# Sex dimorphism in antitumor response of chemotherapeutic drug cisplatin in a murine host-bearing a T-cell lymphoma

Vivekanand Gupta and Sukh Mahendra Singh

**Previously we have demonstrated that in-vivo growth of a murine T-cell lymphoma of spontaneous origin designated as Dalton's lymphoma (DL) shows sex dimorphism (*J Rep Immunol* 2005; 65:17–32). It remained unclear, however, if DL growth in female and male tumor-bearing hosts also shows a sex-dependent differential susceptibility to the antitumor action of cancer chemotherapeutic drugs. In this study we have demonstrated that in-vivo administration of anticancer drugs: cisplatin or doxorubicin to the DL-bearing host results in a sex-dependent different antitumor activity of the drugs, causing a sex dimorphism in the antitumor response of the drugs with respect to tumor growth inhibition. The antitumor effect of both drugs was found to be better in male tumor-bearing hosts compared with female tumor-bearing hosts. The study also shows that DL cells obtained from male and female tumor-bearing hosts display a differential growth response to following treatment with cisplatin *in vitro*. Cell growth regulatory proteins: interleukin-2, interferon-γ, tumor growth factor-β, p53, caspase-activated DNase, vascular endothelial growth factor, and interleukin-2 receptor were found to be involved in the observed sex-specific response of DL cells to the antitumor action of cisplatin. Moreover, gonadal hormones: androgen, estrogen, and their specific antagonists flutamide and tamoxifen were found to directly modulate the cytotoxicity of cisplatin against DL cells *in vitro*. This study, therefore, suggests for the first time that the efficacy of cancer chemotherapeutic may vary in a sex-specific manner in a host-bearing a T-cell lymphoma. *Anti-Cancer Drugs* 19:583–592 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Tumor growth has a complex relationship with the host's immune and endocrine systems [1–3]. Hormones and cytokines, of both host and tumor origin, have been shown to be involved in the regulation of tumor progression in either directions [4–6]. Nevertheless, the mechanism(s) of hormone-dependent differential regulation of tumor progression varies with the etiology of individual tumor clones as well as on the profile of hormones and cytokines present in the tumor microenvironment [6–8]. The existence of sex dimorphism and the associated mechanisms in the progressive growth of several types of tumors still, however, remains obscure.

Nearly 18% of the total human malignancies are of lymphocyte origin [9], and it is considered as one of the most complicated cancers for clinical management [10,11]. Very little information is, however, found in the literature regarding the effect of sex-specific hormones in the regulation of such lymphocytic tumors. Although existence of receptors for sex-specific hormones has been demonstrated on various lymphocytes [12], the effect of sex-specific hormones on the manifestation of sex dimorphism of tumor growth remains unclear to a large extent with respect to tumors of lymphocyte origin.

Using a murine model of a transplantable T-cell lymphoma of spontaneous origin designated as Dalton's lymphoma (DL), we have been investigating several aspects of host–tumor relationship. DL is a T-cell tumor that originated in thymus of DBA strain (H-$2^d$) of mice [13,14]. DL can grow in the form of either an ascitic or a solid tumor [15] and has been reported to possess chromosomal aberrations [16]. Like some lymphoma and leukemia of human origin, DL cells do not metastasize to other lymphoid organs [17]. During the course of our earlier investigation, we observed that the progression of the ascitic DL growth is rapid in syngenic BALB/c (H-$2^d$) mice, causing death of the host in a relatively short time. Furthermore, we have reported that DL growth is associated with thymus regression, modulation of macrophage activation, humoral, and T-cell-mediated immune responses associated with an alteration of TH1/TH2 cytokine balance [18–25].

