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Hematotoxicity and blood glutathione levels after cisplatin treatment of tumor-bearing mice

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Abstract

The involvement of glutathione, a major cellular antioxidant, in cisplatin-mediated development of various hematological changes in mice bearing ascites Dalton lymphoma tumor was investigated. With tumor growth, glutathione levels decreased in blood but increased in tumor cells. Cisplatin treatment of tumor-bearing mice caused a decrease in glutathione levels in blood, ascites supernatant, and tumor cells. Blood hemoglobin, erythrocytes, packed cell volume and leukocytes (eosinophils, basophils, and lymphocytes) were also decreased along with the development of various morphological abnormalities in erythrocytes (microcytes, macrocytes, echinocytes, acanthocytes, etc.) after cisplatin treatment. All these hematotoxic features were noted to be increased more when buthionine sulfoximine (a specific glutathione-depleting agent) was also given prior to cisplatin treatment. However, combination treatment of cysteine (precursor for glutathione synthesis) plus cisplatin resulted in an improvement in the glutathione levels and decrease in hematological toxicities. It is noted that the glutathione levels in blood and abnormalities in erythrocytes and other hematological parameters are inversely related in cisplatin-mediated cancer chemotherapy. It is suggested that blood glutathione may play an important role in the development of cisplatin-mediated hematological toxicity in the host.

Abbreviations: GSH, reduced glutathione; BSO, L-Buthionine (*S*,*R*)-sulfoximine; PBS, phosphate-buffered saline; DL, Dalton lymphoma; RBC, red blood cells; WBC, white blood cells; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)

Introduction

cis-Diamminedichloroplatinum(II), commonly known as cisplatin, is a potent chemotherapeutic agent widely used against various malignancies in animals and humans (Rosenberg, 1985; Jurga et al., 1994; Prasad and Giri, 1994; Go and Adjei, 1999). The molecular mechanism of cisplatin's anticancer activity has been suggested to involve its ability to bind with cellular DNA and render the cell incapable of replication (Pascoe and Roberts, 1974; Pinto and Lippard, 1985; Zamble and Lippard, 1995). Besides its interaction with cellular DNA, changes in the host immune response (Collins and Kao, 1989), cell surface (Prasad

and Sodhi, 1981), tissue calcium and potassium concentrations (Prasad and Giri, 1999), various enzymes (5′-nucleotidase, arginase, cathepsins, lactate dehydrogenase) (Prasad et al., 1999), and mitochondria (Kharbangar et al., 2000) have also been observed and it has been suggested that these changes could also be involved as additional components in cisplatin's mechanism of anticancer activity.

The major limitation in the clinical therapeutic efficacy of cisplatin is the development of dose-limiting side-effects, which include nausea, vomiting, nephrotoxicity (Krakoff, 1979), embryotoxicity (Keller and Aggarwal, 1983), peripheral neuropathy (Hamers et al., 1991), mutagenicity (Giri et al., 1998), and sterility (Aydiner et al., 1997). Cisplatin has also been reported to develop hematotoxicity (Hoagland, 1982; Ohno et al., 1993; Olas and Wachowicz, 1998) in the host, but its relationship with blood glutathione changes has not been clarified.

Glutathione (a tripeptide; L-γ-glutamyl-L-cysteinyl-glycine) is the most widespread cellular thiol and an important intracellular antioxidant. The reduced form of glutathione (GSH) inhibits free radical-mediated injury by eliminating toxic peroxides and protects protein sulfhydryl groups from oxidation by serving as a biological redox reagent (Meister, 1988; Beutler, 1989). GSH also preserves cellular levels of other antioxidants (Meister, 1994) and participates in the detoxification of xenobiotics that cause cellular injury by generating free radicals (Shi et al., 1994). GSH is a nucleophile that can bind to the electrophilic form of platinum. Intracellular platinum–glutathione conjugates have been isolated from tissue culture cells treated with cisplatin (Ishikawa and Ali-Osman, 1993; Mistry et al., 1993).

L-Buthionine-(*S*,*R*)-sulfoximine (BSO) is an inhibitor of γ-glutamylcysteine synthetase, (Griffith and Meister, 1987) and has often been used to deplete cellular GSH. *In vitro* studies demonstrated that cancer cells resistant to alkylating drugs including cisplatin could be made sensitive to these drugs through BSO-induced glutathione depletion (Hamilton et al., 1985; Prasad et al., 1994). Cysteine is the rate-limiting and predominant plasma substrate for the synthesis of the reduced form of glutathione (Ross et al., 1997). Cytosolic cysteine and glutathione are important thiols that function as physiological reducing agents to maintain oxidative balance within cells. As a free amino acid, cysteine is transported efficiently in most cell types (Ross et al., 1997; Van Klaveren et al., 1997).

Erythrocytes (red blood cells, RBC), oval biconcave-shaped cells lacking cellular organelles, are abundant in the blood of mammals. In addition to oxygen transport, they also function as conveyors of nutrients, participate in inter-organ communication, and serve as targets for drugs, environmental xenobiotics, and pathological factors (Pikula et al., 1996). Thus, cisplatin-mediated changes in GSH status within the blood and their effect on erythrocytes should be of special importance because erythrocytes are thought to be particularly susceptible to injury by oxidants (Ross et al., 1997).

The present study aimed to investigate the significance of glutathione in relation to cisplatin's effect on erythrocytes and other hematological parameters in tumor-bearing mice. To understand the involvement of GSH in these effects, GSH level was also specifically depleted or increased using BSO or cysteine, respectively, in some cisplatin treatment conditions.

Materials and methods

Chemicals

Reduced glutathione, L-buthionine (*S*,*R*)-sulfoximine (BSO), and 5,5′-dithiobis(2-nitro-

benzoic acid) (DTNB) were purchased from the Sigma Chemical Company, St. Louis, MO, USA. L-Cystiene was purchased from Central Drug House, Mumbai, India. Cisplatin solution (1 mg/ml of 0.9% NaCl) was obtained from Biochem Pharmaceutical Industries, Mumbai, India. All other chemicals used in the experiments were of analytical grade. Glass double-distilled water was always used to prepare the solutions.

