Erythrocytes as Carriers for Recombinant Human Erythropoietin

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Purpose. The aim of this work was to encapsulate recombinant human erythropoietin (rHuEpo) in human and mouse red blood cells (RBCs) to improve the stability of encapsulated rHuEpo.
Methods. The encapsulation of rHuEpo was achieved by an hypotonic dialysis-isotonic resealing procedure. A radioimmunoassay method was used for the estimation of rHuEpo. The hypoosmotic resistance of carrier erytrhocytes was studied by osmotic fragility measurements. Cell morphology was observed under scanning electron microscopy. Encapsulated rHuEpo was identified by an immunogold labeling assay.
Results. Encapsulation yields were 22% for human RBCs and 14% for mouse RBCs. Cell recovery was around 70%. Carrier-RBCs exhibited a tendency to spherocytic morphology, and showed the typical higher hypoosmotic resistance than normal RBCs. The presence of rHuEpo inside carrier RBCs was identified. The stability of encapsulated rHuEpo seems to be related to the experimental conditions used during the encapsulation procedure. An increase with time of released rHuEpo was observed in carrier-RBC suspensions.
Conclusions. The encapsulation of rHuEpo in RBCs has been achieved for the first time. These carrier RBC-preparations may serve as an alternative sustained cell delivery system for the *in vivo* administration of rHuEpo.

KEY WORDS: red cell delivery systems; recombinant human erythropoietin; erythrocyte morphology; anaemia; peptide drugs.

INTRODUCTION

Erythropoietin (Epo), a sialoglycoprotein hormone (34,000 daltons) mainly produced in the kidney, is the principal physiological regulator of erythroid differentiation. This extremely potent agent induces a very rapid increase of erythropoiesis by means of its high-affinity binding to specific receptors on the surface of hematopoietic progenitor cells (1,2). Recently, Epo has been cloned by recombinant genetic engineering (rHuEpo) (3,4). At present, rHuEpo is being administered, both intravenously and subcutaneously, as a therapeutic agent used in several anaemic processes associated to chronic renal failure, neoplastic diseases, chronic inflammatory processes, etc. (5).

In the last decades it has been widely demonstrated that human and animal erythrocytes (red blood cells, RBC) can be properly engineered to behave as passive carriers of encapsulated chemicals. These transport cell systems have been proposed for dissemination of incorporated active compound through the organism. The major advantages of this strategy are the *in vivo* protection from premature degradation and immunological reactions, as well as widespread distribution via circulatory system (6,7).

In view of the extremely short *in vivo* half-life of rHuEpo (4–9 h), the main goal of this study was to encapsulate rHuEpo in human and mouse RBCs. This objective has been achieved by the first time by using a hypotonic dialysis-isotonic resealing procedure (8). The *in vitro* stability and the released rate of the encapsulated rHuEpo has been determined from rHuEpo-loaded RBCs as an essential requisite for its potential use as an *in vivo* slow releasing system.

MATERIAL AND METHODS

Preparation of Carrier Erythrocytes

Human blood was drawn from healthy volunteer donors by venipuncture into heparinized tubes (10 U/mL blood). Mouse blood was collected from anesthetized CD1 Swiss mice (25–35 g) by retroorbital puncture with heparinized Pasteur pipettes. Whole blood was centrifuged (1000 g, 10 min, 4°C) and the supernatant and buffy coat were removed. Packed cells were washed three times with isotonic saline solution to eliminate leukocytes and other contaminants. In the third washing the hematocrit was adjusted to 80–85%. Bovine serum albumin (BSA; Sigma Chemical, Co.) was added to the washed RBC suspensions (100 mg/mL). A volume of rHuEpo, Eprex, 4000 U/mL, 33.6 μg of protein/mL (i.e. 0.12 units/mg of protein) from Cilag Spain S.A., was added (for the preparation rHuEpo-RBCs) or not (for unloaded-RBCs), to BSA-enriched RBC suspensions to obtain a final activity of 200 U of rHuEpo/mL RBC suspension. Hematocrit was precisely adjusted to 70% with saline solution. The mixtures were introduced into a dialysis bag (Medicell, International LTD, 12–14 kDa) and a hypotonic dialysis, with gently mixing of the bags by rotation in a cell mixer, was performed against 50 volumes of 15 mM sodium phosphate, 15 mM sodium bicarbonate, 20 mM glucose, 2 mM ATP and 3 mM reduced glutation, pH 7.4, for 45 min, at 4°C. Hypotonically dialysed RBCs were then incubated at 37°C for 10 min. 1 vol. of resealing solution (100 mM sodium phosphate, 1.6 M KCl, 190 mM NaCl, 100 mM adenine, 20 mM ATP, 100 mM glucose, 100 mM sodium pyruvate, 4 mM $MgCl_2$, pH 7.4) was added to every 10 vol. of RBC suspension and the cells were reannealed by incubation at 37°C, for 30 min.

