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Hypotonically Loaded Rat Erythrocytes Deliver Encapsulated Substances into Peritoneal Macrophages[1]

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Previous work has shown increased uptake of hypotonically loaded rat RBCs by the spleen and liver "*in vivo*," suggesting that the cells of MPS are involved in their elimination from the circulation. In order to elucidate the mechanism of such elimination, we have undertaken studies on the interaction of such loaded RBCs, in comparison with native RBCs, with peritoneal macrophages. Erythrophagocytosis assays were performed in well plates to which thioglycollate-induced peritoneal macrophages had adhered. Native or loaded ^{51}Cr-RBCs were added under different opsonization conditions to monolayer adherent macrophages, and then the amount of RBCs that were recognized was determined, with separation into adhesion and phagocytosis fractions. Native RBCs are slightly recognized by peritoneal macrophages, about one RBC per macrophage (MΦ). Osmotic treatment of rat RBCs used for encapsulation (independently of the encapsulated substance, ^{125}I-CA or FITC-dextran) produces some modification in the erythrocyte membrane that induces higher recognition of these cells, about three loaded RBCs per macrophage. Consequently, both fluorescent (FITC-Dx) and radioactive (^{125}I-CA) substances previously encapsulated in RBCs were transferred to MΦs. The fluorescence microscopic observations confirmed these results. Moreover, in the case of carrier ^{51}Cr-cells loaded with ^{125}I-CA, the amount of ^{125}I-radioactivity delivered into MΦs was relatively higher than that of ^{51}Cr. The highest ratio, ^{125}I-CA (encapsulated substance)/^{51}Cr-RBCs (carrier cells), present in MΦs means there was a stronger interaction with macrophages of RBCs that carry a higher amount of encapsulated CA, as a function of the heterogeneity of the loaded rat RBCs population previously reported. Finally, the adhesion and phagocytosis of loaded RBCs seem not to involve complement receptors or Fc receptors on the macrophages.

Key words: cellular recognition, drug delivery, drugs encapsulation, peritoneal macrophages, rat carrier erythrocytes.

As a new therapeutic approach, human and animal red blood cells (RBCs) have been used as carrier vehicles for a number of exogenous drugs to be disseminated in an organism. They have the advantages of protecting the active substance from rapid clearance and of avoiding toxic side effects. These erythrocytes are particularly suitable as biological carriers, because of the following: (1) they are naturally biodegradable, (2) their circulating character, (3) autologous cells elicit little or no immune response (*1-3*), and (4) simple encapsulation of drugs in these cells through hypotonic dialysis and isotonic resealing (*4, 5*). The idea of using erythrocytes as a drug delivery system has been successfully explored by several investigators (*6, 7*). Erythrocytes are a natural target of the reticuloendothelial system (RES); consequently, drugs entrapped in RBCs can be easily directed towards the RES (*8, 9*), and also to other organs with different specific treatments in several mammalian species (*10-13*). We previously established conditions which allow an acceptable yield of drug incorporation into rat erythrocytes (*14*), employing encapsulation by means of hypotonic dialysis of markers of different chemical properties, *i.e.* ^{125}I-carbonic anhydrase (M_r=29,000) and fluorescently labeled dextran (FITC-Dx, M_r=19,600) (*15*). Both radioactive and fluorescent labeling allow measurement of the degree of encapsulation in rat and human RBCs, and analysis of the features of the loaded erythrocytes (*16, 17*).

Keeping in mind that the most important criterion for viability is survival in the circulation, rat RBCs loaded with ^{125}I-carbonic anhydrase by means of osmotic treatment were evaluated as a carrier system in an experimental model for biological assays such as the rat (*15*). Loaded RBCs show circulation kinetics and an organ biodistribution similar to those of the native cells, but with accelerated

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Abbreviations: CA, carbonic anhydrase; FCS, fetal calf serum; FITC-Dx, fluorescein isothiocyanate-dextran; GSH, glutathione; IP, intraperitoneal injection; MΦs, macrophages; PBS, phosphate-buffered saline; PBSH, Hanks phosphate-buffered saline; MPS, mononuclear phagocytic system; PIGPA, phosphate inosine glucose pyruvate adenine solution; RBCs, red blood cells.

removal. Their half-life in the circulation (8-10 days) is advantageous for the carrier function. Although the liver is the main target, the spleen shows the most selective removal of loaded RBCs and the ^{125}I-CA encapsulated in them (*15*).