Tumor maintenance and cisplatin treatment

Inbred Swiss albino mice were maintained under conventional laboratory conditions with free access to food pellets (Amrut Laboratory Animal Feeds, Delhi) and water. Ascites Dalton lymphoma tumor is maintained *in vivo* in 10–12-week-old mice by serial intraperitoneal (i.p.) transplantations of 1×10^7 tumor cells per animal (0.25 ml in phosphate-buffered saline (PBS) 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate buffer, pH 7.4). Tumor-transplanted hosts usually survived for 18–20 days.

The therapeutic dose of cisplatin against malignant tumors has been established to be 8–10 mg/kg body weight (Rosenberg, 1985). For each time point a single dose of cisplatin (8 mg/kg body weight, i.p.) was administered to 3 or 4 tumor-bearing mice on the 10th day following tumor transplantation when the tumor was at the logarithmic phase of growth. With the regression of Dalton lymphoma very little ascites is recovered from the hosts after 6–7 days of cisplatin treatment. The treatment schedule for 24, 48, 72, and 96 h has been used previously by us (Prasad and Giri, 1994; Prasad et al., 1998; Kharbangar et al., 2000) and the same treatment schedule was followed here. Since the maximum glutathione depletion (43–45%) by buthionine sulfoximine (BSO) in Dalton's lymphoma cells was found to be after 8 h, in the combination treatment BSO was given 8 h prior to cisplatin. Cysteine was given 30 min prior to cisplatin.

Glutathione estimation

Total GSH was determined in the liver, DL cells, and ascites supernatants collected from the tumor-bearing mice at different stages of tumor growth. GSH was also determined in the DL cells and ascites supernatants from control (untreated tumor-bearing mice) and cisplatin-treated tumor-bearing mice using the method of Sedlak and Lindsay (1968).

Ascites tumor fluid collected from mice was centrifuged (2000*g* at 4°C, 10 min) to separate tumor cells and ascites supernatant. Briefly, 5% homogenates of DL cells or liver were prepared in 0.02 mol/L EDTA, pH 4.7. Total GSH was determined by adding the homogenate/supernatant or pure reduced form of glutathione (100 µl) to 0.2 mol/L Tris-EDTA buffer (1 ml, pH 8.2) and 0.02 mol/L EDTA, pH 4.7 (0.9 ml), followed by 20 µl of Ellman's reagent (10 mmol/L DTNB in methanol). After 30 min of incubation at room temperature, the absorbance was read at 412 nm in Beckman DU-640 spectrophotometer. Samples were centrifuged before the absorbance of supernatants was measured.

Evaluation of hematological parameters

Collection of blood samples. Blood samples from control (untreated, tumor-bearing mice) and cisplatin-treated tumor-bearing mice were collected from the tail vein into a sterilized tube containing heparin 15–20 IU per ml of blood and used for the study.

Blood glutathione estimation. Blood glutathione was determined following the method of Beutler et al. (1963). Briefly, to the 100 µl of freshly collected blood, 0.9 ml water and 1.5 ml precipitating solution (glacial metaphosphoric acid 1.67 g; Na_2EDTA 0.20 g; NaCl 30 g; water 100 ml) were added immediately and mixed well. After 5 min of incubation at room temperature, the reaction mixture was centri-

fuged (3000g at 4°C, 15 min) and to the 500 µl of clear supernatant, 2 ml of 0.3 mol/L phosphate solution and 250 µl DTNB solution (200 mg in 100 ml of 1% sodium citrate solution) were added and mixed thoroughly. Blank was prepared by taking 1 ml phosphate solution (0.3 mol/L), 1 ml water, 0.5 ml precipitating solution, and 250 µl DTNB solution. Both the blank and sample reaction mixtures were read against water at 412 nm in a Beckman DU-640 spectrophotometer. Glutathione concentration was calculated on the basis of a millimolar extinction coefficient of 13.6 and a molecular weight of 307 as follows:

$$\text{OD} = \text{OD sample} - \text{OD blank}$$

$$\frac{\text{OD} \times 2.75 \times 2.75 \times 307 \times 100}{13.6 \times 0.1 \times 0.5 \times 1000}$$

$$= \text{OD} \times 341.42 \text{ mg GSH/100 ml blood}$$

Hemoglobin estimation. Hemoglobin (Hb) content of blood was determined according to Dacie and Lewis (1975). Blood was diluted (1:200) with cyanide–ferricyanide solution (potassium ferricyanide 200 mg; potassium cyanide 50 mg; water 1000 ml; pH 9.6) and allowed to stand for 10 min at room temperature to ensure complete conversion of hemoglobin. The absorbance of the reaction mixture was read at 540 nm in a Beckman DU-640 spectrophotometer. The hemoglobin content was calculated as follows:

$$\text{Concentration (g/dl)} = \frac{A_{540} \times 64\,500 \times \text{ Dilution factor}}{44.0 \times d \times 1000 \times 10}$$

where, A_{540} = absorbance at 540 nm, 64 500 = molecular weight of hemoglobin, dilution factor = 200, when 10 µl blood was diluted to 2 ml; 44.0 = millimolar extinction coefficient; d = layer thickness (cm); 1000 = conversion factor for mg to g; 10 = conversion factor for g/L to g/dl.