For the calculation of the entrapment percentages, aliquots of dialysed/resealed RBC suspensions were frozen at −20°C. The rHuEpo activity of these samples correspond to non-encapsulated rHuEpo plus incorporated rHuEpo. To remove the external rHuEpo, the cells were washed at least three times with a phosphate buffered solution (PBS; 150 mM NaCl, 5 mM sodium phosphate and 5 mM glucose, pH 7.4) at 800 g for 10 min. The rHuEpo activity of the washed rHuEpo-RBCs correspond to the encapsulated rHuEpo. The yield of incorporation (encapsulation percentages) is expressed as rHuEpo activity in the washed rHuEpo-RBCs with repect to the activity (100%) after the reannealing step.

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ABBREVIATIONS: rHuEpo, recombinant human erythropoietin; RBCs, red blood cells; BSA, bovine serum albumin; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; Hb, hemoglobin content; OFC, osmotic fragility curves; SEM, scanning electron microscopy; TEM, transmission electron microscopy; PBS, phosphate buffered solution; CPDA, citrate, phosphate, dextrose and adenine solution; UD, undetectable.

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Erythrocytic indexes, mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and hemoglobin content (Hb) were all measured with a hematological analyzer (HemoAnalyzer 9000+, Menarini-Serono Co.).

Osmotic Fragility Resistance of Carrier RBCs

The osmotic fragility curves (OFC) of native (control) and dialysed-resealed RBCs, either unloaded or rhuEpo loaded RBCs, were determined by adding 25 μL of each RBC suspension to 2.5 mL of a series of hypotonic saline solutions of increasing osmolality (0–300 mOsm/Kg). After 30 min at room temperature, the mixtures were centrifuged (800 g; 5 min) and the absorbance of hemoglobin (540 nm) in the supernatant was measured. Hemolysis values are given as the percentage of total hemolysis of each RBC suspension in water after 30 min (100%).

Estimation of rHuEpo Activity by RIA

For the quantitative determination of rHuEpo activity a commercial radioimmunoassay kit, currently being employed for diagnosis of clinical samples, was used according to the manufacturer's intructions (I^{125} EPO-Trac™ from INCSTAR Corporation-Stillwater, Minnesota, USA). This procedure is a competitive binding disequilibrium radioimmunoassay, which utilizes rHuEpo for both tracer and standards, and a primary polyclonal antibody (goat anti-human Epo serum). Accuracy (percent recovery), sensitivity (limits of detection) and specificity for several serum proteins, including serum albumin (percentage of cross-reactivity) were 103%, < 4 mU/mL and < 0.001, respectively.

Preparation of Samples for Scanning Electron Microscopy (SEM) Observations

Native, unloaded-RBCs and rHuEpo-RBCs from humans and mice were prepared for SEM as described by Bessis (9). Briefly, cells were fixed with 1.5% glutaraldehyde in 144 mM sodium cacodylate solution (pH 7.4, 300 mOsm/kg) for 1 h at 4°C. Fixed cells were washed three times with 144 mM sodium cacodylate and post-fixed with 1% osmium tetroxide in the cacodylate buffer for 30 min, at 4°C. Post-fixed cells were finally washed three times with cacodylate buffer. The cells were dehydrated by resuspension in acetone solutions (50%, 70%, 80%, 90% and 3 × 100%; v/v) and then a critical point drying in liquid carbon dioxide. Platinum-gold coating was performed by a sputter coater (Polaron SEM Coating System). Finally, cell samples were observed with a scanning electron microscope (Carl Zeiss DMS-950) at a beam voltage of 30 kV.

Preparation of Samples and Immunogold Labeling Assay for Transmission Electron Microscopy (TEM) Observations

Native unloaded-RBCs and rHuEpo-RBCs were fixed in 1% paraformaldehyde and 1% glutaraldehyde in 144 mM cacodylate buffer (pH 7.4, 300 mOsm/kg) for 1 h, at 20°C. Fixed cells were washed three times with cacodylate buffer. After the final washing, the samples were dehydrated in 50–100% acetone solutions and then embedded in Unicryl™ acrylic resin (British Biocell International), for 3 days at 40°C. Ultrathin sections were obtained (40–50 nm) and layered onto nickel grids (200 mesh, Gilder, G200-N3). Free aldehyde residues in the sections were blocked by incubating the grids in 20 mM glycine in a standard phosphate buffered solution (PBS-glycine), pH 7.4, for 1h at 20°C. The primary antibody supplied with the commercial rHuEpo RIA kit (Epo-Trac™) was diluted (1:50, v/v) in PBS-glycine for the incubation of the sections (16–24 h, at 4°C). The grids were then incubated in 1:100 (v/v) diluted rabbit anti-goat IgG (secondary antibody) conjugated with colloidal gold (size 15 nm, EM.RAG15, Biocell, Research Laboratories) in 1% BSA-PBS, pH 7.4, for 2 h at 20° C. After washing in PBS (3 × 5 min, 20° C), the sections were slightly stained with 2.5% aqueous uranyl acetate for 5 min, washed in PBS (3 × 5 min, 20° C) and dried at room temperature.

