Studies on Ammocytes: Development, Metabolic Characteristics, and Detoxication of Ammonium

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The possibility of reducing ammonium concentration in the blood of mice with hyperammonemia with ammocytes (erythrocytes loaded with glutamate synthase) and the metabolic characteristics of these cells were studied. Injection of ammocytes into the blood stream of animals with hyperammonemia led to reduction of the blood ammonium concentration within the first 30-120 min and this activity of ammocytes was retained for at least 2 days. Endogenous phosphofructokinase, glucose-6-phosphate dehydrogenase, hexokinase, lactate dehydrogenase, pyruvate kinase, and Na^+,K^+-ATPase in ammocytes remained at the levels of catalytic activities characteristic of intact erythrocytes. Hence, ammocytes are functionally active cells and can be used as a protective system in pathological hyperammonemia, while the method can be regarded as a new technology for medicine and veterinary.

Key Words: *erythrocytes; glutamate synthase encapsulation; ammonium; biotechnology*

Modulation of metabolic processes in humans or animals with medical and experimental purposes by injections of liposomes or microparticles, erythrocytes, or erythrocyte ghosts, loaded with bioactive substances was used during two recent decades. Intracellular substances injected into animal blood in this form react with their immediate environment (plasma) and with toxins hazardous for health preventing their effects. One of the main endogenous toxins in mammalians is ammonium. Its removal from the blood is a new task, heretofore not studied, and a pressing biomedical problem. Ammonium in high concentrations is toxic and causes functional disorders in the CNS, which can lead to coma and death. Numerous diseases (cirrhosis, hepatic encephalopathy, alcohol intoxication, Alzheimer's disease, many genetic disorders) are associated with hyperammonemia. One of the main causes of fatal hyperammonemia in humans, most often developing after transplantation of the lungs and bone marrow, is glutamine synthase (GS) insufficiency [3,5].

Plasma concentration of ammonium in patients with hyperammonemia can be reduced by drugs, but this process takes several days [1,2,7]. No agents capable of eliminating high concentrations of ammonium from the blood within a short time are known up to the present time.

We studied the capacity of ammocytes loaded with GS to metabolically remove ammonium from the blood of mice with hyperammonemia. Ammocytes are erythrocytes with encapsulated bioactive substances intended for reduction of plasma ammonium concentrations. No studies of this kind were described. Changes in functional activity of erythrocytes after their modification as a result of charging with foreign enzyme systems are unknown.

MATERIALS AND METHODS

The study was carried out on mice (26-30 g). Erythrocytes were isolated from pooled mouse blood and loaded with GS using the encapsulation pro-

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TABLE 1. Catalytic Activities of Glycolytic Enzymes and Na^+,K^+-ATPase in Intact and Sham-Loaded Murine Erythrocytes and GS-Loaded Ammocytes (*M*±*m*)

Cell type	HK	G-6-PDH	PFK	PK	LDH	Na^+,K^+-ATPase
Intact erythrocytes	13.50±0.17	112.5±8.3	85.7±1.5	82.5±2.5	558±20	165.7±52.7
Sham-loaded erythrocytes	12.5±0.5*	91.7±3.3*	85.5±2.5	79.2±5.0	410±45*	153.5±11.7
Ammocytes	12.50±0.33*	91.7±2.7*	84.9±1.5	75.5±2.5*	405±32*	150.9±8.2

Note. HK: hexokinase; PFK: phosphofructokinase; PK: pyruvate kinase. Enzyme activity is expressed in ncat/ml cell volume. The means and standard deviations for 4-6 experiments are presented. *$p<0.05$ compared to intact erythrocytes.

cedure, based on hypotonic dialysis, "hardening", and isotonic "sealing" [6].

The blood was collected into a tube with sodium citrate. GS was encapsulated in isolated thoroughly washed erythrocytes. The resultant ammocytes were resuspended in native donor plasma or in 0.9% NaCl and their capacity to eliminate ammonium from the blood of mice with experimental hyperammonemia was evaluated.

Two additional suspensions of purified erythrocytes were prepared for each experiment: native cells subjected to the above treatment except dialysis against hypotonic buffer and sham-loaded erythrocytes subjected to the above treatment without addition of GS into the dialysis sac.

The integrity of cell membranes was evaluated by the release of cytoplasmic enzymes from the cells. Catalytic activities of intracellular enzymes were evaluated by methods of enzyme analyses.

Directly after injection of 0.4 ml ammocytes or unloaded erythrocytes into the lateral caudal vein, the mice were intraperitoneally injected with ammonium acetate (2.5 mmol/kg). This dose did not modify animal behavior and caused moderate hyperammonemia (up to 1.2-1.5 mM) at least during the first 4 h after ammonium injection. The blood was collected from the retroorbital venous plexus 30, 60, and 120 min after injection and ammonium concentration was measured by fluorometry with glutamate dehydrogenase as described previously [4].

