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The combined effect of IDA and glutaraldehyde on the properties of human erythrocytes

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Abstract

The effect of IDA and glutaraldehyde on the properties of human erythrocytes was examined by Electron Spin Resonance spectroscopy and fluorescence measurements. In this study glutaraldehyde was used as the agent linking the drug to the erythrocyte membrane. We have demonstrated that idarubicin (IDA) alone caused only negligible changes of the membrane fluidity. When IDA preincubated erythrocytes were treated with glutaraldehyde, the alterations in the fluidity were observed in the polar parts as well as in the deeper regions of the cell membrane. The incorporation of drug and glutaraldehyde into human erythrocytes also caused conformational alterations of membrane cytoskeletal proteins and changes in the internal viscosity of the cells. Our data suggest that glutaraldehyde in idarubicin-pretreated erythrocytes may potentiate the drug toxicity leading to significant perturbations in the organization of the plasma membrane lipids and proteins.

Keywords: Idarubicin; Glutaraldehyde; Erythrocytes; ESR; Fluorescence

1. Introduction

Idarubicin (IDA, 4-demethoxydaunorubicin) is a widely used cytostatic drug, highly effective in the treatment of various types of malignancies, mainly leukemias. Both IDA and its parent compound daunorubicin (DNR) belong to the class of anthracycline antibiotics. These are aminoglycosides bearing a tetracyclic quinone chromophore. Idarubicin is obtained by glycosidation of 4-demethoxydaunomycinone, a nonnatural aglycone, since all anthracycline-producing microorganisms are generally unable to deoxygenate that position along the biosynthetic pathway leading from the intermediate enzyme-bound polyketide to the final metabolites (Grimm et al., 1994). The small chemical modification results in IDA being a potent inducer of remission when administered intravenously in acute leukaemias, and exhibits interesting antitumour properties also when administered orally (Cersosimo, 1992).

Being very effective as antineoplastic drugs, anthracycline antibiotics, including IDA, are

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highly toxic. Drug administration is associated with severe side effects and thus its clinical usefulness is limited (Mandelli et al., 1991; Vogler et al., 1992; Wiernik et al., 1992; Reiffers et al., 1996). A particularly difficult problem for patients receiving chemotherapeutic agents is the dose limiting toxicity of such drugs that often leads to suboptimal therapy. In recent years, there has been extensive research in the field of drug delivery systems as a means of improving the therapeutic index of chemotherapeutic drugs (Allen, 1997; Sadzuka and Hirota, 1998; Iffert et al., 2000).

A number of investigators have been focusing their attention on red cells as carriers for IDA. Unfortunately, anthracycline drugs, including IDA, diffuse rapidly from erythrocytes (Kitao and Hatori, 1980). This problem has been overcome by covalently linking the drug to the erythrocyte membrane using glutaraldehyde. Lejeune et al. (1994) noted that glutaraldehyde could be used as a cross-linking agent to covalently bind DNR to the proteins (Fig. 1). As IDA differs from DNR by lack of the methoxy group in the C-4 position only, we tried to conjugate the IDA to erythrocyte membrane in the same manner.

The mechanism responsible for anthracycline-antibiotics immobilization in the glutaraldehyde-treated erythrocytes is not well understood. It is still not clear whether glutaraldehyde decreases the permeability of the erythrocyte membrane to anthracycline antibiotics or it chemically binds drugs to different erythrocyte components to potentiate their toxic effect.

Little is also known about the interaction of glutaraldehyde with lipids and proteins in the biological membranes as well as about the combined effects of drug and glutaraldehyde on the cell structure and function.

Thus, in this study we investigated the effect of idarubicin and glutaraldehyde on the structural properties of the human red blood cells. By comparing the effects of idarubicin alone and in the presence glutaraldehyde we have been able to test the importance of the cross-linking agent as a determinant of drug-toxicity in the cells. Alterations in the erythrocytes induced by the tested agents were monitored by electron spin resonance technique.

