Encapsulation of Adriamycin in human erythrocytes

(entrainment/carrier erythrocyte/anthracyclines/glucose-6-phosphate dehydrogenase deficiency)

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ABSTRACT Adriamycin (doxorubicin) was encapsulated in human erythrocytes by means of a dialysis technique involving transient hypotonic hemolysis followed by isotonic rescaling. Up to 1.6 mg of the drug was entrapped per ml of packed erythrocytes, with the efficiency of encapsulation being 60–80%. In vitro incubation of the Adriamycin-loaded erythrocytes in autologous plasma was accompanied by progressive release of unaltered Adriamycin in the medium. The efflux was still evident after 50 hr. The metabolism of encapsulated Adriamycin was restricted to limited formation of the C-13 hydroxylated metabolite, adriamycinol, Adriamycin-NADP+ of encapsulated glucose-6-phosphate dehydrogenase activity. Reductive bioactivation of encapsulated Adriamycin to yield the corresponding aglycone was not observed in a variety of conditions. However, when NADPH ferredoxin reductase and ferredoxin, both purified from spinach leaves, were co-entrapped within erythrocytes and allowed to catalyze electron transfer to Adriamycin intracellularly under N2, a quantitative conversion to 7-deoxyadriamycin aglycone was obtained. Adriamycin-loaded erythrocytes did not show any significant oxidative damage, except for a variable increase of methemoglobin, suggesting some redox cycling between native Adriamycin and its semiquinone radical. Encapsulation of Adriamycin in autologous human erythrocytes may represent a therapeutic strategy for the slow release in circulation of this antineoplastic drug in order to reduce or prevent its adverse effects and especially the delayed cardiotoxicity that limits its use in patients with neoplastic disease.

Adriamycin (doxorubicin) is an anthracycline antibiotic of wide use in antineoplastic chemotherapy because of its remarkable cytotoxicity toward several solid tumors (1–3). Among the adverse effects it produces, delayed cardiotoxicity is certainly the most serious. Accordingly, total dosage of Adriamycin should never exceed 500 mg per m2 of body surface (2), which is usually administered fractionally at 3-week intervals.

It has been reported (4) that cardiotoxicity is almost completely prevented by slow intravenous infusion because of significantly improved pharmacokinetics. However, such a way of administration is not practicable for routine treatment in daily hospital therapeutic schedules. Alternative systems, such as Adriamycin-encapsulating liposomes (5) or use of intravenous peristaltic pumps, seem not to be practically applicable.

Human and animal erythrocytes have been proposed and used as carrier vehicles for a number of chemicals to be disseminated in the circulatory system (6–8). When hypotonic dialysis is used to achieve encapsulation, carrier erythrocytes have been shown in vivo survival (6). Cytotoxic and antineoplastic drugs have also been entrapped in erythrocytes for targeting purposes (9) or to obtain slow release of therapeutic agents (10). Recently, daunomycin (daunorubicin), a close analog of Adriamycin, has been entrapped within human erythrocytes but found to produce marked perturbation of the loaded erythrocytes (11).

To check whether Adriamycin-loaded human erythrocytes are a potentially useful vehicle for time-programmed dissemination via the circulatory system, we investigated the encapsulation potential, the intraerythrocytic metabolism, and the efflux of unaltered Adriamycin by using dialysis-loaded erythrocytes. The results reported in this paper indicate that such therapeutic strategy holds promise on the basis of in vitro studies. In addition to showing the potential for encapsulation, these findings demonstrate (i) limited biotransformation of Adriamycin intracellularly (because of the absence of electron transfers in the mature erythrocyte), (ii) a slow release of the unmodified molecule to the medium, and (iii) an absence of significant erythrocyte damage as assessed by several metabolic parameters.

