METHODS

Filtration Method for Studies of the Kinetics of Hypo-Osmotic Pore Closure in Erythrocyte

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Filterability of erythrocytes through small (3 μ) pores decreases with decreasing osmolarity of suspension medium because of hypo-osmotic swelling of cells. After appearance of lytic pores, erythrocyte filterability increases for some time, while after recovery of membrane integrity it decreases again. We suggest filtration method for studies of the kinetics of hypo-osmotic lytic pores closure. The dynamics of changes in erythrocyte filterability was studied in 2 patients with paroxysmal nocturnal hemoglobinuria and 6 donors (Ht 0.01%, Na phosphate buffer 5 mM, pH 7.4, 35 mOsm, 24°C). The method can be used for studies of erythrocyte membrane characteristics in various diseases and for evaluation of the membranotropic effects of drugs, infusion media, hemolysins, ethanol, *etc.*

Key Words: *erythrocytes; filterability; membrane; hypo-osmotic pore closure*

Like other body cells, erythrocytes are exposed to a variety of stresses throughout life; these exposures lead to membrane damage and even ruptures (pores). The cells can repair these injuries. The mechanism of membrane pore repair is very intricate and remains not quite clear. The process of pore closure is initiated by Ca^{2+} ions entering into the cell through the damaged membrane [9-11,15]. An obligatory condition for pore repair is the presence of the cytoskeleton and proteins capable of phospholipid binding in the presence of Ca^{2+}: annexin A1, dysferlin, and calpain.

Hypo-osmotic hemolysis of erythrocytes is a model system for studies of the kinetics of membrane pore formation and healing [3,7,13,14]. According to some authors [13], the pores in erythrocyte membrane appear in about 10 sec after the cells are placed in a hypotonic solution. Electron microscopy showed that these pores look like fissures or longitudinal ruptures of 200-500 Å and allow the passage of macromolecules through the membrane. These pores are temporary, they exist until the membrane spontaneously closes them (25-250 sec after the beginning of osmotic hemolysis) [13]. It was shown [13] that erythrocytes do not close the pores simultaneously. The pores are open in almost all erythrocytes 15 sec after the cells are placed into hypotonic solution (25 mM NaCl, Na-phosphate buffer 10 mM, pH 7.0), after 25 sec the pores are open in 50% cells, after 30 sec in 18%, and after 60 sec in 8% cells. It was shown later [7] that pore lifetime depends on experimental conditions (temperature, medium osmolarity, pH, Ca^{2+} concentration, *etc.*) and varies from seconds to tens of hours. The pore size and rate of their closing were studied by the following methods.

Ferritin, colloid gold, or stains were added to the solution at different time after the beginning of hemolysis and the counts of erythrocytes containing the

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added components were estimated by electron or fluorescent microscopy [13,14].

Radioactive isotopes ^{22}Na, ^{24}Na, ^{86}Rb, and ^{131}I-labeled albumin were added to the solution before and at different time after the beginning of hemolysis, and the kinetics of their release from erythrocytes was measured [3].

Polyethylene glycols with known diameters of molecules capable of closing the membrane pores and preventing hemolysis were added to the solution. The hydrodynamic radii of pores were evaluated using polyethylene glycols with known diameters of molecules as the osmotic protectors [1].

Electric conduction of erythrocyte suspensions was studied [1,12].

A new method for studies of the kinetics of hypo-osmotic pore closing in erythrocytes is suggested in this paper. The method is based on measurement of filterability of erythrocytes in solutions with low osmolarity through filters with 3-μ pores. This method allows differentiation of cell with open and closed pores.

Erythrocyte capacity to pass through narrow filter pores with a diameter (3 μ) lesser than the diameter of a resting erythrocyte (8 μ) is explained by its excessive surface in comparison with a sphere of the same volume [2,8]. Due to this, the cell can reversibly change its shape (be deformed) if necessary and penetrate through narrow holes (capillaries, filter pores, *etc.*). If the osmolarity of erythrocyte environment is reduced, the concentration of osmotically active matter in the erythrocyte becomes higher than outside it, due to which a volume of water needed to level the osmolarity inside and outside the cell can enter into it. The erythrocyte volume increases and the ratio of cell surface to cell volume decreases. At a certain osmolarity value depending on the initial geometrical parameters of the cell, the erythrocyte turns into a sphere and no longer passes through the filter pores [2,8].