Red blood cell (RBC) counts. Freshly collected blood was diluted (200 times) with RBC diluting fluid and the RBC counting was done in a Neubauer counting chamber. Erythrocyte count values were calculated according to Dacie and Lewis (1975).

$$\text{Red blood cells count per µl} = \frac{N \times 1 \times 200 \text{ (dilution)}}{0.02}$$

$$= N \times 10^4 \times 10^6/\text{L}$$

White blood cell (WBC) counts. Freshly collected blood was diluted by adding 20 µl of blood to 0.38 ml of WBC diluting fluid and white blood cells were counted in the Neubauer chamber. The number of WBC was calculated according to Swarup et al. (1981). White blood cells count per µl = $N \times 10 \times 20$ (dilution) = $N \times 200 = N \times 200 \times 10^6$/L

Differential leukocytes count. A thin and uniform blood film was prepared on a clean slide and was air dried overnight, stained with Leishman's stain the following day. Counting was done under a microscope in a narrow longitudinal strip of the blood. During counting, the number of different types of white blood cells were noted and expressed in percentage as described by Swarup et al. (1981).

Packed cell volume (PCV) determination. Blood was centrifuged (10 000*g* at 4°C, 5 min) and the height of the column of red blood cells was taken as packed cell volume (volume occupied by the red blood cells expressed as a fraction of the total volume of blood) according to Dacie and Lewis (1975).

Scanning electron microscopy. Blood was collected in a clean microcentrifuge tube and fixed in 2.5% glutaraldehyde for 30 min at room temperature. After fixation, the blood was centrifuged (750*g* at 4°C, 5 min). The cell

pellet was washed twice and resuspended in distilled water. A thin film of the suspension was made on a clean coverslip and allowed to air dry. The coverslip was mounted on a brass stub with electroconducting paint and then coated with gold in a fine coat ion sputter. The red blood cells were thoroughly examined and photographed under a Jeol scanning electron microscope operated at 15 kV.

Results

Total GSH level at various stages of tumor growth showed no significant changes in liver, while it increased in Dalton lymphoma cells, reaching maximum on day 10 (Figure 1A). In blood, as compared to day 0 of tumor transplantation which actually refers to normal mice, GSH level decreased during growth of the tumor (Figure 2B). Blood GSH level further decreased in tumor-bearing mice after cisplatin alone or BSO plus cisplatin treatment (Table 1; Figure 2) and an increase was noted after the combined treatment with cysteine plus cisplatin (Table 1; Figure 6). In tumor cells and ascites supernatants, a decreased GSH level was noted initially (24–48 h), which was found to recover during the later periods of cisplatin treatment in both cases (Figure 2A).

Evaluation of various hematological toxicity parameters in tumor-bearing mice showed that the hemoglobin content, erythrocytes, leukocytes, and packed cell volume (PCV) decreased after cisplatin treatment (Table 2; Figure 3). A further decrease in these values was noted after the combination treatment of BSO plus cisplatin. However, combination treatment of mice with cysteine plus cisplatin resulted in a consistent rise in these hematological parameters (Table 2; Figure 3). As compared to normal mice, an increase in eosinophils and neutrophils, and a decrease in basophils, lymphocytes, and monocytes was noted in tumor-bearing mice (Table 3). Cisplatin treatment of tumor-bearing mice caused a significant decrease in basophils (22–64%) and lymphocytes (40–60%), while an increase was observed in neutrophils and monocytes (Table

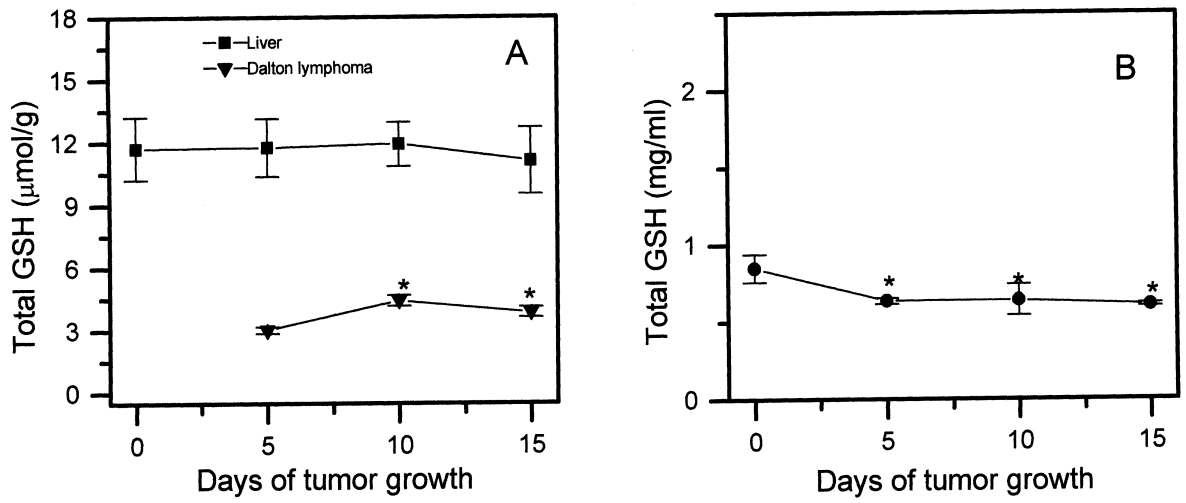

Figure 1. Comparative changes in GSH content (mean ± SD) in liver, Dalton lymphoma (DL) cells (A), and blood (B), at different stages of tumor growth in mice. Statistical analysis, as compared to day 0 (liver and blood) or day 5 (DL cells). Student's *t*-test, $n = 3$; $^{*}p \leqslant 0.05$.

