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Thermally induced transformation of mammalian red blood cells during hyperthermia

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ABSTRACT

The structural and transport characteristics of membranes are mainly determined by the state of the cytoskeleton. The characteristic changes in morphology of human (adult donor and cord) and rat Red Blood Cells (RBC) and of their membrane, induced by hyperthermia (46–51 °C) have been analyzed. Two different types of morphological changes have been observed to take place during hyperthermia in all studied RBC groups. We have observed either formation and exfoliation of spiculas from membrane, resulting in the formation of large (4–5 μm) sphere-like cell body and small (0.5–1.5 μm) vesicles or cell fragmentation with formation of large (3–3.5 μm) vesicles. The two distinct phenomena are likely to be determined by the heterogeneity of the RBC population in terms of cell age. There was noted the difference of cord RBC from the donor ones in temperature value of transformation beginning, as well as the character of deformation and vesicle formation, that may testify to their less thermoresistance. The ultrastructure of the membrane, studied with the freeze-fracturing technique, testifies to an irreversible character of membrane changes. The aggregation of intramembrane particles (IMPs) as a continuous network testifies to the strengthening of the interactions between denatured spectrin and bilayer integral components.

1. Introduction

Warming red blood cells (RBC) to temperatures above +45 °C is widely employed to study both the phenomenon of thermal hemolysis and the structural modifications of cells and their membranes after any subsequent treatment. This is due to the fact that the main RBC skeletal protein – spectrin – undergoes a denaturating thermal transition at 49.5 °C [1]. Transitions in the RBC membrane at, and above this temperature, are irreversible in nature, and result in the loss of some functional properties of the cell. Warming in the range 46–49 °C results in the oligomerization of band 3 protein, and in an increase in its rotatory and lateral mobility [2], and induces alterations in the critical cell volume [3]. Under these conditions the microviscosity of the membrane increases, RBC exhibit marked vesiculation along with other pronounced events [4,5]. A key question, therefore, is what do we witness at temperatures above 50 °C? We have thus studied the RBC morphology and the ultrastructure of the RBC membrane in the temperature range of 46–51 °C.

2. Materials and methods

RBC from human adult donors and from cord blood, prepared with a Glugizir preservative (2 g sodium citrate; 3 g water-free glucose; of about 100 ml bidistilled water), and rat blood treated with heparin, were employed. RBC were separated by centrifugation (10 min at 1500 g) in a phosphate buffer, pH 7.4 (150 mM NaCl + 5 mM Na-phosphate buffer). The RBC pellet was washed three times in a 4-fold volume of buffer, and resuspended at a hematocrit of 20% in PBS. RBC were warmed in a thermostatically-regulated water bath (accuracy of stabilization was ±0.5 °C) by incubating them for 15 min at 46, 47, 48, 49, 50 and 51 °C. The accuracy of the stabilization and of the temperature measurement were additionally monitored using two thermometers (ΔT = 0.1 °C), and differential copper-constantan thermocouple (the second end of the thermocouple at 0 °C), contained within a close-fitting chamber. It was therefore possible to assess the likelihood of development of a temperature gradient and to determine the speed with which the desired temperature could be attained in polyethylene containers containing 1 ml of cell suspension.

For each temperature tested, at the end of the incubation RBC were fixed in a 2.5%-solution of glutaraldehyde (GA) in phosphate buffer (1:1), the temperature of which was similar to that of the cell suspension. For subsequent scanning-microscope studies, RBC were dehydrated for 10–15 min in ethanol preparations of increasing concentrations (30°, 50°, 70°, 90°, 96°), in absolute ethanol (98°) and in acetonitrile. The RBC were covered with a platinum-carbon layer in a vacuum chamber. RBC surfaces were visualized using a REMMA-101A scanning electron microscope ('SELMI', Ukraine) under an accelerating voltage of 20–30 kV. The magnifying power was 3–10 × 10^3-fold.

