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The effect of daunorubicin and glutaraldehyde treatment on the structure of erythrocyte membrane

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Abstract

The effect of daunorubicin (DNR) and glutaraldehyde on erythrocyte membrane structure was examined by Electron Spin Resonance spectroscopy. Human erythrocytes were incubated with daunorubicin and then with glutaraldehyde to prevent drug efflux. We have demonstrated that DNR alone caused changes in membrane fluidity mainly in the hydrophobic regions of the lipid bilayer. When DNR-preincubated erythrocytes were treated with glutaraldehyde, the alterations in fluidity were observed in the polar regions as well as in the deeper regions of the cell membrane. The incorporation of drug and glutaraldehyde into human erythrocytes also caused conformational alterations in membrane cytoskeletal proteins and changes in the internal viscosity of the cells. The results suggest that glutaraldehyde in the drug-pretreated erythrocytes may lead to significant perturbations in the organization of the plasma membrane lipids and proteins.

Keywords: Daunorubicin; Glutaraldehyde; Erythrocytes; Plasma membrane

1. Introduction

The anthracycline antibiotics, which include daunorubicin, possess significant therapeutic activity against several human cancers. However, their use is restricted by severe side effects, mainly suppression of bone marrow function and a cumulative dose-dependent cardiotoxicity (Arcamone, 1988).

These undesirable effects have stimulated the search for new strategies to obtain selective destruction of neoplastic cells. One of the ways to diminish the toxic side effects of drugs and to improve their therapeutic indices is to use various drug delivery systems such as nanospheres, nanocapsules, liposomes or monoclonal antibodies (Gabizon, 1994; Kreuter, 1994; Kulkarni et al., 1995). They may be used for drug targeting to specific tissues or for the slow release of antineoplastic agents.

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Recently, a number of investigators have been focusing their attention on the encapsulation of antineoplastic drugs within erythrocytes (Tonetti et al., 1992; Matherne et al., 1994) or nanoerythrosomes (Lejeune et al., 1994; Moorjani et al., 1996). Erythrocytes appear to be very convenient carriers for anthracycline drugs and other pharmacological agents. They are naturally occurring biodegradable and non-immunogenic. Moreover, carrier erythrocytes circulate well in vivo, can be targeted to specific organs (liver and spleen), and can carry large quantities of encapsulated drugs (Tonetti and de Flora, 1993). Because drugs such as daunorubicin diffuse rapidly from the cells, to prevent the drug efflux, erythrocytes are treated with cross-linking reagents, for example glutaraldehyde (Ataullakhanov et al., 1996).

Previous experiments demonstrated that subsequent treatment of drug-loaded erythrocytes with glutaraldehyde increased specific targeting of the cells to the liver (Tonetti et al., 1992) and decreased the metabolism of the drug inside the erythrocytes (Tonetti et al., 1990).

That carrier erythrocytes, treated with glutaraldehyde, might be useful in targeting antitumor agents is supported by promising results reported from preliminary clinical studies of these carriers, administered to patients with massive hepatic metastases and to dogs with lymphosarcoma (Tonetti et al., 1992; Matherne et al., 1994). These studies, however, did not answer the questions: (1) whether daunorubicin entrapped within glutaraldehyde-treated erythrocytes is able to produce perturbation in the membrane structure and (2) whether glutaraldehyde potentiates the toxic effect of the drug to erythrocytes.

Thus, in this paper, we decided to estimate the intracellular accumulation of daunorubicin in glutaraldehyde-treated human erythrocytes. Using Electron Spin Resonance spectroscopy (ESR) we monitored the combined effect of DNR and glutaraldehyde on the organization of human erythrocyte membranes.

2. Materials and methods

2.1. Chemicals

Daunorubicin (DNR) was purchased from Laboratory Roger Bellon. Spin labels, 5-doxylstearic acid (5-DSA), 16-doxylstearic acid (16-DSA), 4-maleimido-tempo and 4-amino-tempo (TEMPAMINE) were purchased from Sigma. All other chemicals were of the best quality commercially available.

2.2. Preparation of erythrocyte suspensions

Human peripheral blood from healthy donors in ACD solution, was centrifuged at $600 \times g$ for 10 min. After removal of the plasma and buffy coat the erythrocytes were washed three times in phosphate buffered saline, PBS, (5 mM sodium phosphate buffer, containing 0.15 M NaCl, pH 7.4) and suspended in the same medium.

