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In Vivo Survival and Organ Uptake of Loaded Carrier Rat Erythrocytes[1]

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Rat RBCs loaded with ^{125}I-CA by hypotonic dialysis and isotonic resealing were evaluated as a carrier system. Loaded RBCs stored at 4°C remained unlysed (90% survival) allowing release of encapsulated ^{125}I-CA for up to 4 days. Thereafter, cellular lysis increased significantly. IP-injected loaded RBCs reached the maximum level (50%) in circulation at 24 h post-injection. Circulating loaded RBCs showed a half-life of 8-10 days, which was advantageous for carrier function. In contrast to IP-injected free CA, which remained in circulation for only a short time, encapsulated CA showed significant levels in circulation up to 10 days post-injection. The profile of organ uptake with time is essentially not altered for loaded with respect to native cells, being higher the removal of loaded cells and mainly localized in spleen. Nevertheless, liver is the organ with highest elimination capacity for both native and loaded cells, showing its maximum at 24 h post-injection. Concomitantly, the concentration of ^{125}I-CA in all organs studied was highest at this time. These data demonstrate that rat loaded RBCs can potentially be used as a carrier system for long-term dissemination of drug into the organism, with specially increased delivery to the spleen. They also support the use of the rat as an experimental model for biochemical and pharmacological studies in these therapeutic systems.

Key words: carrier erythrocytes, cell targeting, encapsulated carbonic anhydrase, organ uptake, sustained release.

Different delivery systems are currently used in therapy. They have the advantage of protecting the active substance from rapid clearance and avoiding toxic side-effects. Among the many carrier systems proposed (*1*), RBCs have many desirable properties: they are naturally biodegradable and may stay in circulation over prolonged periods of time (*2, 3*); RBCs are easily obtainable and large amounts of material can be entrapped in a small volume of cells; and autologous cells elicit little or no immune response (*4-6*).

Preparation of carrier erythrocytes by hypotonic dialysis affects minimally the biophysical and immunological properties of RBCs, and they have near normal *in vivo* survival. Most data support the conclusion that these lysed and resealed RBCs may be of large value for carrying drugs in various treatments in different mammalian species (*2, 7-13*). The disadvantage of the majority of the other encapsulation methods is that the cells loose their viability when returned to the circulation (*14*).

Among the different species from which hypotonically treated carrier RBCs have been prepared, rat and rabbit have been reported to present some problems for encapsulation (*15*). However, we have previously demonstrated that rat RBCs can bind and/or encapsulate substances of different nature and molecular size, and we have established both the conditions for preparation and the features of rat carrier erythrocytes (*16-19*).

CA was selected here for encapsulation because it is a protein available as M_r marker, easy to label with ^{125}I and supposedly stable in the intracellular medium. CA II is the most widely distributed of the three known soluble isozymes in human tissues. An almost complete absence of CA II in erythrocytes causes "marble bone disease" which, although not common, affects some families (*20*). Thus, encapsulated CA could have a therapeutic application.

For *in vivo* administration, different routes of injection have been used to expose body tissues to carrier cells. Both in human and animals, IP injection of carrier erythrocytes has proved a viable and advantageous alternative to IV injection (*8-10*). We chose to adopt IP administration, since it allows comparative study of chemically modified loaded rat RBCs.

Because the most important criterion for viability is survival in circulation, and because of the limited number of available *in vivo* studies, this work focuses on the

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Abbreviations: CA, carbonic anhydrase; Hct, hematocrit; IP, intraperitoneal; IV, intravenous; PBS, phosphate-buffered saline; PBSH, Hanks phosphate buffered saline; PIGPA, phosphate inosine glucose pyruvate adenine solution; CPDA, citrate phosphate dextrose adenine solution; RBCs, red blood cells.

pharmacokinetics of this drug delivery system, especially on an experimental model in biological assays such as the rat. It examines the *in vitro* and *in vivo* behavior of carrier RBCs and encapsulated CA with respect to cell survival, cell circulation and the diffusion of CA, and the distribution of both among the different organs that play a role in removal of RBCs.