2. Materials and methods

2.1. Chemicals

Idarubicin (IDA) was purchased from Laboratory Roger Bellon (France).

Spin labels, 5-doxylstearic acid (5-DSA), 16-doxylstearic acid (16-DSA), 4-maleimido-tempo (MSL), 4-amino-tempo (TEMPAMINE) were purchased from Sigma (USA). All other chemicals were of the best quality commercially available.

2.2. Preparation of erythrocyte suspensions

Human peripheral blood from healthy donors in ACD (23 mM citric acid; 45.1 mM sodium citrate; 45 mM glucose) 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,

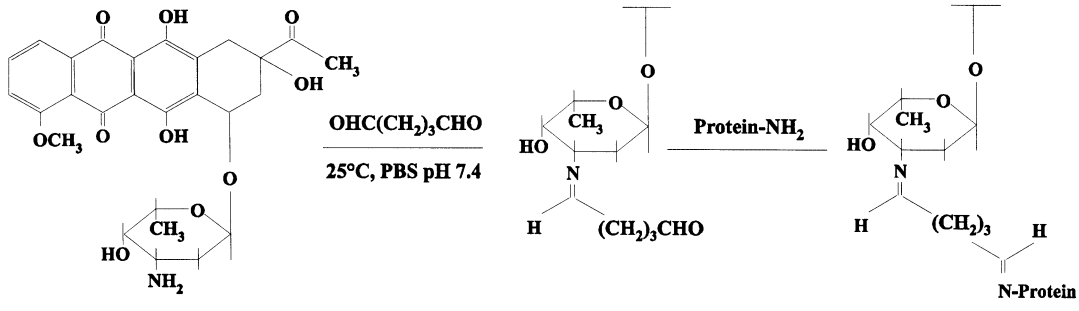

Fig. 1. Covalent binding of daunorubicin to the proteins (Lejeune et al., 1994).

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 idarubicin at a final concentration of 10 μg/ml in the dark for 30 min in a shaking bath at 37°C. After incubation, erythrocytes were washed three times with PBS and then treated with glutaraldehyde.

2.4. Glutaraldehyde treatment

Drug-treated washed erythrocytes were mixed with glutaraldehyde in PBS (final concentrations from 0.0005 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. Simultaneously the erythrocytes with glutaraldehyde (without IDA) were incubated in the same manner.

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. The content of drug in extracellular medium

The level of IDA in the medium was determined by fluorescence analysis (emission at 595 nm, excitation at 450 nm). Measurements were made at room temperature using an LS-5B Perkin Elmer spectrofluorometer.

2.7. 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. The samples of spin labeled erythrocytes and erythrocyte membranes were prepared as described below (Gordon et al., 1989).

2.7.1. Measurement of membrane fluidity

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

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

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

$$a_N = 1/3(T_{zz} + 2T_{xx})$$

$$a'_N = 1/3(T_{\parallel} + 2T_{\perp})$$

T_{zz} (32.4 G) and T_{xx} (6.1 G) 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):

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

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

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

2.7.2. Determination of the conformational changes in plasma membrane proteins

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.

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 to monitor changes in protein conformation and environment within the membrane (Berliner, 1983; Wyse and Butterfield, 1988).

2.7.3. Internal microviscosity

Internal microviscosity was determined using the method of Morse (1986) by introducing 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.

From the spectrum of TEMPAMINE spin label, the rotational correlation time τ_c was estimated using the following equation:

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

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

2.8. Statistical analysis

Data were analyzed by two-tailed Student's *t*-test of paired data.

3. Results

3.1. Determination of the amount of idarubicin entrapped into erythrocytes

Fig. 2 shows the level of IDA in the extracellular medium after incubation of the drug pretreated erythrocytes with the increased concentration of glutaraldehyde. The content of drug was determined by fluorescence study.