There is considerable evidence that RBCs undergo lipid peroxidation *in vivo* and are more rapidly cleared from the circulation (*18*). Damaged or aged erythrocytes are also rapidly removed (*19*). However, the mechanism by which macrophages recognize oxidatively damaged or senescent RBCs remains uncertain. In general it is accepted that the removal is dependent on the binding of antibodies to damaged cell membrane domains and that the phagocytosis depends upon recognition of the antibody-RBC complex by the Fc receptor (*9, 20, 21*). Another important class of blood proteins that can opsonize RBCs for uptake are the complement proteins (*19, 22*). Nevertheless, oxidatively damaged RBCs can be recognized in a direct, antibody-independent way by a macrophage scavenger receptor, which also results in their binding and phagocytosis (*23, 24*), although there is evidence that oxidatively damaged erythrocytes can also be removed by phagocytosis through an antibody-mediated pathway (*25*).

In this sense, the modifications of plasma membranes may represent a general mechanism that marks damaged cells for phagocytosis by macrophages. When erythrocytes are subjected to hypo-osmotic dialysis, they swell. Because these red cells are leaky, it is possible to load them with drugs or proteins before resealing the membrane holes with a hyper-osmotic solution. Resealed erythrocytes are heterogeneous in size and haemoglobin content, but can function and circulate almost normally even if they are injected intraperitoneally (*15, 26*).

Considering the results of our studies on loaded rat erythrocytes, in which the higher removal from the circulation was mainly localized in the spleen and liver (*15*) (both organs having important contents of phagocytic cells), we have analyzed here the phagocytosis of loaded rat erythrocytes by inflammatory rat peritoneal macrophages that present appropriate receptors for erythrophagocytosis. This study could be helpful not only for an understanding of the basis of senescent cell recognition by macrophages but also for establishment of the feasibility of targeting drug-loaded red blood cells to macrophages for specific therapeutic purposes. The encapsulation of fluorescently labeled dextran (FITC-Dx) enables the observation on fluorescence microscopy of the adherence of loaded RBCs to macrophages, and ^{125}I-carbonic anhydrase incorporated to carrier erythrocytes can facilitate the measurement of the phagocytic activity on loaded RBCs. The ^{51}Cr-labeling of native and loaded RBCs permits determination of the percentage of cells which are adhered or phagocytosed in each assay. Moreover, since, as has been discussed, complement components or IgG may play a role in the phagocytic function, the influence of different serum components has been studied.

MATERIAL AND METHODS

Chemicals, Radioisotopes and Materials—Carbonic anhydrase (M_r 29,000) and FITC-dextran (M_r 19,600) were obtained from Sigma Chemical (St. Louis, MO, USA). Na^{125}I (100 mCi/ml) and [^{51}Cr]sodium chromate were purchased from NEN-Dupont (Brussels, Belgium). Carbonic anhydrase was iodinated in our laboratory using chloramine-T immobilized on polystyrene beads (Iodo-Beads; Pierce Chemical, Rockford, IL, USA). RPMI 1640, fetal calf serum (FCS), and the antibiotic-antimycotic mixture were from Gibco Laboratories (Grand Island, NY, USA). ATP and glutathione were from Boehringer Mannheim (Germany). The other chemicals used were of reagent grade and were purchased from Merck (Darmstadt, Germany). The dialysis bag was from Medicell (London, UK). The cell culture cluster dishes (12 wells per plate) were from Costar (Cambridge, MA). Sterile plastic material was from NUNC (Kanstrup, Denmark). Male Wistar rats (250 g) were kept under controlled temperature and humidity conditions, with a day-night cycle according to European Union directives.

