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The Effect of Polyene Antibiotic Amphotericin B on Erythrocyte Cytoarchitectonics and Osmotic Resistance

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We studied the effect of amphotericin B (2.5×10^{-5} and 5.4×10^{-5} M) on osmotic resistance and surface cytoarchitectonics of donor blood erythrocytes. Antibiotic at a concentration of 2.5×10^{-5} M induced most pronounced changes in the studied parameters, which can be related to the specifics of the spatial organization of the cholesterol–amphotericin B complexes at different stoichiometric ratios of the components and their ability to pore formation in the membranes. Cholesterol binding to the polyene antibiotic and the appearance of perforations in the plasma membrane lead to accumulation of reversibly and irreversibly deformed cells and their hemolysis. The appearance of a large number of irreversibly deformed erythrocytes indicates an impaired ability to elastic deformation in the microcirculatory stream, which can lead to disruption of their functions *in vivo* and intravascular hemolysis.

Key Words: *red blood cells; osmotic resistance of erythrocytes; amphotericin B; kinetics of hypoosmotic hemolysis; scanning electron microscopy*

The main function of red blood cells is to transport oxygen from the lungs to the tissues. The oxygen-transporting protein hemoglobin and erythrocyte membranes play the leading role in the regulation of gas exchange. Abnormalities of the hemoglobin structure and impairment of the plasma membrane strength and elasticity negatively affect the physiological function of red blood cells [1].

Sterols, including cholesterol, play an important role in the organization and maintenance of plasma membrane integrity. Cholesterol participates in the coordination of cell processes, affects membrane fluidity, and regulates movement of membrane proteins. Excessive accumulation of cholesterol in membranes contributes to the development of a number of pathological conditions caused by a decrease in plasmalemma elasticity [2].

Changes in the lipid composition of membranes are accompanied by transformational rearrangements of cells. Normally, about 3% of erythrocytes have irregular shape [3]: echinocytic, stomatocytic, dome-shaped, spherocytic, *etc.* [4]; under pathological conditions, their fraction can increase. Laser diffractometry have demonstrated that the transformation of biconvex discocytes into echinocytes is a result of cross-linking between spectrin and hemoglobin [5]. The gas-transport function and hemolysis stability of deformed red blood cells are impaired.

Previous experiments at the Department of Biophysics and Biotechnology of the Faculty of Medicine and Biology of the Voronezh State University showed that polyene antibiotic amphotericin B (AmB) from *Streptomyces nodosus* [6] is a promising probe for cholesterol detection in membranes: cholesterol–AmB associations can fluoresce, which allows visualization of sterol-containing areas of the plasmalemma [7]. The molecular structure of this substance (Fig. 1) is presented by a macrolide lactone ring formed by a rigid lipophilic chain with 7 conjugated double bonds and 7 hydroxylated carbon atoms on the opposite side of the ring. AmB binds to sterol-containing components on the cell membrane and forms pores, which leads to leakage of intracellular cations and causes cell

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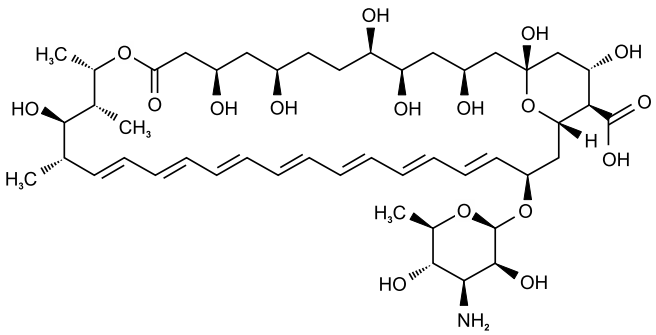

Fig. 1. Structural formula of AmB.

death [8,9]. Due to the presence of a large number of chemically active groups, AmB probably can interact with the protein components of cells. Therefore, not only the fluorescent properties of "cholesterol–AmB" complexes, but also the effect of AmB on the cytoarchitectonics, properties of the plasma membrane, and protein components of erythrocytes are of particular interest.

Scanning electron microscopy (SEM) is used to visualize and analyze the surfaces of objects and is widely applied in biology. In addition to SEM, spectral methods of analysis are convenient and effective for the study of blood components [4].

The aim of this work was to study the impact of AmB on the cytoarchitectonics and osmotic resistance of human erythrocytes.

