BIOPHYSICS AND BIOCHEMISTRY

Structural Transitions in Erythrocyte Membranes in Hereditary Hemochromatosis

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Changes in the thermal transition parameters of the backbone proteins of the erythrocyte plasma membrane, particularly of spectrin and membrane domain of band *3* protein, in patients with hereditary hemochromatosis are demonstrated by differential scanning microcalorimetry. Electron microscopy (the freeze-fracture technique) reveals aggregation of intramembrane particles and their enlargement in erythrocyte membranes from patients in comparison with membranes from healthy donors. These structural changes in the erythrocyte membranes are assumed to contribute to hemolysis and iron overload of the organism.

Key Words: *hereditary hemochromatosis; differential scanning microcalorimetry; erythrocyte membrane*

Hereditary hemochromatosis (HH) is a pathological state that results from disturbed iron metabolism and leads to intracellular accumulation and deposition of iron-containing pigments inducing irreversible structural and functional changes in cells [6,9,19]. The key role in iron metabolism is played by macrophages and erythrocytes [8,10,19]. The role of the macrophage system in the pathogenesis of HH is well established. Macrophages cannot catabolize and effectively store hemoglobin-derived iron [6-9,19]. The role of erythrocytes is poorly understood. However, splenomegaly [2,6,19] and hemolysis [5] in patients with HH point to some defects in the erythrocyte plasma membrane. An increase in serum iron and ferritin contents may result from excessive phagocytosis of defective erythrocytes. Thus, investigation of the structure of erythrocyte membrane gives a new insight into the pathogenesis of HH. In the present study we compared thermal transitions in the erythrocyte membranes from patients with HH and healthy donors.

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MATERIALS AND METHODS

The study was performed on erythrocytes from 22 male patients with HH aged 17-65 years and two boys (9- and 11-year-old). The duration of HH varied from 1 month to 6 years. All patients had symptoms typical of the late stage of the disease: cirrhosis of the liver, diabetes mellitus, splenomegaly, and melanoderma. Laboratory tests revealed specific signs of the disease in all cases: iron metabolism disturbances and the presence of A3 and B7 loci. In half of the patients, ferritin of erythrocyte lysate considerably surpassed the normal level, varying from 12 to 773.8 μg/g Hb (*vs.* 5.9 in the norm). Examination of HH patients revealed intravascular hemolysis usually accompanied by elevation of free plasma hemoglobin.

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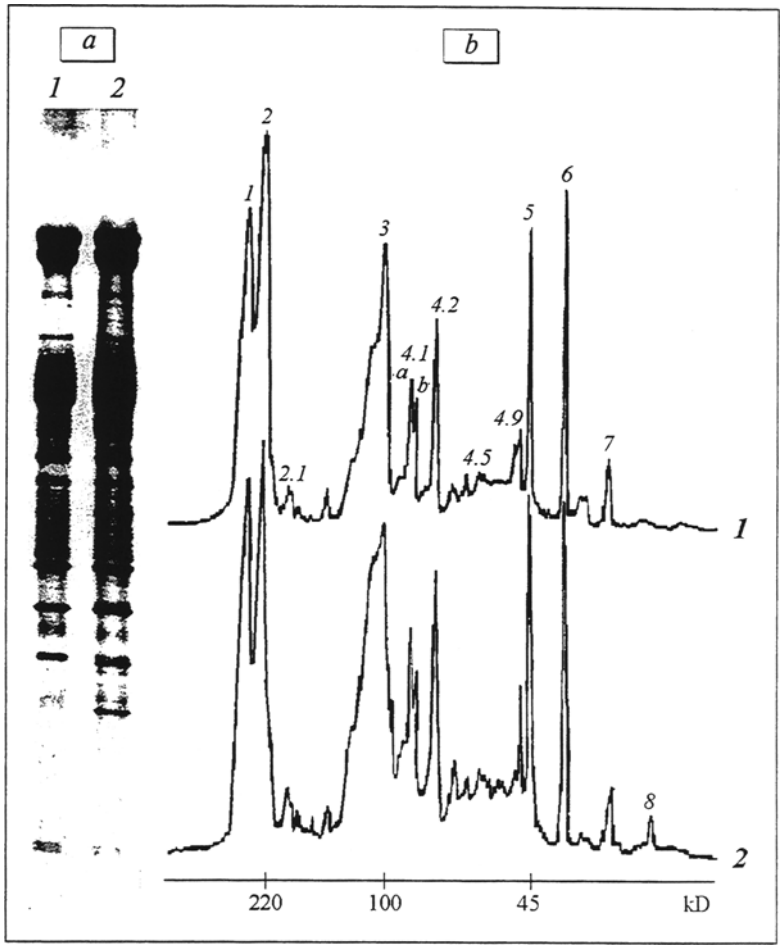