In Vitro Release of the Encapsulated rHuEpo

The *in vitro* release of entrapped rHuEpo was examined in mouse rHuEpo-RBCs loaded at an initial activity of 1000 U rHuEpo/mL RBCs, in the presence of protease inhibitors. The time interval between loaded RBCs preparation and *in vitro* release studies was 15 min. The cell suspensions were prepared in BSA-CPDA solutions at 0.5% hematocrit, and the mixture were incubated for 48 h at 37° C. Aliquots were taken at different times, to estimate the total rHuEpo activity in the RBC suspensions (i.e. encapsulated plus released rHuEpo) as well as the rHuEpo activity in the supernatants (i.e. cumulative amount of rHuEpo released with time to the medium).

RESULTS AND DISCUSSION

rHuEpo Encapsulation in Human and Mouse RBCs

The encapsulation parameters are shown in table I for human and mouse. These include cell recovery, encapsulation yield and erythrocytic indexes (MCV, MCH, MCHC) for native (i.e. non-treated RBCs), unloaded (i.e. dialysed-resealed RBCs in the absence of rHuEpo) and rHuEpo-loaded RBCs.

The incorporation of proteins within RBCs is usually studied by measuring the radioactivity of radio-labeled proteins. To this aim a standard labeling procedure (chloramine T), for the preparation of $[^{125}I]$-rHuEpo was initially used in this study. As it was expected for such an approach (results not shown), or similar ones (for instance Iodine-beads^R from Biorads), these kind of experiments do not mean any direct correlationship between radioactivity measurements rHuEpo activity. Therefore, all the rHuEpo activity results given in this work were estimated by radioimmunoassay measurements of rHuEpo activity (RIA; see Materials and Methods).

The encapsulation of rHuEpo was initially carried out at fixed rHuEpo activity of 200 U/mL of RBC suspension (70% hematocrit) in the dialysis bag. Cell recoveries after the encapsulation process, for either unloaded or loaded RBCs, were similar for humans (65–69%) and mice (63–65%) RBCs. The incorporation of rHuEpo within RBCs was expressed as a percentage of rHuEpo activity in the washed rHuEpo-RBCs with respect to rHuEpo activity (100%) in the reannealed RBC suspension (see Materials and Methods). The encapsulation yield in human RBCs (22.2%) was slightly higher than in mouse RBCs (14.5%; table I). Non specific rHuEpo binding to the RBC membrane was undetectable in both species. As expected,

Table I. Encapsulation Parameters and Erythrocytic Indexes (MCV, Mean Cell Volume; MCH, Mean Cell Haemoglobin; and MCHC, Mean Cell Haemoglobin Concentration) for Human and Mouse Erythrocytes. The RBCs Were Loaded at a Fixed 200 U of rHuEpo/mL RBC Suspension. N = Number of Experiments; UD = Undetectable (Mean ± SEM)

	Human			Mouse		
	Native RBCs	Dialysed/Resealed RBCs		Native RBCs	Dialysed/Resealed RBCs	
	Control	Unloaded-RBCs	rHuEpo-RBCs	Control	Unloaded-RBCs	HuEpo-RBCs
n	24	24	30	26	10	26
Cell recovery (%)	98.5 ± 1.2	65.2 ± 3.0	69.4 ± 9.9	96.5 ± 3.2	65.4 ± 10	63.3 ± 11
Encapsulation (%)	UD	UD	22.2 ± 7.4	UD	UD	14.5 ± 6.8
MCV (fL)	90.9 ± 4.8	75.9 ± 5.6	74.5 ± 4.1	50.5 ± 1.3	44.3 ± 2.4	42.6 ± 1.7
MCH (pg)	31.6 ± 2.5	25.3 ± 0.5	29.1 ± 1.1	16.1 ± 1.4	12.8 ± 2.2	13.8 ± 1.1
MCHC (g/dL)	33.2 ± 1.1	33.4 ± 1.3	31.1 ± 4.6	32.4 ± 1.9	31.8 ± 3.0	32.4 ± 2.6

the analysis of the size distribution of the RBC after the osmotic stress shows a decrease in cell volume (MCV) of unloaded and loaded RBCs, with respect to native RBCs, in both humans and mice (Table I). As a consequence of the simultaneous decrease in hemoglobin content (MCH), the mean cell hemoglobin concentration (MCHC) values remained unchanged in both species (Table I).

