BINDING OF DAUNORUBICIN AND DOXORUBICIN TO ERYTHROCYTES TREATED WITH GLUTARALDEHYDE

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1. ABSTRACT

Intact human erythrocytes can bind anthracycline antibiotics (daunorubicin and doxorubicin) during incubation in the isotonic medium containing the antibiotic. Bound antibiotics can be immobilized in the erythrocytes using glutaraldehyde treatment. It was found, however, that erythrocytes pretreated with glutaraldehyde can uptake daunorubicin and doxorubicin from the incubation medium and do it faster than untreated cells. The antibiotics are readily released from these erythrocytes in the antibiotic-free medium. Our findings provide evidence that immobilization of anthracycline antibiotics in the erythrocytes using glutaraldehyde treatment is connected with the chemical binding of the antibiotics to different erythrocyte components.

2. INTRODUCTION

Being very effective as antineoplastic drugs anthracycline antibiotics are highly toxic[7,12,14,15]. One of the ways to diminish toxic action of anthracycline antibiotics and to improve their therapeutic indexes is to use erythrocytes as drug carrier[1,9–11,18,19]. However, anthracycline antibiotics loaded into erythrocytes rapidly leak from the cell to the surrounding medium[3,8,10,16,18]. Anthracycline antibiotics can be immobilized in erythrocytes using glutaraldehyde treatment[3,9,16,18]. This treatment provides selective trapping of the treated erythrocytes by spleen, liver and lungs and, hence, results in targeting of entrapped drugs to these organs[18]. Doxorubicin-loaded and glutaraldehyde-treated erythrocytes have been shown to be more effective than the doxorubicin solution in prevention of experimental liver metastases in mice[19]. Daunorubicin-loaded and glutaraldehyde-treated erythrocytes have been shown to retain the antineoplastic activity after freezing and thawing[1]. This opens the possibility for storage of the beforehand prepared erythrocytes loaded with anthracycline antibiotics. Erythrocytes loaded with anthracycline antibiotics and then

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treated with glutaraldehyde seem to be extremely promising for therapeutic usage. First results of the clinical trial of doxorubicin-loaded and glutaraldehyde-treated erythrocytes in a patient with massive hepatic metastases and in dogs with lymphosarcoma were reported[13,17].

However, the mechanism responsible for anthracycline antibiotics immobilization in the glutaraldehyde-treated erythrocytes is not well understood. It is not clear if glutaraldehyde decreases the permeability of erythrocyte membrane to anthracycline antibiotics or it chemically binds anthracycline antibiotics to different erythrocyte components. The latter seems more plausible because glutaraldehyde is able to form chemical bonds between substances containing amino groups[6]. Molecules of anthracycline antibiotics contain one amino group; therefore, they can form chemical bonds via glutaraldehyde to different amino-group-containing erythrocyte components. To understand *in vivo* functioning of drug carrier erythrocytes in full detail, it is necessary to know the exact mechanism of glutaraldehyde-mediated immobilization of anthracycline antibiotics in these cells.

In this work, we studied the ability of glutaraldehyde-treated human erythrocytes (GTE) to bind daunorubicin and doxorubicin. It was shown earlier that native erythrocytes are able to bind these antibiotics in a reversible manner[2–5,16,17]. We expected that, if glutaraldehyde treatment decreases the permeability of erythrocyte membrane to anthracycline antibiotics, it would decrease or abolish binding of these antibiotics to erythrocytes.

3. MATERIALS AND METHODS

Pharmaceutic preparation of daunorubicin (rubomycin hydrochloride) was from FAO Ferein (Mosmedpreparaty, Moscow, Russia). Doxorubicin was used as adriablastin, commercially available from Farmitalia Carlo Erba (Montedison Group, Italy). Glutaraldehyde (70% aqueous solution) was from Serva, (Germany).

Erythrocytes were isolated from preserved donor blood by centrifugation, then washed two times by resuspension in a double volume of glucose containing phosphate buffered saline (GPBS) and following centrifugation at 1500g for 10 min. Washed erythrocytes were split into two portions. One portion of erythrocytes was used as control and the other was treated with glutaraldehyde. Control and glutaraldehide-treated packed erythrocytes were brought to the desirable temperature and resuspended to hematocrit about 50% in the solution of daunorubicin (or doxorubicin) in GPBS maintained at the same temperature. The pH values of daunorubicin and doxorubicin solutions were 7.4 and 7.0, respectively. The prepared suspensions of control erythrocytes or GTE (hematocrit 50%) were incubated at constant temperature and periodic stirring. Concentrations of anthracycline antibiotics in the incubation medium were measured during the incubation to evaluate binding of antibiotics to erythrocytes. The release of anthracycline antibiotics was studied using GTE which were loaded with the antibiotic by a 30-min incubation in the antibiotic-containing medium at 24°C. Then suspension was centrifugated at 1500g for 3 min, supernatant was discarded and packed GTE were resuspended in an equal volume of GPBS to hematocrit 50%. In order to evaluate the antibiotic release from the GTE, its concentration in the incubation medium was measured 5 min after the resuspension.