Fig. 1. Electrophoregrams (*a*) and densitograms (*b*) of membrane polypeptides of erythrocyte ghosts from healthy donors (*1*) and patients with hereditary hemochromatosis (*2*). Numeration of electrophoretic protein bands according to T. L. Steck [16].

Fresh venous blood was filtered through mixed microcrystalline and fiber cellulose to eliminate leukocytes, lymphocytes, and thrombocytes. Ghost membranes were prepared by lysing erythrocytes in 5 mM sodium-phosphate buffer, pH 8.0 [14]. Finally, hemoglobin-free ghosts were suspended in isotonic (310 milliosmol) sodium-phosphate buffer, pH 7.4. The concentration of the membranes was measured after drying at 105°C to a constant weight. The membranes were analyzed by electrophoresis [14]. The plots of the excessive heat capacity *vs.* temperature (thermograms) of the erythrocyte membrane suspension was recorded using a DASM-4 differential adiabatic scanning microcalorimeter [1,14]. All measurements were carried out in a 310-milliosmol solution at a heating rate of 1°K/min. The relative specific enthalpy of denaturation (ΔH) was determined after subtracting the baseline and comparing the areas under the experimental thermogram (from 35 to 85°C) standardized to the membrane concentration and a calibration electrical standard. The intensity of the transition was determined as specific heat capacity (C_p^{max}) for a heat absorption peak at the corresponding temperature (T_{max}). The half-width ($\Delta T_{1/2}$) of A and C transitions was determined as a temperature interval at the half-height of the C_p^{max} peaks. For B_1 and B_2 transitions the temperature interval (ΔT) between perceptible minima was measured. The data were processed statistically: the means and standard deviations ($M \pm \sigma$) were calculated, and the significance of differences was evaluated using the Student's *t* test. Erythrocyte membranes were studied by freeze-fracture electron microscopy [3], and computer-assisted quantitative analysis of the distribution of intramembrane particles was carried out using a Sony XC-711P video recorder for image (×330,000) input. The mean size of each particle was calculated as a diameter of a circle with an equivalent area. Particle contours were outlined using the image analysis software. Data files on the particle sizes were processed statistically, and the distribution histograms were constructed.

RESULTS

Electrophoresis of erythrocyte ghosts from HH patients revealed no significant differences in their polypeptide composition compared with those from

healthy donors (Fig. 1). In 6 patients, a decreased content of band *6*, glyceraldehyde-3-phosphate dehydrogenase (35 kD), was noted, and in 2 patients band *8* (20 kD) appeared (Fig. 1, *a*).

Calorimetry. Some calorimetric parameters of erythrocyte membranes are presented in Table 1. A typical thermogram (Fig. 2, *1*) of normal erythrocyte membranes in a 310-milliosmol solution, pH 7.4, has 5 thermal transitions called A, B_1, B_2, C, and D [4]. The thermotropic properties of normal erythrocyte membranes are stable: measurements repeated within one month for the same group of healthy donors revealed no significant changes from the initial thermograms, no individual variations were noted. These thermograms are characterized by equal values of C_p^{max} of A and C transitions and the mean C_p^{max} for B_1 and B_2 transitions (Table 1, Fig. 2, *1*). Moreover, the intensity of visible minima between A and B_1 and between B_2 and C transitions does not exceed the half-height of A and C transitions, respectively, which allows determination of the true $\Delta T_{1/2}$ for these transitions. However, we calculated $\Delta T_{1/2}$ of A and C transitions as two-fold width of their left and right tails, respectively. Such an approach makes it possible to compare $\Delta T_{1/2}$ in cases when the true $\Delta T_{1/2}$ cannot be determined due to low intensities of A and C transitions and/or their broad overlap with B_1 or B_2 transitions, respectively. As seen from Table 1, $\Delta T_{1/2}$ for A transition is lower than $\Delta T_{1/2}$ of C transition. Thermal transitions in the erythrocyte membranes are calorimetrically irreversible: repeated heating to 90°C revealed no heat absorption peaks (Fig. 2, *6*). Thermograms of erythrocyte membranes from HH patients exhibit some peculiar features (Fig. 2, *2-5*): first, the intensity of A transition is reduced by 9% and, therefore, the relation between intensities of A and C transitions varies from sample to sample (Fig. 2, *2*, *5*); second, the difference between $\Delta T_{1/2}$ of A and C transition disappears due to widening of A transition; third, T_{max} of B_1 and C transitions shifts to the right; and, finally, in 2 HH patients with a high erythrocyte ferritin level (2000 μg/g Hb) repeated heating revealed two wide low-intensity thermal transitions at 50 and 60°C (Fig. 2, *7*), which probably attests to unequal immobilization of lipids on protein components in erythrocyte membranes [15] of HH patients and healthy donors. These peaks disappeared after the third heating to 90°C. Repeated calorimetric study (within 1 month) showed that in one-third HH patients the parameters of A, B_1, and C transition changed significantly ($p<0.05$) in comparison with the initial thermograms.