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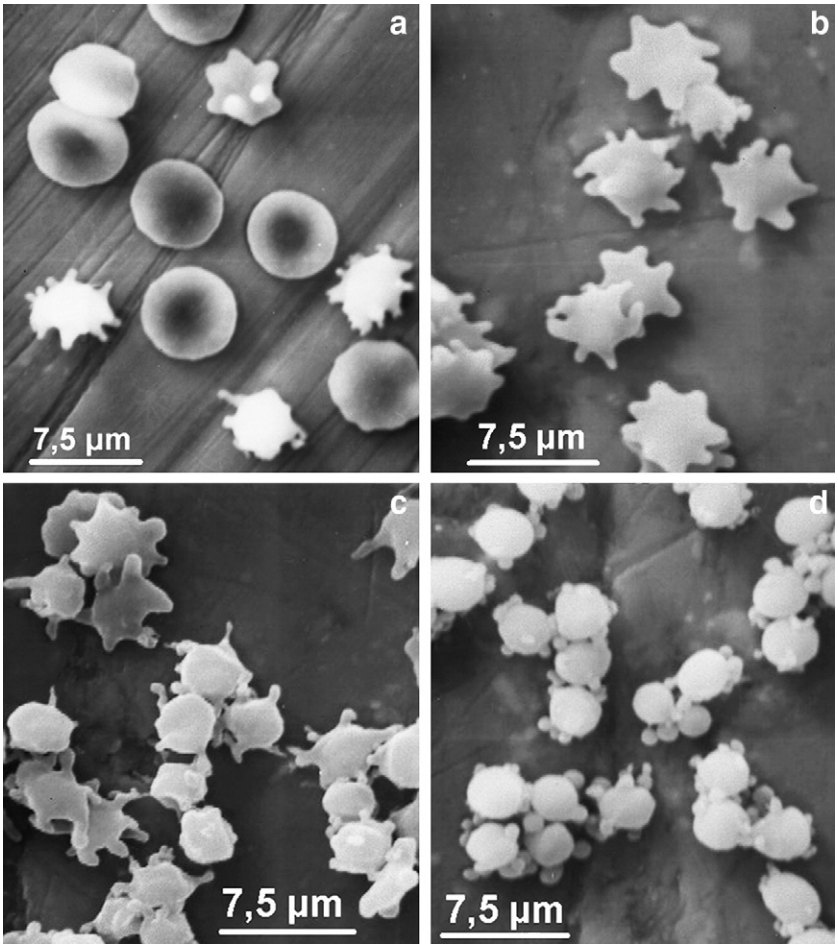

Fig. 1. Stages of surface deformation of rat RBC and formation of spicules (b, c) and development of a sphere-like cell body (c, d) at temperatures: (a) 46 °C, (b) 49 °C, (c) 50 °C, (d) 51 °C.

To analyse membrane morphology, samples were prepared using a freeze-fracture method [6,7]. The RBC were cryofixed using either a sandwich method or in propane, supercooled in liquid nitrogen (solidification point is −187.1 °C). Platinum–carbon replicas, collected from the RBC fractures, were studied with a TEM-125K transmission electron microscope, equipped with a SAI-01A system ('SELMI') for collection and analysis of images, based on a CCD chamber DX-2 and a program package (KAPPA, Germany) with the accelerating voltage of 75 kV.

3. Results and discussion

In order to eliminate artefacts associated with temperature-dependent changes in cell shape while viewing samples under light microscopy, GA was applied to the samples after each experimental procedure. In this way, the morphological state of cells was fixed, facilitating the monitoring of the dynamics of transformation of their surfaces within a temperature range of 46–51 °C.

It is known, that two types of morphological transformation became evident during cell warming in the temperature range of 46–48 °C [5]. We have also observed two types of morphological changes for all studied RBC groups. In the first case, a wavelike deformation, with the appearance of spicules was observed on the cell surface. Further warming of the cells resulted in the formation of sphere-like cell body (4–5 μm in diameter) and small vesicles (0.5–1.5 μm). We demonstrated this by the example of rat RBC (Fig. 1). We believe a threshold for compaction of the membrane skeleton may be the limiting factor, which determines the size of the sphere-like cell body.

