Rh_{null} human erythrocytes have an abnormal membrane phospholipid organization

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1. Rh_{null} human erythrocytes lack the antigens of the Rhesus blood group system, have an abnormal shape and an increased osmotic fragility, and are associated with mild chronic haemolytic anaemia. 2. Studies with phospholipase A₂ and sphingomyelinase C show that the asymmetric distribution of phosphatidylethanolamine (PtdEtn) in the membrane of these cells differs from that found in control cells. The amount of PtdEtn which can be hydrolysed by phospholipase A₂ in the presence of sphingomyelinase C in intact Rh_{null} cells is twice as high as that in normal erythrocytes. 3. In intact Rh_{null} cells all of the phosphatidylcholine (PtdCho) present in the membrane can be readily exchanged with a PtdCho-specific exchange protein, whereas in control cells 75% is readily exchanged and 25% at a much lower rate. This indicates that PtdCho experiences a relatively fast transbilayer movement in the Rh_{null} cells. 4. The observation that the loss of two membrane polypeptides in the Rh_{null} cells leads to abnormal shape, increased osmotic fragility, abnormal PtdEtn distribution and enhanced transbilayer mobility of PtdCho strongly suggests that one or both polypeptides are essential for the maintenance of a proper membrane-membrane skeleton interaction.

In the normal erythrocyte the aminophospholipids are preferentially located in the inner, cytoplasmic layer of the membrane. Evidence is accumulating that erythrocytes that have an abnormal shape as a consequence of alterations in the membrane skeleton also have an abnormal distribution of phospholipids between the two layers of the lipid membrane. In particular, the aminophospholipids have been found in increased amounts in the outer layer. This occurs in sickle cell erythrocytes (Lubin *et al.*, 1981) and spherocytotic erythrocytes (Lubin & Chiu, 1982).

It has recently been observed that when abnormal phospholipid localization occurs, the rate of transbilayer movement of phospholipids is enhanced (Rossi *et al.*, 1982; Franck *et al.*, 1983; Haest, 1982). These observations suggest that an intact membrane skeleton is essential for the maintenance of phospholipid asymmetry in the

erythrocyte. There is evidence that peripheral proteins like spectrin (Mombers *et al.*, 1979) and band 4.1 (Sato & Ohnishi, 1983) can interact directly with phospholipids present in the cytoplasmic leaflet. It is also possible that intrinsic membrane proteins are involved in stabilization of the lipid layer, especially when a direct interaction between these proteins and the membrane skeleton occurs.

Erythrocytes which lack all the antigens of the human Rhesus blood group system have been described by Vos *et al.* (1961), and are denoted Rh_{null} erythrocytes. These cells have an abnormal stomatocytic shape, and show increased osmotic fragility (Sturgeon, 1970). This syndrome is associated with mild chronic haemolytic anaemia. Two membrane polypeptides are apparently absent from Rh_{null} cells. One corresponds to the Rh(D) polypeptide and the other to the R6A binding polypeptide (Ridgwell *et al.*, 1983). Recent evidence suggests that at least one of these polypeptides, the Rh(D) polypeptide, is a trans-membrane protein which appears to be associated

Abbreviations used: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

with the erythrocyte membrane skeleton (Ridgwell *et al.*, 1984). The observation that a shape change occurs in these Rh_{null} erythrocytes prompted us to investigate the organization of the phospholipids of these membranes. The results suggest that the absence of two intrinsic membrane polypeptides has a marked effect on the organization of the lipids in the Rh_{null} erythrocyte membrane.

Materials and methods

Erythrocytes

Rh_{null} erythrocytes from donor A.L., and control normal erythrocytes taken at the same time, were kindly provided by Mr. J. Moulds (Gamma Biologicals, Houston, TX, U.S.A.). Rh_{null} erythrocytes from donor Y.T. were kindly provided by Dr. P. Harden (Red Cross Blood Transfusion Service, Brisbane, Australia). A corresponding age-matched control was obtained from the South West Regional Blood Transfusion Service, Bristol, U.K.

Methods

Osmotic fragility was determined as previously described (Kuypers *et al.*, 1984). Erythrocytes were isolated by centrifugation for 10 min at 2500g and washed three times with 4 vol. of 0.9% NaCl. Cells were suspended in a buffer containing 10 mM-Tris/HCl (pH 7.5), 10 mM- $CaCl_2$, 0.25 mM- $MgCl_2$ and 0.9% NaCl for phospholipase incubations. For the phosphatidylcholine exchange experiments the buffer contained 150 mM-NaCl, 25 mM-glucose, 1 mM-EDTA, 100 i.u. of penicillin/ml, 100 μ g of streptomycin/ml and 10 mM-Tris/HCl, pH 7.4. Incubations with phospholipase A_2 from *Naja naja* and sphingomyelinase C from *Staphylococcus aureus* were carried out as described previously (van Meer *et al.*, 1981). Following the incubations, cells were extracted (Rose & Oklander, 1965), and phospholipids were analysed by t.l.c. (Broekhuysen, 1969) and phosphate analysis (Rouser *et al.*, 1970). Exchange experiments were carried out as de-

scribed previously, using [^{14}C]PtdCho-labelled microsomes from rat liver as the donor system (van Meer & Op den Kamp, 1982).

Results

Fig. 1 shows that the Rh_{null} cells from donor Y.T. are osmotically fragile; 50% haemolysis is observed at 52% iso-osmolality, whereas in control cells this extent of haemolysis is found at 44% iso-osmolality.