Ultrastructural analysis. Figure 3 shows distribution histograms of particle sizes and microphotographs of replicas of PF fracture surface of erythrocyte membranes from donors and HH patients. The mean diameter ($M\pm\sigma$) of particles in normal erythrocyte membranes was 72±16 Å; their number was 3753±277 particles/μ², large particles (>100 Å) constituted no more than 5% (Fig. 3, *a*). No significant differences in these parameters between donors were found. Fracture surface of erythrocyte membranes from patients with HH was characterized by a 28% increased mean diameter (92±24, $p<0.05$) of particles, higher percentage (30-60%) of large particles (>100 Å) (Fig. 3, *b*, *d*), particle aggregation into clusters of various size, and appearance of particle-free spaces (Fig. 3, *e*, *f*). Enlargement and aggregation were ac-

TABLE 1. Parameters of Thermograms of Erythrocyte Plasma Membranes of Healthy Donors and Patients with HH ($M\pm\sigma$)

Transition	Parameters	Donors (n=22)	HH (n=22)
	ΔH of denaturation, kJ/kg	10.2±0.9	10.6±1.2
A	C_p^{max}, J/kg×K	565±35	515±95*
	T_{max}, °C	49.9±0.3	49.8±0.4
	$\Delta T_{1/2}$, °C	3.3±0.2	3.6±0.7*
B_1	C_p^{max}, J/kg×K	345±35	340±35
	T_{max}, °C	56.3±0.5	56.6±0.4*
	$\Delta T_{1/2}$, °C	6.0±0.6	6.1±0.6
B_2	C_p^{max}, J/kg×K	350±20	350±20
	T_{max}, °C	61.8±0.3	62.0±0.6
	ΔT, °C	5.2±0.3	5.2±0.5
C	C_p^{max}, J/kg×K	565±40	560±70
	T_{max}, °C	67.5±0.3	68.2±0.6*
	$\Delta T_{1/2}$, °C	3.7±0.3	3.8±0.4

Note. *$p<0.05$ compared with healthy donors.