MATERIALS AND METHODS

To obtain erythrocyte suspension, cellular components were precipitated from the whole blood of donors obtained at the Voronezh Regional Blood Transfusion Station, a branch of the Emergency Medical Care Hospital No. 1. The whole blood (1 ml) was mixed with 4 ml of 0.9% NaCl and centrifuged at 1500 rpm for 15 min (MPW-340 centrifuge; MPW Med. Instruments). Erythrocytes were washed 3 times with a 4-fold volume of 0.9% NaCl. In our experiments we used erythrocyte suspension with the concentration of 2×10^5 cells/ml, which corresponded to optical density OD_{490}=0.8 [10]. The erythrocyte suspension was incubated for 30 min with AmB solutions in final concentrations of 2.5×10^{-5} and 5.4×10^{-5} M, under sterile conditions at 37°C in a dry-air thermostat TS-1/80 SPU (Smolenskoe Special Design and Technological Bureau of Programmed Control Systems).

Erythrograms were recorded in hypoosmotic medium (0.3% NaCl) immediately after addition of the cell suspension. The light transmission of the samples at λ=490 nm was recorded on UV-2401 PC (Shimadzu) for 300 sec in the kinetic mode at an interval of 1 sec. The dependence of light transmission intensity (T, %) on time (sec) was constructed. The following parameters were estimated on the graph: t_{lat}, latent phase of hemolysis, or phase of spherulation (sec); t_{50}, time of half-hemolysis (sec); t_G, time of complete hemolysis (sec); proportion of osmotically unstable, medium stable, and highly stable erythrocytes in the sample [10].

The surface architectonics of donor blood erythrocytes was studied by SEM. The control and experimental erythrocyte samples were prepared as described elsewhere [11]. The preparations were sputtered with gold and examined under a JSM-6380 LV scanning electron microscope (Jeol) at accelerating voltage of 20 kV at the Common use Center Scientific Equipment of the Voronezh State University.

Structural characteristics of erythrocyte membranes were assessed according to the classification of G. I. Kozinets and J. Simworth [12]. The following cell types were identified: reversibly deformed (5 classes that can restore the pellucid shape: discocytes, discocytes with one outgrowth, with a ridge, with multiple outgrowths, "mulberry" shaped erythrocytes) and irreversibly deformed, or prehemolytic erythrocytes (5 classes: dome-shaped erythrocytes (stomatocytes), spherical erythrocytes, spherocytes with spikes on the surface (echinocytes), "downed ball" erythrocytes, and degeneratively altered erythrocytes).

For a detailed analysis of the nature of changes in the surface architectonics of erythrocytes we calculated a number of indicators: D, number of discocytes (%); RD, number of reversibly deformed erythrocytes (%); ID, number of irreversibly deformed erythrocytes (%); IT, transformation index, which is a quantitative assessment of the ratio of pathological and normal forms of red blood cells: IT=(RD+ID)/E; IRT, index of reversible transformation: RD/E; IIT, index of irreversible transformation: IIT=ID/E.

Experiments were performed in 5-7 replications, analytical determinations for each sample were performed in 4 repetitions. Statistical processing of the experimental results was carried out using Stadia 8.0 Professional software (InCo), the results are presented as $M\pm m$. Normality of distributions was checked using the Kolmogorov, ω^2, and χ^2 tests. Significance of differences in case of normal distribution was assessed by the Student's t test at 5% level of significance [13].

RESULTS

The native sample of erythrocyte suspension (Table 1, Fig. 2) contained ~94% discocytes; ~4% reversibly deformed cells (discocytes with one outgrowth, with a ridge, with multiple outgrowths, mulberry-shaped erythrocytes) and ~2% irreversibly deformed erythrocytes (dome-shaped erythrocytes, spherocytes with smooth surface, with spikes on the surface,

