Determination of Optimal Conditions for Studies of Electrophoretic Mobility of Human Erythrocytes Loaded with Cryoglobulins

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For studies of the charge characteristics of human erythrocytes loaded with cryoglobulins the cells should be stored in Alsever solution with 0.1% sodium azide no longer than 1 week after blood collection. Erythrocytes should be loaded with cryoglobulins by incubation during at least 4 h, the final concentration of cryoproteins in the solution being no lower than 30 μg/ml.

Key Words: *cryoglobulins; electrophoretic mobility; human erythrocytes; optimal conditions*

A wide spectrum of diseases is associated with disorders in Ig metabolism or synthesis, with autoimmunity or hypersensitivity phenomena, or with persistent antigenic stimulation. These diseases are sometimes associated with an appreciable increase in the blood levels of proteins with abnormal thermal solubility (cryoglobulins). Cryoglobulins play an important pathogenetic role in many diseases of the kidneys, skin, and nervous system tissues [1]. However, in low levels they can be present in the blood of normal subjects [1].

Pronounced specificity, characteristic of cryoproteins, is not proven. Experimental data on physical and chemical characteristics of cryoglobulins indicate that abnormal properties of cryoglobulins are largely determined by their charge characteristics, which, in turn, depend on the presence of extra negatively charged residues on the surface of protein molecule.

According to some data, abnormal solubility of cryoglobulins is determined by polar interactions: inhibition of cryoglobulin precipitation with neutral salts attenuating electrostatic interactions [7]; negative Gibbs energy for cryoglobulin reaction with polyethylene glycol at low temperatures [4]; direct relationship between galactosylation level, determining Ig molecule charge, and its cryoactivity [3]. It was also shown that cryoglobulins subjected to enzymatic treatment for removal of carbohydrates from their surface lost the capacity to precipitate after cooling [6].

The aim of our study was to determine optimal conditions for storage of erythrocytes under laboratory conditions and for loading of erythrocyte membranes with cryoglobulins for subsequent comparative study of electrophoretic mobility (EPM) of erythrocytes loaded and not loaded with cryoglobulins.

MATERIALS AND METHODS

Erythrocytes were isolated from donor blood. Whole blood and plasma were centrifuged for 10 min at 1000*g*. Plasma and white blood cells were removed, and erythrocytes were washed 3 times in normal saline.

Cryoglobulins were isolated from the sera of patients with acute carotid ischemic stroke by multiple cold centrifugation followed by dissolving du-

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ring heating [1]. Protein concentration was determined photometrically on a CARY spectrophotometer at λ=280 nm.

Erythrocyte EPM was evaluated by the micromethod [2] on an OPTON flow cytoferrometer.

In order to evaluate the duration of storage and type of medium for storage of erythrocytes, the cells were put into the following media: Alsever solution; Alsever solution with 0.1% sodium azide; phosphate buffered saline (PBS); PBS with 0.1% sodium azide at 4°C for 17 days. Electrophoretic mobility of "intact" (not loaded) erythrocytes was evaluated on days 1, 2, 4, 7, 8, 12, 14, 15, and 17 of incubation.

The optimal duration of incubation for erythrocyte loading with cryoglobulins was determined by adding 0.2 ml cryoglobulin solution (250 μg/ml) to 1 ml erythrocyte suspension (4.0×10^6 cells). The suspension was incubated at 4°C. Samples were taken from the suspension after 15, 30, 60, 120, 180, 240 min, and 16 h of incubation and EPM was evaluated.

RESULTS

A gradual decrease of EPM was observed during the first 7-8 days of storage in all media, indicating a progressive loss of the total charge of erythrocyte membrane (Fig. 1). Significant hemolysis was visually seen in samples with PBS on days 7-8. In Alsever solution massive hemolysis of erythrocytes was observed on days 10-12. Great scatter in EPM values after 8 days for erythrocytes stored in PBS and after 12 days for cells stored in Alsever solution can be due changes in erythrocyte weight because of partial or complete loss of hemoglobin during hemolysis and to changes in erythrocyte membrane charge. Minimum hemolysis was observed in Alsever solution with 0.1% sodium azide.

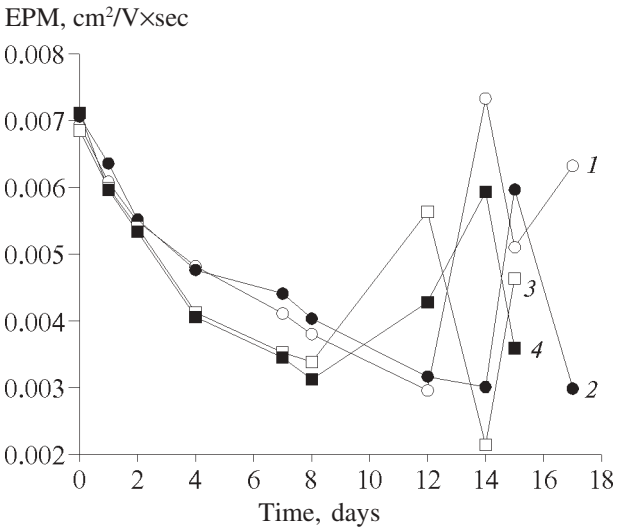

Fig. 1. Chnages in electrophoretic mobility (EPM) of erythrocytes stored *in vitro*. *1*) Alsever solution; *2*) Alsever solution+sodium azide; *3*) PBS+sodium azide; *4*) PBS.