Osmotic Fragility Behavior of Carrier RBCs

Hypoosmotic resistance for native, unloaded and rHuEpo-loaded RBCs is given by the osmotic fragility curves (OFC; Fig. 1). The OFC profiles for native RBCs are sigmoidal in both species (8,10). The shift towards the left of the OFC profiles for the dialysed-resealed RBCs is a confirmation of a properly performed dialysis-isotonic resealing procedure (6–8). As a consequence the H_{50} values (osmotic pressure required for 50% hemolysis) either in unloaded or rHuEpo-loaded RBCs

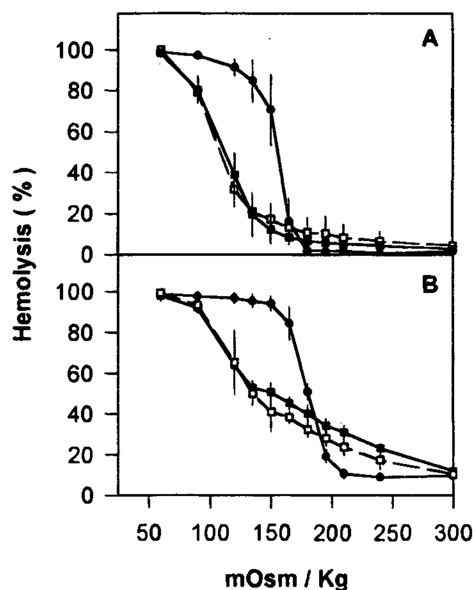

Fig. 1. Osmotic fragility curves (OFC) for human (A) and mouse (B) RBCs. (●) normal or untreated RBCs; (□) unloaded RBCs and (■) rHuEpo-loaded RBCs. The curves are determined from hemoglobin A_{540}. The results are expressed as percentage of hemolysis. Data are mean ± SEM of 15 experiments.

(120 mOsm/kg for humans and 140 mOsm/kg for mice) were lower than those obtained for native RBCs (160 Osm/kg for humans and 180 mOsm/kg for mice). The similarity in H_{50} values for dialysed-resealed RBCs show, as in other proteins, that the presence of rHuEpo during the hypotonic dialysis-isotonic resealing process has no influence on the osmotic resistance of dialysed-resealed RBCs. While native RBCs from human (Fig. 1A), and mouse (Fig. 1B) hemolysed with typical sigmoid-shaped curves their corresponding dialysed-resealed RBCs showed a complex hemolysis relationship. These profiles indicate that mouse dialysed-resealed RBCs are a more heterogeneous RBCs population than human ones.

Morphological Observations of rHuEpo-RBCs

Native human and mouse RBCs show the expected discocyte morphology under scanning electron microscopy (SEM) (Fig. 2A and 2C). However, in unloaded (results not shown) and rHuEpo-loaded human and mouse RBCs, discocytes are coexisting with sphero-stomatocytes and spherocytes (Fig. 2B and 2D). One or two membrane invaginations or endovesicles, can also be observed in both species. It means, in agreement with previous reports (11,12), that the morphology of carrier RBCs suffer some changes as a consequence of native cells being subjected to the dialysis-resealing procedure. No significant differences in the smooth surface of native or rHuEpo-RBCs were observed at a higher magnifications (results not shown).

The presence of the encapsulated rHuEpo within RBCs was identified using an immunogold-labeling rHuEpo assay based on the specific recognition of rHuEpo by a polyclonal antibody (goat anti-human Epo serum). The F_c fragment of the rHuEpo-bound primary antibody can then be recognized by a secondary antibody (rabbit anti-goat IgG) conjugated with colloidal gold particles of 15 nm size.

A slight background labeling was observed in native RBCs (results not shown) or unloaded RBCs (Fig. 3A). The small number of gold particles (black points), either inside or outside native RBCs, are indicative of a negligible unespecific binding. The density of gold particles inside rHuEpo-RBCs is significantly higher, as shown in figure 3B. These observations clearly indicate that the encapsulated rHuEpo is randomly distributed within the cytosol, and not specifically bound to the cell membrane. Observations with human rHuEpo-RBCs (results not shown) were similar to those showed for mouse erythrocytes.