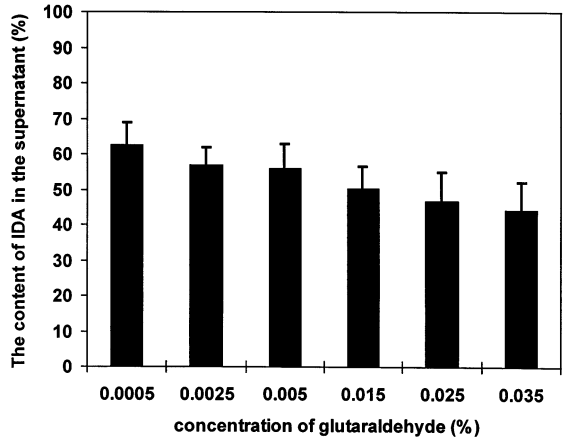

Fig. 2. The content of idarubicin in the extracellular medium after incubation of drug pretreated erythrocytes with glutaraldehyde. The level of idarubicin in the supernatant from erythrocytes without glutaraldehyde was taken as 100%. Values are means and S.D. of seven values from separate experiments.

It has been observed that addition of glutaraldehyde gradually decreases the fluorescence intensity of IDA in the extracellular medium. These data indicated that human erythrocytes treated with glutaraldehyde were able to take up the increased amount of idarubicin from the incubation medium. At a concentration of 0.035% glutaraldehyde, the extracellular level of IDA in erythrocytes was about 50% lower than when cells were incubated with idarubicin only.

3.2. The effect of idarubicin and glutaraldehyde on the membrane lipids fluidity

The membrane fluidity was monitored at the surface as well as in the deeper regions of the lipid bilayer. Pretreated erythrocytes with idarubicin and glutaraldehyde were labeled with two lipid spin labels: 5 and 16 doxylstearic acids.

In Fig. 3, the effects of interaction of the drug and glutaraldehyde with cells on the plasma membrane fluidity near the polar groups are presented. Idarubicin alone at a concentration of 10 μg/ml had no effect on the value of the order parameter

S of spin label 5-DSA. Addition of glutaraldehyde ($>0.0025\%$) to erythrocytes caused a marked increase in the order parameter S both in the samples containing cross-linking agent only as well as in the samples incubated with IDA and aldehyde. However, when erythrocytes were preincubated with drug and then treated with glutaraldehyde, the value of the measured parameter significantly increased in comparison to data with glutaraldehyde alone. Statistically significant changes were observed for the higher concentrations of aldehyde (0.0025–0.035%) ($P < 0.001$).

This indicated that the addition of glutaraldehyde to cells decreased the membrane fluidity at the surface of the lipid bilayer of human erythrocytes. Only low concentrations of aldehyde (0.0005%) showed no influence on the membrane lipids in this region. The large changes were observed for erythrocytes pre-incubated with IDA and then treated with glutaraldehyde, suggesting that glutaraldehyde may potentiate the toxic effect of idarubicin.

The effect of idarubicin and glutaraldehyde on the fluidity of lipids in the hydrophobic parts of erythrocyte membranes was measured using 16-DSA probe. The results are summarized in Fig. 4.

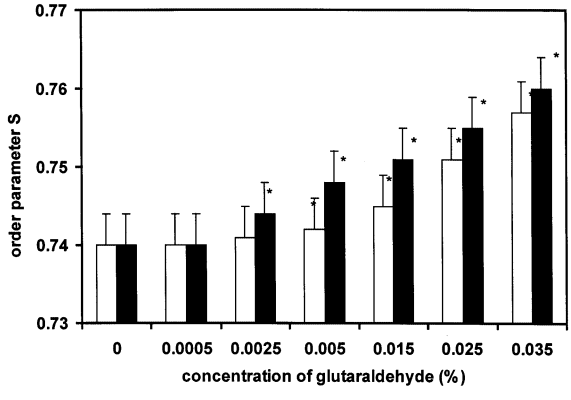

Fig. 3. Effect of idarubicin and glutaraldehyde on the fluidity of the cell membrane at surface regions of the lipid bilayer. The order parameter (S) was derived from the ESR spectrum of 5-DSA in erythrocyte membranes. Open columns (□) represent the samples incubated without IDA, solid columns (■), the samples preincubated with 10 μg/ml IDA and then treated with glutaraldehyde. Each point represents the average ± S.D. of seven experiments. *Values statistically significant in comparison to the control erythrocytes, $P < 0.0001$.

