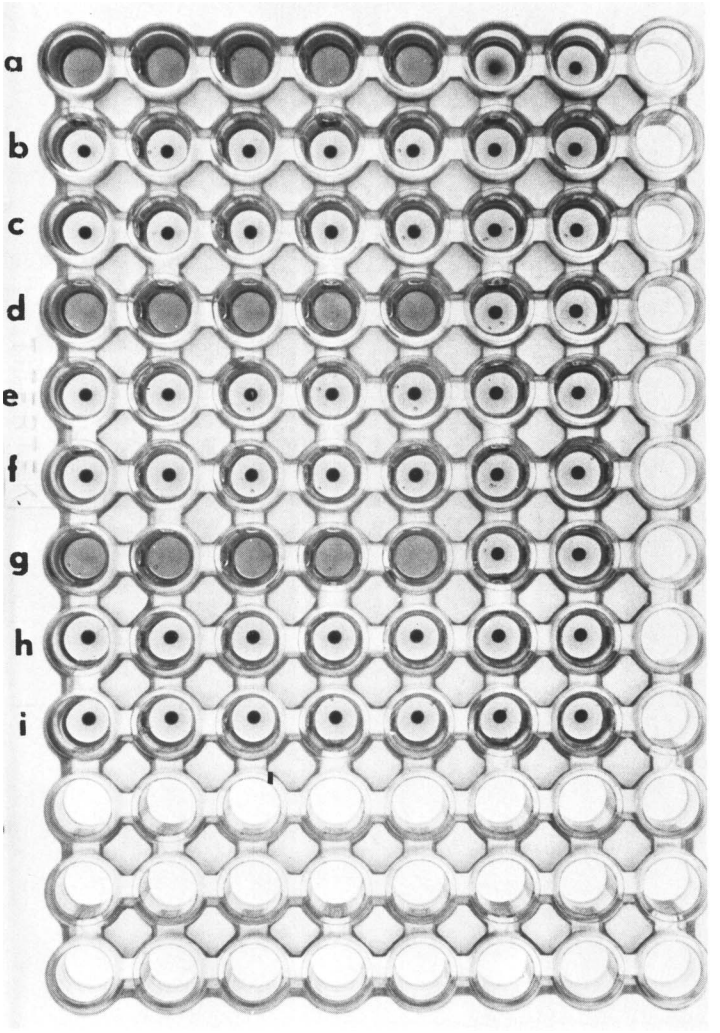

FIG. 5. Hemagglutination inhibition assay. Anti-A typing sera were adsorbed with: (a) nothing, (b) untreated (control) erythrocyte membranes, (c) phospholipase-treated erythrocyte membranes. Anti-B typing sera were adsorbed with: (d) nothing, (e) untreated (control) erythrocyte membranes, (f) phospholipase-treated erythrocyte membranes. Anti-M typing sera were adsorbed with: (g) nothing, (h) untreated (control) erythrocyte membranes, (i) phospholipase-treated erythrocyte membranes.

assumed that phospholipids did not contribute to the antigenic determinants of the Rh_o(D) antigen, but were required for proper orientation of the antigen within the erythrocyte membrane. Degradation of membrane phospholipids might have altered the native conformation of the Rh_o(D) antigen or the membrane environment necessary for the expression of the antigenic determinants at the outer membrane surface, resulting in the inability of these membranes to bind antibody. However, following solubilization, deoxycholate might have bound to the Rh_o(D) antigen in place of the degraded phospholipids and allowed the antigen to reassume its active conformation. Similarly, incorporation of solubilized, phospholipase-treated antigen into phosphatidylcholine liposomes would replace the missing phospholipids and allow Rh_o(D) antigen activity.