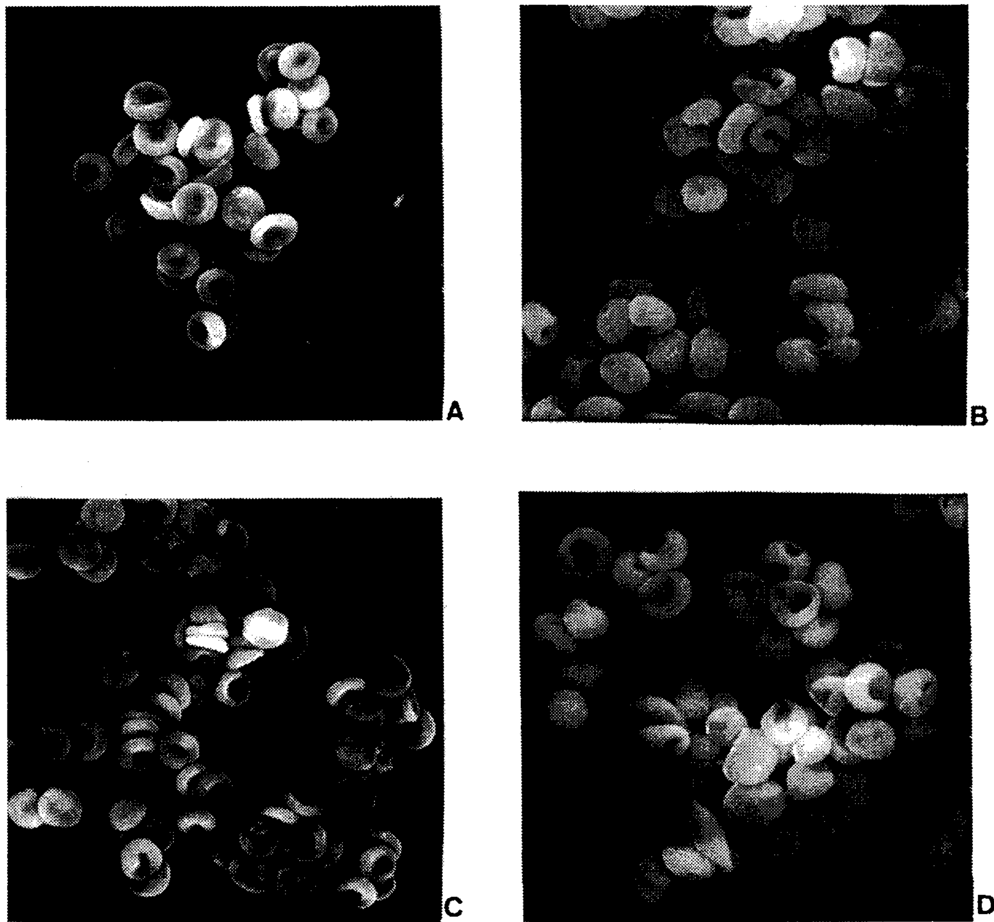

Fig. 2. Scanning electron micrographs of: A) native human RBCs; B) human rHuEpo-loaded RBCs; c) native mouse RBCs and D) mouse rHuEpo-loaded RBCs. (all × 5000).