Buffers and Solutions—Isotonic (300 mOsm/kg) and hypotonic (80 mOsm/kg) PBS, PIGPA, and all solutions of ^{125}I-carbonic anhydrase and FITC-dextran were prepared according to previously established conditions (*15, 16, 27*). The macrophage culture medium was composed of RPMI 1640 supplemented with heat-inactivated FCS (10%) and an antimicrobial solution (1%). PBS-Dulbecco was prepared as usual. All solutions used in culture assays were sterilized by autoclaving or with Millipore filters (0.22 μm).

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

Dialysis Loading Procedure—Packed erythrocytes were subjected to the loading procedure according to previously established conditions (*14, 16*). The marker was added to a RBC suspension to a final concentration of 34.5 μM ^{125}I-CA ($18.82 \pm 1.55 \times 10^8$ cpm/μmol) or 400 μM FITC-Dx. The mixture was dialysed at 4°C for 1 h against hypotonic PBSH (80 mOsm/kg) supplemented with 2 mM ATP and 3 mM GSH. The cells were then annealed (37°C for 10 min) and resealed with 0.5 volume of a hypertonic PIGPA solution (37°C for 30 min), and then washed three times in cold isotonic PBSH (160 g, 5 min). The presence of the fluorescent marker (FITC-Dx) within erythrocytes and macrophage cells was followed by means of fluorescence microscopy. Control cells, equally processed but in isotonic medium, allowed measurement of the non-specific binding of CA and FITC-Dx to RBCs.

^{51}Cr Labeling of RBCs—After the resealing step in the loading cell procedure, ^{125}I-CA loaded erythrocytes were incubated with [^{51}Cr]sodium chromate to radiolabel the cells as described by Morrison *et al.* (*28*). Loosely, 500 μl of a packed loaded RBCs were mixed with 50 μl of a ^{51}Cr solution (100 μCi/μl) for 30 min at 4°C. Thereafter, the cells were washed three to four times in isotonic PBSH to remove unbound ^{51}Cr. Native RBCs were labeled similarly as a control. Aliquots were taken for measurement of radioactive markers (^{125}I-CA and ^{51}Cr-RBCs) with a gamma-radiocounter. Aliquots of a loaded (^{125}I-CA, 5.08 ± 0.09-10^6 cpm/ml) and ^{51}Cr-labeled (5.30 ± 0.16-10^6 cpm/ml) RBC suspension (5-10^9 RBCs/ml, 30% haematocrit) were diluted in isotonic PBSH to an appropriate concentra-

tion ($0.25\text{-}10^9$ RBC/ml) for phagocytosis assays.

Preparation of Peritoneal Macrophages—Macrophage (MΦ) production was induced in rats by IP injection of thioglycollate medium (*29*). The macrophages were collected by peritoneal lavage with sterile NaCl 0.9% and then suspended in culture medium composed of RPMI 1640 medium supplemented with heat-inactivated FCS (10%) and the antibiotic-antimycotic mixture (1%). The total number of cells was determined with a haemocytometer and their concentration was adjusted to 3×10^6 cells/ml. Aliquots (1 ml) were pipetted into wells (12 wells per plate) and then incubated for 2 h at 37°C, under humidified air with 5% CO_2. Non-adherent cells were removed by three-four washings with 1 ml of Dulbecco's medium containing calcium, and monolayer adherent cells were kept for phagocytosis assays (Stage 1, Fig. 1-2). Cell viability was routinely determined before and after each experiment using the trypan blue exclusion test.

Phagocytosis Assay Using ^{51}Cr-Labeled RBC—The procedure was essentially that in Ref. *30*. Adherent peritoneal macrophages ($2.5\text{-}10^6$) in each chamber were covered with culture medium supplemented with 200 μl of a carrier RBC suspension ($0.25\text{-}10^9$ RBC/ml) in the presence or absence of homologous serum or IgG (Stage 2, Fig. 1-2). Control wells were incubated with a equal number of native RBCs under the same conditions. After 2 h incubation, nonadherent/noningested RBC were removed by gentle aspiration followed by three washes with 1 ml of Dulbecco's/Ca^{2+} solution (Stage 3, Fig. 1-2). In the second step (*30*), adherent but non-internalized RBCs were lysed by the addition of 2 ml of 140 mM NH_4Cl/Tris buffer, pH 7.4, for 3 min at 20°C (Stage 4, Fig. 1-2). Quantitation of these interactions was performed through the ^{51}Cr label previously associated with the RBCs, referred to as the adhesion fraction (cpm adhesion, Fig. 1). Finally, the adherent peritoneal macrophages were then removed with 1 ml of 1 M NaOH/Triton X-100 (0.025%). The radioactivity of the internalized ^{51}Cr-labeled RBCs associated with them was taken as the amount of phagocytosis (cpm ^{51}Cr phagocytosis, Fig. 1). In the case of loaded RBCs, the engulfment of carbonic anhydrase by macrophages was measured as ^{125}I radioactivity (cpm ^{125}I engulfed protein).