It was especially intriguing that in the

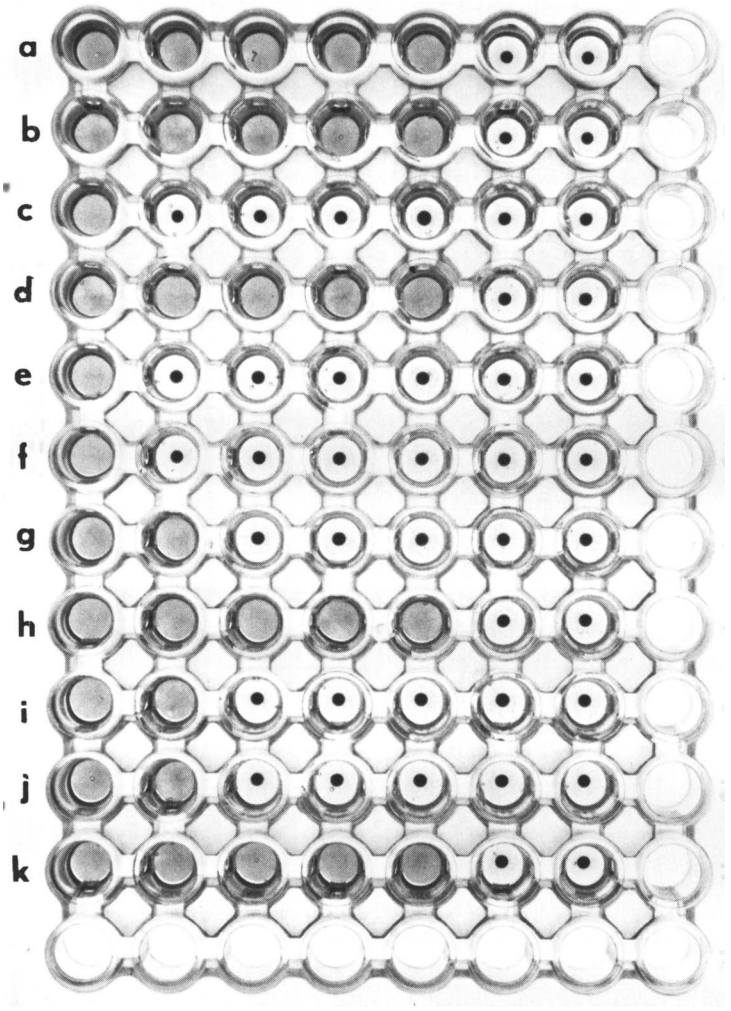

FIG. 2. Hemagglutination inhibition assay. Anti-$Rh_o(D)$ IgG was adsorbed with the following. Rows (a) nothing, (b) phospholipase-treated erythrocyte membranes, (c) untreated (control) erythrocyte membranes, (d) supernatant after centrifugation of phospholipase-treated membranes, (e) solubilized phospholipase-treated erythrocyte membranes, (f) solubilized untreated erythrocyte membranes, (g) liposomes containing erythrocyte membrane proteins, (h) liposomes containing only egg phosphatidylcholine, (i) partially purified $Rh_o(D)$ antigen, (j) phospholipase-treated partially purified $Rh_o(D)$ antigen, (k) phospholipase-treated membranes incubated with bovine serum albumin.

(D) antigen activity. Phosphatidylcholine alone was not nonspecifically inhibiting hemagglutination since protein-free liposomes did not inhibit agglutination (Fig. 2h).

These results indicated that the $Rh_o(D)$ antigen remained bound to the erythrocyte membrane following phospholipase treatment but could no longer bind anti-$Rh_o(D)$ IgG, until the membrane was solubilized. Since it has been reported that the free fatty acids released from phospholipids by phospholipase A_2 remained bound to the membrane (14), it seemed possible that these fatty acids might have been bound to the $Rh_o(D)$ antigen and interfered with the antigen–antibody reaction. Therefore, phospholipase-treated membranes were incubated with defatted bovine serum albu-

