A Comparative Study of Structural Transitions in Erythrocyte Membranes of Adult Donors and Neonates

A. V. Matveev, V. R. Akoev, Yu. S. Tarakhovskii, A. A. Deev, V. M. Bryukhanov,* G. G. Zhadan, and V. L. Shnyrov****

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 123, No. 2, pp. 196-200, February, 1997
Original article submitted November 14, 1995

Differential scanning microcalorimetry and electron microscopy using freeze-fracture technique shows that the erythrocyte plasma membrane of adults differs in several parameters of heat absorption from that of neonates. In particular, electron microscopy demonstrates that the number and diameter of intramembrane particles are smaller in neonatal erythrocyte membranes. The results of this study account for a number of features possessed by neonatal erythrocytes.

Key Words: *erythrocyte membrane; differential scanning microcalorimetry; neonates; freeze fracturing*

In recent years, structural organization of the erythrocyte ghosts from neonates has been attracting the attention of investigators. This is so because neonatal erythrocytes differ from their adult counterparts in several respects. They have a shorter life-span, reduced deformability and mechanical stability, and decreased permeability for water, HCO_3^-/Cl^-, and nonelectrolytes [7]. These differences may be related to the adaptation of erythrocytes to the fetal conditions of gas exchange and circulation. As no differences in the protein or lipid composition were detected between erythrocyte membranes of adults and infants, it was suggested that the differences are mainly due to the special structural organization of the membrane skeleton in neonatal erythrocytes [7]. So far, no studies comparing the structural organization of adult erythrocytes with that of neonatal cells have been undertaken. In the present study erythrocyte membranes of adult donors and neonates are compared using differential scanning microcalorimetry and electron microscopy.

Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino; *Altai State Medical University, Barnaul; **Department of Biochemistry and Molecular Biology, Salamanca University, Spain

MATERIALS AND METHODS

Blood was taken from the umbilical cord of 15 healthy neonates born at term. Glugicir was used as the anticoagulant. Membranes were obtained by the procedure we described previously [8]. At the concluding stage of processing, erythrocyte ghosts were suspended in isotonic sodium phosphate buffer (pH 7.4) of 60 or 310 milliosmol. Electrophoretic examination was performed as previously [8]. The membrane concentration was calculated as the dry weight (drying to constant weight at 105°C). The curves describing the temperature dependence of excessive specific heat absorption of the ghost suspensions (these curves are referred to as thermograms) were recorded with a DASM-4 differential adiabatic scanning microcalorimeter [8]. The relative specific enthalpy of denaturation (ΔH) undergone by the membranes was determined after subtracting the baseline and comparing the membrane concentration-normalized area under the thermogram of the sample (from 35° to 85°C) with the area of an electric calibration marker. The intensity of transition was defined as the maximum specific heat (C_p^{max}, J/kg×K) of the peak at the temperature of the heat absorption peak maximum (T_{max}). The half widths ($\Delta T_{1/2}$) of