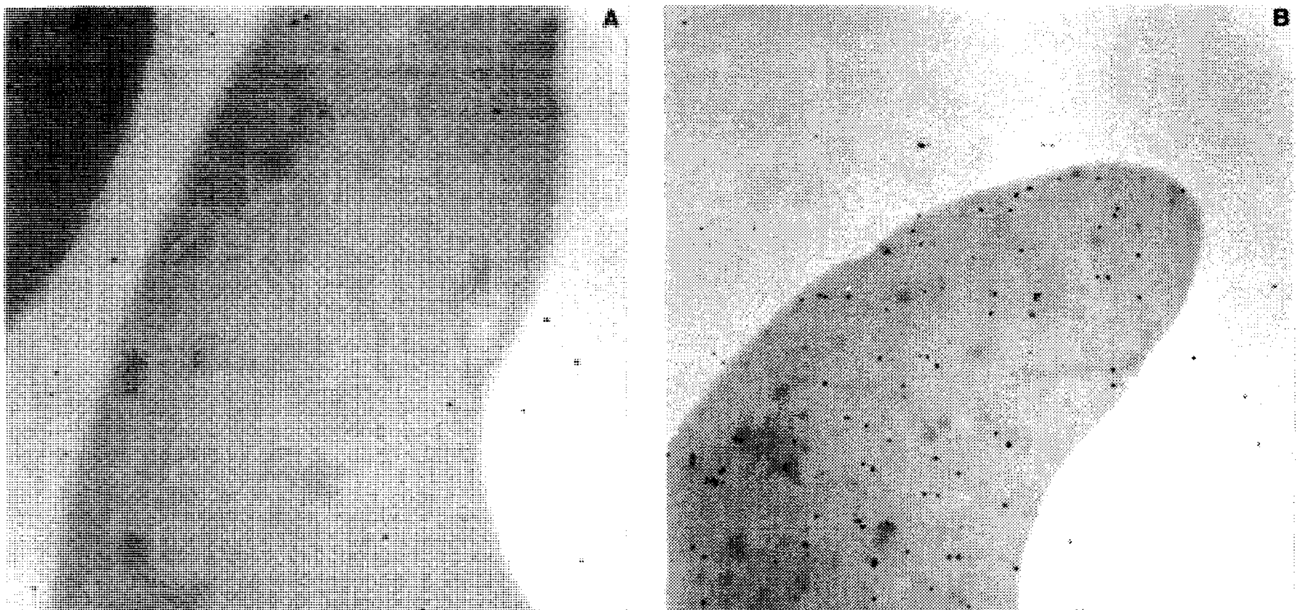

Fig. 3. Transmission electron micrographs of: unloaded mouse RBCs (A) and mouse rHuEpo-loaded RBCs (B). Cell samples were prepared for TEM and immunogold labeled as described in Material and Methods. (all × 30000).

The fact that the very small number of the endovesicles observed in some RBCs do not show any immunogold-labeling suggests that the mechanism of rHuEpo encapsulation may be mostly due to a passive protein diffusion through the membrane pores formed during the hypotonic step in the encapsulation process (13).

In Vitro Stability of Encapsulated rHuEpo

Once the encapsulation of rHuEpo was demonstrated in human and mouse RBCs, the stability of the entrapped rHuEpo was compared with that of rHuEpo solutions under similar *in vitro* conditions. To this purpose, rHuEpo was encapsulated at an initial activity of 200 U/mL RBCs (70% hematocrit). rHuEpo solutions and rHuEpo-RBC suspensions (0.5% hematocrit) were prepared in a CPDA medium and incubated at 37° C for 96 h. rHuEpo activities were estimated by RIA at different times during the incubation. The results are given as percentages of rHuEpo activity present at each time with respect to the activity (100%) at time 0. The time interval between the rHuEpo-RBCs preparation and the *in vitro* analysis of stability is 15 min to avoid the degradation of rHuEpo.

As shown in Figure 4, the activity of rHuEpo in solution decreases dramatically to 25% in less than 30 min, to slowly decrease from this time to reach 18% of activity at 24 h and 5% activity at 96 h of incubation. However, a less pronounced bimodal stability profile was also observed for the rHuEpo-RBCs, in both humans and mice. Here, there was no significant loss in activity during the first 30 min. At 24 h, the activity of the encapsulated rHuEpo had decreased by 50% in humans and 62% in mice, and at the end of incubation period (96 h), 30–35% of the initial rHuEpo was still remaining in both species. Therefore, while the half-life of free rHuEpo in CPDA solutions at 37° C, was extremely short, around 30–45 min, the stability of the entrapped rHuEpo was considerably higher, reaching half-life values of 20 h for mouse and 35 h for human carrier RBCs. These results point out that the encapsulation in RBCs stabilizes the recombinant protein, particularly at the beginning of the incubation period.

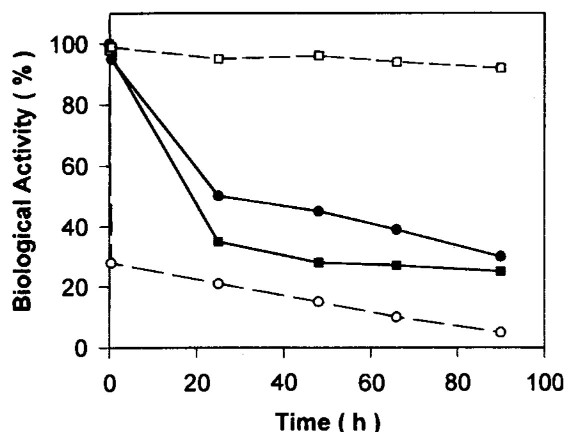

Fig. 4. Time course of *in vitro* stability of rHuEpo solutions in the absence (○) and in the presence (□) of BSA; and of encapsulated rHuEpo in human (●) and mouse (■) RBCs. rHuEpo was encapsulated at an initial activity of 200 units of rHuEpo/mL RBC suspension at 70% hematocrit. Free rHuEpo solutions and rHuEpo-RBC suspensions were prepared in CPDA solutions (327 mg/mL citrate acid, 2.63 g/mL sodium citrate, 2.92 g/mL dextrose, 220 mg/mL sodium biphosphate and 27 mg/mL adenine) and incubated at 37° C. rHuEpo was estimated by radioimmunoassay (RIA). Values are mean of three experiments and agreed within 15%.

It should be noticed that, like most other active peptides in solution, rHuEpo is considerably stabilised by the presence of BSA in the incubation media (14). In fact, as shown in Figure 4, the stability of rHuEpo in solution was constant in the presence of 2.5 mg of BSN/mL CPDA medium. These solutions were then used to compare the stabilizing capacity of RBC encapsulation on rHuEpo stability.

