Different Protein-Lipid Interaction in Human Red Blood Cell Membrane of Rh Positive and Rh Negative Blood Compared with Rh_{null}

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1-anilino-naphthalene-8-sulfonate (ANS) fluorescence measurements have revealed that red blood cell membrane of the Rh_{null} type undergoes a transition at about 16 °C. In contrast, viscosity measurements of the extracted membrane lipids showed the usually observed transition at about 18 °C.

Lower values of titratable sulfhydryl (SH) groups were observed in Rh_{null} membrane using 5,5′-dithiobis-(2-nitro-benzoic-acid) (Nbs_2). In contrast, disulfide bonds in Rh_{null} membrane were estimated to be about 3 times the value of the controls.

Spin labeling experiments using 2-(3-carboxypropyl)-4, 4 dimethyl-2-tridecyl 3-oxazolidinyl-oxyl were carried out with phospholipase A_2 modified membranes. The mobile part of the spectra was significantly increased on the Rh_{null} membrane.

In the presence of D-glucose, infrared spectrometry showed a larger reduction of the intensity of the POO-band in Rh_{null} membrane.

In contrast to controls, binding of the reagent diethylpyrocarbonate resulted in no significant changes of the Rh_{null} membrane as determined by electron spin resonance (ESR) measurements.

D-glucose transport activity was found to be at the upper level of a group of Rh positive and Rh negative persons.

It is suggested that the intensity of the polar protein-lipid interaction is reduced in Rh_{null} membrane.

Introduction

The rhesus antigens belong to the most important antigens in human red blood cells and still cause diverse problems in biochemical characterization. The difficulties compared to other antigens of the red cell membrane are probably caused by still unidentified protein-lipid interactions of these antigens [1–4]. To obtain a better understanding of the Rh antigenicity, a blood which lacks all detectable Rh antigens (D, C, c, C^w, E, e, including the LW-factor), the so-called Rh_{null} blood [5–7], was taken for this investigation. In most of the Rh_{null} cases, a mild hemolytic anemia can be observed [8]. The decreased erythrocyte survival as determined by Cr^{51} labeling is accompanied by an increased osmotic fragility and an increased potassium transport [9–13]. From these data, it can be concluded that the red blood cell membrane is altered in some yet unknown way. Furthermore, sulfhydryl (SH) groups in erythrocyte membrane can be demonstrated to be in a close relationship to sodium and potassium active transport [14], Rh antigenicity [15–17] and protection against hemolysis [4]. No connection could be found to the syndrome of congenital absence of reduced glutathione (GSH), since GSH values were similar in Rh_{null} and controls [9].

It has been demonstrated previously by Smith *et al.* [18] that in contrast to the controls the Rh_{null} erythrocyte membrane showed no significant change in SH group reactivity and ANS-fluorescence after phospholipase A_2 treatment. To study the relation between the activity of Rh antigens, protein-lipid interaction and sulfhydryl groups, these investigations were carried out.

Preliminary results have been reported elsewhere [11, 12].

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Materials and Methods

1-anilinonaphthalene-8-sulfonate (ANS) (Mg-salt) and 5,5′-dithiobis-(2-nitro-benzoic-acid), Nbs_2, were obtained from Serva, Heidelberg, while diethylpyrocarbonate was purchased from Merck-Schuchardt, Hohenbrunn/München. Phospholipase A_2 EC 3.1.1.4, purified from the snake venom of Crotalus, was purchased from Boehringer, Mannheim. ESR spin labels were obtained from Syva Associates, Palo Alto, Calif., USA. All other reagents were of the highest purity available. Freshly drawn ACD blood from Rh positive and Rh negative, healthy volunteers and two persons with the Rh_{null} syndrome was used in this study. The serological data including further details of the investigated Rh_{null} blood have been reported previously [10, 11].