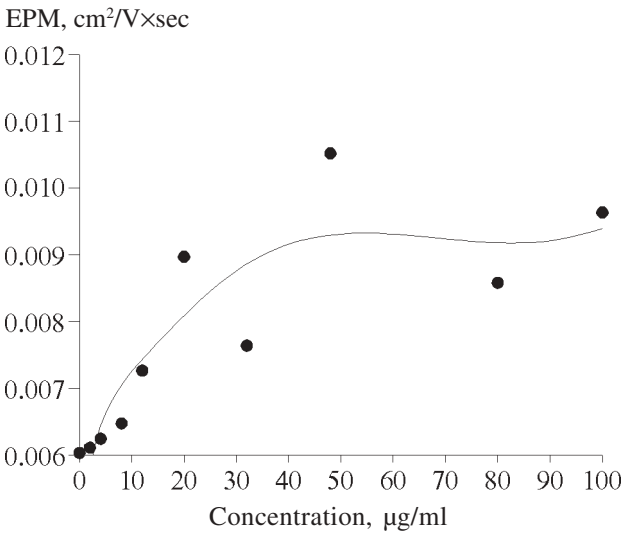

Fig. 2. Relationship between erythrocyte EPM and concentration of loaded protein.

TABLE 1. Cryoglobulin Concentrations for Erythrocyte Loading

Parameter	Sample No.									
	1	2	3	4	5	6	7	8	9	10
Volume of erythrocyte suspension, ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cryoprotein solution, ml	0.0	0.01	0.02	0.04	0.06	0.10	0.16	0.24	0.4	0.5
PBS, ml	0.5	0.49	0.48	0.46	0.44	0.4	0.34	0.26	0.1	0.0
Final concentration of cryoglobulins, μg/ml	0.0	2.0	4.0	8.0	12.0	20.0	32.0	48.0	80.0	100.0

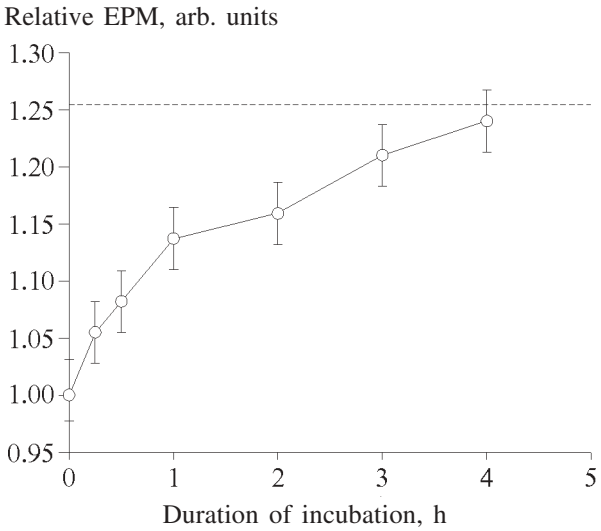

Fig. 3. Relationship between relative EPM and duration of incubation. Intermittent line: relative EPM after 16-h incubation.

In order to detect the effect of the initial concentration of cryoprotein on EPM of loaded erythrocytes, we determined the optimal concentration of loaded protein.

Different volumes of cryoglobulin solution in a concentration of 200 μg/ml and normal saline were added to 0.5 ml erythrocyte suspension (4.0×10^6 cells/μl) to a final volume of 1 ml (Table 1). Erythrocytes were loaded with cryoglobulins over 14-h incubation at 4°C (Fig. 2).

Saturation of the erythrocyte membrane with cryoglobulins (when the curve reached a plateau) was observed at a final concentration of 40 μg/ml and remained unchanged after increasing the concentration to 100 μg/ml (Fig. 2).

Previous data on EPM changes and differences in EPM for different age groups [5] prompted us to calculate the relative EPM (the ratio of EPM for cryoglobulin-loaded and not loaded erythrocytes, Fig. 3), in order to rule out the effect of the proper EPM of uncharged erythrocytes.

Equilibrium in interactions between cryoglobulins and erythrocyte membrane was attained after 4-h incubation (Fig. 3). Negligible changes in EPM during the next 12 h indicate that 4-12 h is a sufficient period for erythrocyte incubation in cryoglobulin solution.

Hence, optimal conditions were determined for studies of the charging characteristics of cryoglobulins for loading of human erythrocytes to be used for evaluation of their EPM.

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