MATERIAL AND METHODS

Chemicals and Radioisotopes—CA (M_r 29,000) was obtained from Sigma Chemical (St. Louis, MO, USA). Na^{125}I (100 mCi/ml) was purchased from NEN-DuPont (Brussels, Belgium). CA was iodinated in our laboratory using chloramine-T immobilized on polystyrene beads (Iodo-Beads, Pierce Chemical, Rockford, IL, USA). Antibiotic-antimycotic mixture to add to CPDA solution was from Gibco Laboratories (Grand Island, NY, USA). ATP and glutathione were from Boehringer Mannheim (Germany). The dialysis bag was from Medicell (London, UK). Other chemicals used were of reagent grade and were purchased from Merck (Darmstadt, Germany).

Buffers and Solutions—PBS was 10 mM phosphate buffer, containing 3.5 mM KCl, 0.5 mM $MgCl_2$, 145 mM NaCl, pH 8.0. Isotonic PBSH was prepared by adding 6 mM glucose to PBS. Hypotonic PBSH was prepared by dilution of isotonic PBSH to 80 mOsm/kg. PIGPA solution contained 30 mM NaH_2PO_4, 30 mM inosine, 30 mM glucose, 30 mM sodium pyruvate, 1.5 mM adenine, 3% NaCl, pH 8.0, and was used to restore isotonicity when mixed with erythrocyte suspensions after dialysis. CPDA solution contained 2.2 mM citric acid, 13.5 mM sodium citrate, 0.2 mM Na_2HPO_4, 0.3 mM adenine, 2.3 mM dextrose, 130 mM NaCl, and 1% antimicrobial mixture. CA (10 mg/ml) and ^{125}I-CA ($5.8 \pm 0.6 \times 10^{11}$ cpm/μmol) stock solutions were prepared to be used as the encapsulation marker.

Preparation of RBCs—Wistar rat blood was collected into heparinized tubes. Blood was centrifuged at $900 \times g$ for 5 min and the plasma and white cells (buffy coat) were discarded. Erythrocytes were washed three times in isotonic PBS (pH 8.0). In the last wash, each fraction was centrifuged at $2{,}500 \times g$ for 5 min to obtain packed erythrocytes (80% hematocrit).

Dialysis Loading Procedure—Packed erythrocytes were subjected to the loading procedure according to previously established conditions (*16*). The marker was added to RBC suspension to a final concentration of 34.5 μM ^{125}I-CA. The mixture (70% hematocrit) was placed in a dialysis bag (molecular size cutoff, 12-14 kDa), then dialysed against hypotonic PBSH supplemented with 2 mM ATP and 3 mM GSH (80 mOsm/kg), while gently mixing by rotation, at 4°C for 1 h. The cells were then annealed (37°C for 10 min) and resealed by addition of 0.5 volume of hypertonic PIGPA solution (37°C for 30 min) and washed three times in cold isotonic PBSH (160 g, 5 min). Particular care was taken to avoid physical damage to the cells. Aliquots were taken for measurement of γ-radiation.

The amount of marker substance entrapped within cells was determined from the specific activity of CA in the encapsulation mixture ($18.82 \pm 1.55 \times 10^8$ cpm/μmol) and expressed as percentage of encapsulation relative to the total amount added. Control cells, equally processed but in isotonic medium, provided a measurement of non-specific binding of CA to RBCs. To study RBC survival and viability after the encapsulation process, the erythrocytes were counted and sized in a hematological analyzer.

Behaviour of Loaded RBCs in Suspension—Efflux of encapsulated substance (^{125}I-CA) was measured to determine the ability of carrier RBCs to retain the marker in storage conditions. Loaded RBCs were stored at 4°C suspended in CPDA solution at 1 : 1 ratio (v/v). The radioactivity was measured in both cell pellet and supernatant to study the diffusion of loaded substance from cells to the outside.

