NAD and Glutathione Modulate Sensitivity of Bone Marrow Cells to Oxidative Stress

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We studied *in vitro* cytostatic effect of doxorubicin on bone marrow cells preincubated with NAD and reduced glutathione. The presence of NAD and glutathione in the medium potentiated membrane toxicity of this antibiotic, but prevented its toxic effects on intracellular pool of pyridine nucleotides during oxidative stress.

Key Words: *oxidative stress; bone marrow; NAD; glutathione*

Oxidative stress leads to considerable changes in cell redox-buffer systems, including oxidation and hydrolysis of nicotinamide coenzymes and oxidation or irreversible loss of glutathione [7,14]. Cytolysis and active transport of pyridine nucleotides and glutathione from cells are accompanied by accumulation of these metabolites in the extracellular space [4,15]. NAD released from cells is utilized by mono-ADP-ribosyltransferases and NAD glycohydrolases [8,15]. Glutathione affects extracellular redox-sensitive sites in membrane protein molecules and modulates their sensitivity to ligands [6,10].

Extracellular glutathione and NAD modulate proliferation and apoptosis, regulate signal cascade pathways, and change cell membrane stability [12,15]. Addition of NAD to the incubation medium induces various changes in lymphoid cells [11]. Activation of lymphocytes depends on glutathione concentration in the extracellular medium [10]. NAD and glutathione probably play a particular role in the bone marrow, where the microenvironment performs important regulatory functions.

Here we studied *in vitro* damages to bone marrow cell (BMC) membranes during oxidative stress in the presence of NAD and reduced glutathione (GSH).

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MATERIALS AND METHODS

Experiments were performed on male outbred albino mice weighing 20-25 g (5 animals in each series).

BMC were isolated routinely [2]. Oxidative stress was induced by incubation of BMC with doxorubicin (DOX) in concentrations of 5×10^{-7}, 10^{-6}, and 5×10^{-6} M. This xenobiotic exhibits pronounced antioxidant activity due to the formation of semiquinone, superoxide, and hydroxyl radicals [3]. Blebbing in the plasma membrane was studied by phase-contrast microscopy [9]. BMC (10^6 cells/ml) were incubated for 5, 15, 30, 45, and 60 min in medium 199 at 37°C and 200 cells in each sample were examined using an immersion objective (×900). The formation of small bubbles (initial blebbing), appearance of large bubbles (terminal blebbing), and development of necrosis were taken into account.

The content of lipid peroxidation (LPO) products in BMC suspension was estimated routinely by the concentration of thiobarbituric acid (TBA)-reactive substances. The sample was incubated with TBA, reaction products were extracted with butanol, and their content was measured spectrophotometrically [1].

The cytotoxic effects of xenobiotics were studied by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, 5 mg/ml, 50 μl) to each sample. After 1.5-h incubation with MTT at 37°C formazan crystals were dissolved with 500 μl 0.1 N HCl-isopropanol. Optical density was measured at

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570 nm on an SF-26 spectrophotometer. The baseline optical density was estimated at 640 nm. The difference between these values was calculated.

In vitro effects of NAD and GSH on BMC were studied after incubation of the cell suspension with these reagents (Reanal) in a concentration of 1 mM. This concentration corresponds to *in vivo* NAD and GSH contents in the extracellular medium during various diseases [15]. The samples for microscopy were taken after 5-, 15-, 30-, 45-, and 60-min incubation.

The results were analyzed by Student's *t* test.

RESULTS

DOX dose-dependently stimulated initial blebbing in plasma membranes of BMC after 45-min incubation. In the highest dose the preparation caused severe cell necrosis (Fig. 1). DOX intensified LPO in BMC membranes (Fig. 2). In further experiments we used DOX in a concentration (10^{-6} M) producing most pronounced membrane-toxic (initial blebbing of plasma membranes and LPO induction) and minor cytolytic effects.

Preincubation of BMC with 1 mM NAD had no effect on LPO, but intensified necrosis of DOX-treated cells. Preincubation of BMC with 1 mM GSH potentiated cell necrosis, promoted initial and terminal blebbing, but inhibited generation of TBA-reactive substances in DOX-treated cells (Table 1).

It should be emphasized that 60-min incubation of BMC with NAD or GSH induced transitory blebbing of plasma membranes or development of cytolysis (Table 2); generation of TBA-reactive substances markedly increased (14.59±0.49 *vs.* 8.60±0.74 μmol/liter in the control, $p<0.001$). The presence of 1 mM GSH in the medium also intensified generation LPO products (14.81±1.08 μmol/liter, $p<0.01$).