Preparation of red cell membrane

The intact red cells were washed three times with 0.9% NaCl and the buffy coats were carefully removed. Membranes were prepared by lysis of the concentrated erythrocytes over 24 h with 10 volumes of 15 mM sodium phosphate buffer (pH 7.5) using the washing procedure of Dodge *et al.* [19]. The membranes were washed first in the described 15 mM buffer until the supernatant became clear and then in 10 mM sodium phosphate buffer (pH 7.5).

According to Fairbanks *et al.* [20], special care was taken at each step of the washing procedure in removing all of the yellow-red precipitate. The white membranes were finally suspended in water, frozen over night, centrifuged once more and lyophilized. The membranes were stored below − 20 °C. The protein concentrations estimated by the method of Lowry *et al.* [21] were about 0.5 mg of protein per mg membrane.

ANS Fluorescence measurements

Fluorescence measurements were generally carried out as described previously [22]. 2 mg of the membrane preparation was suspended in 9 ml 0.9% NaCl. Thereafter, sonication was performed by a Branson S-75 sonifier four times for 15 sec and at 3 amperes output. During the sonication, the temperature was kept between 2 °C and 12 °C. Then 1 ml of 0.1 M sodium phosphate buffer (pH 7.2) was added. Three ml of that suspension was mixed with 0.2 ml of an ANS solution (10^{-3} M in 0.9% NaCl). Fluorescence excitation was obtained at 380 nm, emission at 462 nm. During the single steps of measurement, the temperature was held constant within ± 0.2 °C. Measurements were performed in a Farrand spectrofluorometer type Mark II.

Viscosity measurements of extracted lipids

Viscosity measurements of extracted lipids were carried out in an Ostwald capillary viscosimeter with a water flow speed of 60 s at 20 °C and performed as described previously [23]. Lipid extraction was based on the procedure of Dawson *et al.* [24]. Total lipid and cholesterol determinations were carried out using the given methods [25, 26].

SH group determinations

4 mg of the membrane was suspended for 40 min in 1 ml 0.1 M NaCl solution with sodium dodecylsulfate (SDS) in the ratio 1 : 1 w/w for protein: SDS. Thereafter, 5 ml 0.1 M NaCl and 1 ml of 0.1 M phosphate buffer (pH 8.1) were added.

In cases of membrane suspensions without SDS, 10 mg of red cell membrane was given into 10 ml 0.9% NaCl/0.01 M phosphate buffer solution (pH 8.1). Subsequently membranes were dispersed by means of a Potter-Elvehjem homogenizer.

Performing SH group determination, 3 ml of the suspension was mixed with 0.1 ml of a 10 mM solution of 5,5′-dithiobis-(2-nitro-benzoic-acid) (Nbs_2) in 0.1 M phosphate buffer (pH 7.5) according to the method of Ellman [27]. References were treated similarly, but without Nbs_2. Absorbancy at 412 nm was measured in a Zeiss PMQ II spectrophotometer after 80 min incubation time. In cases of determinations performed in the presence of urea, 8 M concentration of this reagent was added just before SH measurement.

S-S group determination

Reduction of disulfide bonds was performed with SDS treated membranes in presence of 8 M urea at 37 °C for 60 min, following the method of Brown [28]. Excess $NaBH_4$ was destroyed by addition of acetic acid until the pH of 8.1 was reached.

Infrared spectroscopy

5 mg of red cell membrane was homogenized in a Potter-Elvehjem homogenizer. The initial pH of the

suspension was about 6.7. The membranes were then centrifuged for 10 min at 20000 × *g* and the pellet was resuspended in 1 ml of water containing 78 mM NaCl, 17.4 mM sodium docecylsulfate and 3.15 mM D-glucose and was incubated at 25 °C for 30 min [29]. Controls were treated by a similar medium containing no glucose. After the incubation period, the suspension was dialysed for 6 h at 5 °C against water containing a few drops of NH_4OH at a pH of about 8. The material was then lyophilized. After drying, KBr infrared spectra were performed.