Three different initial rHuEpo activities in the dialysis bag (200, 500, and 1000 U/mL RBCs) were used to prepare carrier RBCs containing increasing rHuEpo activities: 38, 85, and 183 U/mL RBCs, respectively. Most of the loss in stability of the preparations mainly occur during the first 24 h of incubation. At this time, 27, 42, 50% of the original activity is still remaining. It means that, the capacity of RBCs to protect the stability of encapsulated rHuEpo, is significantly higher with respect to that observed for rHuEpo solutions in the absence of BSA and lower than that observed for rHuEpo solutions in the presence of BSA (Fig. 4).

To improve the stability of rHuEpo-RBCs, encapsulations were also carried out in the presence of enzyme proteolytic inhibitors in order to prevent the degradation of entrapped proteins (15). Since the nature of RBC proteolytic activity is not well known (16), a general inhibitor cocktail formed by 0.2 mM AEBSF (for serine proteases); 10 μM EDTA (for metalloproteases); 1 μM pepstatin (for acid proteases) and 10 μM leupeptin (broad range) has been used (Calbiochem Co.). The coencapsulation of the protease inhibitors did not affect the encapsulation yield (14–20%). The rHuEpo activities in the RBC preparations were 49, 80 and 190 U/mL, respectively. Stability experiments under similar conditions as above showed that the decrease in stability during 24 h was less pronounced than that observed in the absence of inhibitors. Although not significative, the difference in rHuEpo activities thus observed suggest an improvement of stability of rHuEpo when encapsulation is carried out in the presence of protease inhibitors. This point may be of interest for the *in vivo* administration of rHuEpo-RBCs.

rHuEpo Release from Carrier RBCs

The mechanism of action of rHuEpo is based on its binding to specific receptors on the cell surface of bone marrow erythroid progenitor cells (2). Therefore, to exert its biological effect encapsulated rHuEpo must be sustainlly released from rHuEpo-RBCs. Zolla et at. (1990; 17), have previously demonstrated that membrane pores formed during the hypotonic treatment, which still remain as smaller residual pores after the resealing step of the encapsulation, allow an efflux of the entrapped proteins from the carrier RBCs.

As previously suggested for other encapsulated proteins, the efflux rate of the encapsulated rHuEpo was indirectly related to the hematocrit of the carrier RBC suspensions. A low hematocrit would be more appropriate than a higher one to achieve a maximum release of the encapsulated rHuEpo (17). A low hematocrit value would also be more appropriate from the point of view of a potential *in vivo* administration of rHuEpo RBCs.

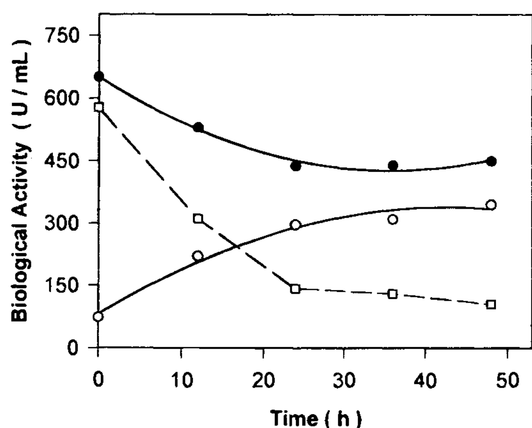

Fig. 5. *In vitro* rHuEpo release from mouse carrier RBCs. rHuEpo-suspensions were prepared in BSA-CPDA (2.5 mg/mL BSA, 327 mg/mL citrate acid, 2.63 g/mL sodium citrate, 2.92 g/mL dextrose, 220 mg/mL sodium biphosphate and 27 mg/mL adenine) and incubated at 37° C. rHuEpo, estimated by radioimmunoassay (RIA), was measured in the whole rHuEpo-RBC suspensions (●, total rHuEpo activity) and in the respective supernatants (○, released rHuEpo activity). (□) calculated values for rHuEpo activity remaining within rHuEpo-RBCs. Values are the mean of three experiments and agreed within 15%.

Therefore 0.5% is the hematocrit here used to study *in vitro* the release of rHuEpo from carrier RBCs.

Figure 5 shows the time course of total and released rHuEpo activity from mouse rHuEpo-RBCs (loaded in the presence of protease inhibitors at an initial activity of 1000 U/mL RBCs), incubated at 37° C, for 48h, in BSA-CPDA solutions at 0.5% hematocrit. The profile for total rHuEpo is similar to that previously observed (Fig. 4). As shown in figure 5, the activity of rHuEpo in the supernatant increases to reach a constant value at 24 h. This could be an indication of a sustained release of the encapsulated rHuEpo over time. The release follows a biphasic profile characterized by a rapid but sustained efflux of the entrapped protein during the first 24 h of incubation, to reach a plateau at 48 h. Figure 5 also shows the calculated values for rHuEpo activity within carrier RBCs (i.e., the difference between total and released rHuEpo activities). Due to the very low hematocrit value (0.5%) it was not possible a direct experimental confirmation of the activity remaining within the RBCs. The sustained release is also supported by the absence of any cellular breakdown of rHuEpo-RBCs, as shown by the constant number of total RBCs (autoanalyzer cell counting; results not shown) observed during the incubation period.