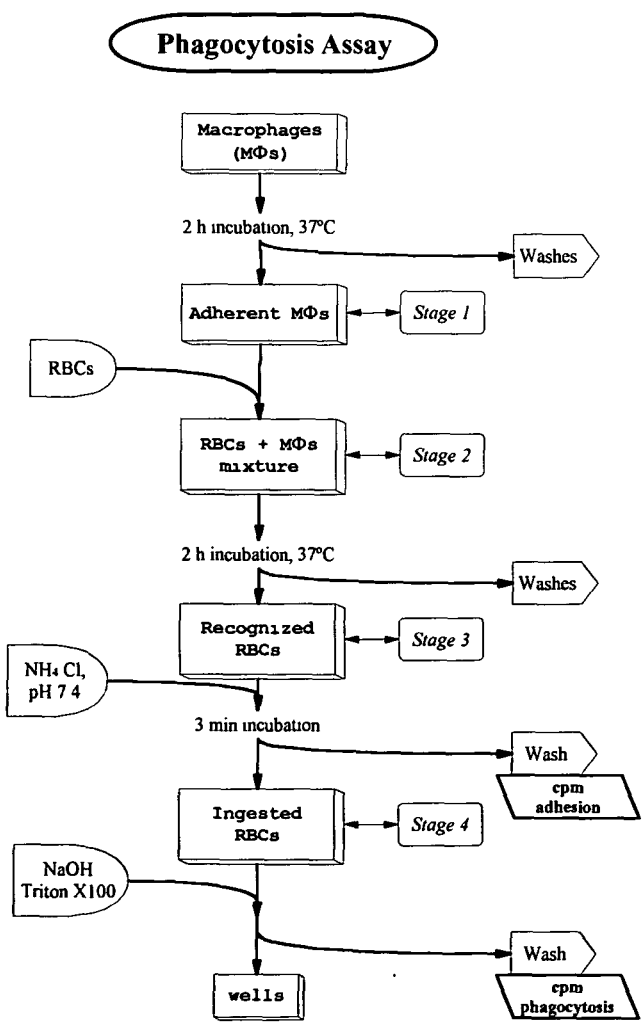

Fig. 1. **Schematic representation of the different stages of the phagocytosis assays.** The reagents added to wells containing MΦs are shown on the left and fractions collected for measurement on the right. Stages 1 to 4 correspond to the images presented in Fig. 3.

Statistical Treatment of Data—Replicate measurements were summarized as means±standard error of the mean (SEM). The significance of differences was evaluated using Student's *t* test.

Fluorescence Microscopy—Peritoneal macrophages were incubated under the same conditions, but were layered over sterile glass coversliped in the wells. The carrier RBCs were loaded with FITC-dextran, therefore they were fluorescently labeled cells. The procedure was performed according to above described phagocytosis assay, and the glass coverslips were treated with formaldehyde (10%) to fix the cells. Random fields on these coverslips were examined microscopically at 1,000×magnification under oil with an Olympus B071 optical microscope with an epifluorescence attachment. Micrographs were taken under illumination either with a Hg lamp (fluorescence) or simultaneously with a Hg lamp and normal light (transmitted white light).

RESULTS AND DISCUSSION

Initially, several ratios of hypotonically loaded rat RBCs and macrophages were assayed, from 10 up to 100 RBCs/MΦ. At higher ratios, although common in the literature (*9, 19, 31*), the dilution of the signal did not allow observation of the existing differences, due to background interference. Therefore, an initial mixture of 20 RBCs/MΦ was chosen, which allowed the study of the behaviour of these carrier RBCs in erythrophagocytosis assays, as described under "MATERIAL AND METHODS," and depicted in Fig. 1.