Oxidative stress is accompanied by a decrease in the concentration of NAD(P)H and changes in the ratio between reduced and oxidized nicotinamide coenzymes in cells [14]. Incubation of BMC with DOX decreased parameters of the MTT-test reflecting the

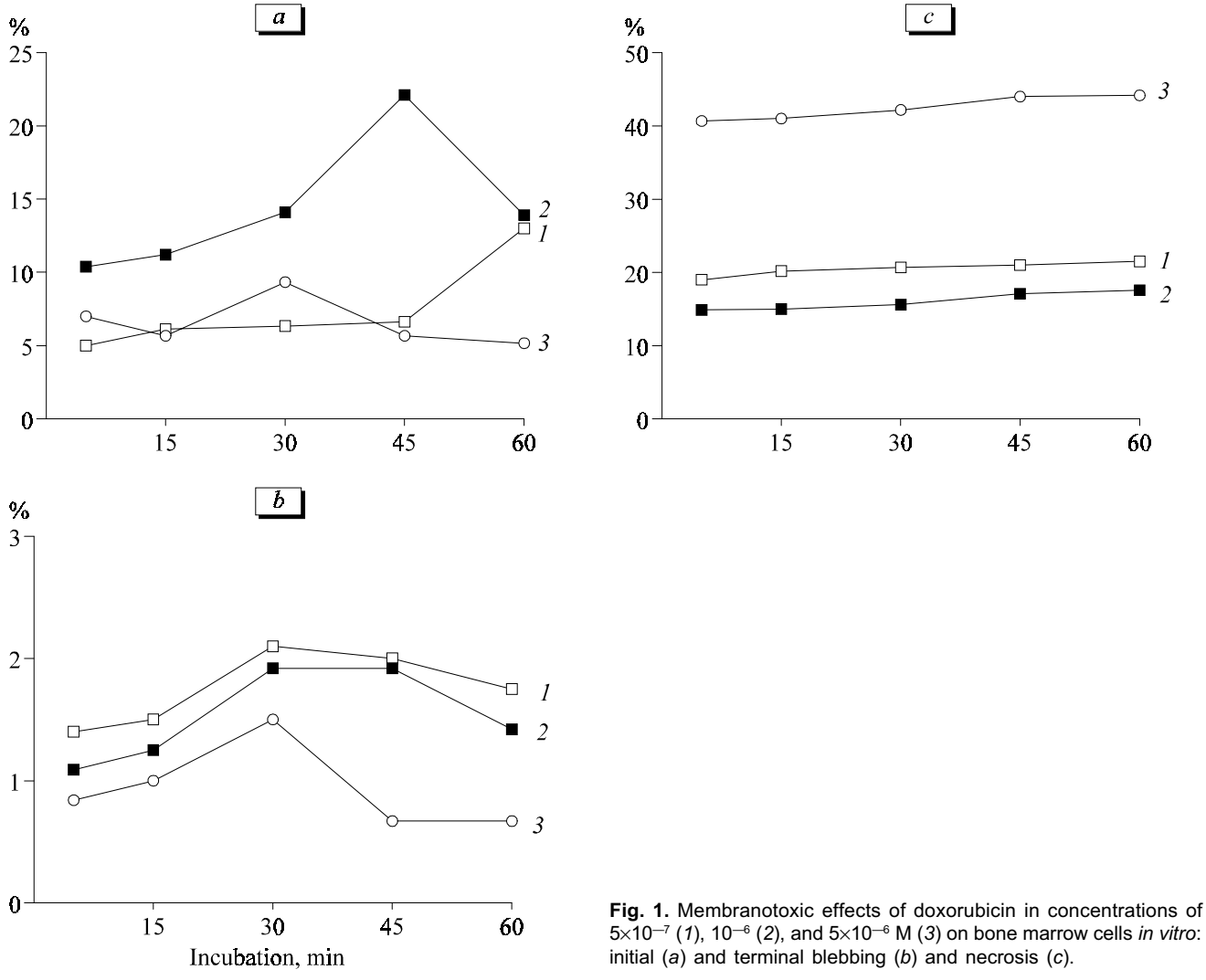

Fig. 1. Membranotoxic effects of doxorubicin in concentrations of 5×10^{-7} (*1*), 10^{-6} (*2*), and 5×10^{-6} M (*3*) on bone marrow cells *in vitro*: initial (*a*) and terminal blebbing (*b*) and necrosis (*c*).

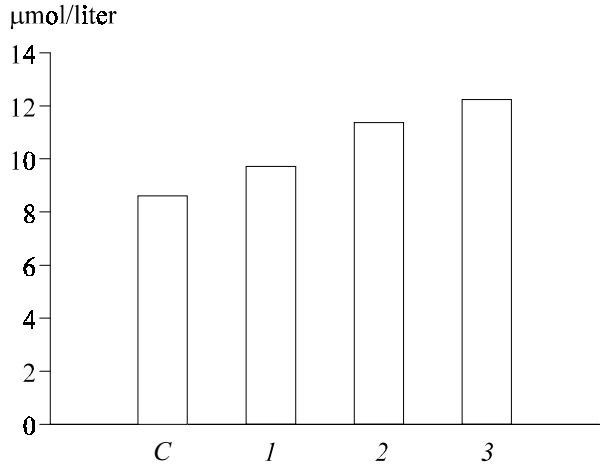

Fig. 2. Induction of LPO in bone marrow cells by doxorubicin in concentrations of 5×10^{-7} (*1*), 10^{-6} (*2*), and 5×10^{-6} M (*3*). Ordinate: concentration of TBA-reactive substances. *C*: control.

content of NAD(P)H and functional state of mitochondria [5]. Incubation with NAD or GSH increased parameters of the MTT-test and decreased DOX toxicity (Fig. 3).