MATERIALS AND METHODS

Materials. The following chemicals were kindly provided as gifts by F. Nicolis and V. Malatesta (Farmitalia): Adriamycin, adriamycinol (13-dihydroadriamycin), 7-deoxyadriamycin aglycone, 7-hydroxyadriamycin aglycone, 7-deoxyadriamycin aglycone. Glucose-6-phosphate, NADP+, hypoxanthine, xanthine oxidase, reduced glutathione (GSH), and ATP were purchased from Sigma. D-[1-14C]Glucose was obtained from the Radiochemical Centre. Ferredoxin:NADP+ oxidoreductase (EC 1.18.1.2) and ferredoxin, both purified from spinach leaves to homogeneity (12), were kind gifts from B. Curti and G. Zanetti.

Blood Samples and Enzyme Assays. Blood samples were obtained after informed consent from normal subjects (both males and females) and from glucose-6-phosphate dehydrogenase (G6PD); d-glucose-6-phosphate:NADP+ 1-oxidoreductase, EC 1.1.1.49)-deficient hemizygous males, of Sardinian ancestry, who were asymptomatic and had the Mediterranean variety of G6PD deficiency (13), as assessed by estimates of erythrocyte G6PD activity in the range of 0.8 to 7 international units × 10-3 (mIU) per g of Hb (14). Heparin was used as anticoagulant and leukocytes and residual platelets were removed according to Beutler et al. (15). Assays of Ca2+-ATPase and of Na+/K+-ATPase activities were carried out as described (16). Ferredoxin reductase activity was assayed according to Zanetti and Curti (12). Calcium and potassium were determined by atomic absorption spectrophotometry as described (17). GSH and ATP were estimated according to Beutler (18). Methemoglobin was determined according to the procedure of Evelyn and Malloy (19) and thiobarbituric acid-reactive material according to the procedure of Stock and Dormandy (20). The hexose

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Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; GSH, reduced glutathione; HMs, hexose monophosphate shunt; IU, international unit(s).
monophosphate shunt (HMS) activity of intact erythrocytes was estimated as reported (21). Osmotic fragility curves were determined according to Sprandel and Zollner (11).

**Dialysis Encapsulation.** Encapsulation of Adriamycin, ferredoxin:NADP⁺ reductase, and ferredoxin within human erythrocytes was achieved by the hypotonic dialysis-isotonic rescaling method (22) essentially according to the procedure of Ropers and coworkers (23, 24). Briefly, washed erythrocytes were placed in a dialysis bag (Spectrapor, Spectrum Medical Industries, Los Angeles, CA; molecular size cutoff, 12–14 kDa) at a final hematocrit of 70% together with either Adriamycin or ferredoxin:NADP⁺ reductase and ferredoxin, or both, dissolved in 0.9% NaCl. Transient hemolysis was achieved by placing the dialysis bag in 10 vol of 10 mM sodium phosphate and 10 mM sodium bicarbonate (pH 7.4) containing 20 mM glucose, and gently mixing by rotation for 20 min at 4°C. Ten milliliters of the lysed erythrocytes were left for 10 min at 39°C, then mixed with 1.0 ml of a rescaling isotonic solution (5 mM adenine/100 mM inosine/100 mM sodium pyruvate/100 mM sodium phosphate/100 mM glucose/12% NaCl) and incubated for 30 min at 39°C. The rescaled erythrocytes were washed three times in 0.9% NaCl and once more in autologous plasma to remove extracellular bound material before being used in the experiments. The percentages of encapsulation were calculated as reported (21). Subsequent incubation of the native, unloaded (i.e., processed as for the entrapment technique, yet without addition of Adriamycin), and loaded erythrocytes was carried out at 37°C at a 50% hematocrit in autologous plasma for various time intervals.