^{51}Cr Labeling—After the resealing step in the loading cell procedure, ^{125}I-CA loaded erythrocytes were incubated with [^{51}Cr]-sodium chromate to radiolabel the hemoglobin as described by Morrison *et al.* (*21*). Loosely packed loaded RBCs were mixed with 50 μl of ^{51}Cr solution (100 μCi/μl) for 30 min at 37°C. Thereafter, cells were washed three to four times in isotonic PBSH to remove unbound ^{51}Cr and used for *in vivo* or *in vitro* experiments. Native RBCs were labeled similarly for control.

A 500-μl aliquot of loaded (^{125}I-CA, $2.54 \pm 0.09 \times 10^6$ cpm) and ^{51}Cr-labeled ($2.65 \pm 0.16 \times 10^6$ cpm) RBC suspension (5×10^9 RBCs/ml) in saline (30% hematocrit) was injected into the intraperitoneal cavity of male Wistar rats (10 weeks old, 250-300 g).

"In Vivo" Survival of Carrier RBCs—To study the carrier RBCs circulation, six groups of 12 rats were IP-injected with either native or loaded ^{51}Cr-labeled cells. Samples of blood (400 μl) were taken from the tail vein of injected animals at designated time points (see figures). Whole blood, cell pellet and plasma were assayed for radioactivity. Cellular recovery (^{51}Cr) and CA (^{125}I) present in circulation was expressed as percent of the amount of radioactivity injected, assuming total blood volume as 6% of body weight. Experiments were run in triplicate, with blood extracted from 3-4 rats at each time.

Tissue Distribution of Injected RBCs—To study the organ distribution of carrier cells, six groups of 15 rats were IP-injected with either native or loaded RBCs. At designated times, 2 rats were killed by cervical dislocation. Liver, lungs, spleen, and kidneys were excised, immediately blotted dry with tissue paper and stored at −70°C. Tissues were minced and the total ^{51}Cr and ^{125}I radioactivity in each tissue was determined with different gates in a γ-counter. Results were expressed as percentage of the total injected radioactivity.

RESULTS AND DISCUSSION

"In Vitro" Stability of Loaded Erythrocytes—Loaded rat RBCs stored in CPDA solution at 4°C were found to have a survival of over 80% during the first 4 days (Fig. 1), lower than that under similar conditions of loaded human RBCs, of which 95% persist after 7 days of conservation (data not shown). This agrees with the lower osmotic resistance (*16*) and the volume increase that rat loaded RBCs suffer during the encapsulation process and the conservation period (Fig. 1), contrarily to human RBCs (*17*). CA efflux kinetics (Fig. 1) are compatible with a rapid diffusion process during the first hours of storage. At longer times, the amount of substance released into the medium evolves in parallel with the loss of intact cells, reflecting release by gradual hemolysis.

These data indicate, as has been discussed before (*16*), that the resealing process of hypotonically treated rat RBCs is of limited efficiency. The efflux of inulin (M_r 5,500) (data not shown) is faster than that of CA, which confirms that the release from the RBC vehicle is enhanced for smaller encapsulated compounds (*3*). These features of rat RBCs represent an advantage, since they would permit a slow and continuous diffusion of encapsulated substances as soon as the carrier RBCs are administered to the organism for *in vivo* applications.

Survival of Carrier RBCs in Circulation—*In vivo* survival of ^{51}Cr-labeled carrier RBCs was evaluated by measuring radioactivity at different times after injection. Figure 2 shows the kinetics of the incorporation, circulation, and disappearance of ^{51}Cr-RBCs administered to rats *via* the peritoneal cavity. The amount of RBCs in circulation is maximum between 20 and 24 h post-injection, even though at 15 h a high proportion has already been incorporated, both for loaded and native RBCs. Analysis of the ^{51}Cr data in the cell pellet as well as whole blood demonstrates that the ^{51}Cr label remains bound to circulating rat RBCs (Fig. 2). At no time could we detect more than minute amounts of ^{51}Cr in the plasma, thus ruling out intravascular hemolysis. Hypotonically treated RBCs showed a behavior in circulation qualitatively analogous to native RBCs (Fig. 2); however, some differences can be emphasized. Treated RBCs are incorporated to a markedly reduced extent in comparison with native ones (*ca.* 48% in blood after 24 h *versus* 85% for native cells, Fig. 2). This important difference in incorporation must be due to modifications of RBCs introduced by the encapsulation process. These levels of circulation are comparable, though slightly lower, to those found for other species (*2, 8, 22, 23*) and very similar to incorporation and kinetics reported for IP-injected canine RBCs (*10, 24*).