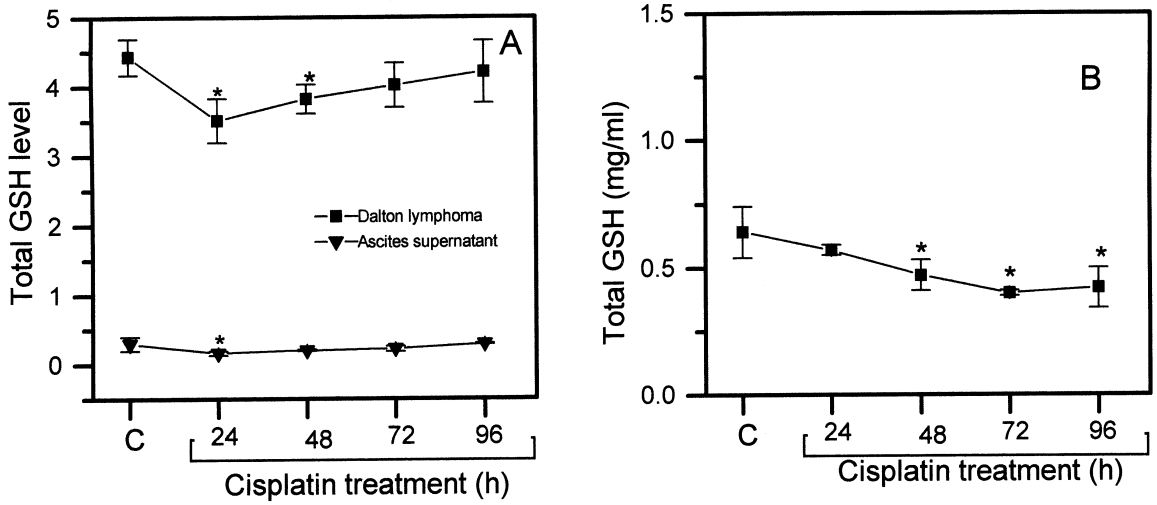

Figure 2. Changes in GSH level (mean ± SD) in Dalton lymphoma cells (μmol/g), ascites supernatant (μmol/ml) (A), and blood (B), collected from mice after cisplatin treatment. C = control (untreated tumor-bearing mice). Statistical analysis, as compared to the respective control: Student's *t*-test, $n = 4$; $^{*}p \leqslant 0.05$.

Table 1. Blood GSH level (mg/ml) in tumor-bearing mice after cisplatin or BSO plus cisplatin treatment

Treatment	GSH (mean ± SD)
Normal	0.85 ± 0.09
Control	0.64 ± 0.10
Cisplatin (24 h)	0.57 ± 0.02
Cisplatin (48 h)	0.47 ± 0.06*
Cisplatin (72 h)	0.40 ± 0.01*
Cisplatin (96 h)	0.42 ± 0.08*
BSO + cisplatin (24 h)	0.45 ± 0.12
BSO + cisplatin (48 h)	0.42 ± 0.08*
BSO + cisplatin (72 h)	0.37 ± 0.06*
BSO + cisplatin (96 h)	0.39 ± 0.05*
Cysteine + cisplatin (24 h)	0.83 ± 0.03*

Normal = blood from the mice without tumor or treatment. Control = blood from the tumor-bearing mice without treatment. BSO = L-buthionine (*S*,*R*)-sulfoximine, (5 mmol/L/kg body weight) was given 8 h prior to cisplatin (8 mg/kg body weight). Cysteine (200 mg/kg body weight) was given 30 min prior to cisplatin. Student's *t*-test, $n = 3$–4, as compared to control: $^{*}p \leqslant 0.05$.

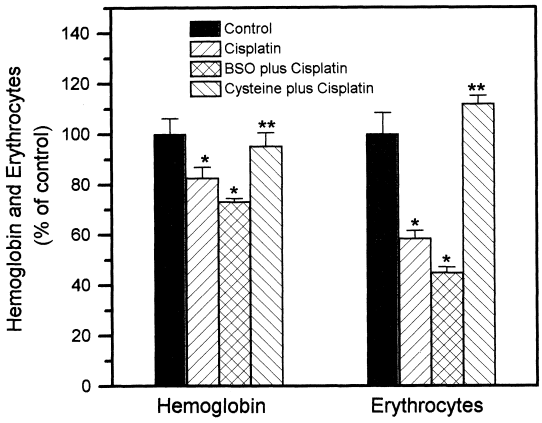

Figure 3. Comparative changes in blood hemoglobin (Hb) and erythrocytes (red blood cells) in tumor-bearing mice treated for 24 h with cisplatin alone, BSO plus cisplatin, or cysteine plus cisplatin. Treatment schedule for BSO and cysteine was the same as described in Table 1. C = control (untreated tumor-bearing mice). Statistical analysis, *as compared to the respective control; **as compared to BSO plus cisplatin treatment: Student's *t*-test, $n = 4$; $^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.05$.

Table 2. Hematological changes (mean ± SD) in the tumor-bearing mice following cisplatin, BSO plus cisplatin, and cysteine plus cisplatin treatment

Treatment	Hb (g/dl)	RBC ($\times 10^{12}$/L)	WBC ($\times 10^{9}$/L)	PCV (%)
Normal	14.74 ± 1.32	7.84 ± 0.59	5.56 ± 0.18	0.54 ± 0.02
Control	13.45 ± 0.84	6.84 ± 0.58	6.85 ± 0.16	0.52 ± 0.02
Cisplatin (24 h)	11.11 ± 0.12*	4.01 ± 0.20*	6.20 ± 0.19*	0.35 ± 0.03*
Cisplatin (48 h)	11.94 ± 0.44*	3.71 ± 0.59*	5.07 ± 0.20*	0.33 ± 0.01*
Cisplatin (72 h)	12.10 ± 0.76	4.15 ± 0.37*	3.21 ± 0.17*	0.38 ± 0.03*
Cisplatin (96 h)	12.75 ± 0.60	5.88 ± 0.96	2.86 ± 0.19*	0.42 ± 0.02*
BSO + cisplatin (24 h)	9.84 ± 0.56*	3.08 ± 0.18*	5.21 ± 0.20*	0.40 ± 0.02*
BSO + cisplatin (48 h)	9.39 ± 0.90*	3.06 ± 0.35*	4.60 ± 0.21*	0.31 ± 0.01*
BSO + cisplatin (72 h)	9.33 ± 0.52*	3.34 ± 0.23*	2.06 ± 0.19*	0.33 ± 0.01*
BSO + cisplatin (96 h)	9.44 ± 0.36*	3.60 ± 0.42*	1.01 ± 0.04*	0.37 ± 0.03*
Cysteine + cisplatin (24 h)	12.80 ± 0.21	7.65 ± 0.74	5.20 ± 0.76*	0.48 ± 0.04

Hb = hemoglobin; RBC = red blood cells; WBC = white blood cells; PCV = packed cell volume. Treatment schedule for BSO and cysteine was the same as described in Table 1. Student's *t*-test, $n = 4$, as compared to control (untreated tumor-bearing mice): *$p \leqslant 0.05$.