With respect to the time of incubation of carrier RBCs with macrophages, on incubation for up to 4 h (data not shown) there was no significant increase from the values for 2 h incubation, even with an increase in the dispersion of results. Hence, 2 h was used throughout.

The viability of macrophages was assessed using the common Trypan Blue dye exclusion method, no significant loss being revealed.

As to interaction of native and loaded ^{51}Cr-RBCs with macrophages (Fig. 2), there was higher recognition of the latter, and the levels in both cases were above those of control samples (RBCs added to wells without MΦs). The

limited recognition of native RBCs (2–4% of added ^{51}Cr-RBCs), mainly in the form of adhesion, may be due to some damage to the cells that occurred during the ^{51}Cr labeling process. This agrees with the *in vivo* behaviour of native RBCs, that show a reasonable lifespan in the circulation (*15*). In the case of the loaded RBCs, the interaction with MΦs was higher under all assay conditions, reaching levels near 12% of the initially added ^{51}Cr-RBCs (Fig. 2). On the other hand, in the presence of heat-inactivated serum as an opsonizing agent the recognition was reduced, approaching the response of native RBCs. This could indicate the involvement of active serum components in the recognition of loaded RBCs, but this proposal was not supported by the results obtained in the absence of any serum (NS, Fig. 2), only slightly lower than in the case of normal serum. The effects of inactive serum could be associated with possible interference by adsorption of denatured serum proteins on the macrophage or RBCs surface.

On the other hand, in a search for appropriate conditions of opsonization prior to the phagocytosis assay, loaded RBCs were incubated with active homologous serum before adding them to MΦs. The recognition signal (PrS, Fig. 2) is not significantly modified with respect to the case without preincubation (S, Fig. 2). In order to study the involvement of Fc or other serum proteins in the recognition of loaded RBCs by MΦs, IgG was added to some wells. The degree of recognition (IgG, Fig. 2) appears to be slightly lower under these conditions, but the difference was not very significant.

The results of recognition are also expressed as the number of RBCs associated with one macrophage (right axis in Fig. 2). The average value was below one for native RBCs and around three for loaded RBCs, results which are similar to those obtained by other authors in phagocytosis assays on diverse modified RBCs (*19, 30, 32*).

The similarity between the NS, S, PrS, and IgG conditions suggests that neither Fc receptors nor complement protein receptors are specifically involved in the interaction of loaded RBCs with macrophages. It is possible that the scavenger receptor could be acting on these cells, similar to what happens with oxidatively stressed RBCs (*24, 33*).

These results as to recognition of carrier RBCs by macrophages were confirmed by using RBCs loaded with a fluorescent substance (FITC-dextran, Fig. 3). At the same time, the recognition was divided into two fractions: adhesion, *i.e.* RBCs are attached to the outer surface of the macrophages, and phagocytosis, *i.e.* RBCs have been engulfed by the macrophages. Later on, the analysis of these two components was also performed quantitatively on ^{51}Cr-RBCs carrying a radioactively labeled substance (^{125}I-CA, Figs. 4 and 5, and Table I).

The loading of RBCs with FITC-dextran permits the observation of the phagocytosis action by means of fluorescence microscopy. Figure 3 shows the cells at different stages that lead to the separate observation of the adhesion and phagocytosis of loaded rat RBCs by peritoneal MΦs. Stage 1 shows a control with only adherent MΦs. Stage 2 corresponds to the sample of FITC-dextran loaded RBCs