A slightly different method was used in sample preparation with diethylpyrocarbonate. After homogenization of 10 mg membrane in 10 ml 0.9% NaCl, diethylpyrocarbonate was added to a final concentration of 1 mM. The pH value during this reaction decreased to about 5.5–5.6 within about 25 min. After 30 min incubation time at 23 °C, the membrane suspension was centrifuged as described above. Thereafter, the pellet was suspended with 0.25 ml 1% SDS-solution, which contained 0.4 µl 1:10 dilution of diethylpyrocarbonate. The protein precipitated during this procedure. After homogenization of the pellet, 1.25 ml of 0.9% NaCl, containing 1.85 µl of 1:10 dilution of diethylcarbonate was added and the suspension was lyophilized. The dry substance was suspended in 1 ml distilled water and dialysed for 6 h against water containing 1 mM final concentration of diethylpyrocarbonate. After dialysis, KBr was added and the sample dried over P_2O_5. Controls were treated similarly but without addition of diethylpyrocarbonate.

Infrared spectra were recorded on a standard Perkin Elmer spectralphotometer, type 521.

Phospholipase A_2 treatment of membranes

Phospholipase A_2 (EC 3.1.1.4) was dissolved according to Simpkins *et al.* [30] in 0.01 M citrate buffer (pH 5.5) at a concentration of 0.1 mg/ml, heated to 100 °C for 10 min and filtered two times through a Millipore filter (0.22 µ). Thereafter, the pH was adjusted with 0.05 M Na_2HPO_4 to pH 7.4, and 0.2 M $CaCl_2$ was added to make the enzyme solution 2 mM in Ca^{2+} ion. The final enzyme solution was found to possess no proteolytic activity [30, 31]. Polyacrylamide gel electrophoresis pattern of enzyme treated membranes revealed no changes as compared to controls. 10 mg of RBC membranes were homogenized in 10 ml 0.1 M Tris/HCL (pH 7.4) and 1 mM $CaCl_2$ with a Potter-Elvehjem homogenizer and incubated at 37 °C for 30, 60 and 120 min with phospholipase A_2 at a concentration of 5 µg enzyme protein to 200 µg membrane protein. The reaction was stopped by centrifugation at 20000 × *g*. The sediment was washed with 0.1 M Tris/HCL (pH 7.4) and then twice with 20 mOsm. phosphate buffer (pH 7.4). Thereafter spin labeling with the label 2-(3-Carboxypropyl)-4, 4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (No. 618, Syva) was performed. Controls were treated identically but without enzyme.

Thin layer chromatography of membrane lipids

Aliquots of the phospholipase A_2 treated membrane suspension were analysed for lipids by thin layer chromatography according to the method of Op den Kamp *et al.* [32] and Goerke *et al.* [33]. Silica gel 60 F_{254} TLC-plates were obtained from Merck, Darmstadt.

Gradient SDS-gel electrophoresis

Gradient slab gel electrophoresis was performed as previously described with 5–15% polyacrylamide gel [34]. About 0.11 mg protein (not heated) was applied to the gels. Electrophoresis was carried out with 30 mA constant current for about 2 h. Staining was performed with Coomassie blue. Absorbance of the gels was measured with a Zeiss PMQ II spectrophotometer at 560 nm.

Diethylpyrocarbonate treatment of membranes

Treatment of the homogenized red cell membranes (10 mg membrane/10 ml 0.9% NaCl) with diethylpyrocarbonate at a final concentration of 1 mM was carried out for 30 min at 24 °C [35]. Thereafter, the pH was adjusted to 6.75 by means of addition of sodium phosphate buffer to a final concentration of 0.01 M. Subsequently the membranes were centrifuged and spin labeled with the fatty acid label (No. 618, Syva).

Recording of ESR spectra

Spectra were recorded on a Bruker B-MN, 155-45 Sl 6 after intense mixing of 0.5 µl of the spin label (10 mM solution of label No. 618 in C_2H_5OH) with the centrifuged membrane (containing about

0.5 mg protein) suspended in 100 µl of respectively the last used buffer.