After appearance of lytic pores, the erythrocyte start passing through filters with narrow pores [4], because the cytoplasm is partially squeezed out from the cell during filtering.

Erythrocyte ghosts (spheres) form after closing of hypo-osmotic membrane pores [6]. These spheres cannot be deformed and hence, cannot be filtered; that is, the filtration method differentiates the cells with open pores from those with closed pores and can be used for studies of the kinetics of hypo-osmotic pore closing in erythrocytes.

MATERIALS AND METHODS

The study was carried out on blood specimens from 6 donors and 2 patients with paroxysmal nocturnal hemoglobinuria (PNH).

The blood was collected from the ulnar vein, heparin serves as the anticoagulant. Blood erythrocyte count was evaluated by a cell counter (COBAS MICROS). Buffer solutions were prepared by adding the needed volume of NaCl to 5 mM Na-phosphate buffer (pH 7.4). Solution osmolarity was measured by a cryo-osmometer (Advanced Model 3250 Osmometer). Erythrocyte filterability was measured by an Ida 1 hemorheometer (patent No. 2052194 of the Russian Federation) developed and made at Laboratory of Physical Biochemistry of Hematological Center. This hemorheometer measures the time of filtration of a fixed volume of fluid (250 μl) under a pressure of 60 mm H_2O [2,8]. Polycarbonate nuclepore filters were 7 μ thick with the mean diameter of pores 3 μ, $3.5\text{-}3.8\times10^5$ pores per working surface. The filtration-osmotic curves were plotted in the following coordinates: buffer solution osmolarity, U (abscissa) and ratio of filtration time for diluted blood (Ts) to filtration time for the same volume of buffer, Tb (ordinate). The blood-buffer proportion was selected so that the measured volume contained 3×10^5 erythrocytes (Ht about 0.01%). The measurements were carried out 30-40 sec after blood dilution in the buffer at 24°C.

RESULTS

Filterability of diluted blood is close to that of buffer under isotonic conditions (Tb/Ts=0.96; Fig. 1). Decrease in medium osmolarity impairs erythrocyte filterability because of hypo-osmotic swelling of cells and their transformation into spheres. Gradual decrease in filterability can be explained by heterogeneity of erythrocyte population. Osmolarity (U) at which the

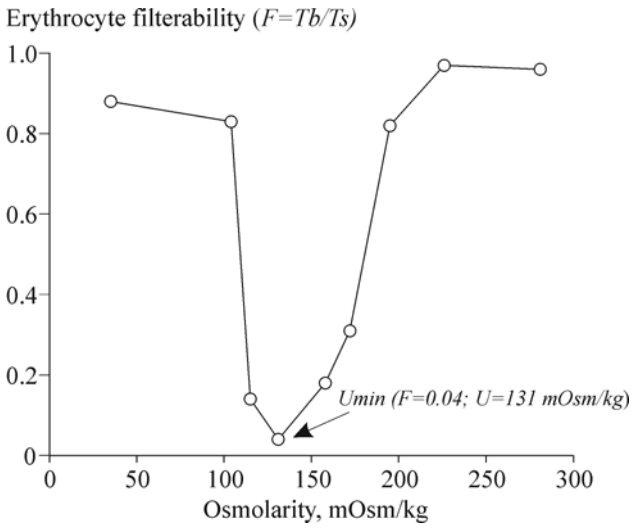

Fig. 1. Filtration-osmotic curve for donor erythrocytes. Measurements were carried out 30-40 sec after blood dilution in a buffer solution (24-25°C, pH 7.4, Ht 0.01%).

filterability is minimum (Umin) corresponds to transformation of all erythrocytes into non-filterable cells (spheres). Blood filterability improves again with further decrease in osmolarity (Fig. 1). The explanation is as follows. At U<Umin, ruptures appear in the erythrocyte membrane and a part of the cytoplasm leaks through these ruptures during cell passage through the filter pore. Hence, cells with damaged membrane easily pass through the filter with narrow pores. This improvement of erythrocyte filterability after mechanical injury to the membrane was described by other researchers [4].