2.3. Treatment of erythrocytes with drug

Three percent erythrocyte suspensions in PBS, pH 7.4, were incubated with daunorubicin at a final concentration of 10 μg/ml in the dark for 70 min in a shaking bath at 37°C. After incubation erythrocytes were treated with glutaraldehyde.

2.4. Glutaraldehyde treatment

Drug-treated washed erythrocytes, were mixed with glutaraldehyde in PBS (final concentrations 0.0025 to 0.035%) and incubated at room temperature, 20–22°C, for 20 min. Subsequently, the samples were taken for fluorescence determination or were labeled with spin labels.

2.5. Membrane preparation

Erythrocyte membranes were obtained by hypotonic lysis according to the procedure of Dodge et al. (1963) at 4°C and then were suspended in PBS, pH 7.4. Protein concentration was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.6. Fluorescence measurements

The level of DNR in the supernatant was determined by fluorescence analysis (emission at 595 nm, excitation at 450 nm). DNR concentration in erythrocytes was also evaluated by fluorescence analysis after extraction of the drug in the presence of 50% EtOH/0.3 N HCl.

2.7. Preparation of spin-labeled erythrocytes and erythrocyte membranes

Erythrocytes suspended in PBS to the hematocrit of 50% were labeled with 5- and 16-doxylstearic acids for 30 min at 37°C in the dark. The lipid spin labels were added to the erythrocyte samples in a ratio of 1:100.

Erythrocyte membranes were labeled with maleimide spin label for 12 h at 4°C also in the dark, in a ratio of 1 mg of label per 25 mg of membrane protein. The spin-labeled samples were washed several times with PBS, pH 7.4 to remove unbound spin labels.

Internal microviscosity was determined using the method of Morse (1986) by the introduction of TEMPAMINE solution (0.1 M) into erythrocyte suspension in PBS in the ratio 1:50 (v/v). The mixture was incubated for 30 min at room temperature. Before measurement, erythrocytes were washed with 5 mM sodium phosphate buffer (pH 7.4), containing 80 mM potassium ferricyanide ($K_3Fe(CN)_6$). Potassium ferricyanide was used as a broadening agent in order to eliminate the signal derived from excess amount of extracellular spin label.

2.8. ESR measurements

ESR spectra were measured at room temperature using a Brücker ESP 300E spectrometer with computerized data acquisition and analysis capabilities. Typical instrumental parameters during these measurements were as follows: modulation frequency 100 kHz, modulation amplitude 2.02 G, time constant 20.48 ms, scan time 400 s, field set 3240 G and scan range 100 G.

From the spectrum of 5-DSA an order parameter (S) was derived by measuring the outer and inner hyperfine splitting $2T_{II}$ and $2T_{\perp}$ (Gordon et al., 1989) using the formula:

$$S = (T_{II} - T_{\perp})(a_N)/(T_{zz} - T_{xx})(a'_N)$$

where $a_N = 1/3(T_{zz} + 2T_{xx})$ and $a'_N = 1/3(T_{II} + 2T_{\perp})$. T_{zz} (32.4G) and T_{xx} (6.1G) are the principal hyperfine splittings that correspond to the main axis of the spin probe (Sauerheber et al., 1980).

For 16-DSA spectra, rotational correlation times were calculated using the formula (Ondrias, 1989):

$$\tau_B = 6.5 \times 10^{-10} \times \Delta H_0[(h_0/h_{-1})^{1/2} - (h_0/h_{+1})^{1/2}],$$

$$\tau_C = 6.5 \times 10^{-10} \times \Delta H_0[(h_0/h_{-1})^{1/2} + (h_0/h_{+1})^{1/2} - 2],$$

$$\Delta\tau = \tau_C - \tau_B$$

From the ESR spectrum of maleimide spin label the ratio of W/S was determined. Maleimide spin label binds covalently to membrane proteins giving rise to ESR absorptions which represent, respectively, weakly (W) and strongly (S) immobilized state of the labeled sites in the membrane proteins. In this study, the W/S ratio has been used as monitor of changes in protein conformation and environment within the membrane (Berliner, 1983; Wyse and Butterfield, 1988).

To determine the internal microviscosity of erythrocytes the spin label TEMPAMINE was used. From the spectrum of this spin label the rotational correlation time τ_R was estimated using the following equation (Morse, 1986):

$$\tau_R = 6.5 \times 10^{-10} \times W_0[(h_0/h_{-1})^{1/2} - 1],$$

where W_0, h_0 and h_{-1} are mid-field line width, mid-field line height and high-field line height, respectively. The internal microviscosity of erythrocytes was calculated from the ratio:

$$\tau_{R(\text{erythrocytes})}/\tau_{R(H_2O)} = \eta_{\text{erythrocytes}}/\eta_{H_2O}$$

where τ_R is the rotational correlation time and η is internal microviscosity. The viscosity of water was taken to be equal to 1 centipoise (cP) (Morse, 1986).