Spin labeling of homogenized membranes with the protein spin labels 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl and 3-((2-(2-maleimidoethoxy)ethyl)carbamoyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (Nos. 111 and 114, Syva) was performed by incubating the homogenized membranes with the label over 3 h according to Simpkins *et al.* [30].

Glucose transport measurements

D-glucose transport activity measurements were performed by the method described by Lacko *et al.* [36, 37]. The erythrocytes were preloaded with 200 mM glucose. The incubation was carried out for five seconds at 20 °C in isotonic phosphate buffer (pH 7.5) at five different [C^{14}]glucose concentrations between 0.5 and 0.25 mM.

Results

ANS fluorescence measurements of red cell membranes over a temperature range reveal a discontinuity similar to that observed with other methods [38–42]. We estimated the fluorescence change at temperatures between 12 °C and 28 °C. The control membrane showed a clear discontinuity which occurred in a range of 16–20 °C with a midpoint at 18 °C (Fig. 1). In comparison, ANS fluorescence of Rh_{null} membrane showed a short discontinuity between 15 °C and 17 °C with a midpoint at 16 °C (Fig. 1). We were interested to find out whether these differences might be also found in the extracted membrane lipids, as observed by viscosity measurements over a similar temperature range [23]. Contrary to the results in red cell membrane, this method yielded a complete congruence in transition temperatures at about 18 °C in the lipids of Rh_{null} and the controls. Thin layer chromatography of the membrane phospholipids did not reveal differences between Rh_{null} and controls. Also, we did not find any significant change in the cholesterol contents of Rh_{null} versus control membranes. Moreover, Sturgeon [9] has reported that the phospholipid content was not significantly changed in Rh_{null} membrane. Thus, there are no apparent changes in the lipid contents of the membranes per se.

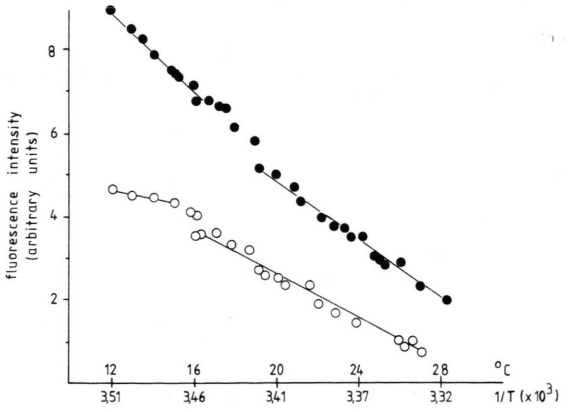

Fig. 1. Fluorometric measurements of Rh pos. and Rh neg. membranes (solid circles) and Rh_{null} membranes (open circles) with 1-anilino-naphthalene-8-sulfonate (ANS) at 390 nm excitation and 465 nm emission.

Table I. SH group determination of SDS treated red blood cell membranes with 5,5′-dithiobis-(2-nitrobenzoic-acid) (Nbs_2). Values in nmol SH/mg protein. Determination in absence and presence of 8 M urea.

	Without urea	With urea	With urea and $NaBH_4$
Rh_{null}	$46.7 \pm 1.2_{(n=4)}$	$52.5_{n=2}$	$69.1 \pm 6.2_{(n=4)}$
Controls	$55.3 \pm 3.0_{(n=6)}$	$58.3_{n=3}$	$63.4 \pm 6.8_{(n=4)}$

Subsequently, we started investigations on the protein part of the Rh_{null} membrane. SDS-gradient gel electrophoresis of both the membrane types did not reveal appreciable differences in the Coomassie blue stained protein pattern (not shown). Rh antigenicity, however, has been found to be dependent on membrane SH-groups [15–17]. Consequently, SH-group determinations of membranes were performed. In Table I, lower values of Rh_{null} membrane SH group contents are shown compared to the control values. Unfolding of the membrane proteins by 8 M urea still showed lower values for the Rh_{null} membrane.

Disulfide bonds were determined after reduction of membrane SH-groups with sodium borohydride ($NaBH_4$) in SDS and urea. It was found that the amount of disulfide bonds in Rh_{null} membrane was about 3 times as high as compared to the controls (Table I).