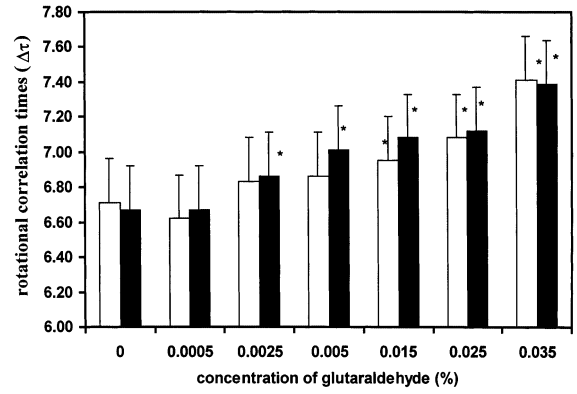

Fig. 4. Effect of idarubicin and glutaraldehyde on the fluidity of the cell membrane at hydrophobic regions of the lipid bilayer. Open columns (□) represent the samples incubated without IDA, solid columns (■), the samples preincubated with 10 μg/ml IDA and then treated with glutaraldehyde. Each point represents the average ± S.D. of seven experiments. *Values statistically significant in comparison to the control erythrocytes, $P < 0.0001$.

There were no changes for erythrocytes incubated with IDA alone. Glutaraldehyde incubated with cells without drug, at concentrations from 0.0015 to 0.035% induced a significant alteration in $\Delta\tau$ in a concentration-dependent manner. These changes were statistically significant in comparison to the control samples. In the case of erythrocytes preincubated with IDA and then treated with glutaraldehyde, the statistically significant changes were also observed at lower concentrations of glutaraldehyde (0.0025–0.015%).

3.3. Changes in the erythrocyte membrane proteins

In order to identify changes of erythrocyte proteins, the isolated membranes were selectively labeled with maleimide spin label that binds predominantly to SH groups of cytoskeletal proteins (Berliner, 1983). The spectrum erythrocyte membranes labelled with maleimide consists of two dominant classes of binding sites: strongly (S) and weakly (W) immobilised. The ratio W/S has been frequently used to correlate protein state. Fig. 6 demonstrates the effect of idarubicin and glutaraldehyde on erythrocyte membrane proteins. Idarubicin without glutaraldehyde did not signifi-

cantly affect the conformation of the membrane proteins. The values of the W/S parameter were, respectively, 3.50 ± 0.48 for the control erythrocytes and 3.54 ± 0.57 for the cells incubated with idarubicin. In the presence of glutaraldehyde a decrease in the W/S ratio was observed. These results imply a stronger immobilization of maleimide spin label in the erythrocyte membranes after incubation with glutaraldehyde. Under the studied conditions we noted similar changes for erythrocytes incubated with glutaraldehyde alone and for the samples preincubated with IDA. The statistically significant values in comparison to the samples incubated without drug and with drug only were observed at higher concentrations of glutaraldehyde.

3.4. Internal microviscosity assay

The internal microviscosity of erythrocytes was determined as described by Morse (1986), using the spin-label method. The data are presented in Fig. 5. The internal microviscosity of control erythrocytes was found to be 2.02 ± 0.61 cP. IDA in tested concentration of 10 μg/ml only negligibly altered the η value. An increase in the internal viscosity of erythrocytes was observed in the presence of enhanced concentrations of glutaraldehyde. However, statistically significant results were noted only for concentrations above 0.0025% glutaraldehyde. When erythrocytes were preincubated with IDA and then treated with glutaraldehyde, a small increase in the internal microviscosity was observed. The differences are extreme at the highest concentration of glutaraldehyde.