3. Results

3.1. Fluorescence studies of the drug concentration

Fig. 1. shows the decrease of fluorescence intensity at 595 nm as a function of glutaraldehyde concentration when 10 μg/ml DNR was added to erythrocytes suspended in PBS buffer. Addition of glutaraldehyde to drug-treated erythrocytes caused the gradual reduction of the extracellular drug concentration with increasing concentration of glutaraldehyde. This treatment suggests that such fluorescence changes are associated with incorporation of daunorubicin into erythrocytes. Fluorescence determinations of the drug level inside the cells also indicated that the percentage of DNR retained in erythrocytes was significantly increased in glutaraldehyde-treated cells in comparison with the respective control group. The percentages of drug remaining in erythrocytes in the presence of 0.005 and 0.025% glutaraldehyde were 7.6 and 26.4% respectively (data not shown).

3.2. Effect of daunorubicin and glutaraldehyde on membrane fluidity

To characterize the alterations in membrane fluidity caused by DNR and glutaraldehyde treatment, erythrocytes were spin labeled with 5- and

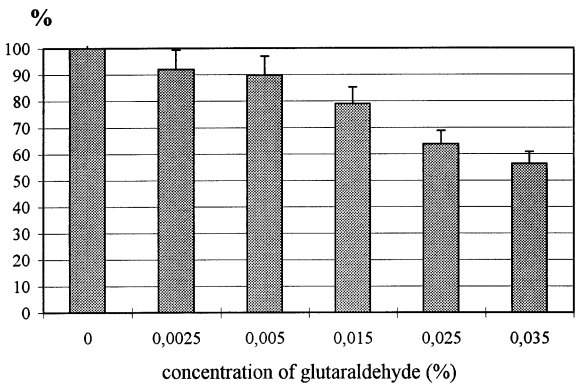

Fig. 1. The content of daunorubicin in the extracellular medium after incubation of drug-treated erythrocytes with glutaraldehyde. The content of DNR was determined by fluorescence analysis.

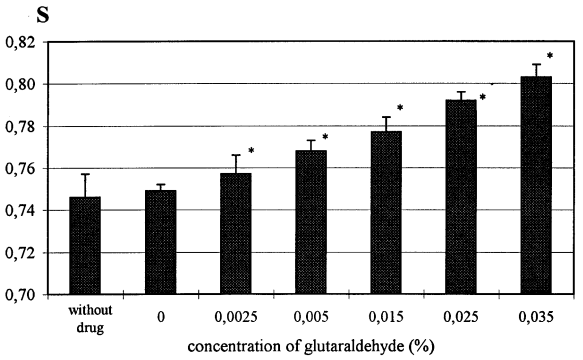

Fig. 2. Effect of daunorubicin and glutaraldehyde on the order parameter of 5-DSA in erythrocyte membranes. The values are means ± S.D. of six experiments. *Values statistically significant in comparison to the samples incubated with drug only, $P < 0.0001$.

16-doxylstearic acids and changes in membrane fluidity were determined by ESR spectrometry. As shown in Fig. 2 incubation of human erythrocytes with daunorubicin alone had no effect on the value of the order parameter S. However, when erythrocytes were preincubated with drug and then treated with glutaraldehyde, the value of S increased significantly for all tested concentrations of glutaraldehyde. This indicated that addition of glutaraldehyde to drug-treated cells decreased the membrane fluidity at the surface of the lipid bilayer of human erythrocytes in a concentration-dependent manner.

Using 16-DSA, the rotational correlation times τ_B and τ_C were estimated and then $\Delta\tau$ was calculated. This spin label reflects the changes in hydrophobic core of the membrane. The effects of DNR and glutaraldehyde on the values of correlation times are presented in Table 1. The correlation times τ_C and τ_B are higher both for erythrocytes incubated with DNR only and for cells incubated with drug and glutaraldehyde than for control cells. Data demonstrated that daunorubicin alone produced a significant alteration in $\Delta\tau$. Low concentrations of glutaraldehyde (0.0025–0.015%) induced only negligible changes in $\Delta\tau$ values of erythrocytes preincubated with the drug. With 0.025 and 0.035% glutaraldehyde, however, the value of $\Delta\tau$ enhanced markedly in comparison to the sample treated with drug only.