The cell survival expressed as the half-life of native and loaded erythrocytes deduced from the curves (Fig. 2) can be estimated at 8 and 9 days, respectively. This value is similar for both population types and corresponds with the literature data for most other species (*10, 11*), being slightly lower than that for mice (*8*). These half-life periods, together with the fraction of cells passing the peritoneum, argue in favor of the use of rat loaded RBCs as carrier systems for pharmacological agents.

Circulation of Encapsulated CA—Figure 3 compares the behavior in circulation of ^{125}I-CA administered either encapsulated in ^{51}Cr-RBCs or in free form. The curves

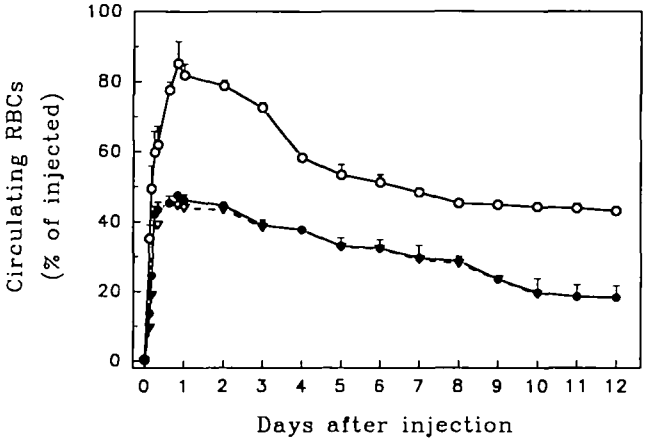

Fig. 2. ***In vivo* survival of ^{51}Cr-labeled rat RBCs.** ^{51}Cr-labeled RBCs, either native or hypotonically loaded, were IP-injected into animals. At different times, samples of whole blood were taken from the tail vein of injected animals. The presence of ^{51}Cr radioactivity in whole blood for native (○) and loaded (●) RBCs as well as ^{51}Cr associated with the cells (▽) are shown. Data (mean±SEM, n=8-10) are expressed as percentage of injected radioactivity, assuming a total blood volume of 16 ml, equivalent to 6% of rat body weight.

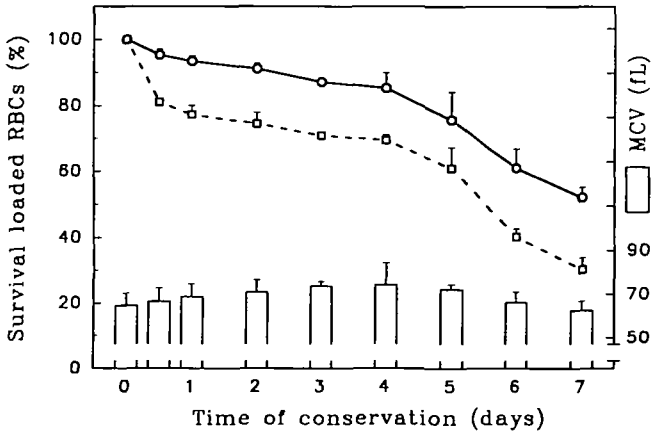

Fig. 1. ***In vitro* survival of CA-loaded rat erythrocytes under storage.** ^{125}I-CA-loaded RBCs were kept at 4°C in CPDA solution (20% Hct). At different times the suspension was hematologically analyzed and radioactivity in cell pellet and supernatant was determined. Survival of cells (○), retention of encapsulated ^{125}I-CA (□) and variation of cell volume (MCV, bars and right axis) are represented. Each value represents the mean of 8-10 measurements±SEM.