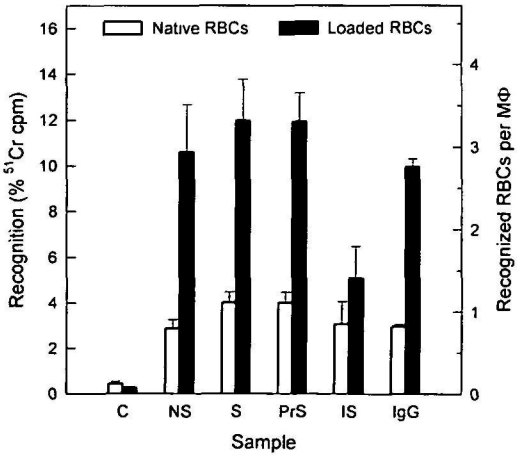

Fig. 2. ***In vitro* interaction of peritoneal macrophages with carrier ^{51}Cr-RBCs under different conditions of opsonization with serum or IgG.** Native or loaded RBCs, both labeled with ^{51}Cr, were added to wells containing adherent MΦs, followed by incubation for 2 h in the presence of different opsonizing agents: (C) control sample: RBCs added to wells containing no MΦs, (NS) no opsonizing agents, (S) serum added simultaneously, (PrS) serum added to RBCs 30 min before mixing them with MΦs, (IS) heat-inactivated serum, (IgG) non-specific IgG. The radioactivity remaining associated with MΦs (stage 3) is expressed as a percentage of the total ^{51}Cr-RBCs added and presented as the mean of 8 measurements ± SEM. On the right-hand axis the recognition data are expressed as the number of RBCs/MΦ.

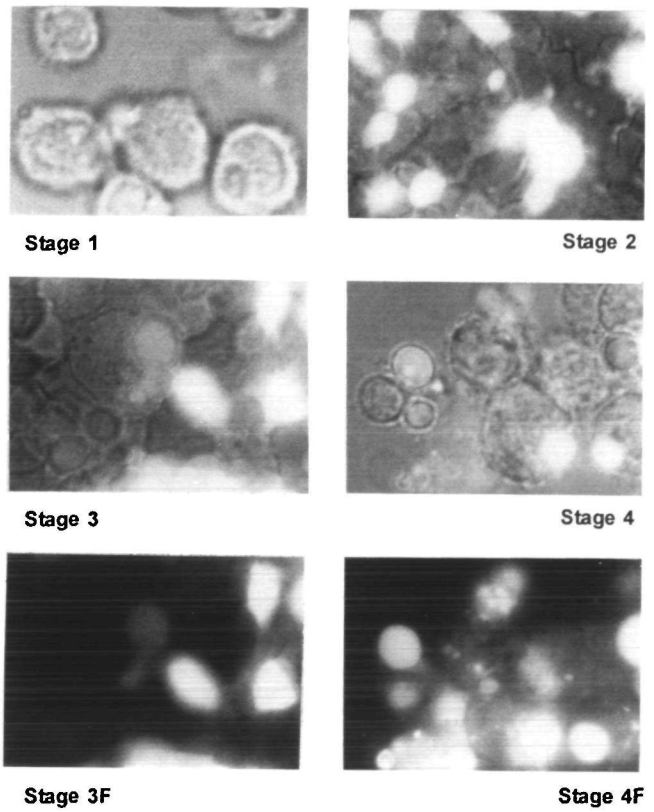

Fig. 3. **Microscopic observation of peritoneal macrophages interacting with RBCs loaded with FITC-dextran.** The photographs were taken 2 h after the addition of RBCs and homologous serum. (Stage 1) monolayer adherent MΦs; (Stage 2) loaded RBCs added over adherent MΦs; (Stage 3) loaded RBCs remaining attached to MΦs (recognition fraction); and (Stage 4) MΦs showing engulfed RBCs (phagocytosis fraction). Stages 3F and 4F correspond to preparations illuminated with a fluorescence lamp only, while 1 to 4 were observed with both light sources.

