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Erythrocytes as Carriers for L-Asparaginase. Methodological and Mouse In-vivo Studies

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Abstract—L-Asparaginase has been encapsulated in Swiss mouse or human erythrocytes by hypotonic haemolysis followed by isotonic resealing and reannealing. The details of incorporation and properties of carrier erythrocytes are presented. When L-asparaginase loaded into ^{51}Cr-labelled erythrocytes, was infused intravenously, the same half-life was found for asparaginase and ^{51}Cr. In addition, L-asparaginase loaded into erythrocytes was much more effective in eliminating plasma asparagine compared with the same dose of free L-asparaginase injected in solution, during a sustained period (14 days).

L-Asparaginase (L-asparagine-amino-hydrolase) has long been used for the treatment of acute lymphoblastic leukaemia (Oettgen et al 1967; Nesbit et al 1981). However, as the L-asparaginase is obtained from a bacteria or plant (*E. coli* or *Erwinia chrysanthemi*), major immunologic disorders, including immunosuppressive activity and intolerance reactions, are frequently encountered (Oettgen et al 1970; Hersch 1971; Land et al 1972; Evans et al 1982). Furthermore, the enzyme is toxic for some normal tissues, principally liver and pancreas (Oettgen et al 1970; Nesbit et al 1981) and has a short plasma life-span. Updike et al (1976), Updike & Wakamiya (1983) and Updike (1985) have reported potential improvement of the therapy using L-asparaginase loaded into red blood cell (RBC) ghosts, which resulted in an increase of their life-span and of the time of L-asparagine elimination from the plasma in monkeys, and a decrease of passive anaphylaxis in the guinea-pig. Similar results were obtained by Alpar & Lewis (1985).

We have developed a new method for encapsulation of xenobiotics into RBC using a dialysis device to create a reversible lysis of the cells (Ropars et al 1983, 1985, 1987). This method was an adaptation of a dialysis method proposed by Dale et al (1977) and Deloach & Ihler (1977) but lysis of erythrocytes was obtained by a continuous flow dialysis system. This treatment leads to a normal in-vivo life-span for lysed and resealed cells. We present here our results for L-asparaginase encapsulation in-vitro for mouse and human RBC and in-vivo in mice.

Materials and Method

Materials

L-Asparaginase (Kidrolase) was obtained from Roger Bellon (Paris, France). CPD (citrate 45, NaH_2PO_4 15, glucose 130 mM pH 7.4) and PIGPAC (Na_2HPO_4 100, inosine 10, glucose 100, pyruvate 100, adenine 5 mM pH 7.0) were obtained from Laboratoires Bruneau (Boulogne Billancourt, France). Whole blood was obtained from volunteers. Swiss mice were purchased from Janvier (St Berthevin, France). The dialyser (Lundia 10 1 N) was purchased from Gambro (Lundia, Sweden). Blood was monitored for haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MHC) and mean cell haemoglobin concentration (MCHC) by means of a TI 660 Cell Counter (Coultronics, Margency, France). Adenosine triphosphate (ATP) and 2.3 bisphosphoglycerate (2.3 BPG) were measured with an enzymatic kit from Sigma Chemical Co. (Laverpilliere, France). All other chemicals were reagent grade.

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Preparation of human carrier erythrocytes

The procedure was similar to that of Ropars et al (1985). Blood collected in CPD (180 mL L^{-1}) was centrifuged (1000 *g* 10 min, 4°C) to remove plasma and buffy coat. Packed erythrocytes were washed three times in NaCl 150 mM (saline) (1:1 v/v). The washed erythrocytes were mixed with L-asparaginase (115 ± 3 units mg^{-1}) at 4000 units mL^{-1}. The haemotocrit was adjusted to 75% using saline. The lysis of erythrocytes was obtained by the continuous flow dialysis system described by Ropars et al (1985). Briefly, the blood flow was fixed at 20, 30 or 40 mL min^{-1} and a hypotonic buffer (glucose 2, NaCl 10 mM, pH 7·4) was run at 180 mL min^{-1} in the other compartment of the dialyser. After collection, the lysed erythrocytes were incubated at 37°C for 10 min, then a resealing solution (PIGPAC–NaCl 12% (1:1·4 v/v)) was introduced into the blood bag (containing 1 vol of resealing solution to 10 vol of lysed RBC) and the cells were incubated at 37°C for 30 min.

