Reversible binding of anthracycline antibiotics to erythrocytes treated with glutaraldehyde

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Human erythrocytes treated with glutaraldehyde can take up anthracycline antibiotics (daunorubicin and doxorubicin) from the incubation medium and do so faster than untreated cells. The antibiotics are readily released when the suspending medium is replaced with an antibiotic-free one. Our findings provide evidence that glutaraldehyde-treated erythrocytes remain capable of reversible binding of anthracycline antibiotics.

Introduction

Being very effective as antineoplastic drugs, anthracycline antibiotics are highly toxic. Sometimes their administration is associated with severe side effects [1–5]. One of the ways to diminish the toxic action of anthracycline antibiotics and to improve their therapeutic indexes is to use erythrocytes or erythrocyte ghosts as drug carriers [6–11]. However, anthracycline antibiotics loaded into erythrocytes rapidly leak from the cell into the surrounding medium [7,8,12–14]. Anthracycline antibiotics can be immobilized inside erythrocytes or erythrocyte ghosts by using glutaraldehyde treatment [8,9,13,14]. 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 [8]. 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 [10]. Daunorubicin-loaded and glutaraldehyde-treated erythrocytes have been shown to retain antineoplastic activity after freezing and thawing [11]. This opens up the possibility of storing pre-prepared erythrocytes loaded with anthracycline antibiotics. Erythrocytes loaded with anthracycline antibiotics and then treated with glutaraldehyde seem to be extremely promising for therapeutic usage. First results of clinical trials of doxorubicin-loaded and glutaraldehyde-treated erythrocytes in a patient with massive hepatic metastases and in dogs with lymphosarcoma were reported [15,16].

However, the mechanism responsible for anthracycline-antibiotic immobilization in the glutaraldehyde-treated erythrocytes is not well understood. It is not clear whether glutaraldehyde decreases the permeability of the erythrocyte membrane to anthracycline antibiotics or if 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 [17]. Molecules of anthracycline antibiotics contain one amino group; therefore they can form chemical bonds via glutaraldehyde to different amino-group-containing erythrocyte components such as proteins (including haemoglobin), cell membrane phospholipids, etc. To understand the *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 the present work we studied the ability of glutaraldehyde-treated human erythrocytes to bind daunorubicin and doxorubicin. It was shown earlier that native erythrocytes are able to bind these antibiotics in a reversible manner [13–15,18–20]. We expected that, if glutaraldehyde treatment decreases the permeability of the erythrocyte membrane to anthracycline antibiotics, it would decrease or abolish binding of these antibiotics to erythrocytes.

Materials and methods

Reagents

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

Experimental procedures

Erythrocytes were isolated from preserved donor blood by centrifugation, then washed twice by resuspension in 2 vol. of glucose-containing phosphate-buffered saline (GPBS[2])

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[2] Abbreviation used: GPBS, glucose-containing phosphate-buffered saline.

followed by centrifugation at 1500 **g** for 10 min. Washed erythrocytes were divided into two portions. One portion of erythrocytes was used as control and the other was treated with glutaraldehyde. Solutions of daunorubicin (1 or 3 mg/ml, pH 7.4) and doxorubicin (0.2 or 0.6 mg/ml, pH 7.0) in GPBS were prepared. Both control packed erythrocytes (haematocrit 0.80–0.85) and glutaraldehyde-treated packed erythrocytes (haematocrit 0.92–1.00) were brought to the desired temperature. The exact values of haematocrit of packed erythrocytes were determined and adjusted to 0.5 by addition of daunorubicin (or doxorubicin) solution maintained at the same temperature. The resulting concentration of the antibiotic (C) in the liquid portion of the erythrocyte suspension immediately after mixing with the antibiotic solution was calculated according to the following equation:

$$C = C_0V/[V + V_0(1 - H)]$$

where C_0 is the concentration of antibiotic in the solution, V_0 is the volume of packed erythrocytes, V is the volume of the antibiotic solution which was added to erythrocytes to give a suspension with haematocrit 0.5, and H is the initial value of haematocrit of the packed erythrocytes. The prepared suspensions of control or glutaraldehyde-treated erythrocytes (haematocrit 0.5) were incubated at constant temperature and with periodic stirring. Concentrations of anthracycline antibiotics in the incubation medium were measured during the incubation to evaluate binding of antibiotics to the erythrocytes. The release of anthracycline antibiotics was studied using glutaraldehyde-treated erythrocytes which were loaded with the antibiotic by a 30 min incubation in the antibiotic-containing medium at 24 °C. Erythrocytes were centrifugated at 1500 **g** for 3 min, the supernatant was discarded and packed erythrocytes were resuspended in an equal volume of GPBS to a haematocrit of 0.5. In order to evaluate the antibiotic release from the erythrocytes, its concentration in the incubation medium was measured 5 min after the resuspension.

Glutaraldehyde treatment

The glutaraldehyde treatment procedure was based on previously described methods [11,13,14]. 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 vol. of GPBS, followed by centrifugation at 1500 **g** for 3 min. GPBS containing 10 mM glycine was used for the first washing in order to remove glutaraldehyde that had not reacted.