**Extraction and Analysis of Adriamycin and Its Metabolites.** Extraction was carried out by treating 0.2 ml of a 50% erythrocyte suspension in 0.1 M sodium phosphate (pH 8.0) or 0.2 ml of the supernatants (either plasma or buffers), with 1.0 ml of methanol/chloroform (1:4, vol/vol). Extraction was performed twice and the organic solutions were pooled, vacuum dried, and dissolved in 1.0 ml of n-butanol. HPLC analysis of the extracts and of the standard compounds was carried out by injecting 10 µl of the butanolic solutions in a Waters model 510 solvent delivery system equipped with a µBondapak C-18 column and monitored by a Shimadzu RF-530 fluorescence detector. Measurements were carried out at 471 nm excitation and at 554 nm emission. Elution was at a flow rate of 2 ml/min with 0.05 M sodium phosphate (pH 3.0) containing 30% (vol/vol) acetonitrile.

**RESULTS**

**Entrapment of Adriamycin in Human Erythrocytes.** The encapsulation potential of Adriamycin in human erythrocytes was found to be remarkably good. Thus, the hypotonic hemolysis–isotonic rescaling procedure allowed up to 1.6 mg to be entrapped per ml of packed erythrocytes, with average contents of 1.0 mg of Adriamycin/ml and average efficiencies of encapsulation of 60–80%. The intraerythrocytic levels of Adriamycin obtained by encapsulation were much higher (~4-fold) than those observed after free diffusion of the molecule into the erythrocyte (Table 1). Most of the intracellular Adriamycin was bound to the membrane, although its progressive leakage seems to rule out tight binding (see below).

**Release of Adriamycin from Loaded Erythrocytes.** The kinetics of efflux of unmodified Adriamycin from loaded normal erythrocytes are shown in Fig. 1. The rate of decrease of Adriamycin within the cells was found to parallel quite satisfactorily the appearance of the unaltered molecule in the supernatant. An obvious requirement for such correlation to be observed was the stepwise replacement of the supernatant, to prevent equilibration between intracellular and extracellular compartments.

| Table 1. Comparative effects of diffusion and encapsulation on the levels and subcellular distribution of Adriamycin in normal erythrocytes |
|-----------------|-----------------|-----------------|-----------------|
|                  | Diffusion        | Encapsulation    |                  |
| Erythrocyte      | Adriamycin, mg/ml| Adriamycin, mg/ml| Hb, mg/ml       |
| fraction         |                 |                 |                 |
| Packed erythrocyte | 353 ± 31        | 0.247 ± 0.026   | 342 ± 24        |
| Membranes        | 0.147 ± 0.014   | 0.218 ± 0.006   |
| Cytosol          | ND 0.107 ± 0.015| ND 0.258 ± 0.039|

Encapsulation was carried out as described at a 2.5 mM concentration of Adriamycin in 0.9% NaCl. In the diffusion experiments, matched erythrocyte suspensions were incubated at a final hematocrit of 70% with 2.5 M Adriamycin in 0.9% NaCl. Incubations were for 60 min at 37°C, and then the erythrocytes were extensively washed with 0.9% NaCl until complete removal of externally absorbed Adriamycin and hemolysed by addition of 10 vol of H2O. The membranes were washed 10 times with H2O. Results are the average of five experiments.

Adriamycinol—i.e., the C-13 hydroxylated metabolite of Adriamycin—was formed to a small extent during the incubation period (see below). Its level in the supernatant was lower than the intracellular level, but both increased in parallel, implying that this metabolite is also released.

**Metabolism of Entrapped Adriamycin Within Erythrocytes.** The initial entrapment of Adriamycin was found to be the same with normal erythrocytes as with cells from subjects who had the Mediterranean variety of G6PD deficiency—i.e., much lower levels of G6PD activity (0.8–7.0 µIU per g of Hb) than those detected in normal subjects (4.8–5.9 µIU per g of Hb). This allowed the metabolism of entrapped Adriamycin to be compared in normal and in G6PD-deficient erythrocytes. As shown in Table 2, encapsulated Adriamycin was found to produce some mild oxidative challenge within the erythrocytes, in agreement with effects previously observed upon incubation of erythrocytes with Adriamycin (25–28): thus, the normal cells showed some stimulation of their HMS activity (although lower than that produced by 100 µM methylene blue; see ref. 21). The G6PD-deficient erythrocytes responded with a significant decrease of intracellular GSH. Incubation for 24 hr caused only a slight decrease in intracellular Adriamycin concentration, with no apparent