The lysed-resealed cells were washed three times (1000 *g*, 10 min, 4°C) in saline (1:1 v/v) and once in autologous plasma (1:1 v/v).

Before and after this procedure, the blood was monitored for haematocrit, MCV and haemogloblin. Extra and intracellular L-asparaginase concentrations were determined either by ^{125}I-labelled asparaginase evaluation or by using the enzymatic method of Cooney et al (1970).

Preparation of mouse carrier erythrocytes

The loading procedure was an adaptation of the method used by Deloach & Ihler (1977) and Dale et al (1977). Briefly, fresh heparinized mouse blood was taken at the brachial artery. RBC were washed at 800 *g* and lysis of erythrocytes was obtained on a 5 mL sample of RBC suspension mixture, in a dialysis bag. Dialysis was performed against 250 mL of hypotonic buffer (Na_2HPO_4-NaH_2PO_4 10, glucose 2,

$NaHCO_3$ 10 mM, pH 7·4) for 45 min at 4°C. The other steps were the same as for the human carrier erythrocyte preparation except that centrifugation was performed at 800 *g*.

Erythrocyte labelling

Normal mouse erythrocytes (M-RBC) were washed three times (800 *g*, 10 min, at 4°C) in saline. Then $Na_2\ ^{51}CrO_4$ was added (1 μCi mL^{-1} at 70% haematocrit) and packed erythrocytes were incubated for 30 min in a water bath at 37°C. After labelling, ^{51}Cr-M-RBC were washed three times in saline and once in autologous plasma. For mouse carrier erythrocytes (M-ASP-RBC), the labelling was initiated after the first post-resealing wash and an additional wash was included in the initial encapsulation procedure.

Osmotic fragility tests

The osmotic fragility of carrier and control erythrocytes was measured taking 50 μL amounts of RBC and equilibrating them in 5 mL of saline in a 0–300 osm kg^{-1} range, at room temperature (20°C) for 30 min. The absorbance at 540 nm of the haemoglobin released into the supernatant after centrifugation at 800 *g* for 10 min at 4°C, was monitored spectrophotometrically. Haemolysis (100%) was defined as the absorbance at 540 nm of the supernatant containing haemoglobin released in the tube at 0 osm kg^{-1} (water).

In-vivo studies

Mice, 30 ± 2 g, were anaesthetized with ether and injected in the tail vein with free L-asparaginase in solution or with normal ^{51}Cr-labelled RBC. Different types of RBC were used; normal RBC, lysed and resealed RBC without L-asparaginase (L-Res-RBC) or L-asparaginase loaded RBC (ASP-RBC).

Before blood infusion, the same volume of blood was taken from the orbital sinus. For free L-asparaginase, the injected dose was diluted with 100 μL saline. When mouse was treated with less than 200 units kg^{-1}, ASP-RBC were diluted into L-Res-RBC.

At different times, groups of three mice were bled. The serial whole blood samples were monitored for L-asparaginase and ^{51}Cr radioactivity; L-asparaginase and L-asparagine concentrations were measured in plasma. The L-asparagine was determined by HPLC (Fauconnet & Rochemont 1978) and L-asparaginase enzymatically (Cooney et al 1970). L-Asparaginase and ^{51}Cr were corrected to the same haematocrit (40%) for all samples.

Results

Dialysis bag encapsulation yields

Using an L-asparaginase concentration of 4 mg mL^{-1} a loading efficiency of 14·1 ± 1·5% was achieved for mouse RBC. Under the same conditions, human RBC loading was more efficient with recoveries of 20·2 ± 2% (Table 1). No enzyme was found in washed control erythrocytes prepared as for carrier RBC but without dialysis, indicating that the drug association with carrier was not due to membrane binding.

Continuous flow dialysis encapsulation

The kinetic parameters of dialysis have an effect on the entrapment of L-asparaginase and the haematological indices of human resealed RBC (Table 2). A compromise between L-asparaginase entrapment and haematological indices of resealed RBC was obtained at the 30 mL min^{-1} blood flow rate, at which we measured the L-asparaginase entrapment versus the total initial L-asparaginase concentration introduced in the RBC suspension (Fig. 1).