An alternative response, which was with higher probability observed with human cord-blood, was the development of deep lateral strangulations of the cells, which bring about fragmentation of RBC and formation of large vesicles (3–3.5 μm in diameter) (Fig. 2 b, c).

We have observed the difference of cord RBC from those of adult donors in a decrease of temperature of morphological change beginning, as well as deformation and vesicle formation character (Fig. 2 b, d), that may testify to their less thermoresistance. In fact, hemoglobin of cord blood RBC has been shown to possess lower thermal stability [8], and to be more susceptible to oxidation to met-hemoglobin (met-Hb) during heating when compared to adult donors' RBC hemoglobin. Thermal hemolysis studies indicate that RBC with increased met-Hb content are more prone to heat-induced hemolysis [9].

It should be noted that, despite such significant impairments in the morphology of the cell itself (Fig. 2 c; 4 b), the degree of hemolysis was not that substantial, amounting to 4.5% in adult donor and cord blood RBC, and 5.3% in rat blood. These data testify to the fact that the lipid bilayer possesses the capacity to reseal for Hb molecules and thus to repair defects that occur during RBC fragmentation.

Freeze-fracture studies of the fine structures of the RBC membranes revealed the distribution of transmembrane proteins within the plane of the membrane fracture (PF- and EF-surfaces) in the form of the so-called IMPs[1] (Fig. 3).

[1] The membrane of intact RBC is known to split under fracturing within the hydrophobic region, thereby uncovering two relatively smooth PF- and EF-surfaces with typical chaotic distribution of intramembrane particles (IMPs). IMPs have been demonstrated to represent integral membrane proteins [10]. Their distribution can be considered to reflect the dynamics of protein-lipid interactions and to be determined by the structural and functional state of cell and its membrane.