In summary, the demonstration of the encapsulation of rHuEpo in RBCs, as well as the morphological properties and release of rHuEpo from carrier red cells, suggests the possibility of using rHuEpo-RBCs as sustained delivery cell systems for the *in vivo* purposes. To this aim, the pharmacokinetic behaviour and biological effect of carrier RBCs are presently being investigated after *in vivo* administration to mice of rHuEpo-RBCs (18).

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REFERENCES

1. E. Goldwasser, Erythropoietin and the differentiation of red blood cells. *F. Proc.* **34-13**:2285–2292 (1975).
2. H. Youssoufian, G. Longmore, D. Neumann, A. Yoshimura, and H. F. Lodish, Structure, function, and activation of the erythropoietin receptor. *Blood* **9**:2223–2236 (1993).
3. F. K. Lin, S. Suggs, C. H. Lin, J. K. Browne, R. Smalling, J. C. Egrie, K. K. Chen, G. M. Fox, F. Martin, Z. Stabinsky, S. M. Badrawi, P. H. Lai, and E. Goldwasser, Cloning and expression of the human erythropoietin gene. *Proc. Nat. Acad. Sci. USA* **82**:7580–7584 (1985).
4. K. Jacobs, C. Shoemaker, R. Rudersdorf, S. D. Neill, R. J. Kaufman, A. Mufson, J. Seehra, S. S. Jones, R. Hewick, E. F. Fritsch, M. Kawakita, T. Shimizu, and T. Miyake, Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature* **313**:806–810 (1985).
5. J. L. Spivak, Recombinant human erythropoietin and its clinical application. *Interf. Cyt.* **12**:11–13 (1989).
6. M. Magnani and J. R. DeLoach, *The Use of Resealed Erythrocytes as Carriers and Bioreactors,* Plenum Press, New York, 1992.
7. J. R. DeLoach and J. L. Way, *Carrier and Bioreactor red blood cells for drug delivery and targeting, vol.92,* Great Britain, Advances in the Biosciences. Pergamon, 1994.
8. C. Ropars, M. Chassaigne, M. C. Villereal, G. Avenard, C. Hurel, and C. Nicolau, Resealed red blood cells as a new blood transfusion product. *Biblthca. Haemat.* **51**:82–91 (1985).
9. M. Bessis, *Corpuscles. Atlas of red cell shape,* Springer-Verlag, New York, 1973.
10. C. Bailleul, M. C. Borrelly-Villereal, M. Chassaigne, and C. Ropars, Modification of partial pressure of oxygen (P_{50}) in mammalian red blood cells by incorporation of an allosteric effector of hemogloblin. *Biotechnol. Appl. Biochem.* **11**:31–40 (1989).
11. C. A. Kruse, G. W. Mierau, and G. T. James, Methotrexate loading of red cell carriers by osmotic stress and electric-pulse methods: ultrastructural observations. *Biotechnol. Appl. Biochem.* **11**:571–580 (1989).
12. R. E. Droleskey, K. Andrews, L. Chiarantini, and J. DeLoach, Use of fluorescent probes for describing the process of encapsulation by hypotonic dialysis. *Adv. Exp. Med. Biol.* **326**:73–80 (1992).
13. J. F. Hoffman, On red blood cells, hemolysis and resealed ghosts. *Adv. Exp. Med. Biol.* **326**:1–15 (1992).
14. R. K. Scopes, *Protein purification: principles and practice,* Springer-Verlag, New York, 1982.
15. G. Falcioni, R. Gabbianelli, A. M. Santroni, A. Concetti, L. Zolla, and M. Brunori, Entrapment of protein protease inhibitors in red blood cells. *Biotechnol. Appl. Biochem.* **16**:269–274 (1992).
16. J. R. Harris, Some high-molecular-weight oligomeric proteins and enzymes of reticulocytes and erythrocytes. In J. R. Harris. (ed.), 1. *Erythroid cells,* Plenum Press, New York, 1990, pp. 251–298.
17. L. Zolla, G. Lupidi, M. Marcheggiani, G. Falcioni, and M. Brunori, Encapsulation of proteins into human erythrocytes: a kinetic investigation. *Biochim. Biophys. Acta* **1024**:5–9 (1990).
18. M. I. Garín, R. M. López-González, and J. Luque, Pharmacokinetics properties and *in vivo* biological activity of recombinant human erythropoietin encapsulated in red blood cells, (submitted for publication, 1995).