**Fig. 1.** Efflux of loaded erythrocytes and appearance in the supernatant of unmodified Adriamycin. After entrapment of Adriamycin, erythrocytes were incubated at 37°C in autologous plasma at a 50% hematocrit. At the time intervals corresponding to each sampling, the supernatant was removed and replaced by an equal volume of native plasma. Levels of Adriamycin in the supernatant (▲) were estimated in the pooled plasma fractions removed at each sampling; ●, erythrocyte Adriamycin; ■, total Adriamycin.
difference between normal and G6PD-deficient cells (Table 2). The only clearly detectable metabolite under these conditions was adriamycinol, the levels of which were consistently lower in the G6PD-deficient than in the normal erythrocytes.

**Mechanisms Mediating Intracellular Metabolism of Erythrocyte-Entrapped Adriamycin.** The lower rate of formation of adriamycinol in the G6PD-deficient than in the normal erythrocytes (Table 2), coupled with the effects on HMS activities and on GSH levels, implicates aldehyde reductase as the only enzyme involved in the erythrocyte metabolism of encapsulated Adriamycin (29). Anthracycline biotransformation can also occur in animal cells via one- or two-electron reduction of the molecule (30–32). This reaction results in quinone methide formation and subsequent loss of the amino sugar moiety (33). It is favored in *vitro* by anaerobic conditions since oxygen can either compete with Adriamycin for the reducing enzyme or reoxidize the semiquinone intermediate. No appreciable formation of Adriamycin aglycones was detected in Adriamycin-loaded erythrocytes in a variety of experimental conditions including incubation under N₂, under CO, and in the presence of hypoxanthine.

Several approaches were made to determine why the entrapped Adriamycin was poorly biotransformed. First, experiments with hemolysates (either containing or lacking membranes) confirmed the failure to observe significant aglycone formation even under anaerobic conditions of incubation. If hypoxanthine and xanthine oxidase (which catalyzes the reduction of Adriamycin) were added to the hemolysate, the aglycone was produced in good yields, in either air or N₂. However, attempts at coentrapping xanthine oxidase with Adriamycin in erythrocytes were unsuccessful, because of the low potential of encapsulation of this protein. Encapsulation in human erythrocytes of another anthracycline reducing system (33–35)—i.e., ferredoxin:NADP⁺ reductase purified from spinach leaves (12), was obtained under conditions that avoided oxidative inactivation of both ferredoxin and the ferredoxin reductase—i.e., under N₂. This enzyme system has been recently demonstrated to catalyze reduction of daunomycin (an analog of Adriamycin), followed by loss of the daunosamine moiety from the molecule and by extensive formation of aglycones (33). By analogy, Adriamycin would be expected to yield the 7-deoxyadriamycin aglycone. In our experimental system, under anaerobic conditions, we observed almost quantitative conversion of entrapped Adriamycin to the aglycone (Table 3). A strict requirement for this process to take place was availability of glucose to erythrocytes as a source of NADPH for ferredoxin reduction, which in turn enables reduction of Adriamycin.

**Properties of the Adriamycin-Loaded Erythrocytes.** Osmotic fragility curves of human erythrocytes following encapsulation of Adriamycin, although being consistent for progressive changes occurring during incubation, were not significantly altered as compared with native and with unloaded erythrocytes (Fig. 2). A number of metabolic properties of the three matched erythrocyte populations—i.e., native, unloaded, and Adriamycin-loaded—incubated for 24 hr at 37°C, are reported in Table 4. The only significant difference in the Adriamycin-containing erythrocytes was an apprecia-

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**Table 2.** Metabolism of encapsulated Adriamycin in normal and G6PD-deficient erythrocytes