Glutaraldehyde treatment procedure was based on the earlier described method[1,3,16]. Erythrocytes were mixed with an equal volume of 0.35% glutaraldehyde solution in GPBS and the mixture was incubated at room temperature for 15 min. Erythrocytes then were washed four times by resuspension in 10 volumes of GPBS followed by centrifugation at

1500g for 3 min. GPBS containing 10 mM of glycine was used for the first washing in order to eliminate unreacted glutaraldehyde.

To determine concentrations of daunorubicin or doxorubicin chloroform extracts were prepared from supernatants obtained by centrifugation of samples of the erythrocyte suspension as it was described earlier[2–4]. Concentrations of antibiotics in the chloroform extracts were determined spectrophotometrically. To this end, optical density of chloroform extracts were measured in 1 cm quartz cells using pure chloroform as a blank. Measurements were made at the wavelength of the corresponding maximum of antibiotic visible spectrum (485 nm for doxorubicin and 500 nm for daunorubicin). For calibration of the measurements standard solutions of daunorubicin or doxorubicin in chloroform were used.

4. RESULTS

GTE retain their ability to bind anthracycline antibiotics. Moreover, compared with untreated erythrocytes, GTE bind anthracycline antibiotics faster. Figure 1 shows the uptake of daunorubicin from the incubation medium by GTE versus untreated erythrocytes. These results were obtained on erythrocytes of five different donors. Under our experimental conditions, untreated erythrocytes bind about 70% of daunorubicin from the medium within 30 min, in agreement with the data reported earlier[2,3,5], whereas GTE uptake about 85% of daunorubicin within only 5 min. In contrast to untreated cells[2,5], GTE seem

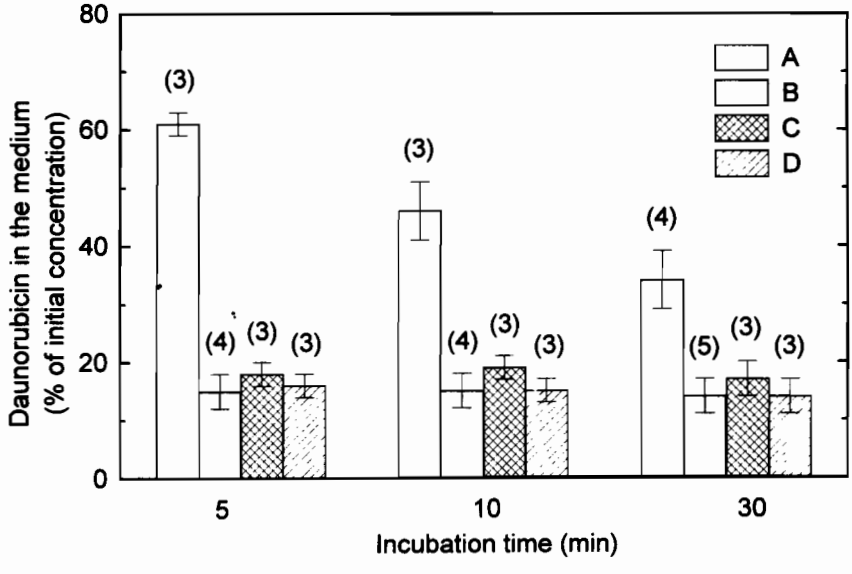

Figure 1. Binding of daunorubicin to glutaraldehyde-treated erythrocytes (GTE). (A) control (untreated) erythrocytes, 24°C, initial daunorubicin concentration in the medium, 0.81±0.02 mg/ml; (B) GTE, 24°C, initial daunorubicin concentration in the medium, 1.00±0.02 mg/ml; (C) GTE, 24°C, initial daunorubicin concentration in the medium, 3.00±0.04 mg/ml; (D) GTE, 4°C, initial daunorubicin concentration in the medium, 1.00±0.02 mg/ml. Number of experiments, each performed on erythrocytes of one individual donor, are indicated in parentheses. In total, erythrocytes of five donors were used.

to bind daunorubicin independently of the initial antibiotic concentration and low temperature.

Doxorubicin showed a similar pattern of binding to GTE. Figure 2 demonstrates the results of a representative experiment arbitrarily chosen of two. Note that GTE bind doxorubicin faster than untreated cells. However, the effect is less pronounced than that observed with daunorubicin.

Anthracycline antibiotics bound to GTE are partially released from the erythrocytes after replacement of the incubation medium by fresh, antibiotic free solution. This release is completed within about 5 min after the replacement, and since then antibiotic concentration in the medium remains almost unchanged for at least an hour. The results of two separate experiments that demonstrate the release of daunorubicin from erythrocytes, treated with glutaraldehyde and then loaded with daunorubicin, are presented in Table 1. After each replacement of the incubation medium, significant amounts of daunorubicin appear in the medium due to its release from the GTE.