Ammocyte viability in the bloodflow of mice was evaluated by flow cytofluorometry. Erythrocytes were preincubated with FITC and injected to animals into the lateral caudal vein. The animals were decapitated 5, 15, 30, and 120 min after injection of erythrocytes, the blood was collected, erythrocytes were isolated from pooled blood and analyzed by flow cytofluorometry.

RESULTS

The conditions of GS encapsulation into erythrocytes were selected in preliminary experiments. Incorporation of the enzyme into cells depended on the composition of hypotonic medium, GS concentration in this medium, hematocrit, pore size in the dialysis membrane, duration of dialysis, conditions of cell hardening, medium composition, and temperature at which the cells were sealed. The following conditions were found to be the optimal for cell sealing after GS loading: incubation of 1.4 ml ammocytes for 30 min at 37°C and constant stirring at 100 rpm in 0.7 ml 30 mM Na_2HPO_4 (pH 7.4) containing 30 mM inosine, 30 mM glucose, 30 mM glutamate, 1.5-30 mM pyruvate, 1.5 mM adenine, 3 mM $MgCl_2$, 3% NaCl, and 6.6 mM mercaptoethanol. The efficiency of the entire procedure was characterized by encapsulation of up to 16% GS with 70% cells retained.

During GS encapsulation, the cells lost up to 20% endogenous soluble glycolytic and conjugated enzymes: hexokinase, glucose-6-phosphate dehydrogenase (G-6-PDH), lactate dehydrogenase (LDH) (Table 1). However, activities of membrane-bound enzymes (phosphofructokinase and Na^+,K^+-ATPase) remained unchanged (Table 1). This suggested that plasma membrane retained normal ATP-dependent sodium pump and geometrical integrity. In addition, analysis showed stable content of ATP in GS-loaded ammocytes and erythrocytes without the

TABLE 2. Changes in Ammonium Concentration in Mouse Plasma during Different Periods after Injection of GS-Loaded Ammocytes (*M*±*m*)

Min after injection	Plasma ammonium concentration, mmol/liter	
	control (0.9% NaCl and glucose)	experiment (mixture of ammocytes, 0.9% NaCl, and glucose)
30	0.94±0.16 (*n*=4)	0.52±0.09* (*n*=4)
60	0.84±0.02 (*n*=2)	0.57±0.00 (*n*=2)
120	0.95±0.26 (*n*=4)	0.22±0.05* (*n*=4)

Note. *$p<0.01$ compared to the control.

enzyme, which was an additional evidence of functional activity of these cells. Ammocytes were capable of retaining enzyme activity of GS *in vivo* for at least 2 days.

The results of measurements of ammonium concentrations in the blood of mice 30, 60, and 120 min after simultaneous injections of ammonium (intraperitoneally) and GS-loaded ammocytes (intravenously) are presented in Table 2. Blood ammonium concentrations decreased almost 2-fold 30 min after ammocyte injection and 4-fold after 120 min in comparison with ammonium concentration in controls. The lifespan of injected erythrocytes in the blood of mice was evaluated by fluorescence of FITC binding exclusively to membrane proteins. The percentage of erythrocytes surviving in circulation of mice was at the level of 50% for 48 h.

The study showed that ammocytes retained their integrity, energy metabolism parameters, activities of encapsulated enzymes, and capacity to decrease ammonium content in the blood stream of animals with hyperammonemia. They can be used *in vivo* as a protective system in pathological hyperammonemia and this method can be regarded as a new biotechnology for medicine and veterinary.

REFERENCES

1. G. T. Berry, N. D. Bridges, K. L. Nathanson, *et al.*, *Arch. Neurol.*, **56** No. 4, 481-484 (1999).
2. A. Chiu, S. Tam, W. Y. Au, *et al.*, *Liver Transpl.*, **11**, No. 3, 353-355 (2005).
3. A. Y. Ho, A. Mijovic, A. Pagliuca, and G. J. Mufti, *Bone Marrow Transplant.*, **20**, No. 11, 1007-1008 (1997).
4. E. Kosenko, V. Felipo, C. Montoliu, *et al.*, *Metab. Brain Dis.*, **12**, No. 1, 69-82 (1996).
5. G. R. Lichtenstein, Y. X. Yang, F. A. Nunes, *et al.*, *Ann. Intern. Med.*, **132**, No. 4, 283-287 (2000).
6. L. Rossi, S. Serafini, F. Pierige, *et al.*, *Expert Opin. Drugs Deliv.*, **2**, No. 2, 311-322 (2005).
7. J. F. Rueda, C. Caldwell, and D. C. Brennan, *Ann. Intern. Med.*, **128**, No. 11, 956-959 (1998).