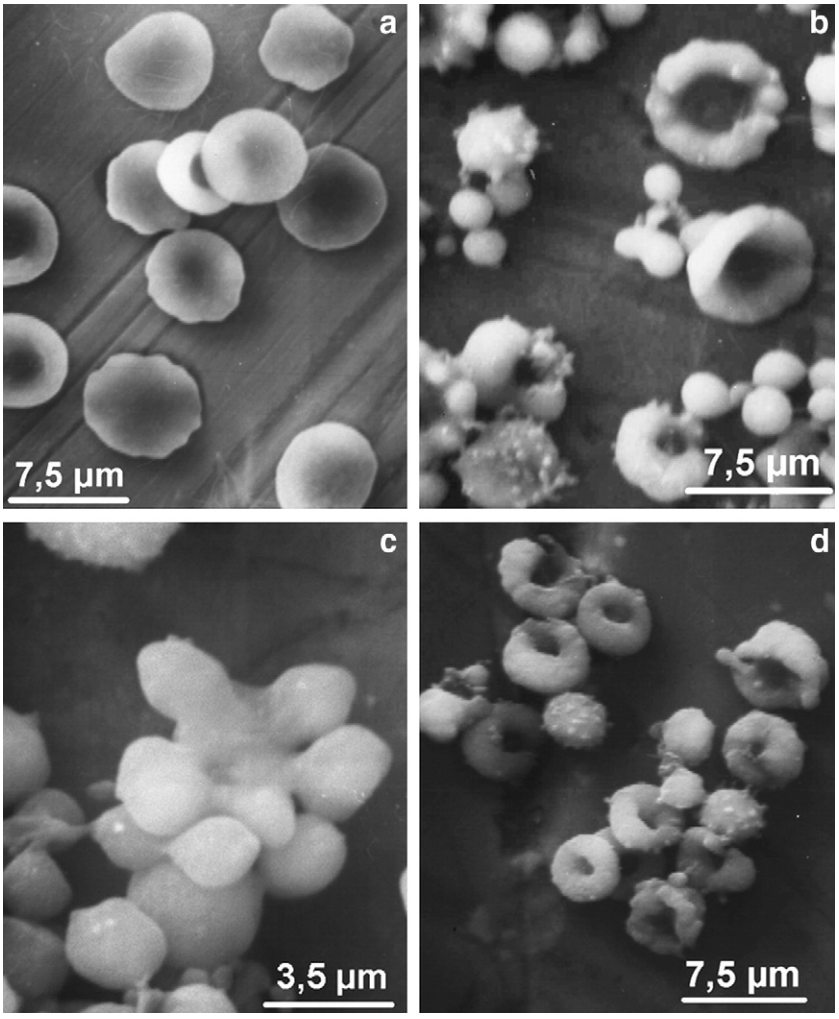

Fig. 2. Sequence of fragmentation of cord blood RBC (a–c) with a formation of large vesicles with the diameter of up to 3.5 μm at temperatures: (a) 46 °C, (b) 48 °C, (c) 50 °C; (d) donor RBC at 50 °C.

Exposure of cells to a temperature of +50 °C for 15 min, followed by lowering of the temperature to 37, 20 or 4 °C induced strong aggregation of the IMP (Figs. 4a and 5a). We observed two types of PF-surface fractures in the RBC membrane or its fragments, characterised by differences in the distribution of IMP. In the first type, IMP aggregation occurred in the form of large clusters and was accompanied by appearance of protein-free areas on the membrane surface (Fig. 4, a), whereas in the second, aggregation of particles in the form of a continuous network was apparent (Fig. 5, a). The morphology and dimensions of the network, formed by

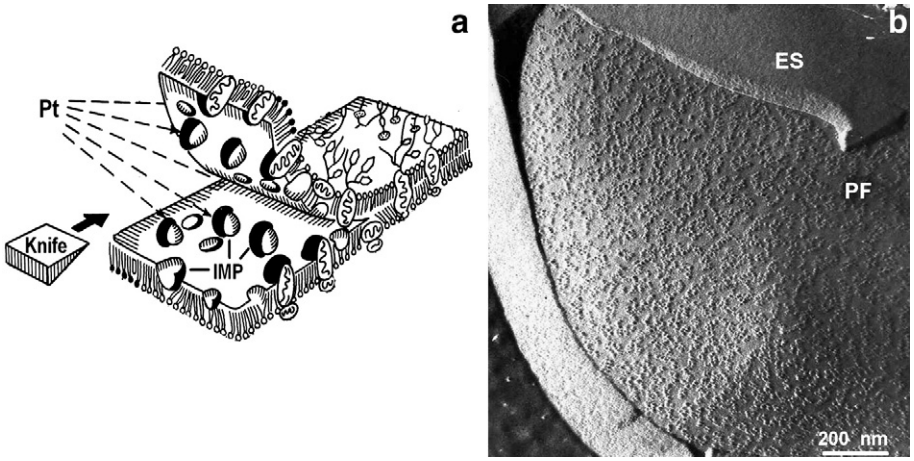

Fig. 3. Nature of membrane fractures at hydrophobic regions (Pt — evaporated material) (a). Morphology of RBC membrane, revealed by the freeze-fracture method (ES — external surface) (b).