At U≥Umin, erythrocyte filterability does not depend on the duration of incubation (Fig. 2, *a*), but it depends on the time of incubation at U<Umin (Fig. 2, *b*). Filterability of a blood sample gradually decreases at U<Umin. This can be explained by gradual closing of membrane pores after hypotonic hemolysis and formation of erythrocyte ghosts (spheres) [6]. These cells block filter pores, which impairs filterability of the erythrocyte suspension.

The dynamics of erythrocyte filterability depends on medium osmolarity (Fig. 2, *b*). At lower osmolarity (35 mOsm), erythrocyte filterability decreases slower. This is in line with the data indicating that the size of membrane pores increases with decreasing medium osmolarity, while the rate of pore closing decreases [7]. The kinetic curve for 115 mOsm is plotted lower than the curve for 35 mOsm osmolarity (Fig. 2, *b*). This can be explained by closing of most pores by the start of measurement (31 sec) at osmolarity of 115 mOsm.

Figure 3 shows the dynamics of filterability for erythrocytes from healthy individuals and patients with PNH at 35 mOsm osmolarity: erythrocytes from PNH patients passed the filter slower than donor erythrocytes (Fig. 3).

The pores appear in the lipid bilayer as a result of mechanical injuries, electroporation, application of pore-forming toxins, reduction of osmotic pressure, LPO, *etc.* One of the most typical and well studied examples of biological membrane destabilization is hypo-osmotic hemolysis of erythrocytes [3,7,13,14]. This phenomenon includes hypo-osmotic swelling of the cells at the initial stage. During swelling, the cell membrane stretches, this causing an increase of membrane tension. At a certain threshold tension, the pores appear in the cell membrane. Pore size is sufficient for the release of molecules of low-molecular-weight substances and hemoglobin. No complete mechanical destruction of the cell (like that of a soap bubble) ensues. The hypo-osmotic pores are temporary and are closed after some time.

We suggested from these facts that the dependence of erythrocyte filterability on medium osmolarity and filtration duration can be explained by erythrocyte swelling leading to deterioration of their filterability with reduction of the medium osmolarity from the normal level to Umin (Fig. 1); by the formation of hypo-osmotic pores leading to improvement of erythrocyte filterability at U<Umin (Fig. 1), and by closing of the hypo-osmotic pores leading to the formation of cells not filtered at U<Umin (Fig. 2, *b*) and reducing erythrocyte filterability.

Hence, measurement of erythrocyte filterability at U<Umin shows the rate of hypo-osmotic pore closure.

The results suggest a simple and available method for studies of the kinetics of hypo-osmotic pore clos-