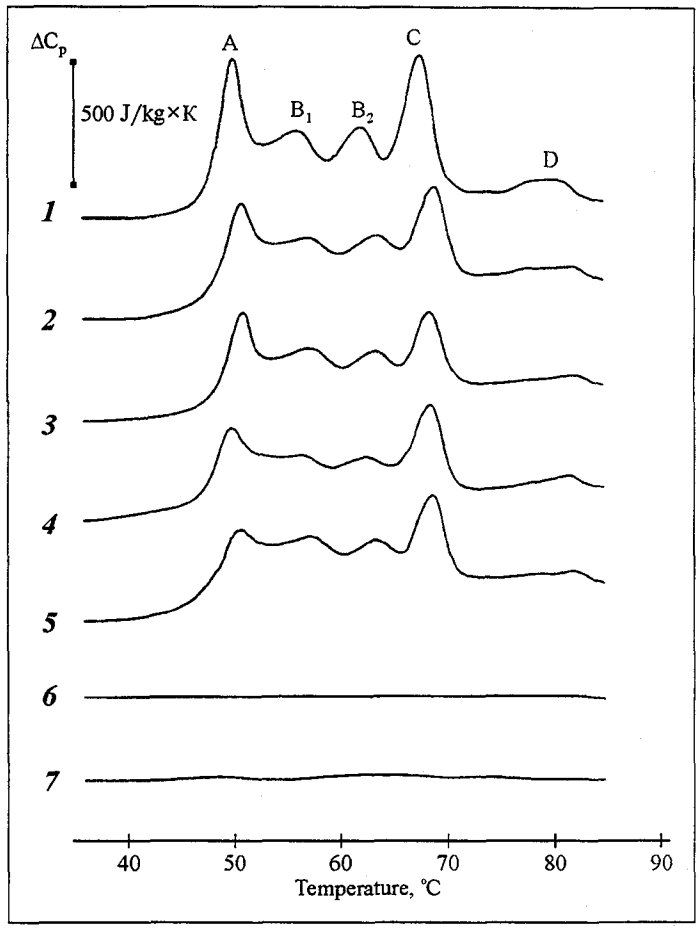

Fig. 2. Typical thermograms of erythrocyte plasma membranes of donors (*1*) and patients with hereditary hemochromatosis (HH, *2-5*); repeated heating of erythrocyte membranes of donors (*6*) and patients with HH (*7*).

companied by a decrease in the mean number of particles to 2960±724 particles/μ² ($p<0.05$) and in preparations with very large particles to 1581± 705 particles/μ² (Fig. 3, *d*). It should be noted that the degree of aggregation and the proportion of large particles varied considerably in different fracture surfaces even in the same membrane preparation (Fig. 3, *d*, *e*).

Thermal transitions result from thermal denaturation of certain membrane domains composed of the erythrocyte framework proteins [4,11,14,20]. It has been previously shown that A transition is due to denaturation of spectrin (bands *1* and *2*, Fig. 1, *b*), which does not immediately interacts with the lipid bilayer and actin (band *5*); B_1 transition occurs as a result of unfolding of ankyrin (band *2.1*) and band *4.1* and *4.2* proteins; denaturation of cytoplasmic and membrane fragments of band *3* protein, an anion transporter, is responsible for B_2 and C transitions, respectively; D transition is a result of band *7* protein unfolding [4,11,14,20] and membrane fragmentation (our data). We suppose that the observed changes in thermograms point to changes in structural organization of the erythrocyte membrane proteins in HH. First, changes in the heat absorption nearby A and B_1 transitions attest to changes in the structure of the spectrin-actin and spectrin-ankyrin-protein *4.1*-protein *4.2* domains of the erythrocyte membrane skeleton. This follows from the presence of aggregates and particle-free spaces in the membrane, since changes in the structural state of the spectrin network are responsible for aggregation [18]. Second, the rise of T_{max} of C transition attests to structural changes in the membrane part of band *3* protein. This corresponds to the increase in the diameter of intramembrane particles, since particle formation is determined predominantly by band *3* protein [18]. It should be noted that individual peculiarities of thermograms revealed by repeated measurements in HH patients and structural heterogeneity of their membranes, i.e., various degree of aggregation and the presence of particles of different sizes even in one specimen, probably result from several reasons. On the one hand, microscopy shows that structural defects involve unequal areas of the membrane skeleton; on the other, different numbers of abnormal erythrocytes are simultaneously present in the circulation because of different rate of elimination of both normal and defective erythrocytes from the blood.

It has been proved that defects of the spectrin network and band *3* protein, an anion transporter,