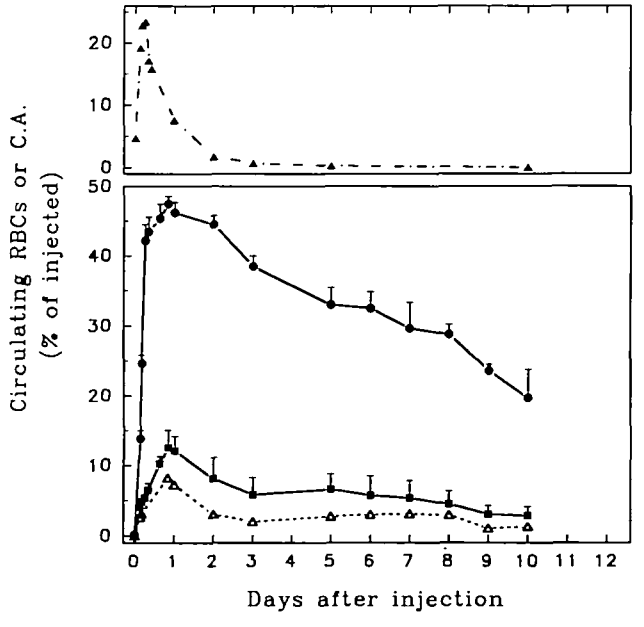

Fig. 3. ***In vivo* behavior of CA-loaded rat erythrocytes.** ^{51}Cr labeled, ^{125}I-CA-loaded RBCs were administered intraperitoneally, and blood samples were taken from the tail vein. The presence of ^{51}Cr radioactivity in whole blood (●) and levels of ^{125}I-CA in whole blood (■) and plasma (△) are shown. Top panel shows, for comparison, the circulation of ^{125}I-CA administered in free form (▲), instead of within RBCs. Data points are the means of 5-6 values±SEM.

corresponding to encapsulated ^{125}I-CA levels in blood (Fig. 3, bottom) show kinetic behavior very similar to the ^{51}Cr-RBCs, with the maximum level in circulation also after 20 to 24 h and a slow and continuous descent in parallel of the levels within cells and in plasma. These data contrast to those obtained when the ^{125}I-CA was injected in free form, where the level in circulation (23% of injected protein, Fig. 3, top) reached a maximum at 6 h, and fell rapidly to only 8% at 1 day after injection, and to near zero after 2 days. This means, in agreement with reports for other substances (*3, 9, 10*), that protein administered in free form is rapidly metabolized and excreted also in rat.

From global analysis of the curves in Fig. 3, it is possible to deduce that a fraction of injected loaded cells, whose heterogeneity as a cellular population has already been shown (*17, 18*), is able to progress from peritoneal cavity to bloodstream, carrying ^{125}I-CA, and the labeled protein diffuses continuously from carrier cells to plasma. The maintenance of steady, appreciable amounts of ^{125}I-CA in the plasma indicates that the rat RBCs are effective as agents for slow and continuous release of exogenous substances in circulation.

