LIPID-PROTEIN INTERACTIONS OF ERYTHROCYTE MEMBRANES: Comparison of Normal O,Rh(D) positive with the Rare O,Rh_{null}

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SUMMARY

Phospholipase A_2 modification of lipid-protein interactions of normal O,Rh(D) positive erythrocyte membranes increased the fluorescence intensity of the membrane bound probe, 1-anilinonaphthalene-8-sulfonate (ANS) and increased the N-1-[^{14}C]-ethyl maleimide ([^{14}C]-NEM) labeling of sulfhydryl groups in two proteins of molecular weight >200,000. In marked contrast, phospholipase A_2 modification of the rare phenotype O,Rh_{null} membranes resulted in no significant increase in ANS fluorescence or labeling of sulfhydryl groups by [^{14}C] NEM. Since the O,Rh_{null} erythrocytes demonstrated an increased osmotic fragility and decreased survival time, the fluorescence and sulfhydryl labeling data support the conclusion that hydrophobic bonding between β-fatty acid side chains and non-polar regions of asymmetric proteins is necessary for maintaining the native structure of the O,Rh(D) positive membrane. Comparative studies with phospholipase C or D implied that ionic bonding played a similar though less important structural role in both membranes.

INTRODUCTION

The erythrocyte membrane has served as a convenient model to study lipid-protein interactions of membranes. Chapman and Dodd (1) have recently reviewed the use of spectroscopic monitoring of chemically and enzymatically modified membranes to delineate the topography of membrane surfaces. Weidekamm *et al.* (2), using 1-anilinonaphthalene-8-sulfonate (ANS) to monitor phospholipase A_2 induced alterations of lipid-protein interactions of erythrocyte membranes, suggested that the architecture of some membrane proteins depends on the nature and state of membrane lipids. In contrast, circular dichroism, proton magnetic resonance measurements, and electron spin resonance studies of phospholipase C modified erythrocyte membranes suggested that removal of the phosphorylated amines of the phospholipids produced gross

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structural changes in the lipid regions of the membrane with no effect detectable on the conformation of the membrane proteins (3, 4).

Recently Senhauser *et al.* (5) reported an example of the phenotype Rh_{null} in which all known Rh-Hr determinants were absent. This rare phenotype was associated with a markedly shortened red cell life span in vivo as demonstrated by a persistently elevated reticulocyte count, increased incubated osmotic fragility, and a decrease erythrocyte survival time as determined by ^{51}Cr labeling. Thus the decreased survival time and increased osmotic fragility suggested a structural defect in the membrane of the O,Rh_{null} erythrocyte. This unique red cell offered an opportunity for the comparative assessment of lipid-protein interactions between phenotypically normal O,Rh(D) positive erythrocyte membranes and the membranes of erythrocytes apparently lacking these antigenic determinants. In order to evaluate whether lipid-protein interactions were altered in the O,Rh_{null} membrane, phospholipids of normal O,Rh(D) positive and O,Rh_{null} membranes were enzymatically modified with phospholipase A_2, C, or D. The effect of the phospholipase modification was monitored by fluorescence spectroscopy using ANS. ANS is an anionic probe which in aqueous solution exhibits a low intensity green emission (6). When the probe is in a non-polar environment, the emission is enhanced with a concomitant hypsochromic shift and a narrowing of the band width (7). These changes provide a means of following alteration of the native structure of the membrane induced by enzymatic modification of lipid-protein interactions.

MATERIALS AND METHODS

Membranes were prepared from washed O,Rh(D) positive and O,Rh_{null} erythrocytes (previously stored in standard ACD solution for 3 days at 4°C) by hypotonic lysis in 5 mM sodium phosphate buffer, pH 8 (8). The membranes were washed six times in 5 mM sodium phosphate buffer, pH 8. At each step of the washing procedure a small opaque cream-colored button was discarded after tilting the centrifuge tube and allowing the loose membrane

pellet to slide off the tightly packed button. This precaution minimized contamination of the membrane preparation with proteinases (9). Phospholipase A_2 was partially purified from *Naja naja* venom obtained from Sigma Chemical Co., St. Louis, Mo. (10). Phospholipase C (from *C. welchii*) and phospholipase D (from cabbage) were from Calbiochem Co., San Diego, Calif. Phospholipase A_2 modifications were performed as described by Weidekamm *et al.* (2). Modification of membrane preparations with phospholipase C or phospholipase D were as described by Eling and Di Augustine (11). Controls were treated in an identical fashion but without enzymes.