Evidently, there is some change in the membrane proteins which, however, cannot be easily detected with a conventional SDS gel electrophoresis method

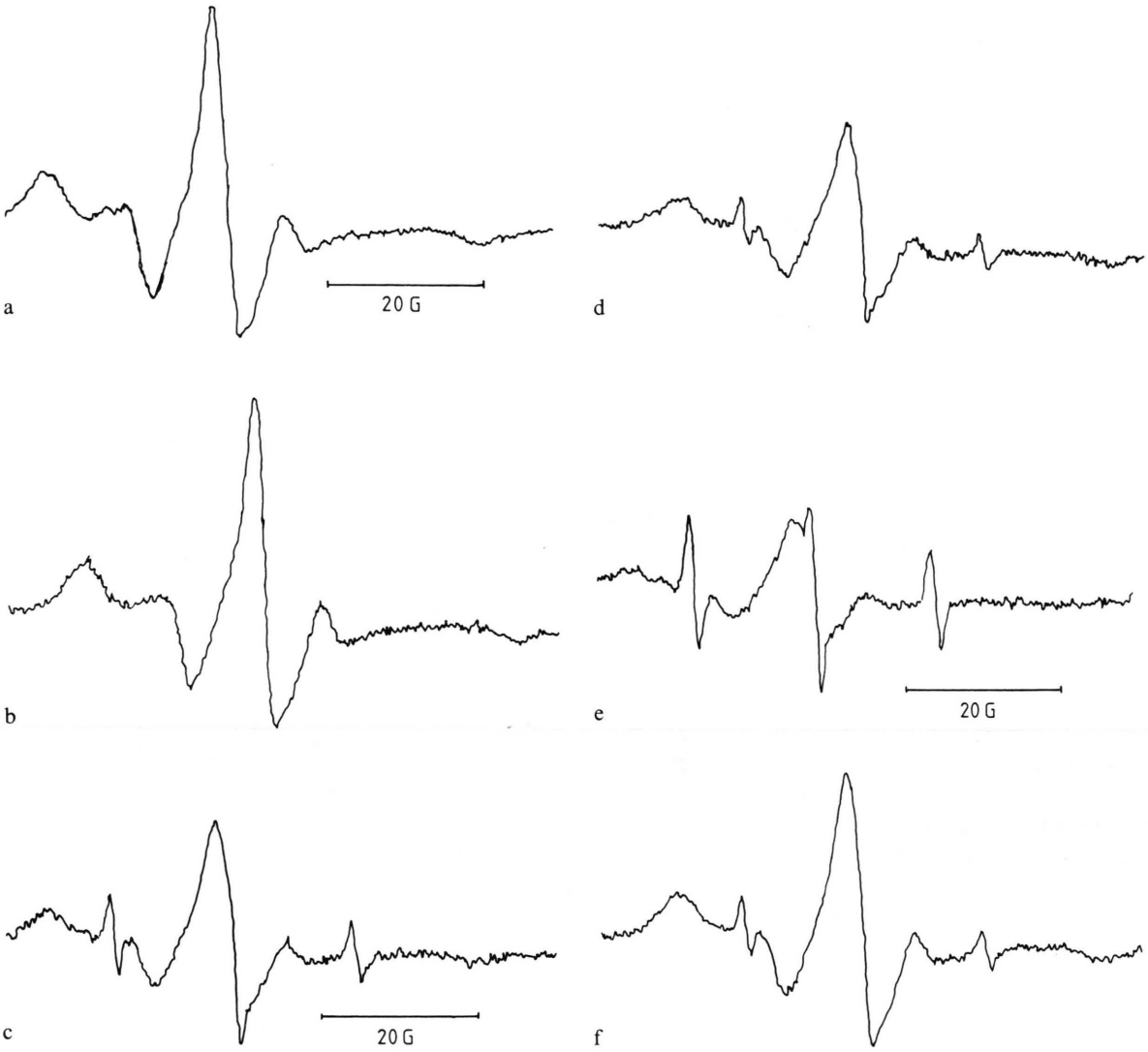

Fig. 2. ESR spectra of lyophilized red blood cell membranes with the lipid spin label (No. 618, Syva) after phospholipase A_2 treatment. Fig. a, c and e represent the spectra of Rh_{null} membranes, Fig. b, d and f the controls. Time of phospholipase treatment: Fig. a and b 30 min, Fig. c and d 60 min, Fig. e and f 120 min.