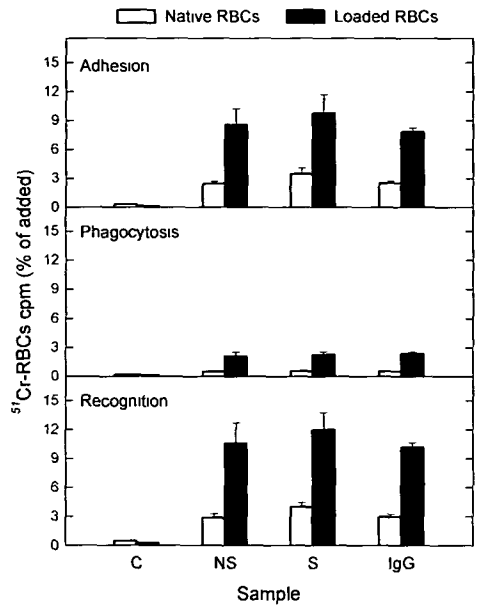

Fig. 4. **Adhesion and phagocytosis fractions of carrier ^{51}Cr-RBCs with peritoneal macrophages.** Either native or loaded RBCs were added to MΦs. After 2 h incubation (see legend to Fig. 2 for opsonizing conditions), the erythrocytes were lysed (adhesion fraction) and finally the MΦs were also lysed to measure the internalized RBCs (phagocytosis fraction). The recognition data are the sums of adhesion and phagocytosis. Values represent percent ^{51}Cr retained with respect to total added, and are given as the means of 5 measurements ± SEM.

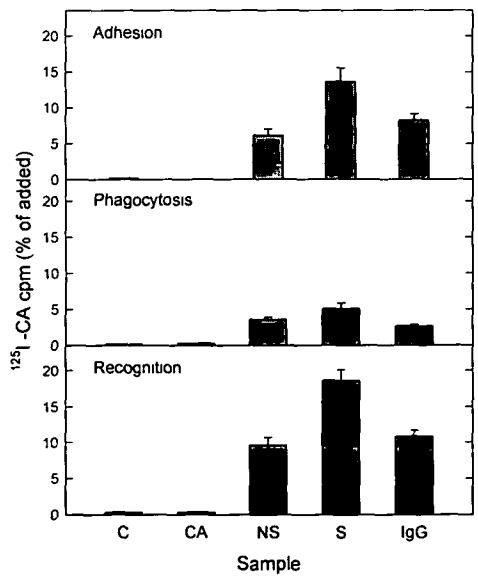

Fig. 5. **Distribution of ^{125}I-carbonic anhydrase previously encapsulated in RBCs after their interaction with MΦs.** The samples and assay were the same as in Fig. 4, except that the ^{125}I radiolabel of carbonic anhydrase was detected (CA: non-encapsulated CA was added to MΦs; see legend to Fig. 2 for the other conditions). The values represent percent ^{125}I-CA retained with respect to total added, and are given as the means of 5 measurements ± SEM.

added over adherent MΦs, where the heterogeneity of the loaded rat RBCs can be observed (*16*), as well as the complete coating of MΦs by RBCs, which proves the adequate ratio of cells. In the next step, RBCs not attached to macrophages have been removed and the adherence to MΦs of carrier fluorescent RBCs can be clearly observed (stage 3 and 3F, Fig. 3). Finally, in stage 4, the FITC-dextran delivered into MΦs through phagocytosis of the carrier RBCs is shown. These pictures give an idea of the extents of adherence and phagocytosis that osmotically treated RBCs undergo, and the delivery of carried substance into the MΦs is clearly manifested.

TABLE I. **Ratio of ^{125}I (cpm)/^{51}Cr (cpm) in the different fractions on the recognition of ^{51}Cr-labeled rat erythrocytes loaded with ^{125}I-carbonic anhydrase by rat peritoneal macrophages ($n=5$).**

Interaction	No serum	Serum	IgG
Adhesion	0.70	1.38	1.04
Phagocytosis	1.70	2.28	1.11
Recognition	0.90	1.55	1.06

The initial sample of RBCs had a ratio of 0.75.

The quantitation of the ^{51}Cr-radioactivity associated with carrier RBCs at each stage of the procedure gave the results for the adhesion and phagocytosis fractions shown in Fig. 4. The proportion of loaded RBCs that appears to be adhered to MΦs is about 9% of the added ^{51}Cr-RBCs; no significant differences between in the presence or absence of serum or IgG were observed ($p>0.1$) (Fig. 4, top). The fraction of phagocytosed ^{51}Cr-RBCs (Fig. 4, middle) is very low, only about 2-3% of the added cells. Total recognition, obtained as the sum of the adhesion and phagocytosis fractions, is limited to *ca.* 12%. This agrees with the reasonable circulation of these carrier cells *in vivo* (*15*).