N-1-[^{14}C]-ethyl maleimide (New England Nuclear Corp., Boston, Mass.) was used for chemical modification of exposed sulfhydryl groups (12). The labeled membrane preparations were solubilized in 1% SDS and the disaggregated chains separated electrophoretically in SDS-polyacrylamide gels (9). The control and modified membranes were electrophoresed in duplicate, one gel was stained with Coomassie Brilliant Blue (9) and scanned with a Gilford Gel Scanner and the other sliced into 1 mm slices for determination of radioactivity (13).

The phospholipid composition of the extracted lipids from the modified and control membranes was determined by thin layer chromatography and quantitated by a micro phosphorus procedure (14).

Fluorescence was measured with an Aminco-Bowman spectrofluorimeter fitted with a 150-W Hanovia high pressure xenon lamp and an RCA 4472 photomultiplier. Excitation was at 365 nm with a band-pass of 20 nm, while emission was at 467 nm. Spectra were run from 400 to 650 nm at 27 C in 0.1 M sodium phosphate buffer, pH 7.4. ANS (Mg salt) was from Nutritional Biochemicals and was used without further purification. Solutions contained 2×10^{-6} M ANS, as determined from the molar absorptivity of 4.95×10^{3} M^{-1} cm^{-1} at 350 nm (15). Protein concentrations were determined by a modified micro Folin procedure and ranged from 0.25 to 0.55 mg per ml (16). Emission spectra for the modified and control membranes were run with and without ANS. The fluorescence intensity of the membrane-ANS complex was determined by sub-

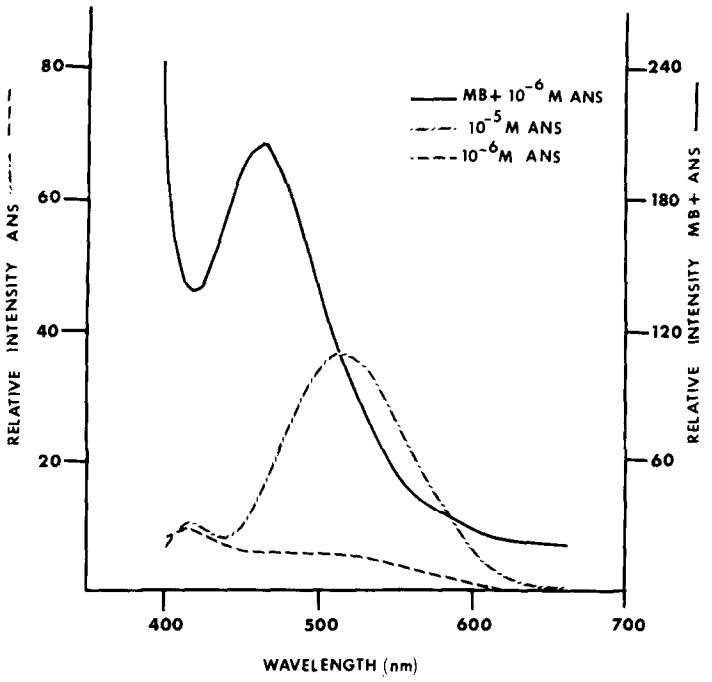

Figure 1. Fluorescence emission of ANS (10^{-5} and 10^{-6} M) and ANS (10^{-6} M) associated with O,Rh(D) positive erythrocyte membrane (MB) (0.39 mg protein/ml). Excitation at 365 nm. Excitation band width 20 nm. Spectra not corrected.

tracting the fluorescence intensity of the suspension without ANS from that with ANS, assuming a negligible contribution from the unbound dye. Each modified and control preparation was normalized to fluorescence intensity per mg of protein.

RESULTS

Typical spectra obtained using ANS are shown in Figure 1. In buffer, the dye fluoresces minimally; however, in the presence of the membrane suspension a large enhancement of fluorescence is observed. The contribution of unbound ANS was less than 5% as seen by comparing the fluorescence intensity at 467 nm of the ANS alone to that of the ANS-membrane complex.