Using this tumor model, we have recently reported that the growth of the DL showed sex dimorphism, which was found to be dependent on sex-specific hormones: testosterone, progesterone, prolactin, and cytokines: interleukin (IL)-4, IL-10, and tumor necrosis factor (TNF)-α leading to a sex-dependent differential survival

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of tumor cells [23,24]. This led us to hypothesize that the sex-dependent differential profile of tumor-growth-regulating hormones and cytokines may influence the therapeutic efficacy of chemotherapeutic drugs in a sex-dependent manner. Moreover, our survey of the literature revealed that there are no reports regarding the sex influence on the chemotherapeutic efficacy of anticancer drugs with respect to any malignancy of lymphocyte origin. Thus, in view of the aforesaid observations regarding the sex-specific differential regulation of the growth of DL, in this investigation we were interested to study if the chemotherapeutic action of antitumor drug cisplatin also displays a sex dimorphism in a DL-bearing host.

Cisplatin was used as a representative chemotherapeutic drug for understanding sex dimorphism of its antitumor activity, considering the wide spectrum of anticancer actions of cisplatin [26,27]. Nevertheless, cisplatin has also been reported to show therapeutic efficacy against hematological cancers [28,29]. We have also reported earlier the antitumor action of cisplatin against DL [30]. To the best of our knowledge, this is the first report of its kind to demonstrate that T-cell lymphoma shows a sex-specific differential susceptibility to the cytotoxic action of cisplatin. The study also attempts to understand the possible mechanisms underlying this phenomenon.

## Materials and methods

### Mice and tumor system

Pathogen-free inbred adult male and female mice of BALB/c (H-$2^d$) strain were used at 8–12 weeks of age. The mice received food and water *ad libitum* and were treated with utmost human care in an approved and certified animal room facility of the Banaras Hindu University at the Institute of Medical Sciences. DL is maintained in the ascitic form by serial transplantation in BALB/c mice or in an in-vitro cell culture system by serial passage as described earlier [23]. A stock of DL cells is also maintained in a cryopreserved state for reference purposes. In most of the experiments, the cells obtained from the ascetic fluid where the yield of DL cells is higher were used. In some in-vitro experiments as indicated elsewhere DL cells from the *in vitro* serially passaged stock for approximately 10 times were also used to remove the effect of hormones. Mice were transplanted intraperitoneally (i.p.), with $1.0 \times 10^5$ DL cells per mouse in 0.5 ml phosphate-buffered saline (PBS). Tumor cells were obtained from the tumor-bearing host on day 10 after DL transplantation.

### Reagents

Tissue culture medium RPMI-1640 and fetal calf serum were purchased from Hyclone (Logan, Utah, USA). MTT [3–(4,5–dimethylthiazol-2yl)-2,5diphenyl tetrazolium bromide] and most of the other chemicals were purchased from the Sigma Chemical Co. (St Louis, Missouri, USA). All the reagents used in the experiments were determined to be endotoxin-free by Limulus amoebocyte lysate assay (sensitivity limit: 0.1 ng/ml). Antibodies against IL-2, IL-2 receptor (IL-2R), interferon (IFN)-γ, vascular endothelial growth factor (VEGF), p53, caspase-activated DNase (CAD), tumor growth factor (TGF)-β, and β-actin were purchased from Imgenex (San Diego, California, USA) and Chemicon (Chandlers Ford, UK). Secondary antibodies conjugated to alkaline phosphatase were obtained from Bangalore Genie (Bangalore, India). BCIP/NBT was purchased from Amresco (Solon, Ohio, USA). All the cell cultures were carried out at 37°C in a $CO_2$ incubator (Sheldon, Cornelius, USA) having 5% $CO_2$ in air in a humified atmosphere in culture medium supplement with 20 mg/ml gentamycin, 100 μg/ml, streptomycin, and 100 IU penicillin (Himedia, Mumbai, India).

### Protocol for in-vivo treatment

Mice in a group of six each were transplanted i.p. with the DL ($1 \times 10^5$ cells/mouse in 0.5 ml of PBS). The DL cells were harvested from mice 10 days after the tumor transplantation. The mice were injected i.p. with 0.5 ml of PBS alone or containing cisplatin or doxorubicin in a single dose of 5 mg/kg body weight, 72 h after the transplantation of DL cells following a protocol standardized earlier in our laboratory [20,30].