<table>
<thead>
<tr>
<th>Erythrocyte</th>
<th>GSH, μmol per g of Hb</th>
<th>HMS activity, nmol per ml of erythrocytes per hr</th>
<th>Adriamycin, mg per ml of erythrocytes</th>
<th>Adriamycinol, mg per ml of erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5.5 ± 0.5</td>
<td>185 ± 22</td>
<td>0.74 ± 0.13</td>
<td>0.049 ± 0.011</td>
</tr>
<tr>
<td>G6PD-deficient</td>
<td>4.6 ± 0.6</td>
<td>2.8 ± 0.7</td>
<td>0.71 ± 0.15</td>
<td>0.006 ± 0.003</td>
</tr>
</tbody>
</table>

Erythrocytes were incubated for 24 hr at a 50% hematocrit in autologous plasma containing 5 mM glucose at 37°C with gentle stirring. No change of plasma was made. Results are the average of three experiments with erythrocytes from different subjects.

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**Table 3.** Metabolism of encapsulated Adriamycin in unloaded and in ferredoxin and ferredoxin:NADP⁺ reductase-loaded erythrocytes

<table>
<thead>
<tr>
<th>Erythrocyte</th>
<th>Adriamycin</th>
<th>7-Hydroxyadriamycin aglycone</th>
<th>7-Deoxyadriamycin aglycone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unloaded</td>
<td>1.488</td>
<td>0.006</td>
<td>0.004</td>
</tr>
<tr>
<td>Loaded</td>
<td>0.073</td>
<td>0.062</td>
<td>1.520</td>
</tr>
</tbody>
</table>

Encapsulations were carried out as described, but under N₂, to avoid inactivation of ferredoxin and of ferredoxin reductase. Measurements of Adriamycin and of the corresponding aglycones were made after incubation of the erythrocytes for 24 hr at a final 50% hematocrit in phosphate-buffered saline containing 5 mM glucose at 37°C under N₂. No change of buffer was made. Values are expressed as mg per ml of erythrocytes.
superoxide. As postulated by Bates and Winterbourn (41), superoxide dismutase would drain this two-step process, thereby accelerating methemoglobin formation yet removing the Adriamycin semiquinone efficiently and thus preventing conversion to the aglycone (33). Aglycone formation can occur in Adriamycin-loaded erythrocytes, however, if the reduction rate is greatly enhanced by co-entrapping ferredoxin reductase and the cells are depleted of oxygen. Accordingly, the enhanced concentration of methemoglobin implies redox cycling of Adriamycin, although with <30% oxidation during a 24-hr incubation, the reaction is relatively slow. It is unlikely to represent a problem in vivo since Adriamycin efflux from the loaded cells would be considerably faster than during in vitro incubation and methemoglobin formation would be correspondingly less. The only other metabolic change observed was conversion to adriamycinol, catalyzed by aldehyde reductase and driven by the hexose monophosphate shunt. Again, this was slow and unlikely to be significant in vivo.

The sum of the findings in the present experiments support the concept of using Adriamycin-loaded erythrocytes as suitable vehicles for slow release of the drug to peripheral targets. The most important findings from this standpoint are (i) the high encapsulation potential, (ii) the limited extent of biotransformation of the entrapped Adriamycin because of lack of specific enzyme systems and of a favorable balance of redox reactions preventing extensive semiquinone formation, (iii) probably as a consequence of ii, the observed absence of cytotoxic oxidative effects within the loaded erythrocytes, (iv) the slow release of unaltered Adriamycin from loaded erythrocytes. Assessing the in vivo life-span of the Adriamycin-loaded erythrocytes in an animal model, and especially in the mouse, whose erythrocytes are similar to human cells as concerns susceptibility to encapsulation (6), is an important prerequisite before clinical trials. Furthermore, the pharmacokinetics of Adriamycin in this new drug delivery system deserve elucidation, since slow release of the drug in circulation might improve its antitumor activity while reducing adverse effects and especially cardiotoxicity (4).

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