Table 1
Effect of daunorubicin and glutaraldehyde on the rotational correlation times for erythrocytes labelled 16-DSA[a]

Sample	$\tau_C \times 10^{-10}$ s	$\tau_B \times 10^{-10}$ s	$\Delta\tau \times 10^{-10}$ s
Control erythrocytes	18.86 ± 3.42	13.78 ± 3.95	5.08 ± 0.73
Erythrocytes plus daunorubicin	22.49 ± 0.81	15.85 ± 0.63	6.64 ± 0.46
Erythrocytes preincubated with drug (10 μg/ml) plus glutaraldehyde:			
0.0025%	23.32 ± 1.65	16.83 ± 1.68	6.49 ± 0.06
0.005%	23.64 ± 1.91	17.16 ± 1.82	6.47 ± 0.3
0.015%	23.97 ± 1.22	17.23 ± 1.4	6.74 ± 0.31
0.025%	26.13 ± 0.86*	18.56 ± 0.84*	7.57 ± 0.15*
0.035%	27.98 ± 1.96*	20.22 ± 1.96*	7.76 ± 0.64*

[a] Each point represents the average ± S.D. of six experiments.
* Values statistically significant in comparison to the samples incubated with drug only, $P < 0.0001$.

These results revealed that daunorubicin decreased the lipid fluidity in the hydrophobic regions of erythrocyte membranes. In this study, only the highest concentrations of glutaraldehyde increased the diminution of membrane fluidity in the drug-treated erythrocytes.

3.3. Changes in the W/S ratio

The effect of daunorubicin and glutaraldehyde on the erythrocyte membrane proteins was investigating employing the protein-specific spin label, maleimide. This spin label was used to monitor changes of the skeletal proteins and organization of membrane by calculation of the W/S ratio. As shown in Fig. 3, daunorubicin in the absence of cross-linking agent, glutaraldehyde, evidently decreased the W/S ratio. The addition of glutaraldehyde to drug-treated erythrocytes caused the further reduction of the W/S ratio. The above data imply that both DNR alone and the combined effect of drug and glutaraldehyde induced conformational changes in skeletal proteins which led to increased protein–protein interaction.

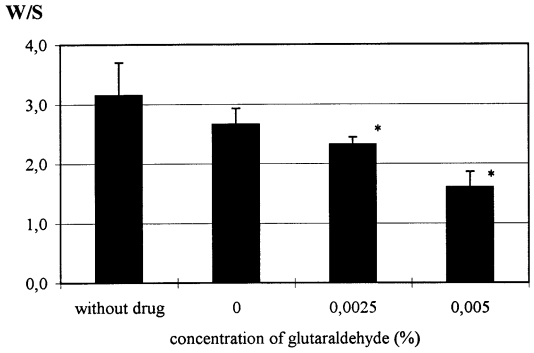

Fig. 3. Effect of daunorubicin and glutaraldehyde on the W/S ratio of maleimide in erythrocyte membranes. Means ± S.D. of six experiments. *Values statistically significant in comparison to the samples incubated with drug only, $P < 0.01$.

3.4. The effect of drug and glutaraldehyde on the internal viscosity

In this paper we also tried to determine the effect of DNR and glutaraldehyde on the internal microviscosity of erythrocytes. These results are summarized in Table 2. The rotational correlation

Table 2
Effect of daunorubicin and glutaraldehyde on the rotational correlation times of TEMPAMINE in erythrocytes[a]

Sample	$\tau_R \times 10^{-10}$ s	η[cP]
Control erythrocytes	27.28 ± 1.57	5.40 ± 0.31
Erythrocytes plus daunorubicin	28.03 ± 1.17	5.47 ± 0.23
Erythrocytes preincubated with drug (10 μg/ml) plus glutaraldehyde:		
0.0025%	32.84 ± 2.84*	6.46 ± 0.57*
0.005%	37.85 ± 6.69*	7.80 ± 0.91*
0.015%	39.29 ± 7.37*	7.66 ± 1.54*

[a] Values are means ± S.D. of six experiments.
* Values statistically significant in comparison to the samples incubated with drug only, $P < 0.0001$.

time (τ_R) of TEMPAMINE in erythrocytes exposed to 10 μg/ml daunorubicin was $28.03 \pm 1.17 \times 10^{-10}$ s. This corresponds to an internal microviscosity of 5.47 ± 0.23 cP. The values of τ_R of TEMPAMINE in erythrocytes exposed to drug and then treated with glutaraldehyde increased gradually with increasing concentration of aldehyde. In the presence of 0.005 and 0.015% glutaraldehyde, the τ_R were $37.85 \pm 6.69 \times 10^{-10}$ and $39.29 \pm 7.37 \times 10^{-10}$ s respectively. This gives values of 7.80 ± 0.91 and 7.66 ± 1.54 cP for these samples. The results demonstrate that DNR alone did not affect the internal microviscosity of human erythrocytes. However, the subsequent treatment of drug-treated erythrocytes with glutaraldehyde produced a significant enhancement of internal microviscosity.