### Study of tumor progression and survival of tumor-bearing mice

Tumor growth was monitored by measuring the increase of the body weight of control and experimental groups of DL-bearing mice, following a method described earlier [23]. DL-bearing male and female mice in a group of six each were administered PBS (0.5 ml) alone or containing cisplatin or doxorubicin (5 mg/kg body weight) i.p. 72 h after DL transplantation. Similarly, age- and weight-matched normal male and female mice were also administered PBS, cisplatin or doxorubicin (5 mg/kg body weight). Both normal and DL-bearing mice were weighed regularly to monitor tumor progression till day 20 after tumor transfer. Change in body weight of normal mice with or without cisplatin or doxorubicin administration over a period of 20 days was insignificant, with an initial decrease of less than 5% around day 10, which returned to normal by the 20th day (data not shown). The final weight change of normal mice was subtracted from the increase of the body weight of DL-bearing mice to indicate the final change in body weight. The percent increase in body weight was calculated as follows:

$$\text{Increase in body weight } (\%) = \frac{W_f - W_i \times 100}{W_i},$$

where $W_f$ = weight of mice on day 20 of tumor transplantation and $W_i$ = weight of mice on day 1 of tumor transplantation.

DL-bearing control or experimental mice were allowed to live under normal conditions until death.

#### Dalton's lymphoma cell survival assay

Tumor cell survival was assayed according to a method described earlier [30]. DL cells were seeded ($1.5 \times 10^5$ viable cells 200 µl/ml) in a 96-well tissue culture plate in medium alone or containing the indicated doses of hormones in the presence or absence of their antagonist with or without cisplatin for 72 h. Cell survival was measured by MTT assay, as described in the following section.

#### MTT assay

MTT assay was carried out to estimate cell survival, following a method described by Mossman [31]. MTT was dissolved in PBS at a concentration of 5.0 mg/ml. Fifty microliters of MTT solution was added to each well of the culture plate containing 200 µl medium and incubated at 37°C for 4 h. The plate was then centrifuged at 100*g* for 5 min at 4°C (Remi, New Delhi, India). The supernatant was then carefully removed without disturbing the dark blue formazan crystals. One hundred microliters of the dimethyl sulfoxide (99.7% v/v) was added to each well and mixed thoroughly to dissolve the formazan crystals. The plates were then read on a microplate reader (Labsystems, Helsinki, Finland) at a wavelength of 540 nm. Readings were presented as absorbance at 540 nm.

#### Morphological evaluation of apoptotic cells

The apoptotic DL cell population was enumerated by a method described earlier [19]. DL cell suspension was smeared on a slide and air-dried, fixed in methanol, stained with Wright staining solution, mounted in glycerin and analyzed under light microscope (Carl Zeiss, Gottingen, Germany) at × 450 magnification. Apoptotic cells were identified on the basis of morphological features that included contracted cell bodies, condensed, uniformly circumscribed, and densely stained chromatin; or membrane-bound apoptotic bodies containing one or more nuclear fragments. The percentage of apoptotic cells was determined by counting more than 300 cells in at least three separate microscopic fields.

#### Percent DNA fragmentation

Induction of apoptosis in DL cells was also confirmed by a quantitative determination of DNA fragmentation, following a method given by Sellins and Cohen [32] with slight modifications [19]. DL cells ($1.0 \times 10^6$ cells/ml) were lysed in 0.5 ml of 50.0 mmol/l Tris–Cl buffer, pH 7.4, containing 10 mmol/l EDTA and 0.2% (v/v) Triton X-100 and the fragmented DNA was separated from intact chromatin in a microfuge tube (labeled as B) by centrifugation at 13 000*g* at 4°C for 10 min. Supernatant containing the fragmented DNA was transferred to another microfuge tube (labeled as T). A volume of 0.5 ml of 25% trichloroacetic acid (TCA) was added to each T and B tube and vortexed vigorously. DNA was precipitated overnight at 4°C and collected at 13 000*g* at 4°C for 10 min. Supernatant was discarded and 80 µl of 5% TCA was added to each pellet. DNA was hydrolyzed by heating at 90°C for 15 min. At this stage a blank was included containing 80 µl of 5% TCA. Then, 160 µl of freshly prepared diphenylamine reagent (150 mg diphenylamine in 10 ml glacial acetic acid, 150 µl concentrated $H_2SO_4$ and 50 µl of acetaldehyde solution) was added and the tubes were allowed to stand overnight at room temperature to develop color. Of this colored solution, 100 µl was transferred to the wells of a 96-well flat-bottomed enzyme-linked immunosorbent assay (ELISA) plate (Greiner, Frickenhausen, Germany) and absorbance was measured at 600 nm in a microtiter ELISA plate reader (Labsystems). The percentage of DNA fragmentation was calculated as:

$$\text{DNA fragmentation } (\%) = [T/(T+B)] \times 100,$$

where $T$ = absorbance of fragmented DNA and $T + B$ = absorbance of total DNA.

#### Enzyme-linked immunosorbent assay for detection of tumor growth factor-β, interleukin-2, and interferon-γ in the ascitic fluid

A standard ELISA was performed to detect the presence of indicated proteins in the ascitic fluid following a method described earlier [30]. In brief, 96-well microtiter plates (Greiner) were coated with 10 µl of test samples containing 10 µg of protein and incubated overnight at 4°C. In negative control, text samples were not added to the wells of ELISA plate and plates were processed for subsequent steps in the same way as described for experimental sets. The plates were then washed with 0.15 mol/l PBS containing 0.1% (v/v) Tween-20 (PBS–Tween). Unbound sites were saturated with PBS containing 1% bovine serum albumin. The plates were again washed with PBS–Tween followed by addition of 50 µl of antibodies against the indicated proteins at a concentration of 20 µg/ml. The plates were incubated at 37°C for 60 min followed by washing and incubation with 50 µl of secondary antibodies, conjugated with alkaline phosphatase, at a concentration of 4 µg/ml. The plates were then incubated at 37°C for 60 min followed by addition of 50 µl of *p*-nitrophenyl phosphate (1 mg/ml) in enzyme substrate buffer. The absorbance was read after 10 min at 405 nm in an ELISA plate reader (Labsystems). Data are represented as absorbance at 405 nm. ELISA for cytokines was compared with standard preparations of the respective cytokines obtained from the National Institute for Biological Standards and Control (Potters Bar, UK).

#### Western immunoblot analysis

Samples of cell lysates were processed for western blotting following a method described earlier [19]. Cells after washing with PBS were lysed 50 µl lysis buffer

(20 mmol/l Tris–Cl, pH 8.0, 137 mmol/l NaCl, 10% v/v glycerol, 1% v/v Triton X-100, 2 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 20 μmol/l leupeptin containing aprotinin at 0.15 U/ml) for 20 min at 4°C. Protein content in each sample was determined by standard Bradford method [33] and the samples thus prepared were heated to 100°C for 3 min on a boiling water bath in 1 × sodium dodecyl sulfate (SDS) gel-loading buffer [0.5 mol/l Tris–Cl (pH 6.8), 100 mmol/l β-mecaptoethanol, 20% SDS, 0.1% bromophenol blue, and 10% glycerol]. Thirty micrograms of Triton X-100 solubilized proteins was resolved on a 10% SDS–polyacrylamide slab gel at 20 mA in Tris–glycine electrophoresis buffer [25 mmol/l Tris–Cl, 250 mmol/l glycine (pH 8.3), and 20% SDS]. The separated proteins were transferred onto a nitrocellulose membrane (Sartorius, Gottingen, Germany) (8 h at 125 mA), immunoblotted with antibodies against p53, CAD, VEGF, IL-2R, and β-actin (2 μg/ml) and probed with a secondary antibody: anti-rabbit IgG conjugated to alkaline phosphatase (1 μg/ml) and detected by a BCIP/NBT solution (Amresco). Band intensities were determined by using Quantity One software (Bio-Rad, Regents Park, New South Wales, Australia). The immunoblots thus obtained were captured on a gel documentation image analysis system (Bio-Rad) and intensity of bands was analyzed by Quantity One software (Bio-Rad).