0007-4888/97/0002-0170$18.00

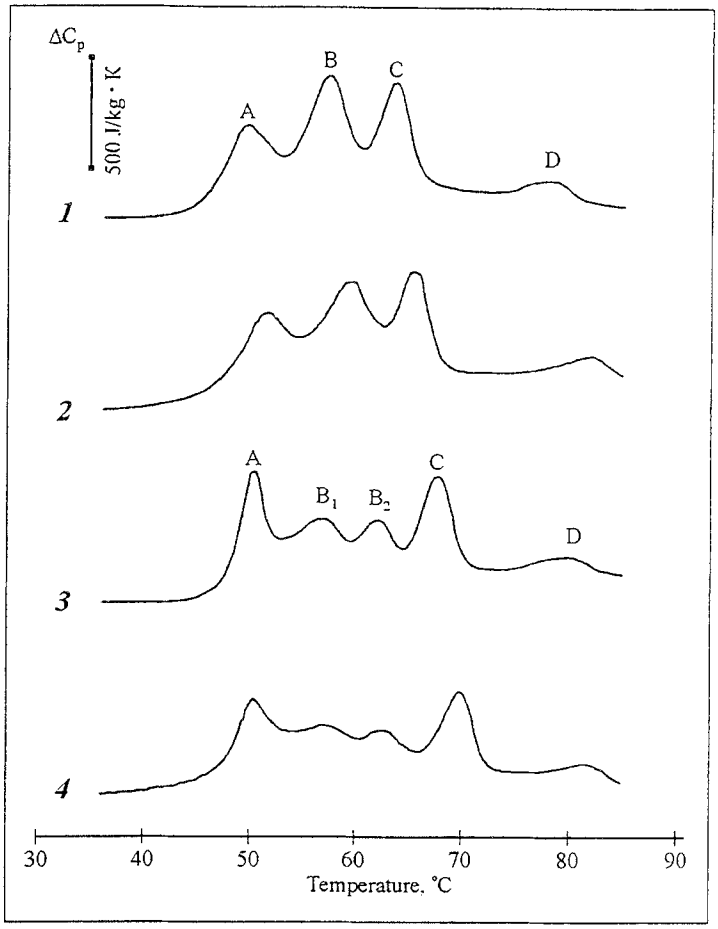

Fig. 1. Temperature dependence of excessive specific heat absorption by suspensions of erythrocyte membranes from adult donors (*1* and *3*) and neonates (*2* and *4*) in 60 milliosmol (*1* and *2*) and 310 milliosmol (*3* and *4*) media.

transitions A and C were defined as the doubled width of the left front of transition A and as the doubled width of the right front of transition C at the half of the peak. The latter method had to be used as it is not possible to determine the true widths of these transitions for neonatal erythrocytes because the right front of transition A has a low intensity and overlaps transition B_1. For transitions B_1 and B_2, the temperature range (ΔT) between visible minima of the transitions was determined. The data were statistically analyzed using Student's paired and independent *t* tests. Unless otherwise stated, statistical significance is at the $p<0.05$ level. Numerical values of thermogram parameters are presented as $M\pm\sigma$.

Election macroscopic examination was performed using the freeze-fracture technique [1]. High-speed cryofixation was carried out in cooled propane, and the platinum-carbon replicas obtained were viewed under a JEM-100B microscope (Jeol). Photomicrographs of the replicas, magnified 300,000-fold, were entered into a computer through a SONY XC-711P video camera for quantifying the distributions of intramembrane particles. The average size was calculated for each particle as the diameter of a circle with an area equivalent to the particle. The boundary contours of particles were identified using a specially designed software for image analysis. The particle size data were analyzed statistically.

RESULTS

Thermograms of donor erythrocytes show four thermal transitions: A, B, C, and D (heat absorption peaks), in a medium of 60 milliosmol, but transition B separates into transitions B_1 and B_2 in a 310 milliosmol medium [2]. Figure 1 shows typical thermograms of adult donor and neonatal erythrocyte ghosts in 60 milliosmol (curves *1* and *2*) and 310 milliosmol (curves *3* and *4*) media.

As seen from Table 1, there are no differences between adult and neonatal erythrocyte membranes in the ΔH of membrane denaturation, the intensity of transition A, or the ΔT of transition B. The T_{max} of all three transitions, the $\Delta T_{1/2}$ of transition A, and ΔT of transition B are significantly higher in neonatal erythrocyte membranes, whereas the intensities of transitions B and C are significantly lower. In the 310 milliosmol medium, all transitions are of significantly lower intensity in neonatal erythrocyte membranes, whereas the T_{max} of transitions B_2 and C and

the $\Delta T_{1/2}$ of transitions A and C are significantly higher. It is of special note that the increase in osmolarity from 60 to 310 milliosmol involved a rise in the mean T_{max} of transition A in donor membranes and a fall by 1.1°C in the T_{max} of neonatal membranes.

Heat absorption by erythrocyte membranes primarily depends on the structural state of proteins in the erythrocyte membrane skeleton. As shown in several studies, in donor membranes transition A is due to the denaturation of spectrin and actin which do not interact directly with the bilayer; transition B, to the denaturation of ankyrin, of proteins in bands *4.1* and *4.2*, and of the cytoplasmic domain in band *3* protein; transition C, to the denaturation of the membrane domain in band *3* protein; and transition D, to the denaturation of band *7* protein [2,8,9,12]. In a 310 milliosmol medium, transition B separates into two transitions — B_1 in which ankyrin and *4.1* and *4.2* band proteins are denatured, and transition B_2 in which the cytoplasmic domain of band *3* protein is denatured. According to our data, transitions of neonatal erythrocyte membranes are due to denaturation of the same proteins as in the corresponding transitions of donor erythrocyte membranes. The differences we detected in heat absorption parameters between donor and neonatal erythrocyte membranes provide direct evidence for the existence of structural differences between these membranes in certain membrane proteins and/or in how these proteins interact in the membrane.