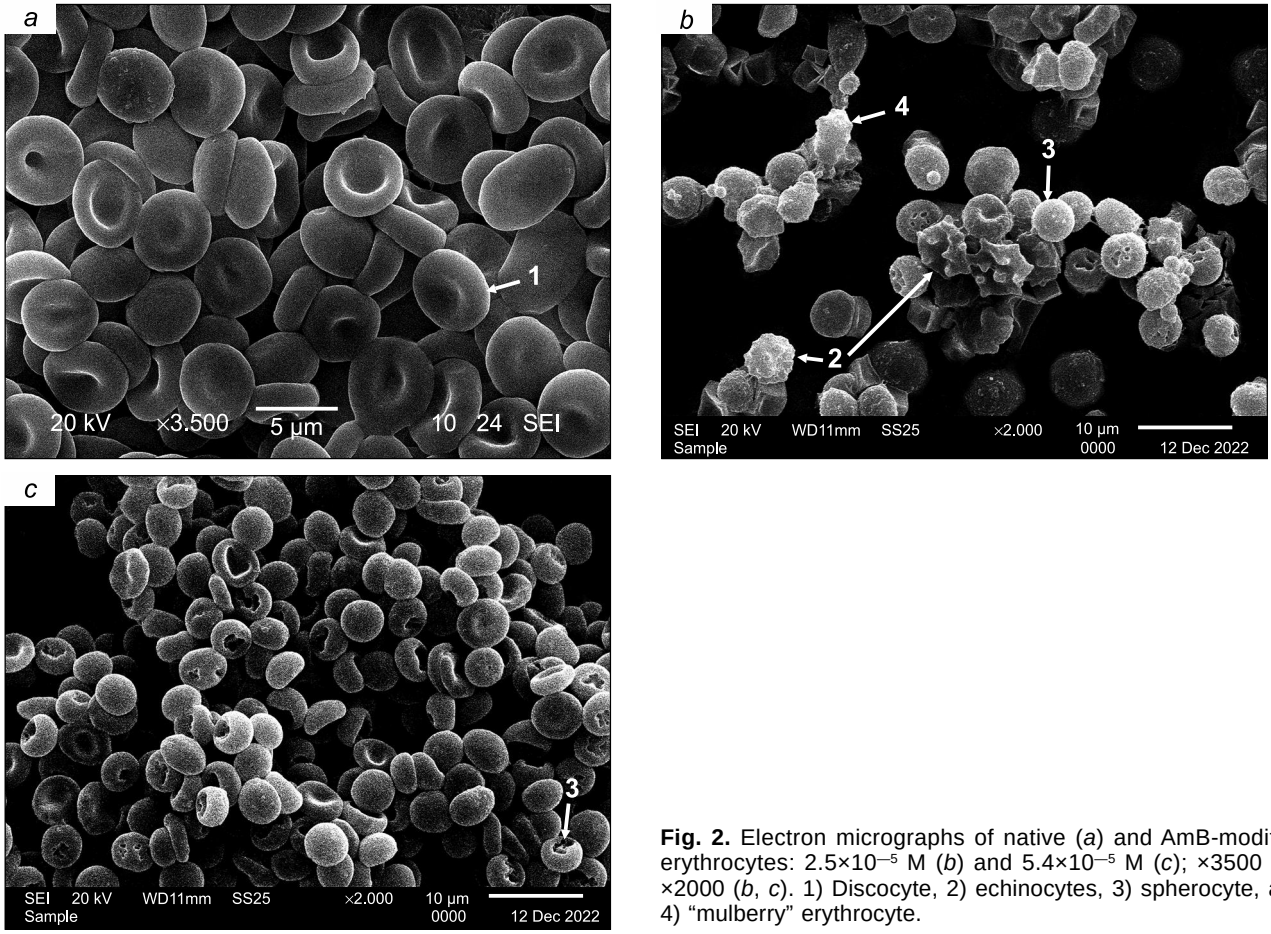

Fig. 2. Electron micrographs of native (*a*) and AmB-modified erythrocytes: 2.5×10^{-5} M (*b*) and 5.4×10^{-5} M (*c*); ×3500 (*a*), ×2000 (*b*, *c*). 1) Discocyte, 2) echinocytes, 3) spherocyte, and 4) "mulberry" erythrocyte.

TABLE 1. Parameters of Cytoarchitectonics of Donor Red Blood Cells before and after Exposure to AmB (*M*±*m*)

Parameter	Control	Erythrocytes+ AmB (2.5×10^{-5} M)	Erythrocytes+ AmB (5.4×10^{-5} M)
Discocytes, %	93.90±2.09	4.08±0.43*	8.58±1.10*
Reversibly deformed erythrocytes, %	4.21±1.05	19.10±2.36*	12.00±1.46*
Irreversibly deformed erythrocytes, %	2.00±0.67	25.40±1.83*	25.60±1.19*
IT	0.065±0.015	12.00±1.21*	5.50±1.08*
IRT	0.044±0.011	4.94±0.54*	1.50±0.18*
IIR	0.020±0.007	7.03±0.81*	3.99±1.04*

Note. *$p<0.05$ in comparison with the control (native samples).

erythrocytes in the form of a "deflated ball", degenerative forms of erythrocytes). Transformation indices are presented in Table 1; these indices corresponded to the morphological picture of red blood cells in a healthy person [12].