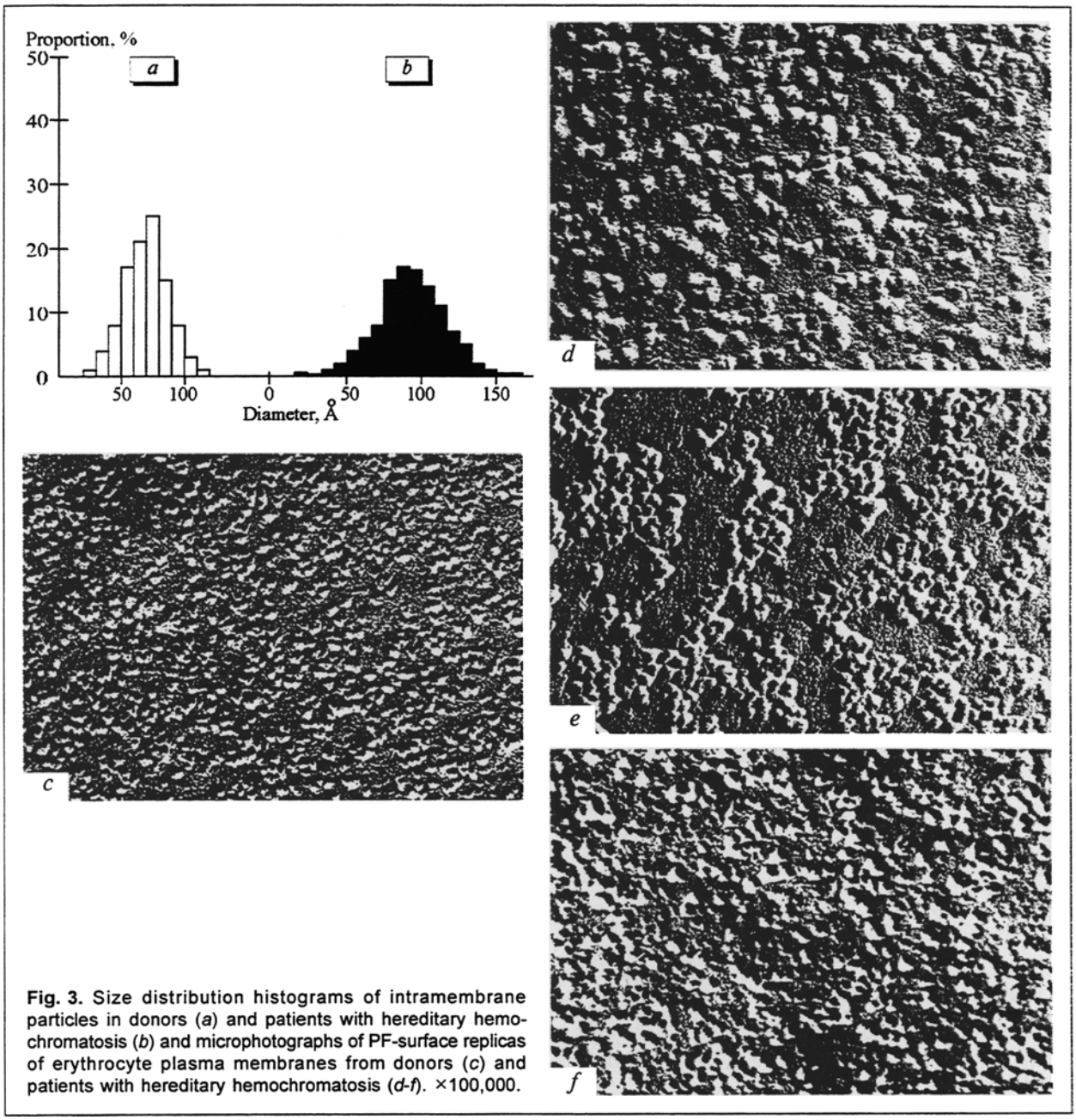

Fig. 3. Size distribution histograms of intramembrane particles in donors (*a*) and patients with hereditary hemochromatosis (*b*) and microphotographs of PF-surface replicas of erythrocyte plasma membranes from donors (*c*) and patients with hereditary hemochromatosis (*d-f*). ×100,000.

restrict the life-span of erythrocytes in the circulation [7,10], since they either result in fragmentation of the membrane and hemolysis or, due to aggregation of band *3* protein, lead to the appearance of antigenic determinants and opsonization with IgG and complement [17], which promotes their recognition and phagocytosis by macrophages [10,17]. We believe that our data on the presence of the defects in the erythrocyte membrane are useful for the understanding of the pathogenesis of HH, since they not only explain the development of splenomegaly and hemolysis in HH but also suggest that defective erythrocytes along with macrophages are involved into the development of hepatic siderosis. We have hypothesized that phagocytosis of defective erythrocytes increases iron delivery to the macrophage system, which, against the background of reduced storing capacity of macrophages for hemoglobin-derived iron [6-9], results in elevation of serum iron and blood ferritin. Such a mechanism probably underlies iron overload of the liver at the late stages of the disease characterized by the absence of transferrin receptors on hepatocytes [13]. Under these conditions ferritin is the main and the most effective iron transporter to hepatocytes. Finally, it should be noted that in HH similar structural defects probably occur in other

cell populations, since band *4.1* and *4.2* and band *3* proteins are expressed practically in all cells of the organism [7].

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