Table 1 summarizes the effect of phospholipid modification on the interaction of ANS with O,Rh(D) positive and O,Rh_{null} erythrocyte membranes. Removal of the β-fatty acid from the phospholipids of the O,Rh(D) positive

Table 1. Effect of phospholipid modification on ANS interaction with O,Rh(D) positive and O,Rh_{null} erythrocyte membranes. Relative fluorescence was calculated as fluorescence intensity/mg protein as described in the text.

Treatment	O,Rh(D) positive		O,Rh_{null}	
	Rel. Fluorescence	% change	Rel. Fluorescence	% change
Control	71.1		79.5	
Phospholipase A_2	122.6	+72	71.5	-10
Control	60.1		88.8	
Phospholipase C	43.0	-28	48.5	-45
Control	54.3		68.0	
Phospholipase D	36.8	-32	41.3	-39

membranes by phospholipase A_2 resulted in a 72% increase in ANS-membrane fluorescence. In marked contrast, phospholipase A_2 modification of the O,Rh_{null} membranes resulted in a 10% decrease in ANS-membrane fluorescence. Phospholipase A_2 modification of the O,Rh(D) positive membranes resulted in conversion of 50 to 60% of the lecithin and 75 to 80% of the phosphatidyl ethanolamine and phosphatidyl serine to the corresponding lysophospholipids in agreement with the results of others (2). Phospholipase A_2 modification of the O,Rh_{null} membranes resulted in the formation of similar amounts of lysophospholipids; however, an increase in lysolecithin (approximately 10 to 15%) was observed in the control O,Rh_{null} preparation when compared with the control O,Rh(D) positive preparation (unpublished). Whether this may have resulted from intrinsic phospholipase A-like activity of the O,Rh_{null} membranes remains to be determined. Phospholipase C modification resulted in a 28% decrease in fluorescence intensity of the O,Rh(D) positive membranes and a 45% decrease for the O,Rh_{null} membranes. In both membrane preparations approximately 60 to 65% of the total phospholipid phosphorus was liberated as a consequence of phospholipase C modification. Phospholipase D modification resulted in decreases in fluorescence intensity of 32% and 39% for the

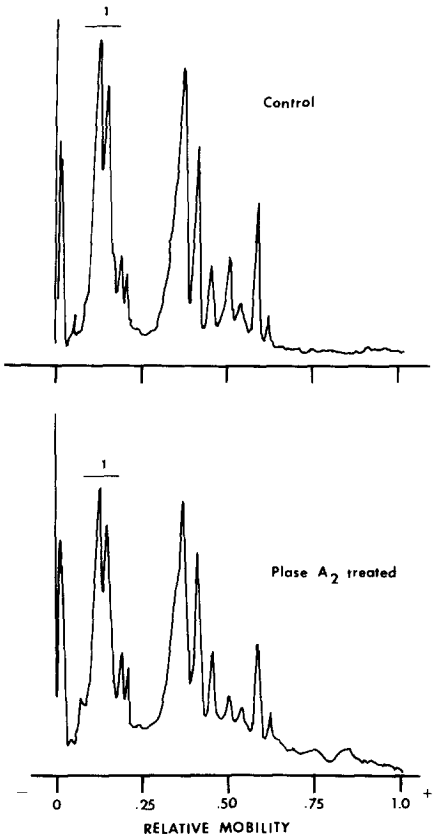

Figure 2. O,Rh(D) positive erythrocyte membrane polypeptides separated by SDS-gel electrophoresis. Control pattern (top) shows major polypeptides of native membrane. Plase A_2 treated pattern (bottom) shows polypeptides after modification of native membranes. Region 1 indicates polypeptides (molecular weight >200,000) labeled by [^{14}C]-NEM (see text).

O,Rh(D) positive and O,Rh_{null} membranes respectively. In both modified preparations, phosphatidic acid, the product of phospholipase D modification, increased approximately 40%. In a series of experiments not shown, phospholipase modification of A,Rh(D) positive membranes gave results similar to those observed with the O,Rh(D) positive membranes.