[18]; this was also proven by our own experiences. Thus, there are small changes in the membrane proteins, which should be followed up by other methods.

At this stage of the investigation, different spectroscopic methods were applied, owing to their sensitivity to small changes in membrane structure. Protein spin labels were used first, because of obvious changes observed in the titratable SH-groups. Labelling with maleimide spin labels (No. 111 and 114, Syva), however, did not yield appreciable differences between controls and Rh_{null} membrane. After the use of phospholipase A_2 in membrane treatment, no differences were observed in the ESR spectra of maleimide spin labeled membranes.

Finally, lipid spin labeling using the stearic acid spin label No. 618 (Syva) resulted in the spectra presented in Fig. 2a–f. In contrast to the controls the mobile components of the spectrum of the Rh_{null} membrane become progressively increased during phospholipase A_2 treatment. This effect was

Table II. Order parameter S and parameter A/B of the ESR spectra of human erythrocyte membranes with the spin label No. 618 (Syva) in absence and presence of diethylpyrocarbonate. Controls are compared with Rh_{null}. Significant different values of order parameter S ($p < 0.01$) are: controls compared with Rh_{null}; controls compared with those in presence of diethylpyrocarbonate.

		Membranes without diethylpyro-carbonate	Membranes with diethylpyro-carbonate (1 mM)
Rh_{null} ($n = 3$)	A/B	1.132 ± 0.003	1.130 ± 0.001
	S	0.7085 ± 0.0004	0.7095 ± 0.0028
Controls ($n = 6$)	A/B	1.123 ± 0.003	1.159 ± 0.012
	S	0.7026 ± 0.0009	0.7134 ± 0.0026

obtained in both lyophilized membrane and freshly isolated membrane specimen.

In experiments using the reagent diethylpyrocarbonate, the same lipid spin label has been applied in both types of red cell membrane. Studies with this reagent have been carried out previously [35]. From these experiments it was proposed that histidine-imidazole or lysine residues interacting with lipid phosphate may be modified by the reagent, resulting in an alteration of polar lipid-protein interaction.

Congruently, our present results show, that an increase in order parameter S [43] of the lipid polar head group region occurred in the presence of diethylpyrocarbonate (Table II). In contrast, no change in the ESR spectral parameter was found with diethylpyrocarbonate treated Rh_{null} membrane. In addition, measurements of the parameter A/B according to the denotification of Butterfield *et al.* [44] have revealed that this ratio is slightly increased in untreated Rh_{null} membrane. The ratios A/B as well as the order parameters of Rh_{null} and Rh_{null} modified by diethylpyrocarbonate are thus in between ratios and order parameters of the controls and the controls modified by diethylpyrocarbonate.

Infrared spectroscopy of both the membrane types did not show any clear differences (Fig. 3a and b). As previously reported [29], however, loading of the membrane with D-glucose results in a distinct intensity reduction of the POO^- band at 1200–1250 cm^{-1}. This effect is more evident in the Rh_{null} membrane (Fig. 3c and d). The glucose transport activity in Rh_{null} blood approached values close to the highest values found for 12 control persons (Table III). This transport function is thus not impeded in Rh_{null} membrane.