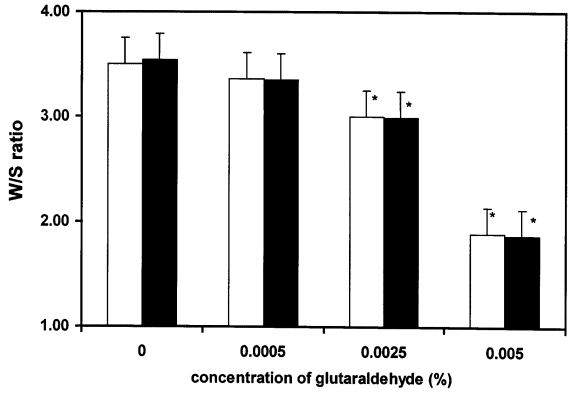

Fig. 6. Effect of idarubicin and glutaraldehyde on the erythrocyte membrane proteins. The maleimide spin label was used to monitor changes of the skeletal proteins and organization of membrane by calculation of the W/S ratio. Open columns (□) represent samples incubated without IDA, solid columns (■), the samples preincubated with 10 μg/ml IDA and then treated with glutaraldehyde. Each point represents the average ± S.D. of seven experiments. *Values statistically significant in comparison to the control erythrocytes, $P < 0.05$.

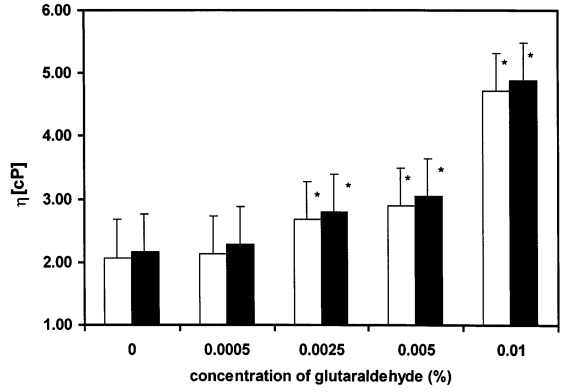

Fig. 5. Effect of idarubicin and glutaraldehyde on the internal microviscosity of erythrocytes. Alterations were determined by ESR spectroscopy using the TEMPAMINE spin label. Open columns (□) represent the samples incubated without IDA, solid columns (■), the samples preincubated with 10 μg/ml IDA and then treated with glutaraldehyde. Each point represents the average ± S.D. of seven experiments. *Values statistically significant in comparison to the control erythrocytes, $P < 0.0001$.

4. Discussion

Anthracyclines, such as DNR and IDA are administered to patients by intravenous injection. Drugs and their metabolites, especially idarubicinol (Fukushima et al., 1994; Hempel et al., 1997) which remain in the blood for longer periods, may interact with the erythrocyte membrane and affect its structure and functions. It is also well known that cancer chemotherapy remains still largely nonspecific, and that drugs are toxic for tumour cells as well as for normal cells. We have been interested in new strategies to diminish the toxic side effects of drugs and to improve their

therapeutic indexes. Liposomes, monoclonal antibodies and nanoerythrosomes are used as drug carriers for the optimal delivery of pharmacological agents. Erythrocytes have also been proposed as biological carriers of a number of different agents to target the drugs to specific organs (DeLoach and Barton, 1981).

In present study we focused on idarubicin, one of the new analogous of anthracycline antibiotics. We determined changes in the erythrocyte lipid and protein organization after incubation with drug and glutaraldehyde, which was used as crosslinker. We are interested mainly in the plasma membrane organization. It is a dynamic structure, which interfaces the cell with the environment and co-ordinates most intercellular communications. Many of the properties of cancer cells, such as invasiveness and growth features, are mediated at least in part by the plasma membrane. Thus, the influence of anticancer drugs on cellular membranes can provide a new understanding of drug cytotoxic mechanisms. Our previous in vitro studies (Szwarocka et al., 1997) demonstrated that IDA is rapidly transported into erythrocytes. The rate of uptake reached equilibrium in about 2 min. We also found that only $\sim 30\%$ of the drug penetrated into the cells. In this paper we estimated that human erythrocytes treated with glutaraldehyde could take up IDA from the incubation medium faster than untreated cells. At the highest concentration of glutaraldehyde (0.035%), the content of IDA in erythrocytes was about 50% higher than in erythrocytes incubated with IDA only. Our previous studies (Szwarocka et al., 1996) and studies of other workers (Matherne et al., 1994; Ataullakhanov et al., 1996) have revealed similar data for the entrapping of daunorubicin and doxorubicin.