4. Discussion

These results and our previous studies (Szwarocka et al., 1996) have demonstrated that daunorubicin at concentrations of 0.1–20 μg/ml did not induce changes in the fluidity of erythrocyte membranes at the surface of the lipid bilayer and only higher doses of drug (10–20 μg/ml) significantly decreased the membrane fluidity of hydrophobic regions of membranes. In this paper, the experiments performed with glutaraldehyde have indicated increased incorporation of the daunorubicin into human erythrocytes in the presence of this agent. It resulted in the decreased fluidity at the surface of erythrocyte membranes and higher rigidification of hydrophobic areas of the lipid bilayer. These data suggest that elevated level of intracellular daunorubicin in glutaraldehyde-treated erythrocytes also efficiently modified the lipid fluidity at the cell surface. Moreover, incubation of drug-pretreated erythrocytes with glutaraldehyde potentiated the extent of the conformational alterations in the skeletal membrane proteins as well as leading to an enhanced internal microviscosity of erythrocytes.

A number of observations on the interaction of anthracycline antibiotics with human erythrocytes have shown that these drugs can exert noticeable effects at the level of the plasma membrane. It has been demonstrated that daunorubicin entrapped in human erythrocytes caused marked perturbations of the loaded cells (Sprandel and Zöllner, 1985) and remarkably altered the erythrocyte morphology (Arancia et al., 1988; Tonetti et al., 1992).

Glutaraldehyde is known to cause non-specific cross-linking involving membrane phospholipids and proteins (Heusinkveld et al., 1977; Komorowska et al., 1982). This agent also cross-links hemoglobin molecules causing an increase in both the intracellular viscosity of hemoglobin solution and the internal viscosity of the cells (Noji et al., 1991). Other experiments with glutaraldehyde and anthracyclines have revealed that daunorubicin in the presence of glutaraldehyde, may be covalently conjugated to the nanoerythrosomes, nEryt (Lejeune et al., 1994; Moorjani et al., 1996). Using fluorescence microscopy they observed that the nEryt-G-DNR complex was rapidly adsorbed into the cell membrane. Furthermore, the complex was more active than free daunorubicin both in vitro and in vivo, by an unknown mechanism. According to Moorjani et al. (1996), the higher cytotoxicity of the nEryt-G-DNR complex is mediated by slow release of drug at the cell surface by hydrolysis of the glutaraldehyde linking arm and by a subsequent increase the concentration of free daunorubicin in the vicinity of the cells.

Further investigations are required to better define the cytotoxicity and therapeutic value of slow release anticancer drugs, incorporated or associated with drug carrier systems such as nanoerythrosomes or erythrocytes. Preliminary studies have shown that encapsulation of doxorubicin in glutaraldehyde-treated erythrocytes significantly reduces peak plasma concentration and prolongs higher plasma concentration of the drug after its administration to clinically normal dogs (Tonetti et al., 1992), reduces the drug side effects, predominantly cardiotoxicity, and causes remission of lymphosarcoma in dogs (Matherne et al., 1994). On the other hand, slow release of free drug may also extend the interaction of the drug with glutaraldehyde and alter the response of erythrocytes to the action of this drug. Our experiments in vitro imply that this interaction took place in human erythrocytes. Under studied con-

ditions, glutaraldehyde indirectly influenced the organization of erythrocyte membrane. This cross-linking agent caused the potentiation of drug damage to erythrocyte membrane lipids and proteins.

In our opinion, these results do not exclude the possibility of using erythrocytes as carriers of anthracycline antibiotics. Moreover, both these findings as well as data previously reported (Szwarocka et al., 1996) suggest that erythrocytes may play an active role in the distribution of low doses of daunorubicin and other anthracyclines. However, glutaraldehyde treatment, especially at higher doses, is not neutral to human erythrocytes in vitro and probably also to the cells used in clinical trials as carriers of anticancer drugs. Thus, the general use of glutaraldehyde to prepare glutaraldehyde-treated carrier erythrocytes awaits further studies.

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