Table 3. Differential leukocytes counts (mean % ± SD) in the blood of tumor-bearing mice following cisplatin, BSO plus cisplatin, or cysteine plus cisplatin treatment

Treatment	Eosinophils	Neutrophils	Basophils	Lymphocytes	Monocytes
Normal	1.56 ± 0.16	16.53 ± 1.40	2.25 ± 0.41	75.54 ± 2.53	4.12 ± 0.61
Control	6.57 ± 1.54	65.80 ± 6.93	0.78 ± 0.13	26.24 ± 3.46	1.44 ± 0.17
Cisplatin (24 h)	5.04 ± 1.25	82.61 ± 2.89*	0.43 ± 0.03*	10.06 ± 0.56*	1.86 ± 0.14*
Cisplatin (48 h)	4.89 ± 0.98	78.14 ± 1.11*	0.34 ± 0.03*	14.67 ± 0.84*	1.96 ± 0.19*
Cisplatin (72 h)	6.32 ± 0.19	77.86 ± 5.96*	0.27 ± 0.08*	13.72 ± 1.47*	1.83 ± 0.28
Cisplatin (96 h)	6.77 ± 0.36	79.09 ± 2.10*	0.61 ± 0.03*	11.65 ± 0.78*	1.88 ± 0.36
BSO + cisplatin (24 h)	4.25 ± 0.55*	76.01 ± 6.86	0.58 ± 0.03*	17.13 ± 7.18*	2.03 ± 0.45*
BSO + cisplatin (48 h)	3.91 ± 0.46*	75.84 ± 1.20*	0.50 ± 0.02*	18.06 ± 1.99*	1.69 ± 0.12
BSO + cisplatin (72 h)	4.63 ± 0.84	71.13 ± 0.44	0.40 ± 0.01*	21.82 ± 2.21	2.02 ± 0.19*
BSO + cisplatin (96 h)	4.16 ± 0.91*	73.14 ± 0.75	0.45 ± 0.09*	20.85 ± 0.95*	1.40 ± 0.10
Cysteine + cisplatin (24 h)	1.86 ± 0.31*	81.42 ± 2.10	0.58 ± 0.08*	13.61 ± 2.42*	2.53 ± 0.97*

Treatment schedule for BSO, cysteine, and cisplatin was the same as described in Table 1. Student's *t*-test, $n = 4$, as compared to respective control (untreated tumor-bearing mice): *$p \leqslant 0.05$.

3). Eosinophils decreased slightly during 24–48 h of cisplatin treatment but recovered during later periods (72–96 h). Except for eosinophils and lymphocytes, an improvement in the number of different leukocytes was noted in the mice treated with cysteine plus cisplatin (Table 3).

Scanning electron microscopic observations revealed the development of various types of cisplatin-induced morphological abnormalities in erythrocytes. These include the microcytes, acanthocytes, scalloping, shistocytes, stomatocytes, ovalocytes, elliptocytes, echinocytes, spherocytes, and burr cells (Figure 4). The number of normal erythrocytes decreased significantly after cisplatin and BSO plus cisplatin treatment (Table 4). Of the various types of observed morphologically abnormal erythrocytes, the frequencies of elliptocytes (about 30%), echinocytes (about 20%), and acanthocytes (about 10%) were the more prominent (Table 4).