Incubations of Rh_{null} cells with phospholipase A_2 from *N. naja* resulted in the hydrolysis of PtdCho as shown in Table 1. The extent of hydrolysis appeared to be somewhat lower in

Fig. 1. Osmotic fragility of Rh_{null} erythrocytes. A continuous dilution procedure (Kuypers *et al.*, 1984) was used to measure osmotic fragility of normal ($\circ$) and Rh_{null} ($\square$) erythrocytes. The data are plotted as percentage haemolysis against the osmolality, expressed as a percentage of iso-osmolality (150 mosm).

Table 1. Treatment of normal and Rh_{null} human erythrocytes with phospholipase A_2 and sphingomyelinase C. Control erythrocytes from two donors (control 1 and control 2) and Rh_{null} erythrocytes (donor Y.T.) were treated with phospholipase A_2 and sphingomyelinase C. The Table shows the percentage of the total membrane PtdCho, PtdEtn, PtdSer and sphingomyelin hydrolysed.

Erythrocytes	Enzyme	Phospholipid hydrolysed (%)			
		PtdCho	PtdEtn	PtdSer	Sphingomyelin
Control 1	Phospholipase A_2	67.4	—	—	—
Control 2	Phospholipase A_2	62.7	6	—	—
Control 1	Phospholipase A_2 + sphingomyelinase C	83.0	21.6	—	80.0
Control 2	Phospholipase A_2 + sphingomyelinase C	80.2	25.4	—	81.7
Rh_{null}	Phospholipase A_2	56.5, 46.3	—	—	0, 2
Rh_{null}	Phospholipase A_2 + sphingomyelinase C	79.0, 76.9	43.8, 45.7	?	78.0, 77.9

comparison with control cells, but subsequent incubations with sphingomyelinase C from *S. aureus* showed that in the variant as much PtdCho is present in the outer layer of the membrane as in control cells. The amount of sphingomyelin present in the outer layer is also not changed. However, a difference is found in the accessibility of PtdEtn in the two cell types. Whereas in control cells around 20–25% of the PtdEtn is hydrolysed in intact cells, 40–45% of this lipid is accessible for enzymic attack in Rh_{null} cells. The data are corrected for hydrolysis occurring in lysed cells. In control cells and variant cells incubated with phospholipase A₂ alone, haemolysis is limited to 2–3%, but on prolonged incubation of the variant cells with both phospholipase A₂ and sphingomyelinase C together, haemolysis up to 15% is observed. This in itself suggests the presence of a modified and probably weakened structure in the Rh_{null} erythrocyte membrane. The data on PtdSer hydrolysis are not reliable because of haemolysis; a limited but variable extent of hydrolysis of this phospholipid was observed.

The membrane of human erythrocytes has a characteristically slow transbilayer movement of phospholipids (van Meer & Op den Kamp, 1982). Experiments with a PtdCho-specific exchange protein show that in control normal cells two pools of PtdCho occur (Fig. 2). Of the PtdCho, 75% is readily exchangeable and is located in the outer layer of the membrane. The remaining PtdCho (25%) present in the inner layer is not exchange-

able or exchanges at a much slower rate. In contrast, all of the PtdCho in the Rh_{null} cells is readily exchangeable. This suggests that the transbilayer movement of PtdCho is much faster in Rh_{null} cells than normal cells.

Discussion

The two polypeptides that are missing in the membrane of Rh_{null} erythrocytes probably have a structural or membrane-stabilizing function, since their absence leads to increased fragility and partial deregulation of phospholipid asymmetry and transbilayer movement, and the condition is associated with mild haemolytic anaemia.

It is clear from previous studies (Ridgwell *et al.*, 1983) that both the Rh(D) and R6A binding polypeptides are exposed to the outside because both are red cell antigens and also their thiol groups are accessible for labelling at the outside of intact erythrocytes. The Rh(D) polypeptide remains associated with the membrane skeleton (Ridgwell *et al.*, 1984). This polypeptide, at least, spans the erythrocyte membrane. Until now, membrane destabilization of the type observed here (increased osmotic fragility together with enhanced phospholipid transbilayer movement and loss of phospholipid asymmetry) has been noted either in association with a modification of the skeleton protein network underlying the membrane (Franck *et al.*, 1983; Haest, 1982), or when the membrane skeleton is uncoupled from the membrane (P. F. H. Franck, E. M. Bevers, B. Lubin, P. Comfrurius, D. T.-Y. Chiu, J. A. F. Op den Kamp, B. Roelofsen, R. F. A. Zwaal & L. L. M. van Deenen, unpublished work). En(a⁻) human erythrocytes which lack the major sialoglycoprotein (α , syn.: glycophorin A), do not show a loss of phospholipid asymmetry (van Meer *et al.*, 1981).

These results suggest that the coupling of the Rh(D) polypeptide (and perhaps the R6A binding polypeptide) has a significant role in maintaining the stability and phospholipid asymmetry of the erythrocyte membrane.

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Fig. 2. Exchange of erythrocyte PtdCho

Cells were incubated with exchange protein and microsomal membranes as PtdCho donor. The extent of PtdCho exchanged, expressed as a percentage of the total erythrocyte PtdCho present, was determined at various time points for normal (○) and Rh_{null} (×) cells. The horizontal broken line shows the amount (75%) of readily exchangeable PtdCho in normal cells.

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