To elucidate the change of physicochemical properties of the membrane structure in the process of interaction of idarubicin and glutaraldehyde with erythrocytes, the membrane fluidity was measured under various conditions. Idarubicin, at the tested concentration (10 μg/ml) had no significant effect on the lipid fluidity, at surface and at deeper regions of erythrocyte membranes. ESR studies have shown the existence of changes both in lipids and proteins of the membrane when cells were incubated with glutaraldehyde. The decrease of the lipid fluidity was noted. Dumas et al. (1997) observed similar data. They demonstrated that the membrane fluidity, measured by a method based on the kinetics of pyrene dodecanoic acid excimers formation, decreased as the glutaraldehyde concentration increased. Preincubation of red blood cells with IDA and then incubation with glutaraldehyde caused higher changes in the fluidity of the lipid bilayer in comparison to the erythrocytes incubated with glutaraldehyde only.

The analyses of the spectra of maleimide attached to erythrocyte membrane were performed by the calculation of W/S ratio, which is a very sensitive indicator of the physical state of proteins in the membrane (Butterfield, 1982; Fung, 1983). Maleimide is bound covalently, predominantly to spectrin and actin, also to bands 4.1, 2.1 and the cytoplasmic side of band 3 protein (Wyse and Butterfield, 1988; Butterfield and Palmieri, 1990). Small perturbations in the nearest proximity of the label bound to membrane proteins may produce significant changes in the maleimide spectra used for the monitoring of conformational changes of proteins. Our data indicated that glutaraldehyde alters the conformation of membrane proteins at the concentration of or above 0.0025%. The decrease in the W/S parameter was observed in probes with glutaraldehyde and in the samples preincubated with IDA and then treated with glutaraldehyde.

It has been shown that changes in erythrocyte plasma membranes (Bartosz and Leyko 1980; Morse 1985) are responsible for alterations of microviscosity (η) inside the cells. We also determined this parameter in erythrocytes incubated with idarubicin and glutaraldehyde. Our results showed that glutaraldehyde enhanced the microviscosity of erythrocytes preincubated with IDA and then treated with glutaraldehyde, as well as in the samples incubated with aldehyde only.

These observations correspond very well with our previous report (Szwarocka et al., 1996). We demonstrated that DNR and glutaraldehyde caused statistically significant alterations in the organization of the membrane cytoskeletal proteins, membrane lipids and in the internal viscosity of the human erythrocytes.

The changes detected in these parameters indicate the deformability of red blood cells. Dumas et al. (1997) noted that exposure of erythrocytes to glutaraldehyde progressively decreased the elongation index values with the increased concentration of glutaraldehyde. The deformability of erythrocytes was significantly reduced, i.e. the cells became more rigid and less deformable.

The above observations indicated that use of glutaraldehyde offers a novel strategy for antineoplastic treatment. With this cross-linking agent much more of the drug was entrapped to the red cells. These results create the possibility of using erythrocytes as carriers of IDA. However, the data suggest that glutaraldehyde in the drug-pretreated erythrocytes may also produce significant perturbations in the organization of the plasma membrane lipids and proteins and alterations in the viscosity of the cells, especially at higher doses. 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). Only small concentrations of glutaraldehyde are not toxic to human erythrocytes. Thus, further investigations are required to better define the cytotoxicity and therapeutic value of drug as well as glutaraldehyde.

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