Although the phagocytosis fraction is small, it is enough to allow the movement of the encapsulated substance (^{125}I-CA) towards the intracellular medium of MΦs (Fig. 5). It must be noted that free ^{125}I-CA is not incorporated into MΦs (CA, Fig. 5); this means that the amounts of ^{125}I-CA determined for the other samples in Fig. 5 are exclusively due to ^{125}I-CA carried within the loaded RBCs. Moreover, it has been established that encapsulated ^{125}I-CA is not adsorbed on the membrane, but is inside the RBCs (*16*).

The amount of CA in the adhesion fraction was up to 12% of the added CA, which was higher than in the phagocytosis fraction, in parallel with the results for carrier ^{51}Cr-RBCs in Fig. 4. The levels of ^{125}I-CA carried by loaded RBCs in the different fractions, adhesion, phagocytosis and total recognition, were increased when serum was present, with significant differences ($p<0.01$, $p<0.1$, and $p<0.01$, respectively) with respect to both the absence of serum and the presence of IgG (Fig. 5).

When using CA-loaded RBCs, in general terms, the profiles of both radioactive labels (^{51}Cr for RBCs and ^{125}I for CA) are similar, as has been shown in the previous figures. However, there are some changes in the proportions of both isotopes in each sample during the process, which are best appreciated by analyzing the ^{125}I/^{51}Cr ratio, as shown in Table I. Firstly, it can be clearly seen that for all samples the phagocytosis fraction contains, in proportion, more ^{125}I-CA than ^{51}Cr-RBC, as compared to the adhesion fraction. Second, samples incubated in the presence of serum also show a higher ratio than those treated without serum or with IgG. Both effects can be attributed to a stronger interaction with macrophages of those cells that carry a

higher amount of encapsulated CA, since it has already been verified (*16, 17*) that the loaded rat RBC population is heterogeneous with respect to both substance content and cell fragility, and that these cells more heavily altered are the same as those that carry the higher amount of substance. In the same context, the results with serum could indicate a partial role of serum components in the process that, although it would not be reflected in the recognition of the total population of loaded RBCs, could show up in the more selective interaction with the most altered (and heavily loaded) cells.

Carrier RBCs can be modified by several other procedures. Of all them, loaded RBCs are the most stable in the circulation (*16*), while chemically crosslinked RBCs (*34*) and highly biotinylated RBCs (*35*) are rapidly removed from the bloodstream, in accordance with their marked recognition by peritoneal macrophages.

Currently, liposomes are also fulfilling their promise as drug delivery vehicles with general applications, because they exhibit reduced toxicity and enhanced efficacy compared with their free counterparts (*36*). Conventional liposomes have limited therapeutical use because of their rapid uptake by phagocytic cells (*37*), whereas sterically stabilized liposomes that exhibit more specific targeting or polymorphic liposomes that exhibit high reactivity to membranes have been referred to as "stealth" liposomes because of their reduced recognition and uptake by the immune system (*38*). Hypotonically loaded RBCs deliver a substance similarly to these other systems, while they seem devoid of the limitations imposed on crosslinked RBCs and liposomes by their rapid elimination.

The results reported in this paper follow the previous finding that proteins and dextran can be encapsulated in erythrocytes, in which they are stable for at least four or five days (*15, 16*). In the present work it was shown that these loaded cells suffer certain opsonization by homologous serum or IgG, and subsequent phagocytosis occurs, whereby the encapsulated substance (both CA and FITC-Dx) is delivered to the MΦs. In addition, the amount of CA found in these cells was several times higher (2.5) than that which corresponds to the number of recognized RBCs, apparently due to preferential phagocytosis of the most heavily loaded RBCs.

In summary, hypotonic loading of RBCs has some effect on the membrane that induces an increase in their macrophage recognition and makes the use of this system for targeting substances towards RES cells feasible. Moreover, the reasonable stability in the circulation of loaded cells supports the use of these loaded RBCs as carrier systems for the distribution of pharmacological agents in the systemic circulation (*15*). The combination of loading with cell surface modification may allow modulation of the mechanism and the efficiency of the delivery of active substances to cells of the RES, or the capacity to release them in the systemic circulation.

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