A NEW CHEMOTHERAPEUTIC AGENT: L-ASPARAGINASE ENTRAPPED IN RED BLOOD CELLS

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The results of an investigation aimed at the prodution of a new therapeutic form of L-asparaginase loaded erythrocytes are presented.

We found that transport of asparagine into erythrocytes can be described by Michaelis-Menten's equation with the following kinetic parameters: Km = 2.5 mM and Vmax = 0.24 mmol/h per litre of cells.[1] The L-asparaginase introduction into the cells does not significantly change the parameters of asparagine transport, and leads to the destruction of entering L-asparagine. However, L-asparagine degradation by L-asparaginase loaded erythrocytes is limited by slow asparagine transport into erythrocytes, so the activity of an asparagine degrading system necessary for therapeutic effectiveness (30 IU/l for man[2]) cannot be reached. On the other hand a continuous leakage of the entrapped enzyme is observed in vitro,[1] probably due to damage of the cells. This process could explain therapeutic effect of asparaginase loaded erythrocytes in the circulation.

The pharmacokinetics of L-asparaginase in solution or entrapped in erythrocytes were studied using normal CBB6F1 male mice. L-asparaginase was introduced by either the method of reversible hypoosmotic lysis,[1] or dialysis,[3] or by a method of stepwise dialysis developed in our laboratory.[4] Comparison of these methods has shown that simple reversible lysis is characterized by the least entrapment efficacy and dramatically alters the biochemical parameters of the loaded cells. Maximal entrapment is achieved by dialysis. Our method (stepwise dialysis) provides 30% less entrapment efficacy but the biochemical properties of the produced cells are significantly better as well as their viability in vivo (Table 1 and Figure 1A). Furthermore, a marked activity of L-asparaginase is observed in plasma up to 26th day after injection (Figure 1B). The erythrocytes loaded by the method of reversible hypoosmotic lysis are being destroyed in a bloodstream and the half-life time of the enzyme entrapped in erythrocytes is only slightly higher than that of the free enzyme in plasma ($t_{1/2}$ = 1 day).

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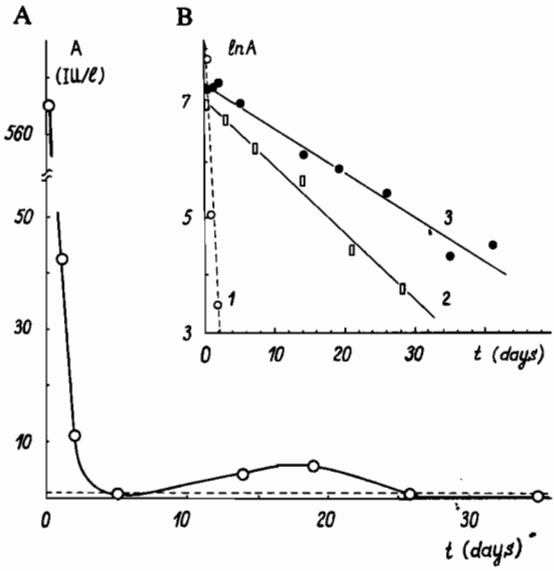

Figure 1. A: L-asparaginase activity changes : 1 - in normal mouse plasma after i/v injection of free enzyme solution ($t_{1/2}$ = 7.2 hours); 2 - in erythrocytes after i/v injection of L-asparaginase loaded erythrocytes prepared by dialysis method ($t_{1/2}$ = 6 days); 3 - in erythrocytes after i/v injection of L-asparaginase entrapped in erythrocytes by stepwise dialysis ($t_{1/2}$ = 9 days). B: L-asparaginase activity changes observed in plasma after i/v injection of erythrocytes loaded by stepwise dialysis.
L-asparaginase dose injected was 5.7 IU/mouse when free enzyme solution or erythrocytes loaded by stepwise dialysis were used and 2.5 IU/mouse when erythrocytes loaded by conventional dialysis method were used.

A dyalisis laboratory setup was constructed to produce aseptically the L-asparaginase loaded erythrocytes which provides continuos counterflow circulation of the cell supsension with added L-asparaginase and the solution for dialysis.