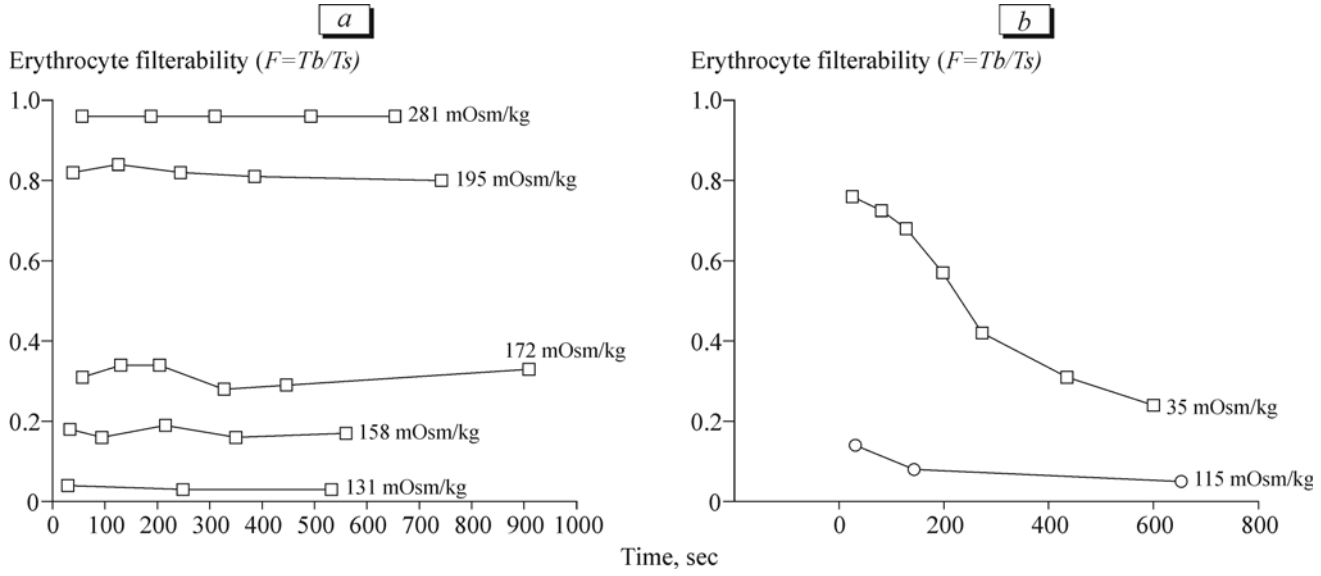

Fig. 2. Dynamics of donor erythrocyte filterability at different osmolarity of the medium (24-25°C, pH 7.4, Ht 0.01%). *a*) U≥Umin; *b*) U<Umin.

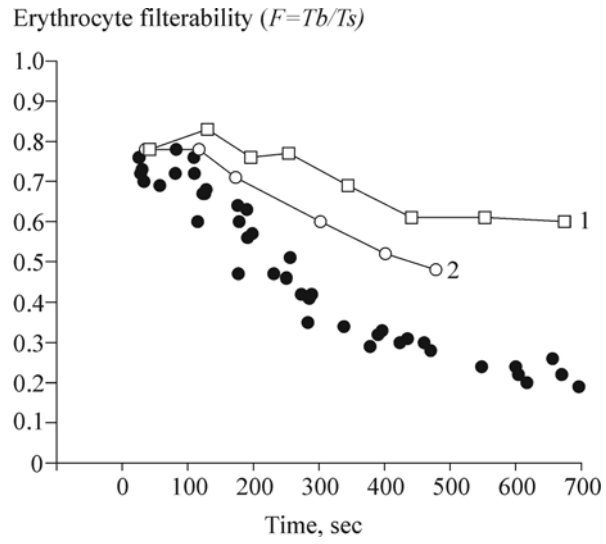

Fig. 3. Dynamics of filterability of erythrocytes from healthy donors and PNH patients at osmolarity of 35 mOsm/kg (24-25°C, pH 7.4; 3×10^5 erythrocytes in measured volume). Dark symbols: donors (*n*=6). *1*) PNH patient 1; *2*) PNH patient 2.

ing in erythrocytes: measurement of the relationship between filterability of blood diluted in hypotonic medium and duration of filterability.

Analysis of the kinetics of hypo-osmotic pores closing in erythrocytes in various hemolytic anemias is expected to explain the causes of erythrocyte hemolysis and provide new data on the mechanism of membrane pore closing.

We showed inhibition of lytic pore closing in erythrocytes in PNH, which is presumably related to changes in erythrocyte membrane characteristics. The greater part of erythrocytes in the blood of PNH patients contains no glycosylphosphatidylinositol (GPI), an glicoprotein binding various proteins to membrane.

The filtration method for studies of the kinetics of hypo-osmotic pore closing in erythrocytes suggested in this paper can be used for studies of membranotropic effects of drugs, infusion media, hemolysins, ethanol, *etc*. It is rapid, simple, and inexpensive.

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