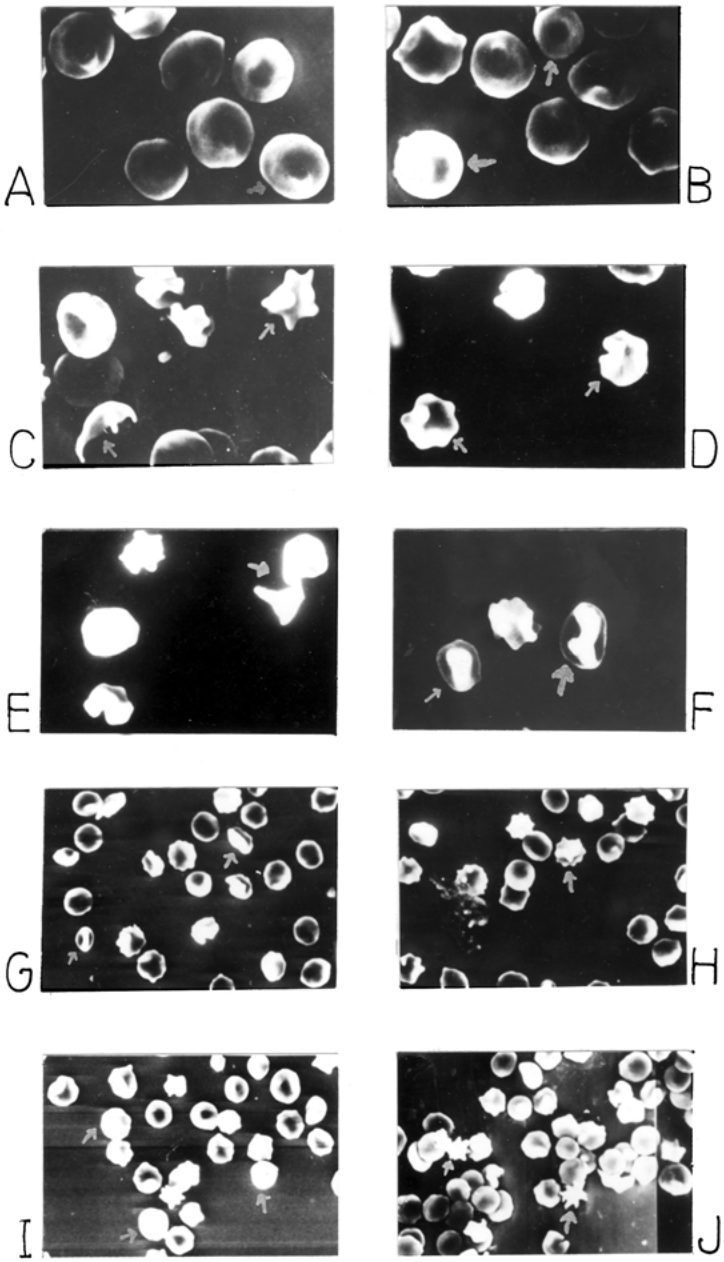

Figure 4. Scanning electron microscopic images of erythrocytes represent various types of morphologically abnormal erythrocytes induced by cisplatin. (A) Normal control erythrocytes or normocytes (having smooth surface and biconcave in shape, arrow). (B) Microcytes and macrocytes (having lesser or larger diameter than the normocytes, arrows). (C) Acanthocytes (showing few horn-like projections over the surface, arrow). (D) Scalloping type (with folded membrane, arrow). (E) Shistocytes (showing damaged and fragmented membrane, arrow). (F) Stomatocytes (showing a slot-like structure at the centre of the cell, arrow). (G) Ovalocytes (cells with oval shape, arrows) and elliptocytes (cells having an elliptical shape, arrow). (H) Echinocytes (cell showing serrated-projections distributed evenly over the cell surface, arrow). (I) Spherocytes (cells having spherical shape and having lost their biconcave nature, arrow). (J) Burr cells (showing spiny projections over the surface, arrows). Original magnification: (A–F) $\times 2000$; (G–J) $\times 1100$.

Table 4. Cisplatin-mediated development of various morphological abnormalities (mean %) in the erythrocytes

Treatment	Normocytes	Macrocytes	Microcytes	Spherocytes	Stomatocytes	Shistocytes	Acanthocytes	Echinocytes	Ovalocytes	Elliptocytes	Scalloping	Burr cells
Normal	92.6 (5.34)	0.10 (0.01)	–	0.10 (0.01)	–	1.0 (0.21)	1.0 (0.016)	1.0 (0.08)	0.1 (0.01)	0.10 (0.01)	4.00 (0.20)	–
Control	82.8 (6.16)	0.10 (0.01)	0.20 (0.01)	0.10 (0.01)	0.20 (0.01)	0.30 (0.01)	2.70 (0.24)	3.80 (0.16)	0.30 (0.01)	8.20 (0.25)	0.10 (0.02)	1.20 (0.20)
CP (24 h)	52.6 (2.13)*	3.50 (0.40)*	6.20 (0.02)*	0.32 (0.02)*	3.50 (0.40)*	0.32 (0.02)	7.80 (0.20)*	20.60 (0.49)*	0.98 (0.05)*	0.98 (0.04)*	1.30 (0.45)*	1.90 (0.32)
CP (48 h)	44.0 (3.43)*	0.40 (0.02)*	1.24 (0.16)*	0.43 (0.04)*	0.33 (0.02)*	0.80 (0.02)*	9.72 (0.33)*	32.0 (2.05)*	0.43 (0.04)*	4.68 (0.12)*	1.70 (0.24)*	4.27 (0.78)*
CP (72 h)	58.6 (1.47)*	1.08 (0.01)*	0.19 (0.02)	0.17 (0.01)*	0.18 (0.01)	0.16 (0.01)*	1.44 (0.36)*	3.06 (0.25)*	1.26 (0.28)*	31.30 (0.90)*	1.08 (0.06)*	1.48 (0.24)
CP (96 h)	72.0 (6.53)	0.22 (0.02)*	0.88 (0.01)*	0.22 (0.02)*	0.22 (0.02)	0.88 (0.04)*	3.74 (0.33)*	13.92 (0.53)*	0.22 (0.02)*	1.54 (0.28)*	0.44 (0.02)*	5.72 (0.41)*
BSO + CP (24 h)	35.6 (3.92)*	2.49 (0.20)*	1.49 (0.09)*	0.16 (0.02)*	0.69 (0.04)*	1.66 (0.32)*	4.48 (0.40)*	9.16 (0.46)*	1.66 (0.32)*	39.10 (1.63)*	0.16 (0.02)*	3.35 (0.28)*
BSO + CP (48 h)	49.00 (2.61)*	3.60 (0.32)*	3.00 (0.40)*	0.68 (0.04)*	0.34 (0.04)*	0.68 (0.08)*	7.10 (0.28)*	10.00 (0.81)*	1.70 (0.41)*	17.10 (0.51)*	0.34 (0.02)*	6.46 (0.40)*
BSO + CP (72 h)	52.52 (6.32)*	2.69 (0.32)*	0.50 (0.04)*	0.16 (0.02)*	0.50 (0.02)*	1.30 (0.24)*	6.00 (0.32*)	12.00 (0.81)*	1.80 (0.16)*	19.00 (1.23)*	0.33 (0.03)*	3.20 (0.37)*
BSO + CP) (96 h	59.65 (1.75)*	0.21 (0.02)*	1.00 (0.16)*	0.63 (0.03)*	1.47 (0.39)*	0.84 (0.04)*	4.80 (0.20)*	10.00 (0.40)*	1.47 (0.20)*	13.00 (0.52)*	0.63 (0.04)*	6.30 (0.37)*

CP = cisplatin; BSO = L-buthionine (*S*,*R*)-sulfoximine (5 mmol/kg body weight) was given 8 h prior to cisplatin (8 mg/kg body weight) treatment. Number in parentheses shows the SD of the respective mean value.