By using this setup the parameters influencing on efficacy of the process have been assessed:

1. Maxiimal entrapment is achieved when the suspension hematocrit is 50-70%.
2. Dialysis time necessary to provide maximal incorporation of the agent as well as a maximum of its intracellular activity depends on L-asparaginase concentration in the medium. When the latter is increased from 100 to 2000 IU/ml of suspension these parameters change from 30 to 90 min and from 90-70% to 30-50% of the initial specific activity in the mixture, respectively.

A procedure was developed to preserve these aseptically loaded erythrocytes at 4°C.[5] We carried out the experiments to show that the overall L-asparaginase activity of the suspension of erythrocytes loaded by our method does not change dramatically during storage for 18 days, with only 10-20% of the activity being released from the cells into the medium. The intracellular ATP concentration decreases not more than 50% during the storage period and free hemoglobin level in the medium increases to approximately 1% of total hemoglobin (Figure 2). These numbers allow us to hope that the L-asparaginase loaded erythrocytes are suitable for clinical usage after 7 day storage.

Table 1. Comparison of in vitro biochemical parameters of intact versus asparaginase loaded erythrocytes prepared by different methods*

Parameters	Control erythrocytes	Loading methods: Reversible hypoosmotic lysis	Dialysis	Stepwise dialysis
ATP (mmol/l cells)	1.66±0.99	2.02±0.09	1.64±0.07	1.70±0.05
GSH (mmol/l cells)	2.11±0.08	0.45±0.09	1.87±0.09	2.01±0.08
G-6-P (μmol/l cells)	89.4±5.9	19.0±3.2	68.6±4.7	35.6±1.8
Glucose utilization rate (mmol/h/l cells)	1.07±0,04	2.17±0.03	1.54±0.04	1.05±0.02
Initial K^+ in erythrocytes (mmol/l cells)	101,6±6.7	17.9±6.0	65.1±3.7	80.5±5.2
K^+ leakage rate (%/h)	1.12±0.06	-1.95±0.06	1.77±0.05	0.86±0.04
Initial Hb (g/l cells)	300.8±4.5	157.3±4.7	305.0±4.0	299.8±2.4
Hemolysis rate (%Hb/h)	0.35±0.04	0.25±0.03	3.72±0.35	1.81±0.19

* Loaded human erythrocytes or their paired control were washed three times with a solution containing 113 mM NaCl; 3 mM KCl; 1.2 mM Na_2HPO_4; 1 mM $MgCl_2$; 50 mM Tris-HCl; 2 mM $CaCl_2$ and 5 mM glucose. Then they were resuspended with the same solution up to hematocrit 45% and incubated during 2-4.5 hours at 37°C. During incubation samples were taken to measure the parameters indicated (per litre of erythrocytes). Means of seven experiments and standard deviations are presented.

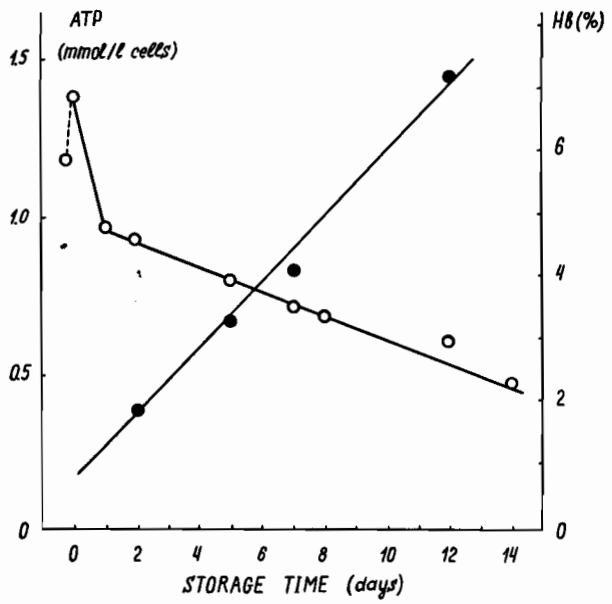

Figure 2. Kinetics of intracellular ATP concentration (o) and hemoglobin level in the medium (as % of total suspension hemoglobin) (●) during storage of erythrocytes loaded with L-asparaginase using the laboratory dialysis system.

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