5. DISCUSSION

Our results suggest that treatment of erythrocytes with glutaraldehyde as such does not abolish or decrease their ability to bind anthracycline antibiotics. Consequently, one may conclude that glutaraldehyde treatment does not decrease the permeability of erythrocyte membrane to these antibiotics. Compared with untreated erythrocytes, GTE uptake anthracycline antibiotics more rapidly. Anthracycline antibiotics are amphifillic; therefore, it is plausible to suggest that glutaraldehyde treatment of erythrocytes diminishes the

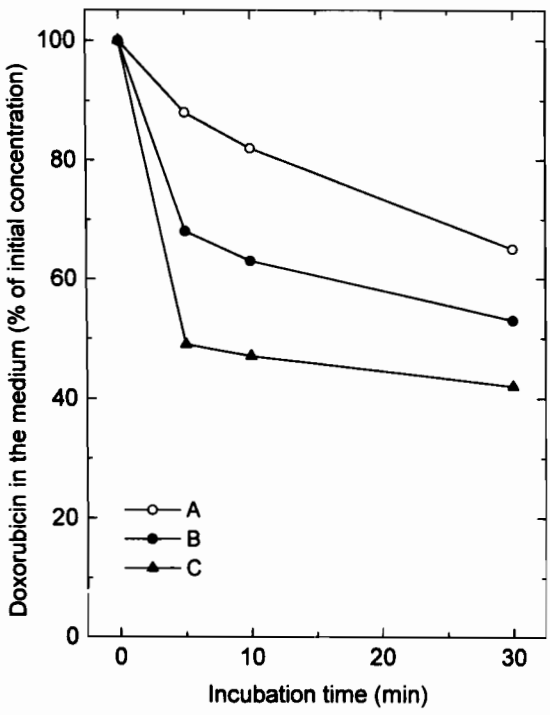

Figure 2. Binding of doxorubicin to glutaraldehyde-treated erythrocytes (GTE) at 18°C. (A) control (untreated) erythrocytes, initial doxorubicin concentration in the medium, 0.17 mg/ml; (B) GTE, initial doxorubicin concentration in the medium, 0.19 mg/ml; (C) GTE, initial doxorubicin concentration in the medium, 0.57 mg/ml.

Table 1. Release of daunorubicin from glutaraldehyde-treated human erythrocytes (GTE) in a series of successive replacement of the incubation medium. GTE were previously loaded with daunorubicin by incubation at 24°C for 30 min in the daunorubicin containing medium (hematocrit, 50%). The initial concentration of daunorubicin in the medium was 1 mg/ml

	Medium daunorubicin (mg/ml)	
Replacement of the medium	Exp. 1	Exp. 2
1-st	0.087	0.11
2-nd	0.068	0.074
3-rd	0.060	0.046

amount of the polar groups on the cell surface and facilitates the interaction between anthracycline antibiotics and hydrophobic lipid compartment within the cell membrane.

Binding of anthracycline antibiotics by erythrocytes pretreated with glutaraldehyde is reversible. The finding that erythrocytes treated with glutaraldehyde and then loaded with daunorubicin rapidly release daunorubicin after replacement of the incubation medium (Table 1) supports this suggestion. In a series of successive replacements of the incubation medium, each replacement resulted in the substantial appearance of daunorubicin in the medium. This finding provides evidence that we observed the release of daunorubicin rather than the contamination of the packed cells by the remaining incubation medium. By contrast, there is no similar leakage of the antibiotics from those erythrocytes that were first loaded with anthracycline antibiotics and then treated with glutaraldehyde[3,16,18]. It is conceivable that glutaraldehyde treatment of erythrocytes loaded with anthracycline antibiotics results in the irreversible chemical binding of the antibiotics to different erythrocyte components.

Specific activities, toxicities, and therapeutic indexes of various anthracycline antibiotics are very different in spite of the apparent slight differences in their chemical structures. Therefore, it is plausible that modification of the structure of anthracycline antibiotics caused by their immobilization within erythrocytes via glutaraldehyde could also modify properties of the original drugs. Moreover, such a modification may significantly depend on details of the glutaraldehyde treatment procedure. Modification of the drug during the glutaraldehyde treatment may explain the occurrence of some unexpected side effects caused by administration of doxorubicin-loaded and glutaraldehyde-treated erythrocytes[13,17]. It is our opinion, however, that the results obtained do not exclude the possibility for the use of erythrocytes as carriers of anthracycline antibiotics. First of all, the preparation of anthracycline antibiotic carrier erythrocytes do not necessarily imply the treatment with glutaraldehyde. Daunorubicin loaded erythrocytes prepared without glutaraldehyde treatment were shown to increase the specific activity[10,11] and decrease toxicity[1] of daunorubicin. Next, strict standardization of the glutaraldrhyde treatment procedure can unify glutaraldehyde treated carrier erythrocytes and, hence, decrease the probability of unexpected side effects of their usage.

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