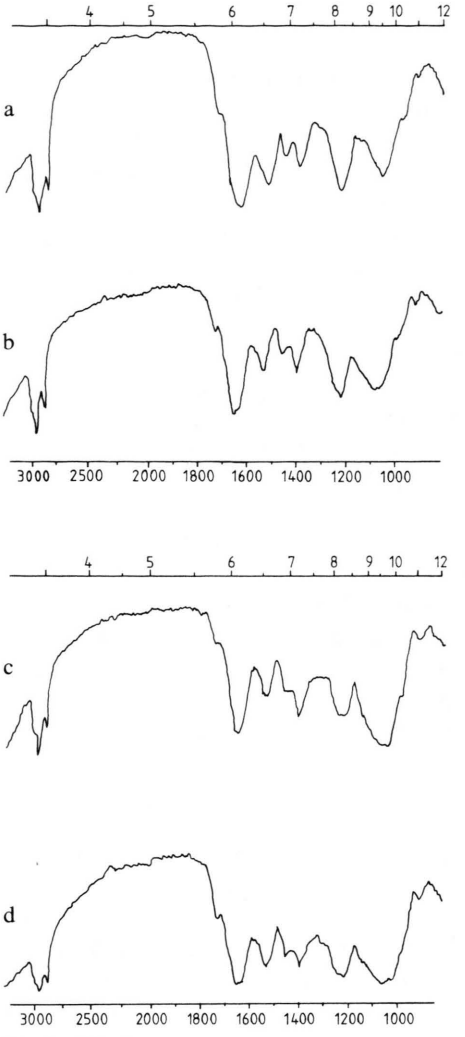

Fig. 3. KBr infrared spectra of red cell membranes. Fig. 3a and 3c represent Rh_{null} membranes, 3b and 3d represent controls. Fig. 3a–3d demonstrate spectra in absence (3a and b) and in presence (3c and d) of D-glucose (3.15 mM glucose concentration in the incubation medium).

Table III. D-glucose transport activity measurements in human erythrocytes of a group of 12 control persons [36] compared with one Rh_{null} blood. Measurements of each blood were performed 3 times. [S], millimolar D-glucose concentration. V, velocity of D-glucose transport. Means ± S.D. are shown.

$1/[S]$	$1/V$ controls [31]	$1/V$ Rh_{null}
2.00	2.6240 ± 0.0885	2.2690 ± 0.0425
1.00	1.5285 ± 0.0686	1.3726 ± 0.0700
0.66	1.1659 ± 0.0599	1.0426 ± 0.0677
0.50	0.9378 ± 0.0387	0.8606 ± 0.0352
0.40	0.8949 ± 0.0445	0.7640 ± 0.0309

Discussion

The importance of phospholipids and protein reactive groups for Rh antigenicity has been reported previously, and it has been stated that lipid-protein interaction is necessary for antigen-antibody reaction in the Rh system [1–4, 15, 17].

The work of Smith *et al.* [18] gives evidence that a structural basis exists for the Rh_{null} disease which is due to an altered conformation within the membrane. It was concluded [18] that phospholipid protein ionic bonding would not play a primary role in the differences of Rh D and Rh_{null} membrane.

Contrary to this view, in the present paper we show that a change of polar lipid protein interaction does indeed play a significant role in both types of membrane.

The phenomenon of phase transition in biological is a process involving the polar/apolar interface. It can be used conveniently as an approach for investigating lipid-protein interaction. Using ANS fluorescence measurement which has been introduced previously in membrane phase transition studies [45], it was observed that the transition of the Rh_{null} membrane is shifted by about 2 °C to lower termperature. Transition of the extracted membrane lipids, however, was identical in both the membrane types. Tighter lipid packing induced by interaction with the membrane proteins with corresponding increased steric restriction of the motion of the phospholipid headgroups is said to elevate the transition temperature [41]. One could suppose that in the case of the Rh_{null} membrane, polar lipid-protein interaction may be less tight. It should be noted, however, that both polar [46] and polar [41, 47] lipid protein interaction have been found to increase or decrease [48] the lipid phase transition temperature.