Table 1. Haematological and encapsulation parameters of mouse and human erythrocytes. Entrapment was obtained after 45 min by the dialysis bag method. The erythrocyte suspensions were adjusted to 70% haematocrit and 4 mg mL^{-1} of L-asparaginase (115 ± 4 units mL^{-1}) (means ± s.d.). MCV = mean cell volume. MCH = mean corpuscular haemoglobin. MCHC = mean cell haemoglobin concentration. n = number of experiments.

	Mice (n = 8)		Human (n = 16)	
Parameters	Before	After	Before	After
RBC recovery (%)	95 ± 2	59 ± 5	100 ± 2	68 ± 2
Asp yield (%)	0	14·1 ± 1·3	0	20·2 ± 2
MCV (fL)	50 ± 1	48 ± 1	94 ± 2	79 ± 1
MCH (pg)	17·7 ± 1·0	16·1 ± 1·1	32·0 ± 0·8	25·6 ± 0·9
MCHC (g dL^{-1})	33·0 ± 1·5	33·2 ± 1·7	34·2 ± 1·1	33·1 ± 0·7
ATP (μmol g^{-1}Hb)	1·9 ± 0·3	3·0 ± 0·1	4·4 ± 0·3	5·9 ± 0·1
2.3 BPG (μmol g^{-1}Hb)	16·4 ± 1·4	23·0 ± 1·4	9·3 ± 2·2	16·4 ± 0·8

Haematological properties of carrier erythrocytes

The haematological properties of human or mouse carrier erythrocytes including MCV, MCH, MCHC, ATP and 2.3 BPG concentrations and osmotic fragility have been measured (Table 1, Fig. 2). Some small modifications occurred including decreased MCV and MCH, but these remained within the normal range, ATP and 2.3 BPG concentrations increased, which is relevant to the PIGPAC rejuvenation effect. The increased osmotic stability may be related to the decreased MCV and intracellular osmotic pressure.

Life-span of carrier erythrocytes

Using a total initial L-asparaginase concentration of 2 or 4 mg mL^{-1}, the half-lives of ^{51}Cr-labelled ASP-RBC were 10·7 ± 0·7 and 9·2 ± 0·7 days, respectively. When control ^{51}Cr-Res-RBC or ^{51}Cr-RBC were injected into mice, half-lives of 10·4 ± 0·7 and 12·3 ± 0·7 days were observed, respectively. Thus, any modification of the ASP-RBC life-span was

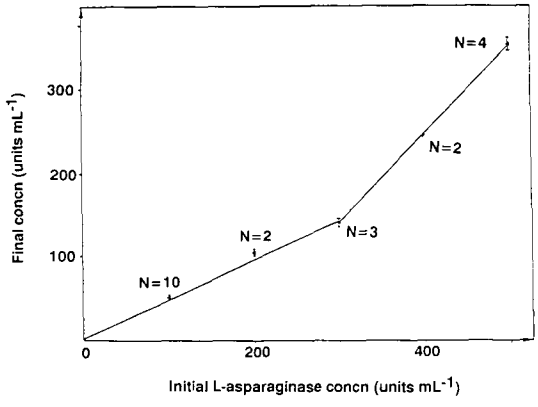

FIG. 1. Effect of the total initial L-asparaginase concentration introduced in the RBC suspension on L-asparaginase entrapment using continuous flow dialysis encapsulation. The dialysis flow rate was fixed at 30 mL min^{-1} and L-asparaginase concentration was measured by an enzymatic method (means ± s.d., N: number of experiments).

Table 2. Influence of blood flow during continuous flow dialysis encapsulation on L-asparaginase entrapment and haematological parameters of the resealed RBC.

Blood flow (mL min^{-1})	MCV (fL)	MCH (pg)	MCHC (g dL^{-1})	ASP (units mL^{-1})
20	76±0·3	23·8±0·2	31·1±0·1	48·7±1·4
30	78±0·4	24·9±1·2	32·5±1·2	46·2±2·5
40	80·5±1·4	25·5±0·8	31·7±0·7	38·8±1·6
Controls	93·8±2·6	30·4±1·1	32·3±0·6	

not due to the asparaginase loading step, but to the dialysis procedure. In addition, a good post-transfusion RBC survival at 24 h was obtained for carrier RBC (82, 86 and 90% for ^{51}Cr-ASP-RBC, ^{51}Cr-L-Res-RBC and ^{51}Cr-RBC, respectively).