Disc-gel electrophoresis of SDS-solubilized membrane preparations was used to evaluate possible alterations of membrane proteins as a consequence of phospholipase modification. Typical densitometer tracings of the O,Rh(D) positive control and phospholipase A_2 modified preparation are shown in Figure 2. As shown, the control and modified preparations were identical with the excep-

tion of a diminished band at relative mobility of 0.5. Similar tracings were obtained with phospholipase C or phospholipase D modified preparations. In all cases the patterns obtained with the O,Rh_{null} controls and modified preparations were similar to those observed with the O,Rh(D) positive preparations. These results suggest that membrane proteins were not hydrolyzed by any proteolytic enzymes contaminating the phospholipase preparations and support the conclusion that changes in fluorescent intensity were the result of alterations in the lipid-protein interactions of the native membrane. Exposed sulfhydryl groups of control and modified membranes were labeled with [^{14}C]-NEM and subjected to SDS-gel electrophoresis. A twofold increase in labeling of two proteins with a molecular weight >200,000 (Figure 2) was observed for the phospholipase A_2 modified O,Rh(D) positive membranes. In marked contrast, phospholipase A_2 modification of O,Rh_{null} membranes resulted in no increase in sulfhydryl labeling with [^{14}C]-NEM. No significant increase in incorporation of label was observed for the phospholipase C or phospholipase D modified O,Rh(D) positive membranes. The results with the O,Rh(D) positive membranes are in agreement with those of others who used NEM in electron spin resonance studies of phospholipase A_2 and phospholipase C modified erythrocyte membranes (4, 17).

DISCUSSION

Recent studies which have demonstrated the importance of sulfhydryl groups (18) and phospholipid (19) for Rh activity strongly suggest that the Rh antigen is lipoprotein in nature. The present study provides information on the nature of the lipid-protein interactions.

Phospholipase C or D modification of either O,Rh(D) positive and O,Rh_{null} membranes produced similar changes in ANS fluorescence and sulfhydryl labeling indicating similar phospholipid-protein ionic bonding for both membrane types. Further, these results imply that the Rh antigen does not interact with either moiety cleaved by phospholipase C or D in a structurally important manner.

The fluorescence data (72% increase in ANS-membrane fluorescence) suggest that the O,Rh(D) positive membrane assumes a more "open" conformation resulting in increased binding of ANS following phopholipase A_2 modification in agreement with Weidekamm *et al.* (2). This increased binding could result from removal of β-fatty acids passively covering ANS binding sites or from alteration of β-fatty acid-protein interactions involved in maintaining native membrane structure. However, the [^{14}C]-NEM studies which showed increased sulfhydryl exposure of two specific proteins would favor the latter alternative. The interaction of β-fatty acids with asymmetric regions of protein in the membrane supports the fluid mosaic model recently proposed by Singer *et al.* (20).

In contrast, phospholipase A_2 modification of the O,Rh_{null} erythrocyte membranes failed to demonstrate this increased ANS-membrane fluorescence and the increased labeling of the two proteins by [^{14}C]-NEM. Since the electrophoretic patterns and the extent of phospholipase A_2 modification (as measured by lysophospholipid formation) were similar for both preparations, our data suggest basic differences in the β-fatty acid-protein interactions maintaining native membrane structure of normal erythrocyte membranes carrying the Rh-Hr antigenic determinants and the O,Rh_{null} membranes, which lack such determinants.

Levine *et al* (21) have suggested that the synthesis of normal Rh components is suppressed in Rh_{null} cells. More recently Levine *et al* (22) have posed a model which assumes that the Rh antigen is absent, although not stating whether structurally or immunologically so, and that this absence contributes to the anemia associated with Rh_{null} cells. However, in our studies no major differences in structural components were observed in the SDS-gel electrophoretic patterns of O,Rh(D) positive and O,Rh_{null} membranes. Whether the Rh antigen is present in Rh_{null} cells but immunologically unreactive because of an altered conformation in the membrane rests on further physical biochemical studies. In contrast to the conclusion of Nicolson *et al* (23) the present studies tend to support a structural role for the Rh-Hr antigenic determinants and may in

part explain the decreased *in vivo* erythrocyte survival often associated with the absence of these antigenic determinants (5,22,24). Thus the Rh_{null} phenotype may represent one example of a class of diseases having a structural basis *not* due to addition or deletion from the membrane but to an altered conformation within the membrane.

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