Typical microphotographs showing replicas of the PF surface of donor and neonatal erythrocyte membrane fractures are shown in Fig. 2. The PF surface accommodates intramembrane particles in whose formation band *3* protein takes part [12]. The distribution histograms of intramembrane particles (Fig. 3) indicate that the mean diameter of these particles in neonatal membranes is greater than in donor membranes (98 Å *vs.* 72 Å; $p<0.01$), and in neonatal membranes the proportion of large particles

TABLE 1. Parameters of Heat Absorption by Membranes of Adult and Neonatal Erythrocyte Ghosts in Sodium Phosphate (pH 7.4) ($M\pm\sigma$)

Transition	Parameter	Adult donors	Neonates
60 milliosmol			
	ΔH of denaturation, kJ/kg	10.8±1.9	10.4±1.4
A	C_p^{max}, J/kg×K	385±55	370±45
	T_{max}, °C	48.7±0.4	50.9±0.4*
	$\Delta T_{1/2}$, °C	5.3±0.4	6.3±0.5*
B	C_p^{max}, J/kg×K	605±90	480±75*
	T_{max}, °C	56.2±0.5	58.8±0.4*
	ΔT, °C	8.0±0.4	7.9±0.5
C	C_p^{max}, J/kg×K	585±105	490±70*
	T_{max}, °C	63.3±0.6	64.9±0.2*
	DT, °C	3.9±0.4	4.7±0.6*
310 milliosmol			
	ΔH of denaturation, kJ/kg	10.2±0.9	8.61±2.19
A	C_p^{max}, J/kg×K	575±40	415±90*
	T_{max}, °C	49.9±0.3	49.8±0.5
	$\Delta T_{1/2}$, °C	3.2±0.2	3.9±0.6*
B_1	C_p^{max}, J/kg×K	340±35	270±65*
	T_{max}, °C	56.3±0.5	56.7±0.5
	ΔT, °C	5.9±0.5	6.0±0.7
B_2	C_p^{max}, J/kg×K	350±20	265±70*
	T_{max}, °C	61.8±0.3	62.2±0.3*
	ΔT, °C	5.2±0.3	5.5±0.3
C	C_p^{max}, J/kg×K	575±45	420±100*
	T_{max}, °C	67.5±0.3	69.1±0.6*
	ΔT, °C	3.7±0.3	4.3±0.8*

Note. *$p<0.05$ compared with adults.