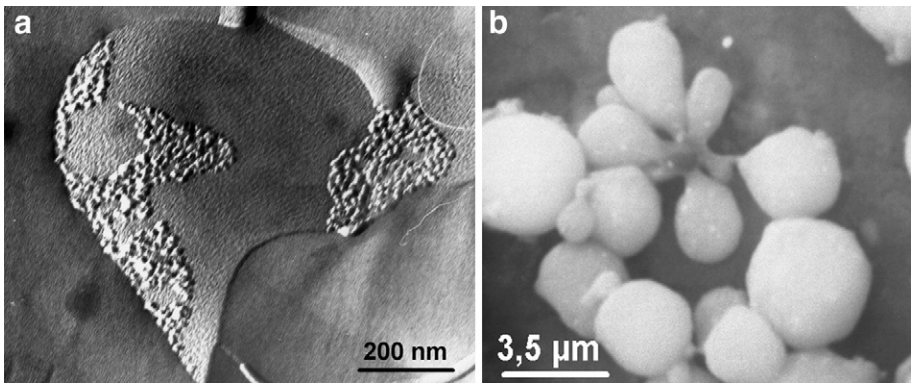

Fig. 4. RBC, incubated at 50 °C for 15 min (which induces thermal denaturation of spectrin), then cooled to 4 °C: (a) intensive irreversible aggregation of IMP with development of large, protein-free, lipid areas, that is typical for a spectrin-free vesicle; (b) fragmentation of RBC after spectrin thermal denaturation and subsequent cooling. Formation of large vesicles.

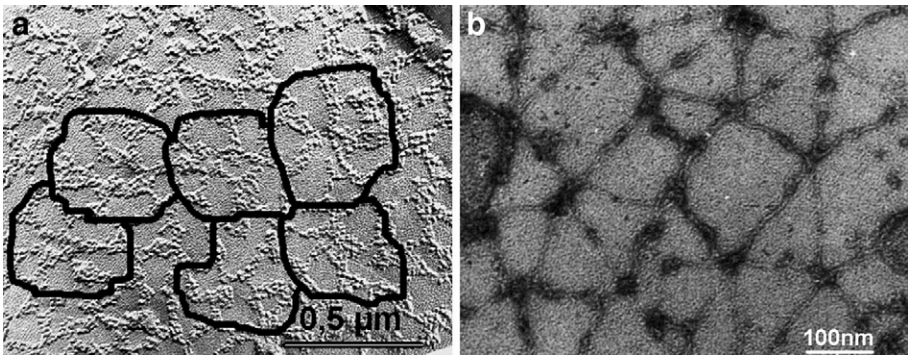

Fig. 5. Characteristic aggregation of IMP in the form of a continuous network in RBC membranes warmed to 50 °C, and then cooled to 4 °C (a). Membrane skeletal network (b) [11].

transmembrane proteins on the surface of the fracture (Fig. 5, a), are in agreement with the morphological pattern and dimensions of the elementary membrane-skeletal cells described elsewhere [11] (Fig. 5, b).

It is known that, under normal conditions, the rotatory and lateral mobilities of membrane proteins are strictly limited by the spectrin-actin membrane-skeleton, and that changes in the dynamic behaviour of the band 3 protein in the membrane can only occur with disruption of the interactions between the membrane-skeleton and the membrane. Data reported elsewhere [12] as well as our own studies [7] have shown that the membrane/membrane-skeleton system is relatively stable under physiological conditions, with no evident lateral re-distribution of IMP in the membrane plane.

The demonstration of two patterns of IMP aggregation is indicative of the existence of two groups of vesicles: small (up to 1–1.5 μm), spectrin-free vesicles, and larger ones, containing denaturated spectrin. An additional temperature effect (+4 °C) caused an intensive IMP aggregation in the first case (Fig. 4, a) and the IMP aggregation in the form of continuous network in the second one (Fig. 5, a). The second pattern is somewhat unexpected and differs, in fact, from the classical examples of aggregation caused by the action of membrane-tropic substances [13], or by hypothermic temperatures [7] or, to a lesser extent, by low pH [12].

In conclusion, the data obtained allow us to speculate that:

- thermal denaturation of spectrin and subsequent lowering of temperature (along with other effects) bring about irreversible changes in RBC morphology;
- the lipid bilayer possesses the capacity to reseal for Hb molecules and thus to repair defects that occur during RBC fragmentation;
- the observed under hyperthermia IMP aggregation in the form of continuos network, similar to a cytoskeletal spectrin one may testify to the strengthening of membrane protein affinity with spectrin molecules.

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