Life-span of L-asparaginase loaded into erythrocytes

When ^{51}Cr-ASP-RBC was injected into mice, identical half-lives were determined for L-asparaginase levels or ^{51}Cr radioactivity (Table 3). When mice were treated with 1000 or 200 units kg^{-1} of RBC-encapsulated L-asparaginase, half-lives of the enzyme, 9·2±0·5 and 10·7±0·7 days, respectively, were greater than that following an injection of free L-asparaginase, (3 h) (Table 3, Fig. 3). The differences observed for the in-vivo recovery after 24 h between ^{51}Cr and L-asparaginase levels may be explained by a heterogenous loading of mice RBC, the most fragile of them containing an increased enzyme level.

L-Asparaginase elimination effect

When mice were treated with an enzyme dose as used in classical acute lymphoblastic leukaemia therapy (1000 units kg^{-1}), the L-asparaginase loaded into RBC lowered the plasma asparagine to essentially undetectable levels during the 14 days period of observation, as compared with 3 days when free L-asparaginase was injected (Table 3). For mice treated with 200 units kg^{-1}, the same result was obtained for L-asparaginase loaded into RBC but free L-asparaginase efficacy lasted 2 days only (Table 3).

Table 3. In-vivo studies of L-asparaginase encapsulated in RBC. Mice were injected with L-asparaginase in RBC or free in solution.

	Life-span RBC		Life-span of L-asparaginase	
Units kg^{-1}	Recovery at 24 h (%)	Half-life	Recovery at 24 h (%)	Half-life
ASP-RBC 1000	85±1	9·0±0·4 d	63±1	9·2±0·5 d
ASP-RBC 200	80±1	10·6±0·8 d	2·5±1	10·6±0·6 d
Free ASP 1000				2·4±0·8 h
Free ASP 200				3·3±0·8 h

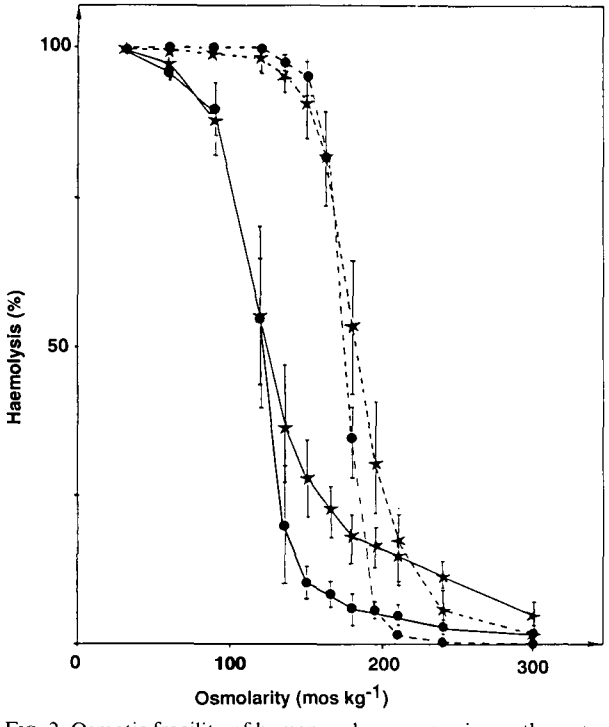

FIG. 2. Osmotic fragility of human and mouse carrier erythrocytes. ●—● human carrier, ★—★ mouse carrier, ●- - -● human control and ★- - -★ mouse control erythrocytes.

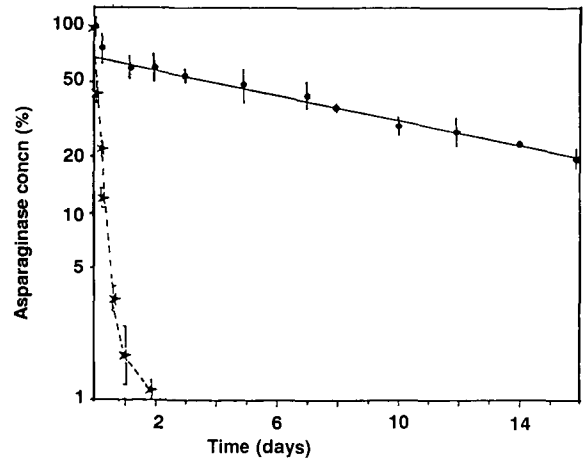

FIG. 3. Pharmacokinetics of L-asparaginase in the mouse. Mice were injected with 1000 units kg^{-1} of L-asparaginase encapsulated into RBC (●) or free in solution (★). Means±s.d. of six mice.