Thus, the presence of NAD and GSH in the incubation medium potentiates DOX-induced damages to cell membranes, which is manifested in enhanced blebbing and necrosis of cells. However, these changes do not correspond to the effects of NAD and GSH on free radical oxidation of membrane lipids, which confirms the absence of direct causal relations between oxidative stress and blebbing in the plasma membrane of damaged cells [9]. At the same time, previous studies showed that oxidation of thiol groups in cytoskeletal and transmembrane proteins can induce blebbing in cell membranes [13].

The pathogenesis of oxidative stress includes changes in quantitative and qualitative characteristics of intracellular pyridine nucleotides resulting from their oxidation and hydrolysis [14]. The GSH-induced increase in MTT-test parameters (*e.g.*, under conditions excluding the possibility of direct reduction of MTT in the extracellular medium) and suppression of DOX cytotoxicity indicate normalization of utilization and hydrolysis of intracellular pyridine nucleotides. The effect of extracellular NAD on nucleotide-depleting

TABLE 1. *In Vitro* Modulation of DOX (10^{-6} M) Cytotoxicity after Preincubation of BMC with NAD (Numerator) and GSH (Denominator) (*M*±*m*)

Parameter		Incubation, min				
		5	15	30	45	60
Blebbing	initial	10.25±1.50** 10.50±0.94*	11.00±1.87*** 10.17±2.16****	13.63±2.60**** 5.50±1.51	7.13±1.61**** 5.50±1.97	4.33±1.43 2.83±0.74
	terminal	0.83±0.34 5.00±0.75**	0.67±0.21 4.88±0.36*	2.17±0.54 5.88±0.72**	1.83±0.41 5.83±2.05	1.25±0.17 5.67±2.15
Necrosis		19.88±1.92** 18.00±1.47**	20.38±1.92** 18.75±1.38*	20.83±2.41*** 19.63±1.04**	21.63±1.82** 19.63±0.72**	23.13±1.21** 20.25±1.19**
Malonic dialdehyde, μmol/liter				9.00±1.29 4.17±0.86**		

Note. Here and in Table 2: *p<0.001, **p<0.01, ***p<0.02, and ****p<0.05 compared to the control.

TABLE 2. *In Vitro* Membranotoxic and Cytotoxic Effects (% of Total Cell Count) of NAD (Numerator) and GSH (Denominator) on BMC (*M*±*m*)

Parameter		Incubation, min				
		5	15	30	45	60
Blebbing	initial	10.67±1.95** 5.67±0.89**	12.00±2.32*** 7.00±2.47	5.50±0.35 6.33±2.27	5.67±1.24* 2.50±0.94	4.00±1.22 1.50±0.35
	terminal	1.50±0.61 3.17±0.89****	2.00±0.35 4.83±1.02***	2.33±0.89 5.67±0.41**	4.83±1.08 3.83±0.82	2.50±0.71 4.00±0.94
Necrosis		19.17±2.01** 16.83±2.07***	21.17±2.01** 18.17±1.67**	24.17±1.67** 19.67±1.14**	26.33±0.89* 20.17±0.74**	29.50±0.35* 24.17±1.24**

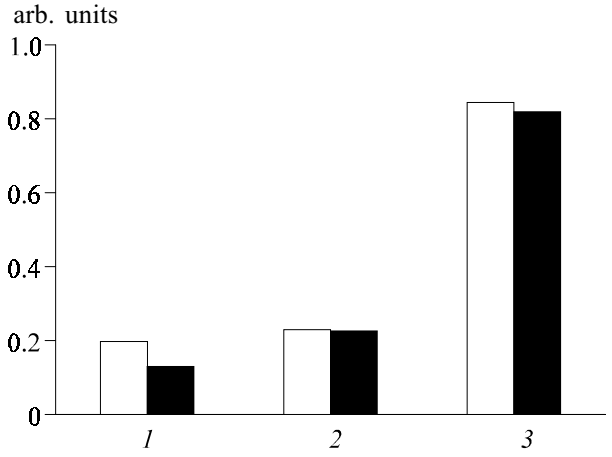

Fig. 3. Modulation of cytotoxicity of 10^{-6} M doxorubicin after incubation of cells with NAD and GSH. Ordinate: cell activity in MTT-test. No treatment (*1*) and incubation with NAD (*2*) and GSH (*3*). Light bars: without doxorubicin. Dark bars: doxorubicin.

activity of DOX confirms the possibility of NAD transport into cells mediated by membrane mono-ADP-ribosyltransferases and NAD glycohydrolases [8,15].

Thus, the presence of NAD and GSH in the extracellular medium in concentrations similar to those observed during intensive cytolysis changes the sensitivity of hemopoietic cells to oxidative stress *in vitro*, which is probably related to modulation of enzymatic mechanisms underlying transmembrane transport of NAD and/or modification of redox-sensitive sites in protein molecules.

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