Analysis of the kinetic parameters of hypoosmotic hemolysis of native erythrocytes (Fig. 3) revealed 3 cell subpopulations in intact erythrocyte suspension: unstable (up to 40% of cells in the sample), medium-stable (~25% of cells), and highly stable (~35% of cells). Unstable erythrocyte subpopulation was characterized by the absence of the latent phase of hemolysis, t_{50} was 6 sec, t_G was 9 sec. In the medium stable fraction of native erythrocytes, the latent phase of hemolysis lasted 6 sec, t_{50} was 17 sec, and t_G was 32 sec. The stable red blood cell subpopulation underwent hemolysis within 10 sec, t_{50} was 83 sec, and t_G was 285 sec.

Exposure of human erythrocyte suspensions to AmB at a concentration of 2.5×10^{-5} M for 30 min

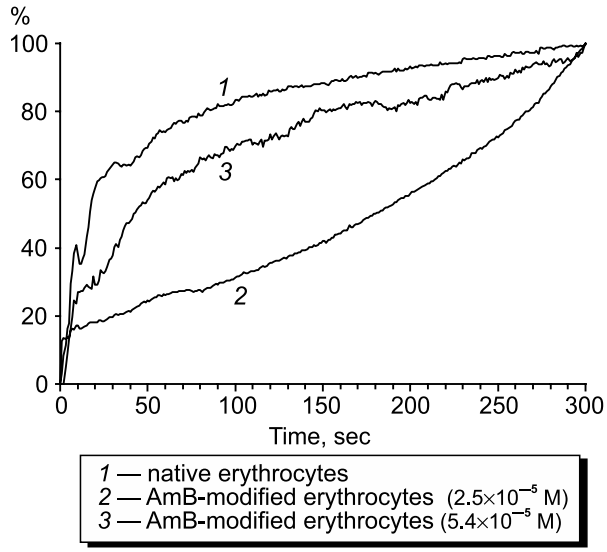

Fig. 3. Hypoosmotic hemolysis of native and AmB-modified erythrocytes.

(Table 1, Fig. 2, *a*) resulted in a decrease in the proportion of discocytes and an increase in reversibly deformed cells, irreversibly deformed erythrocytes, IT, IRT, and IIT values.

Significant changes in the kinetics of hypoosmotic hemolysis were observed in the samples incubated with the polyene antibiotic. The erythrogram of the modified cells instead of the classical S-curve was described by an exponential function $y=a^x$ (Fig. 3). No clear-cut subpopulations of erythrocytes with different resistance to hypoosmotic medium could be distinguished. The latent phase of hemolysis (spherulation phase) was absent; t_{50} was 200 sec, t_G was 300 sec.

After modification of erythrocytes with AmB solutions in a concentration of 5.4×10^{-5} M (Table 1, Fig. 2, *b*), we observed a decrease in the level of discocytes, an increase in the proportion of reversibly and irreversibly deformed cells and the transformation indices (IT, IRT, and IIT). Increasing the concentration of the modifying agent resulted in the following changes in the kinetics of hypoosmotic hemolysis of erythrocytes. The shape of the erythrogram of the modified erythrocytes approached that of the control one. Individual erythrocyte subpopulations were still unidentifiable, but the spherulation phase was recorded (t_{lat}=3 sec); t_{50} was 42 sec, t_G was 290 sec.

Thus, exposure of erythrocyte suspensions to AmB in the studied concentrations caused an increase in the number of reversibly and irreversibly deformed cells in the samples and a decrease in hypoosmotic resistance of red blood cells. We observed a significant increase in the number of spherocytes, echinocytes, and "mulberry" erythrocytes in comparison with the control samples. These forms are characterized by impaired elasticity of the erythrocyte membrane, which is reflected in the kinetics of osmotic hemolysis. It should be noted that the lower concentration of AmB caused more pronounced changes in both osmotic resistance and surface cytoarchitectonics of donor blood erythrocytes. This can probably be explained by the peculiarities of the spatial organization of cholesterol–AmB complexes at different stoichiometric ratios of the components and their ability to pore-formation in the membranes.

The appearance of a large number of irreversibly deformed erythrocytes reflects disturbances of the system stability at the level of the whole cell, which causes changes in its functional state and, consequently, the ability to elastic deformation in the microcirculatory bed [14]. Our findings indicate that the contact of polyene antibiotic with the components of red cell membranes can lead to disruption of their functions *in vivo* and induce intravascular hemolysis.

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