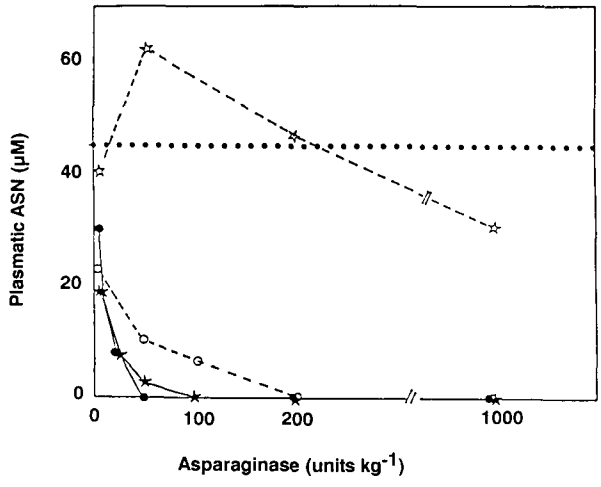

FIG. 4. Effect of circulating L-asparaginase on plasma asparagine. Mice were treated with various levels of L-asparaginase encapsulated into RBC (closed symbols) or free in solution (open symbols). Plasma asparagine levels were measured, by HPLC, 1 (●, ○) or 5 days (★, ☆), after infusion.

In other experiments mice were treated with enzyme levels decreasing from 1000 to 5 units kg^{-1}, loaded into RBC or free in solution. After 5 days, the plasma L-asparaginase level had returned to normal for mice treated with less than 200 units kg^{-1} free in solution. For mice treated with ASP-RBC the enhanced efficacy of L-asparaginase was observed at all doses used (Fig. 4).

Discussion

Erythrocyte ghosts were originally proposed by Ihler et al (1973) as biodegradable carriers of exogenous therapeutic enzymes. For L-asparaginase, most of the reported work (Updike et al 1976; Updike & Wakamiya 1983; Updike 1985) used erythrocytes prepared by a direct dilution method that produces ghosts with a decrease in their haemoglobin or other protein contents and a reduced life-span. The present work has allowed us to test a new procedure for L-asparaginase loading in carefully lysed erythrocytes using a hypo-osmotic dialysis method and resealing in the presence of PIGPAC as a rejuvenating solution. Under these conditions, ASP-RBC presented cellular indices similar to those of normal erythrocytes. The cells also showed in-vivo survival similar to that of normal mouse RBC. In addition, the L-asparaginase activity was not destroyed by erythrocyte enzymes as shown by the similar life-span of L-asparaginase and ^{51}Cr when the ^{51}Cr-labelled L-asparaginase loaded RBC were injected into mice, or the in-vitro stability of L-asparaginase loaded into human RBC (results not shown).

L-Asparaginase encapsulation using a continuous flow dialysis system results in a therapeutic level of the enzyme available within a range of 100 to 300 mL of packed RBC. However, if human in-vivo studies show similar results as in the mouse, this therapeutic level could probably be decreased.

Alpar & Lewis (1985) and Ataullakhanov et al (1985) have demonstrated that the membrane of native human erythrocytes is permeable to asparagine. Therefore, two mechanisms of ASP-RBC action are possible. In the first, plasma asparagine is destroyed after penetration into the RBC. In the second a low L-asparaginase level may be sufficient to destroy plasma asparagine for a sustained time. However, when mice were injected with less than 50 units kg^{-1} of free asparaginase in solution, the plasma asparagine remained detectable even after one day. It thus appears that, the major mechanism for the enzyme activity is the first of these. Furthermore, Updike (1985), using a guinea-pig passive anaphylaxis model showed that a decreased anaphylaxis reaction resulted after loading asparaginase into RBC. It may be assumed that this is the result of a protective effect against the humoral reactivity of circulating anti-asparaginase antibodies.

Our results indicate that encapsulated enzyme behaves like a circulating bioreactor with the erythrocyte membrane protecting the enzyme against plasma proteases and circulating antibodies. The membrane permeability for the substrate maintained the enzymatic activity in erythrocytes.

In-vivo trials of L-asparaginase loaded in erythrocytes may be expected to show improved therapeutic activity of this enzyme.

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