Student's *t*-test, $n = 3–4$, as compared to respective control: $*p \leqslant 0.05$.

Correlation of blood GSH levels and the frequency of normal and abnormal erythrocytes after cisplatin treatment revealed that as the blood GSH decreases, the number of normal erythrocytes also decreases but the average number of abnormal erythrocytes increases significantly (Figure 5). However, in the combination treatment of cysteine plus cisplatin when blood GSH level was increased, the morphological abnormalities in erythrocytes decreased (Figure 6).

Discussion

Glutathione (a tripeptide; L-γ-glutamyl-L-cysteinyl-glycine) is the most abundant non-protein thiol in cells. Under physiological conditions more than 98% of intracellular glutathione is maintained in reduced form (GSH) by glutathione reductase (Wang and Ballatori, 1998). GSH performs a variety of essential functions in mammalian cells including the detoxification of free radicals, metals, and other electrophilic compounds (Wang and Ballatori, 1998). It was observed that GSH levels in DL cells increased with tumor growth, reaching maximum on day 10 when they proliferated actively; thereafter, it decreased slightly over the next 4–5 days when the tumor growth rate should have declined, but still it was much higher than on day 5 (Figure 1A). During the growth of Ehrlich ascites tumor an increase in GSH level in tumor cells has also been reported, being maximum on approximately day 7 of tumor growth (Estrela et al., 1992). Cancer cells can generate large amounts of hydrogen peroxide, which may contribute to their ability to mutate and damage normal tissues and, moreover, facilitate tumor growth and invasion (Szatrowski and Nathan, 1991).

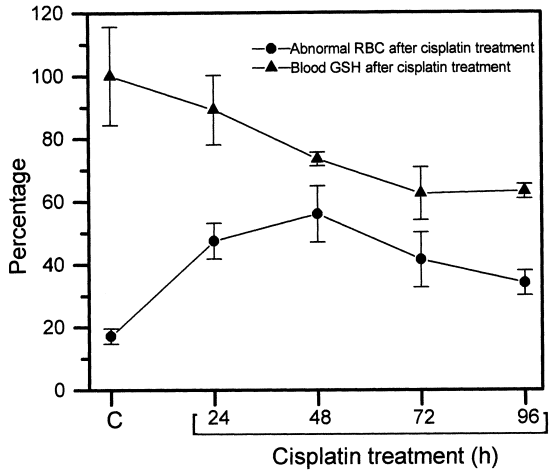

Figure 5. Percentage changes in abnormal RBC and total blood GSH level in tumor-bearing mice after cisplatin treatment. The overall relationship between the changes in these two parameters is inversely related (correlation coefficient, $r = -0.3692$), showing a statistically significance relationship ($r = -0.916$; $p < 0.05$) from control to 48 h of the treatment. C = control (untreated tumor-bearing mice).

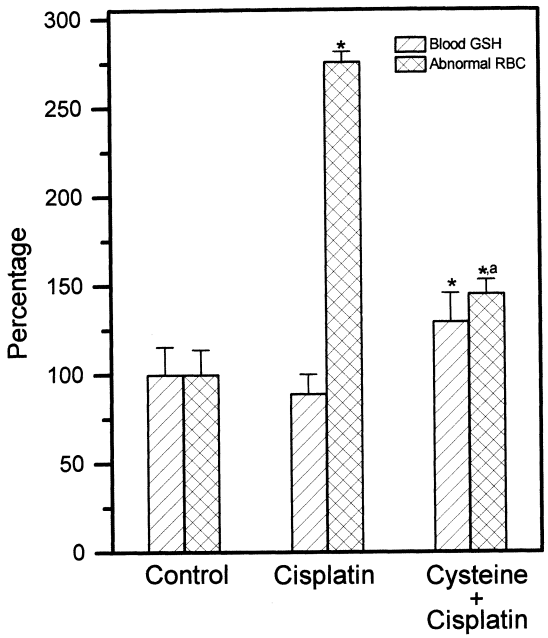

Figure 6. Changes in the frequency of morphological abnormalities in RBC in tumor-bearing mice under conditions of decreased blood GSH level (cisplatin treatment, 24 h) or restored blood GSH level (cysteine plus cisplatin treatment, 24 h). Statistical analysis, *as compared to respective control, [a]as compared to cisplatin treatment: Student's *t*-test, $n = 4$; $^{a}*p \leqslant 0.05$.

The resistance of many cells to oxidative stress is also associated with high intracellular levels of GSH (Estrela et al., 1995). The observed increase in GSH in the tumor cells may also suggest its involvement in facilitating proliferation and metabolism of tumor cells in the host. In fact, it has been reported that elevation of intracellular GSH in tumor cells is associated with mitogenic stimulation (Shaw and Chou, 1986) and GSH controls the onset of tumor cell proliferation by regulating protein kinase C activity and intracellular pH (Terradez et al., 1993). A decrease in the rate of cancer cell proliferation and a decrease of GSH level in the tumor has also been noted (Estrela et al., 1992). Thus, it may be suggested that the changes in the GSH level in DL cells with tumor growth in the mice may reflect the changes in the rate of tumor cell proliferation accompanied by changes in the antioxidant machinery.