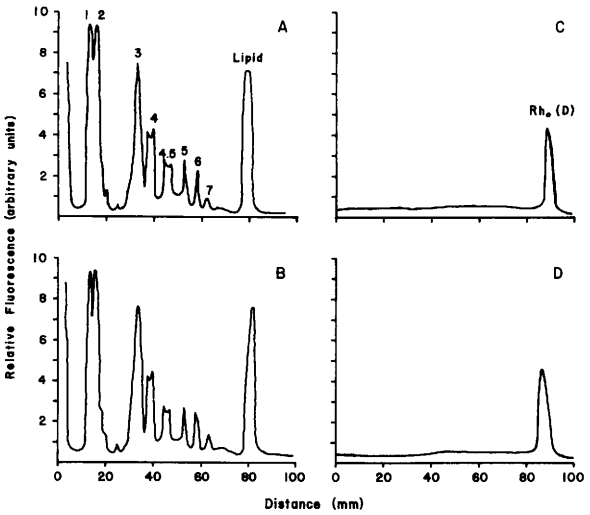

FIG. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of: (A) 50 μg of protein from untreated (control) erythrocyte membranes, (B) 50 μg of protein from phospholipase-treated erythrocyte membranes, (C) 10 μg of protein from partially purified Rh_o(D) antigen fractions, (D) 10 μg of protein from partially purified Rh_o(D) antigen fractions treated with phospholipase.

min, which has been shown to extract free fatty acids from phospholipase-treated membranes (14). However, as seen in Fig. 2k, incubation of treated membranes with 2 ml of phosphate-buffered saline containing 6% bovine serum albumin for 1 hr at 37° did not unmask the Rh_o(D) antigen activity.

The effect of phospholipase A_2 on other blood group antigens, besides the Rh_o(D) antigen, was studied to determine if they would also be inactivated. As seen in Fig. 4, incubation of membranes bearing the Rh(c) or Rh(E) antigens with phospholipase A_2 resulted in the loss of these antigen activities (Figs. 4c and g) compared to control membranes (Figs. 4b and f). However, like Rh_o(D) antigen activity, these antigen activities were also recovered following solubilization of phospholipase-treated membranes with sodium deoxycholate (Figs. 4d and h). Similarly, LW antigen activity was abolished by phospholipase A_2 (Fig. 4k), but was recovered following membrane solubilization (Fig. 4l). In contrast, blood groups A,B, and M were not affected by phospholipase A_2 since phospholipase-treated membranes retained as much antigenic activity (Fig. 5c, f, and i) as control membranes (Figs. 5b, e, and h). The N antigen activity of membranes was also not affected by phospholipase (results not shown).

Discussion. Hughes-Jones *et al.* have previously demonstrated that treatment of erythrocyte membranes with phospholipase A_2 resulted in loss of Rh(c), Rh(D), and Rh(e) antigen activities (3). Similarly, Green reported loss of membrane Rh_o(D) antigen activity following extraction of erythrocyte membranes with butanol, which could be partially regenerated by replacing phosphatidylcholine or phosphatidylethanolamine (1, 2). More recently, however, Litten *et al.* (7) and Plapp *et al.* (6) reported that phospholipase A_2 did not effect the activity of purified Rh_o(D) antigen. Our results confirmed these findings and provided a possible explanation for these seemingly conflicting results.

Phospholipase A_2 degraded phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (Fig. 1). This pattern of phospholipid hydrolysis was similar to the pattern obtained by Hughes-Jones *et al.* (3), Phospholipase A_2 was able to degrade the aminophospholipids on the inner surface of the erythrocyte membrane, as well as phosphatidylcholine on the outer surface, since it had access to both membrane surfaces following preparation of erythrocyte

membrane-bound state all three of the Rh antigens investigated, as well as the LW antigen, were inactivated by phospholipase A_2 while the A,B and M,N blood group antigens were not affected. The Rh antigens (4–7) and the LW antigen (4) appear to be composed predominantly of protein. However, the A and B antigens are glycolipids (14), while the M and N antigens are glycoproteins (15). In both the A,B and M,N antigenic groups the carbohydrate moieties contribute to antigenic expression. In view of these structural differences, the present results suggested that phospholipids play a more crucial role in maintaining the conformation of protein antigens in the membrane, such as the Rh and LW antigens, than the glycolipid and glycoprotein antigens. Furthermore, these results lend more support to our previous findings of a close structural relationship between the $Rh_o(D)$ antigen and the LW antigen (4).

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