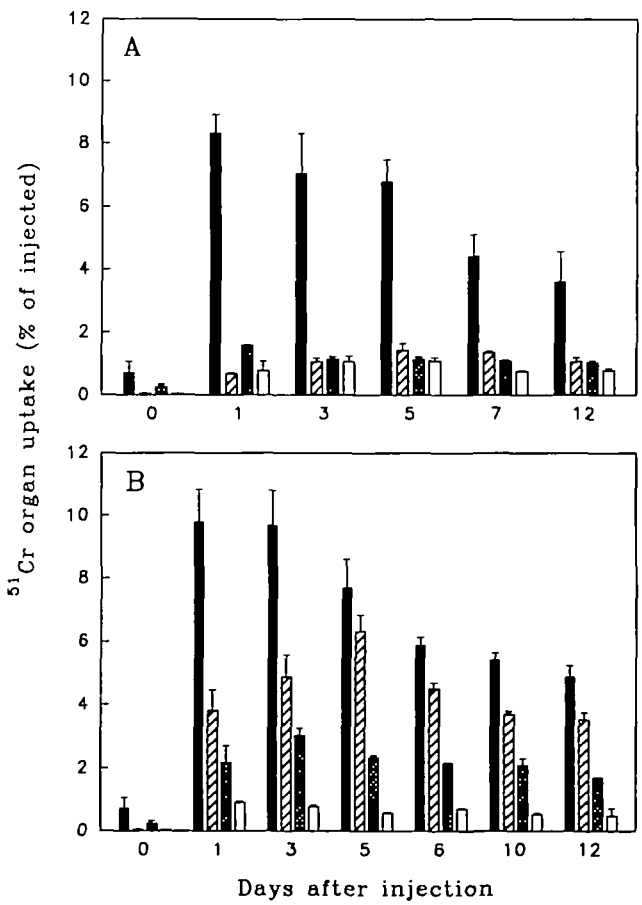

Fig. 4. **Organ uptake of injected ^{51}Cr-labeled rat RBCs.** ^{51}Cr-labeled rat RBCs were IP-injected and samples of whole blood were taken from the tail vein at different times after injection. The percentage of ^{51}Cr radioactivity localized in different organs after injection of either native (A) or loaded (B) RBCs is shown. Data are expressed as percentage of injected ^{51}Cr radioactivity recovered in the liver (filled bars), spleen (striped), kidneys (shaded), and lungs (open bars); mean±SEM, n=5-6.

Organ Distribution of Carrier RBCs—The final fate of both carrier cells and encapsulated substance was investigated by determining the organ distribution of radioactive tracers. Time-dependent organ uptake of native and loaded rat RBCs is presented in Fig. 4.

The total amount of ^{51}Cr-labeled native RBCs that are removed by peripheral tissues at 24 h after injection is close to 10% of the injected dose (Fig. 4A). This, added to the level of RBCs remaining in circulation at that time (85%, Fig. 3), indicates that the native RBCs penetrate the peritoneal barrier easily. On the contrary, the lower percentage of hypotonically treated RBCs in circulation (48%) with only slight increase in the corresponding data of uptake by tissues at 24 h (near 15%, Fig. 4B) indicates that these hypotonic-isotonically treated cells were retained by the peritoneum. Furthermore, the ^{125}I/^{51}Cr ratio, indicative of effectively loaded cells, decreases over three times between the injected cell suspension (1.077) and any of the samples subsequently obtained from circulation (0.285 at most). This means that some cell subpopulation present in the CA-loaded RBC preparation is preferentially retained by the peritoneum. This heterogeneity in the behavior of loaded cell population is discussed further in a following section.

Liver is the main organ involved in removal of RBCs from circulation, showing the predominant removal of native cells (Fig. 4A) and only slightly higher uptake of loaded RBCs (Fig. 4B). This effect is better appreciated in Table I, which gives the ratio between uptake of loaded and native RBCs. The highest value (8-10%) of radioactivity uptake by this organ was observed at 24 h (Fig. 4), decreasing progressively and almost in parallel for the two cell types at longer times, as shown by the constancy of ratios in Table I.

In that the liver is the organ responsible for removing damaged RBCs (*9*), the similarity in the level of uptake for both cell types, could mean either that the modifications suffered by the cells during the encapsulation process do no affect liver recognition, or that the ^{51}Cr labeling alters both cell types to a similar degree, and this alteration results in the apparently elevated level of removal from bloodstream observed with both kinds of RBCs. It has been reported that administration of human RBCs loaded and treated with glutaraldehyde resulted in extensive accumulation of carrier cells in the liver (*7, 25*), and similarly for other species (*12, 13*).

In contrast to the liver, the uptake of RBCs by the spleen is considerably elevated for hypotonically loaded cells (Fig. 4), reaching levels five times higher than those for native

TABLE I. **Comparative uptake of loaded and native ^{51}Cr-RBCs by organs as a function of time after injection.** Values are expressed as the ratio of cpm of ^{51}Cr from loaded to cpm of ^{51}Cr from native RBCs (Fig. 4, B and A, respectively; n=4-6).