Polar lipid-protein interaction is brought about by functional groups in membrane protein bound electrostatically to lipid polar headgroups. Such protein reactive groups may be charged either negatively or positively. Working with the reagent diethylpyrocarbonate, we have found evidence [35] that this reagent interferes with lipid-protein interaction in the red cell membrane. Positively charged groups (Histidine-imidazole, lysine) could be candidates for such interaction, whereby the discontinuities in red cell membrane ANS fluorescence as well as glucose transport activity over a temperature range were largely diminished [35]. These discontinuities were ascribed to membrane lipid transition [22]. Since diethylpyrocarbonate is a protein reagent, the influence on the lipid transition temperature could not normally be anticipated; such influence, however, could be readily explained by polar lipid-protein interaction between charged amino groups and lipid phosphate. Such interaction has previously been suspected to occur [49].

There is a distinct influence of 1 mM diethylpyrocarbonate on the control membranes as revealed by the spin labeling data. No influence at all, however, was found on the Rh_{null} membrane (Table II). This gives strong evidence that the polar lipid-protein interaction is changed in the latter case. If such polar lipid protein interaction is changed, one would assume that the availability of polar rests from membrane protein is changed sterically (Fig. 4). An indication for different steric protein conformation is given by the smaller values of sulfhydryl groups and the higher values of disulfide bridges in Rh_{null} membrane, which can be one of the reasons for the lack of RH antigenicity.

An increase of red cell membrane disulfide groups was found to be correlated with higher phospholipid cleavage rate by phospholipase A_2 [50, 51]. Consistently, we found significant increases of free spin label after phospholipase A_2 treatment in Rh_{null} membrane. The action of phospholipase A_2 is thus more evident in the Rh_{null} membrane. We interpret this finding as an indication of loosening the membrane structure at the phospholipase A_2 interaction site at the membrane interface.

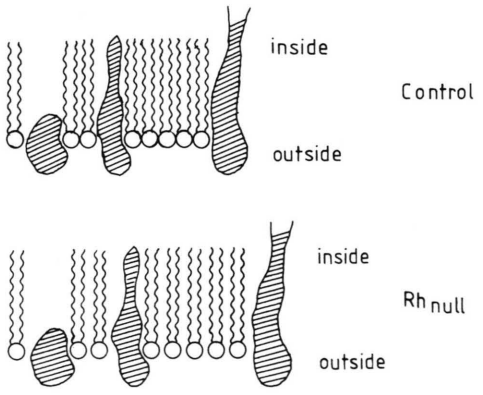

Fig. 4. Scheme of red blood cell membrane model of Rh_{null} and Rh D (control). The different steric conformation is illustrated by wider distances of phospholipids and proteins in the Rh_{null} membrane.

Polar lipid-protein interaction will be changed by phospholipase A_2 treatment of the control membrane, resulting in an exposition of new binding sites for ANS molecules at the interface. As such the increase in ANS fluorescence found by Smith *et al.* [18] may be interpreted. We conclude that polar lipid-protein interaction is reduced in Rh_{null} membrane, so that an exposition of new binding sites to ANS molecules during treatment with phospholipase A_2 does not occur. Concomitantly, an increase in ANS fluorescence was not found [18]. The small decrease in transition temperature (of 2 °C) in the Rh_{null} membrane as compared to the Rh D membrane may be brought about by a better hydration of the phospholipid polar head groups, which cannot interact that closely with the membrane protein polar groups.

Summarizing, the following results are leading to the view presented in Fig. 4:

1) Unchanged ANS fluorescence response after phospholipase A_2 treatment of Rh_{null} membrane, which probably means no further exposition of new binding sites for ANS molecules.

2) Transition temperature shift of 2 °C to lower temperatures in Rh_{null}, probably provoked by wider distances of phospholipid polar head groups.

3) Reduced amount of stearic acid spin label molecules fixed in the Rh_{null} membrane after phosphlipase A_2 treatment.

4) Order parameter of stearic acid spin label molecule is significantly increased after diethylpyrocarbonate treatment of control membrane, while there is no significant change in Rh_{null} membrane. This should be attributed to weaker lipid-protein interaction in the Rh_{null} membrane.

5) High glucose transport rate, probably due to lower polar lipid-protein interaction.

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