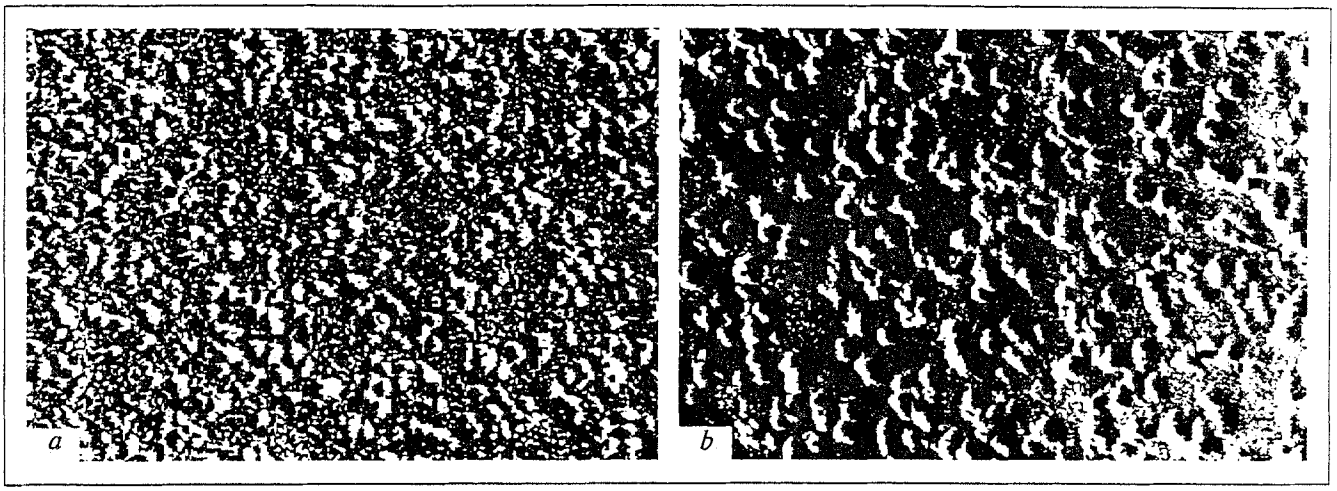

Fig. 2. Microphotographs showing replicas of the PF surface of adult (*a*) and a neonatal (*b*) erythrocyte membrane fractures, ×100,000.

(>100 Å in diameter) is much higher (50% on average) than in donor membranes (≤5%). The total number of particles was 3700±200 per μ^2 in donor membranes and 2700±900 per μ^2 in neonatal membranes, which is significantly less; the mean number of particles in some neonatal membranes was 1900± 200 per μ^2 ($p<0.01$). Granulofilamentous and fibrillary structures on the PF surface of neonatal membranes have been described [5], but no such structures were detected in our study.

It should be noted that the ultrastructural changes we observed did not always correlate directly with particular changes in the thermograms. Thus, a comparison of thermograms of the samples showing the greatest differences in ultrastructural parameters such as the number, size or aggregation of intramembrane particles, failed to reveal any special abnormalities of heat absorption.

Our findings explain several specific features of neonatal erythrocytes. In particular, considerable differences we detected between neonatal and donor erythrocyte membranes in heat absorption parameters of transitions A and B_1, i.e., in protein structures of the membrane's spectrin network (spectrin, ankyrin, actin, *4.1* and *4.2* band proteins), probably underlie the previously demonstrated differences in the properties of neonatal erythrocytes from their adult counterparts [7]. Structural differences exist in the spectrin network, as evidenced by the identified ultrastructural differences, since protein aggregation in the erythrocyte membrane is controlled by the spectrin network [11]. Previously, neonatal erythrocyte membranes were found to contain areas free of spectrin [10], and this also correlates with the particle aggregation and the presence of particle-free areas detected in the present study.

Several other features of neonatal erythrocyte membranes are associated with their reduced permeability for HCO_3^-, Cl^-, and water, which is determined by the properties of the membrane domain in band *3* protein [9] denatured in transition C [6, 8,9]. As shown earlier, falls in the intensity and enthalpy of transition C denaturation in the case of donor membranes are accompanied by inhibition of the anion-transporting function of this protein [9]. It has also been shown that aggregation of band *3* protein may lead to changes in the erythrocyte membrane permeability [10]. Our finding that the intensity of transition C and the aggregation of particles were reduced agrees with the reported reduced transport of anions [7]. Moreover, the aggregation of band *3* protein may alter antigenic determinants of the erythrocyte plasma membrane [4], which may explain why the hemolysis and phagocytosis of erythrocytes in neonates are more pronounced than in adults [7].

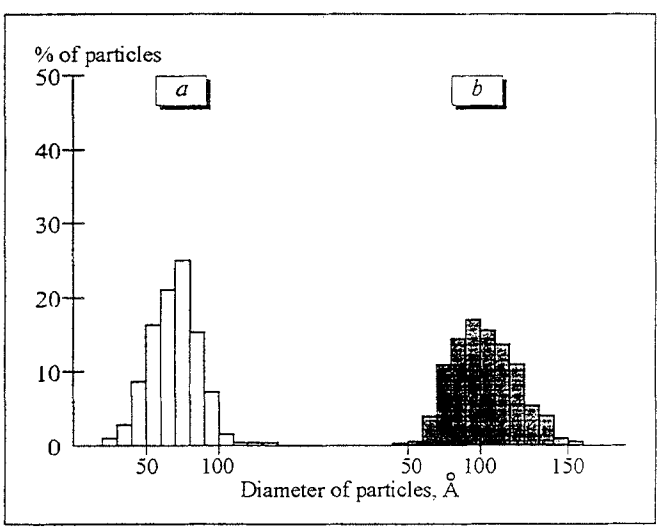

Fig. 3. Histograms showing the distribution of particles on the PF surface of adult (*a*) and neonatal (*b*) erythrocyte membrane fractures.