Days post-IP	^{51}Cr in rat injected with loaded RBCs/ ^{51}Cr in rat injected with native RBCs			
	Liver	Spleen	Kidney	Lung
1	1.18	5.42**	1.31	1.18
3	1.36	4.36**	2.72***	0.74
5	1.13	4.50***	2.09***	0.52**
12	1.36	3.18***	1.70**	0.61

Significance of differences (loaded *vs.* native RBCs) is indicated by asterisks: *p<0.1, **p<0.01, ***p<0.001.

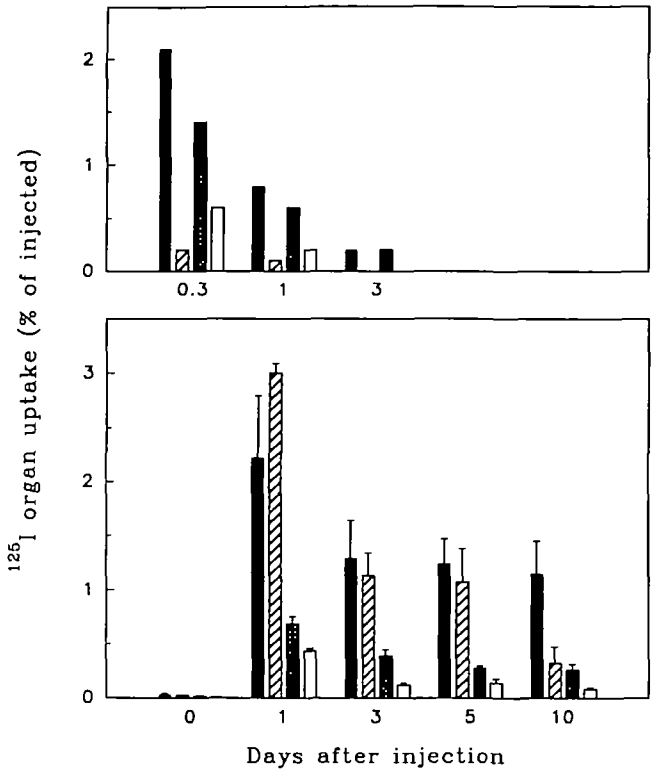

Fig. 5. **Organ uptake of ^{125}I-carbonic anhydrase loaded rat RBCs.** ^{125}I-CA was encapsulated into rat RBCs. The loaded rat RBCs were then labeled with ^{51}Cr and IP-injected. The percentage of ^{125}I radioactivity localized in different organs after injection of the loaded rat RBCs is shown. Data (mean±SEM, n=5-6) are expressed as percentage of injected ^{125}I radioactivity recovered in the liver (filled bars), spleen (striped), kidneys (shaded), and lungs (open bars). Top panel shows comparative results for CA administered in free form.

RBCs in the first days (Table I). In mice, the spleen also removes the highest amounts of cells when these are loaded by dialysis (*9*) without any other treatments. This higher uptake of loaded RBCs may be related to rheological features of these carrier cells; hypotonically treated RBCs have an increased cell size (*17*), which together with other changes in mechanical properties could affect their passage through and interaction with the splenic capillary bed. Similar results have been found as a consequence of oxidative stress induced by hydrogen peroxide treatment (*22*).

For both native and loaded RBCs, the time-dependence is similar and the maximum splenic uptake of ^{51}Cr-RBCs takes place after 5 days. The difference between both types of cells decreases numerically with time, but it is significant even after 12 days (Table I). This dissimilar uptake of loaded and native cells by spleen could play a role in targeting of rat carrier RBCs to this organ.

The activity of kidneys in RBC removal is also different, showing an increment between 1.3 and 2.7 times in the uptake of loaded with respect to native RBCs (Table I). The maximum uptake of native RBCs is reached in the first day, while for loaded cells the activity increases up to the 3rd day, and remains significantly higher than that of control cells up to 12 days post-injection.