Determination of anthracycline antibiotics

Chloroform extracts of daunorubicin or doxorubicin were prepared from supernatants obtained by centrifugation of samples of the erythrocyte suspension. To this end, 3 ml of chloroform and 0.1 ml of 1.6 M potassium bicarbonate solution were added to 1 ml of supernatant. The mixture was vigorously shaken and centrifuged at 1000 **g** for 3 min. After centrifugation, a 2 ml sample from the chloroform layer (the lower layer in the centrifuge tube) was carefully drawn with a glass syringe. Concentrations of antibiotics in the chloroform extracts were determined spectrophotometrically. To this end, the absorbance of chloroform extracts was measured in 1 cm-pathlength quartz cells using pure chloroform as a blank. Measurements were made at the wavelength of the corresponding maximum of the antibiotic visible spectrum (485 nm for doxorubicin and 500 nm for daunorubicin). The absorption coefficients of antibiotics in chloroform were determined experimentally and appeared to be 16.7 ml/mg for doxorubicin and 19.4 ml/mg for daunorubicin. Antibiotic concentrations in the incubation medium were calculated using following equation:

$$A = 3D/E$$

where A is the concentration of antibiotic in the incubation medium (mg/ml), D is the absorbance of the chloroform extract, 3 is a correction factor describing the dilution of antibiotic by chloroform extraction and E is the absorption coefficient (absorbance of a 1 mg/ml antibiotic solution).

Results

Erythrocytes treated with glutaraldehyde retain their ability to bind anthracycline antibiotics. Moreover, compared with untreated erythrocytes, glutaraldehyde-treated erythrocytes bind anthracycline antibiotics faster. Table 1 shows the uptake of daunorubicin from the incubation medium by glutaraldehyde-treated versus untreated erythrocytes. These results were obtained on erythrocytes from 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 previously [14,18,19], whereas glutaraldehyde-treated erythrocytes take up about 85% of daunorubicin within only 5 min. In contrast with untreated cells [18,19], glutaraldehyde-treated erythrocytes seem to bind daunorubicin independently of the initial antibiotic concentration and at low temperature.

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

Table 1 Binding of daunorubicin to glutaraldehyde-treated human erythrocytes

'I' is the daunorubicin concentration in the medium (mg/ml); 'II' is the bound daunorubicin (% of total).

Incubation time (min)	Control erythrocytes		Glutaraldehyde-treated erythrocytes					
	24 °C		24 °C		24 °C		4 °C	
	I	II	I	II	I	II	I	II
0	0.81 ± 0.02[a] (4)[b]	0	1.00 ± 0.02 (5)	0	3.00 ± 0.04 (3)	0	1.00 ± 0.02 (3)	0
5	0.50 ± 0.02 (3)	39 ± 2	0.15 ± 0.03 (4)	85 ± 3	0.53 ± 0.05 (3)	82 ± 2	0.16 ± 0.0 (3)	84 ± 2
10	0.38 ± 0.06 (3)	54 ± 5	0.15 ± 0.03 (4)	85 ± 3	0.54 ± 0.05 (3)	81 ± 2	0.14 ± 0.02 (3)	85 ± 2
30	0.27 ± 0.04 (4)	66 ± 5	0.14 ± 0.02 (5)	86 ± 3	0.48 ± 0.08 (3)	83 ± 3	0.14 ± 0.02 (3)	86 ± 3

[a] Means ± S.D.
[b] Number of experiments, each performed on erythrocytes from one individual donor (in total, erythrocytes from five donors were used).

Anthracycline antibiotics bound to glutaraldehyde-treated erythrocytes 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 after that the antibiotic concentration in the medium remains almost unchanged for at least 1 h. 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 3. After each replacement of the incubation medium, significant amounts of daunorubicin appeared in the medium, owing to its release from the erythrocytes.

Table 2 Binding of doxorubicin to glutaraldehyde-treated human erythrocytes at 18°C

'I' and 'II' have the same meaning as in Table 1.

Incubation time (min)	Control erythrocytes		Glutaraldehyde-treated erythrocytes			
	I	II	I	II	I	II
0	0.17	0	0.19	0	0.57	0
5	0.15	12	0.13	32	0.28	51
10	0.14	18	0.12	37	0.27	53
30	0.11	35	0.10	47	0.24	58

Table 3 Release of daunorubicin from glutaraldehyde-treated human erythrocytes in a series of successive replacements of the incubation medium

Glutaraldehyde-treated erythrocytes were previously loaded with daunorubicin by incubation at 24 °C for 30 min in the daunorubicin-containing medium (haematocrit 0.5). The initial concentration of daunorubicin in the medium was 1 mg/ml.

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

Discussion

Our results suggest that treatment of erythrocytes with glutaraldehyde does not, in itself, make them 'impermeable' to anthracycline antibiotics. Compared with untreated, glutaraldehyde-treated erythrocytes take up anthracycline antibiotics more rapidly. Anthracycline antibiotics are amphiphilic; therefore, it is plausible to suggest that glutaraldehyde treatment of erythrocytes diminishes the amount of polar groups on the cell surface and facilitates the interaction between anthracycline antibiotics and the 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 3) supports this notion. 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 leakage of the antibiotics from those erythrocytes that were first loaded with anthracycline antibiotics and then treated with glutaraldehyde [8,13,14]. 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. It is therefore 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 depend significantly on the details of the glutaraldehyde treatment procedure, properties of glutaraldehyde *per se* (e.g. the degree of its polymerization) etc. 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 [15,16]. It is our opinion, however, that the results obtained do not exclude the possibility of using erythrocytes as carriers of anthracycline antibiotics. First of all, the preparation of anthracycline antibiotic carrier erythrocytes does not necessarily require treatment with glutaraldehyde. Daunorubicin-loaded erythrocytes prepared without glutaraldehyde treatment were shown to increase the specific activity [6,7] and decrease the toxicity [11] of daunorubicin. Next, strict standardization of the glutaraldehyde treatment procedure can render glutaraldehyde-treated carrier erythrocytes more uniform and, hence, decrease the probability of unexpected side-effects of their usage.

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