REFERENCES

1. V. L. Borovyagin, A. G. Sabelnikov, Y. S. Tarahovsky, and I. A. Vasilenko, *J. Membr. Biol.*, **100**, No. 3, 229-242 (1977).
2. J. F. Brandts, K. A. Lysko, A. T. Schwarts, *et al.*, *Colloq. Int. C.N.R.S.*, No. 246, 169-175 (1976).
3. R. B. Gun and R. G. Kirk, *J. Membr. Biol.*, **27**, No. 3, 265-282 (1976).
4. M. B. Kay, *Gerontology*, **37**, No. 1, 5-32 (1991).
5. J. Kurantsin-Mills and L. S. Lessin, *Pediatr. Res.*, **18**, No. 10, 1035-1041 (1984).
6. K. F. Lysko, R. Carlson, R. D. Taverna, *et al.*, *Biochemistry*, **20**, No. 19, 5570-5576 (1981).
7. L. Matovcik and W. Mentzer, *Clin. Haematol.*, **14**, No. 1, 203-221 (1985).
8. V. L. Shnyrov, S. N. Orlov, G. G. Zhadan, and N. I. Pokudin, *Biomed. Biochim. Acta*, **49**, No. 6, 445-453 (1990).
9. J. W. Snow, J. Vincentelli, and J. F. Brandts, *Biochim. Biophys. Acta*, **642**, 418-428 (1981).
10. K. T. Tokuyasu, R. Schekman, and S. J. Singer, *J. Cell Biol.*, **80**, No. 2, 481-486 (1979).
11. J. A. Ursitti, D. W. Pumplin, J. B. Wade, and R. J. Bloch, *Cell Motil. Cytoskeleton*, **19**, No. 4, 227-243 (1991).
12. G. Zhadan, E. Villar, and V. L. Shnyrov, *Biochem. Soc. Trans.*, **22**, No. 3, 386S (1994).

Experimental Validation for the Use of Recombinant Prourokinase and Its Immobilized Forms in the Treatment of Postoperative Fibrinoid Syndrome in Ophthalmology

E. V. Boiko, V. F. Danilichev, and S. V. Kol'tsova

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 123, No. 2, pp. 201-204, February, 1997
Original article submitted October 20, 1995

The specific therapeutic effectiveness of a single intravitreal injection of native and immobilized recombinant prourokinase is assessed in lensvitrectomized rabbits with the fibrinoid syndrome. The agents almost completely lyse the fibrin clot within 1-8 h. Heteroprotein conjugates of protein kinase with molecular weight of 900 kD and high fibrinolytic activity at 2-20-fold lower doses of the enzyme are preferable. Native and immobilized prourokinase are recommended for clinical use in case of intraocular fibrin formation after lensvitrectomy and other operations.

Key Words: *lensvitrectomy; intraocular fibrin production; prourokinase; immobilized fibrinolytic enzymes*

New-generation fibrinolytics — prourokinase (PU) and tissue plasminogen activator — have been recently developed and are now widely used in clinical practice [4,13]. Polymeric immobilized forms of the activators are preferable due to prolonged effect and the ability to accumulate in the pathological focus. They minimize the risk of side effects as a result of decrease in dosage and the number of drug injections at the same therapeutic effectiveness [3,10,12]. Fibrinolytics are widely used in ophthalmology for the treatment of intraocular hemorrhages and massive fibrin exudation after surgery [9,11]. The presence of the blood-aqueous barrier necessitates intraocular administration of the enzymes, which requires low drug doses and prolonged effect to prevent toxicity and repeated injections [1,2,6,8].

Our objective was to study the effectiveness of immobilized PU — soluble heteroprotein conjugates of a preset molecular weight and composition based

Military Medical Academy; Institute of High-Molecular-Weight Compounds, Russian Academy of Sciences, St. Petersburg

0007-4888/97/0002-0174$18.00