Cisplatin treatment of tumor-bearing mice resulted a decrease in GSH levels in tumor cells initially (24–48 h), which was found to recover during the later periods of treatment (Figure 2A). The reasons for this variation of GSH level in tumor cells are not clearly understood, however, it may be that the initial cisplatin-mediated decrease of GSH in tumor cells weakens their antioxidant defense and facilitates their killing but some surviving cells try to strengthen their antioxidant defense by recovering/taking more GSH

In contrast to an increase of GSH in DL cells, a significant fall in blood GSH levels was observed during tumor growth (Figure 1B). However, as compared to the 10th day, blood GSH levels did not change during the 11th, 12th, 13th, 14th, and 15th days of tumor growth. Cisplatin treatment of tumor-bearing mice caused a decrease in blood GSH levels (Table 1). This fall in blood GSH in tumorous condition and cisplatin treatment may result in a decreased antioxidant capacity in these mice. Evidence for low blood GSH has been reported in a variety of diseases including diabetes (Murakami et al., 1989), HIV infection (Staal et al., 1992), cystic fibrosis (Roum et al., 1993), renal failure (Durak et al., 1994; Ross et al., 1997), and cancer (Della Rovere et al., 2000). Decreases in blood GSH may also be due to a decrease in available substrates for GSH synthesis (Navarro et al., 1999). Here also the decrease in blood glutathione levels with the progression of tumor growth could be due to an increased uptake of GSH amino acid substrates by tumor cells from the plasma. It has been reported from our laboratory that cisplatin treatment of tumor-bearing mice results in a decrease in protein concentration in many tissues (Prasad et al., 1999). This means that the availability of substrates (amino acids) for GSH may be affected and it should be decreased, resulting in the lower supply of GSH to erythrocytes in blood. Low levels of blood GSH may also result in impaired glutathione transport to other tissues. Earlier we have found cisplatin-mediated decrease of GSH in kidney, bone marrow, and DL cells (Prasad et al., 1998; Giri et al., 1998) which may be because of decreased synthesis and/or transport to these tissues.

Myelosuppression resulting in leukopenia and thrombocytopenia is a frequent and major complication of cancer chemotherapy (Hoagland, 1982). Thus, various hematological parameters are usually monitored during antineoplastic therapy (Doll and Weiss, 1983). Acute dose-dependent depression in leukocytes (leukopenia), erythrocytes (anemia), and platelets (thrombocytopenia) has been reported after treatment with cisplatin in Fischer 344 rats (Ohno et al., 1993). In the present study also we found that cisplatin treatment caused an inhibitory effect on the number of peripheral leukocytes, RBC, and hemoglobin in tumor-bearing mice (Table 2) and a correlation has been noted with cisplatin-mediated changes in blood GSH levels and various hematological parameters (Figure 3).

As compared to normal mice, hemoglobin and erythrocytes decreased slightly in tumor-bearing mice (Table 2), but cisplatin treatment of tumor-bearing mice caused a greater decrease in hemoglobin as well as erythrocytes (Table 2; Figure 3), which may be indicative of an anemic condition in these mice. Pal et al. (1993) have reported that the growth of Ehrlich ascites carcinoma in Swiss mice was accompanied by a decrease in hemoglobin and RBC values. This observation assumes significance as anemia is a common finding in malignancy (Price and Greenfield, 1958) and chemotherapy often aggravates the situation because of suppressive effect of majority of the anticancer agents on erythropoiesis (Doll and Weiss, 1983). The present observations indicate that cisplatin may also involve similar effects in the hosts.

It has been suggested that decreased blood antioxidant capacity, indicating impairment of erythrocyte antioxidant defenses, may contribute to increased erythrocyte fragility, decreased erythrocyte life span, and anemia (Durak et al., 1994). Blood glutathione is largely (about 99%) contained within erythrocytes (Michelet et al., 1995; Navarro et al., 1999) and decreased blood GSH level should mostly affect the erythrocytes. This suggestion of decreased antioxidant capacity and increased fragility in RBC is supported by the observation of development of various types of cisplatin-induced morphological abnormalities in erythrocytes (Figure 4; Table 4) that indicate the significance of GSH in RBC. Interestingly, with the decrease in blood GSH levels, the percentage of abnormal erythrocytes increases significantly (Figures 5 and 6), which suggests that blood glutathione levels and the development of various cisplatin-induced abnormalities in erythrocytes are inversely related. Erythrocytes are anucleated cells in which DNA, the molecular target of cisplatin action, is lacking. Moreover, the observed increase in percentage of abnormal erythrocytes with the decrease in GSH levels may suggest that glutathione could be one of the target molecules of cisplatin action in erythrocytes. Olas and Wachowicz (1998) have also reported that the intracellular level of GSH plays an important role in the action of cisplatin on blood platelets.

To understand this view clearly, a selective GSH-depleting agent, BSO, was also used with cisplatin and showed that most of the hematological changes induced by cisplatin alone were further aggravated in these treatments (Tables 1–4), which should be because of greater decrease of blood GSH. However, when the GSH levels were increased in the combination treatment with cysteine (a precursor for GSH synthesis), a consistent rise in hemoglobin as well as RBC was noted (Tables 1 and 2; Figure 3) with a decrease in morphological abnormalities in RBC (Figure 6). The possible mechanisms of cisplatin-mediated changes in GSH levels cannot be explained from the present results, but it may also involve, at least partly, changes in some glutathione-related enzymes. We have noted a cisplatin-mediated decrease in glutathione *S*-transferase, catalase, and gutathione peroxidase activity in liver and DL cells (unpublished results). Cisplatin–glutathione conjugates have been shown to be formed intracellularly and actively pumped out of the cell by the MRP/GSx pump (Ishikawa and Ali-Osman, 1993). Thus, the observed decrease in blood GSH should facilitate more uptake of cisplatin in the cells, thereby resulting in abnormalities in erythrocytes and other hematological features. Ohno et al. (1993) reported that carboplatin- and cisplatin-mediated hematological toxicities were significantly enhanced by whole-body hyperthermia (WBH) and suggested that increased drug uptake may be associated with the WBH-induced increase in hematotoxicity caused by cisplatin.

Thus, our study demonstrates that changes in blood GSH levels should be one of the main

determining factors in cisplatin-induced hematotoxicity in the host during cancer chemotherapy.

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