In lungs, however, the behavior is particular, the uptake level being very small (below 1%) for loaded cells (Fig. 4),

TABLE II. **Time course of ^{125}I (cpm)/^{51}Cr (cpm) ratio in organ uptake after injection of ^{51}Cr-labeled erythrocytes loaded with ^{125}I-carbonic anhydrase (n=4-6).**

DAYS post-IP	^{125}I-Carbonic anhydrase/^{51}Cr-RBCs ratio			
	Liver	Spleen	Kidney	Lung
1	0.227	0.787	0.318	0.477
3	0.133	0.231	0.128	0.156
5	0.161	0.169	0.121	0.228
10	0.212	0.086	0.124	0.148

and even lower than removal of control RBCs, with ratios that decrease from 1.2 to 0.5 during the first 5 days (Table I).

Finally, despite the significant increment in the spleen uptake of loaded cells, the total uptake level for all tissues assayed increases by only 5% of the injected dose for loaded with respect to control RBCs. Hence, the application of rat RBCs as carriers cannot be ruled out.

Organ Distribution of Encapsulated ^{125}I-CA—The organ distribution of encapsulated ^{125}I-CA (Fig. 5, bottom) is generally similar to that for the ^{51}Cr-labeled carrier RBCs (Fig. 4B), thus reflecting the uptake of intact loaded cells. A remarkable difference is the increased CA level in spleen at day 1.

The ratios between ^{125}I-CA (Fig. 5, bottom) and ^{51}Cr-RBCs (Fig. 4B) are shown in Table II and explain globally the behaviour of each organ with respect to the selective uptake of the loaded cells. All organs show the highest ^{125}I/^{51}Cr ratio at 24 h, indicating a faster removal of the RBCs that have encapsulated more CA. Additionally, the highest value of all is that for spleen at 1 day post-injection, which is in accordance with this organ having the highest selectivity for uptake of loaded cells. This result also agrees with previous work (*17-19*) using independent techniques, which showed that within a heterogeneous loaded population, those cells with a higher amount of encapsulated substance correspond to cells that appear more altered in their surface properties and morphology.

It is remarkable that the lungs, in spite of having much lower uptake activity (Fig. 4), also present a rather high ^{125}I/^{51}Cr ratio, indicative of a selectivity for the heavily loaded RBC subpopulation, similarly to spleen.

In accord with the data of radioactivity in circulation (Fig. 3), the uptake by tissues of ^{125}I-CA injected in free form is completely different to that of encapsulated CA (Fig. 5, top and bottom, respectively). At 1 day, when the maximum levels of encapsulated CA are found in tissues, those for free CA are already decreasing and account for a total of only 1.7% for all four tissues assayed. This, together with the 8% found in circulation (Fig. 3, top), gives less than 10% of the injected dose of free CA, demonstrating the prolonged circulation brought about by the carrier RBC. Moreover, CA injected in free form is preferentially eliminated by liver and kidneys, with a negligible uptake by spleen, in contrast to the RBC-encapsulated protein.

Conclusions—When administered by IP-injection, rat RBCs loaded by hypotonic dialysis and isotonic resealing display an incorporation rate and half-life in circulation that would justify their use as a potential sustained delivery system. Despite previous reports indicating that the peritoneum was an adequate route for injection of treated RBCs, the results presented here show that the peritoneal barrier represents an important reduction in the adminis-

tered dose. The *in vivo* results reveal that a moderate fraction of hypotonically loaded RBCs is able to reach the circulation and that these cells are eventually removed mainly by the liver and the spleen. The spleen shows more selective behavior in the uptake of loaded cells, which could make it a good target for this type of carrier cells. Additionally, this organ is able to retain the highest amount of encapsulated CA.

Therefore, rat carrier RBCs have a potential for use in drug therapy, supporting the feasibility of this experimental animal as a model for pharmacokinetic assays. As an example, these results will permit us to address the possibility of restoring normal activity in CA-defective organs of patients by administration of RBCs loaded with CA.

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