### Statistical analysis

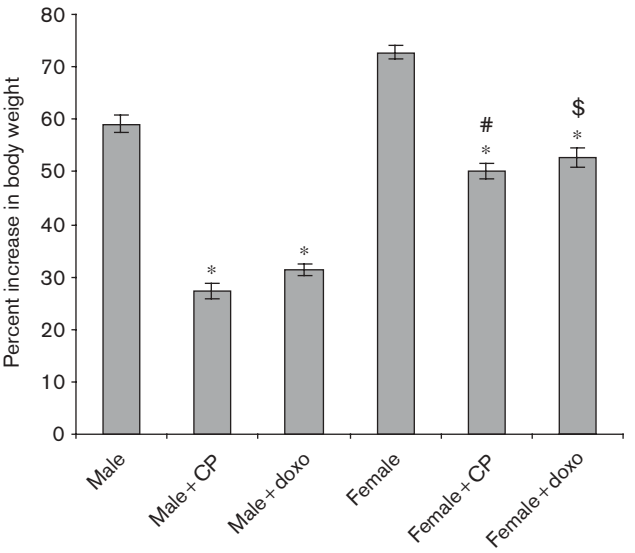
All experiments were conducted in triplicate at least three times. The statistical significance of differences between test groups was analyzed by one-way analysis of variance using the all pairwise multiple comparison procedure (Duncan's method). The level of variation in ELISA for cytokines was determined by calculating coefficient of variation, defined as the respective SD divided by the overall mean and expressed as a percentage.

## Results

### Sex-dependent tumor growth inhibition following in-vivo administration of cisplatin or doxorubicin

DL-bearing male and female mice in group of six each were administered with PBS alone or containing cisplatin or doxorubicin (5 mg/kg body weight in a single dose) as indicated in the Materials and methods followed by monitoring of tumor growth (Fig. 1) and the survival of the tumor-bearing host (Table 1). As shown in Fig. 1, a sex dimorphism was observed in the growth kinetics of the DL in male and female mice, with the female mice showing an accelerated tumor growth compared with that in the male mice. Administration of cisplatin or doxorubicin to both male and female tumor-bearing mice resulted in a significant tumor growth inhibition. The magnitude of the inhibition of tumor growth upon cisplatin/doxorubicin administration was, however, significantly higher in case of male tumor-bearing mice as compared with the female tumor-bearing mice. In a parallel set of experiments, the survival time of female and male tumor-bearing mice following PBS, cisplatin or doxorubicin administration was recorded (Table 1). The life span of male DL-bearing mice following cisplatin or doxorubicin administration was significantly prolonged compared with that observed in the case of female tumor-bearing mice.

**Fig. 1**




Therapeutic efficacy of cisplatin (CP) and doxorubicin (doxo) exhibits dependence on the sex of the tumor-bearing host. DL-bearing male and female mice in a group of six each were administered PBS (0.5 ml) alone or containing cisplatin or doxorubicin (5 mg/kg body weight) into the peritoneal cavity 72 h after DL transplantation. Similarly, age- and weight-matched normal male and female mice were also administered PBS, cisplatin, or doxorubicin (5 mg/kg body weight). Both normal and DL-bearing mice were weighed regularly to monitor tumor progression till day 20 after tumor transfer. Change in body weight of normal mice with or without cisplatin or doxorubicin treatment over a period of 20 days was insignificant (data not shown). The weight change of normal mice was subtracted from the increase of the body weight of DL-bearing mice and the mean of these values are shown ± SD. $^{*}P < 0.05$ vs. values of respective sex without cisplatin treatment. $^{\#}P < 0.05$ vs. values cisplatin-administered male tumor-bearing mice. $^{\$}P < 0.05$ vs. values cisplatin-administered female tumor-bearing mice. PBS, phosphate-buffered saline; DL, Dalton's lymphoma.

### Effect of in-vivo administration of cisplatin to male and female tumor-bearing mice on the survival of Dalton's lymphoma cells

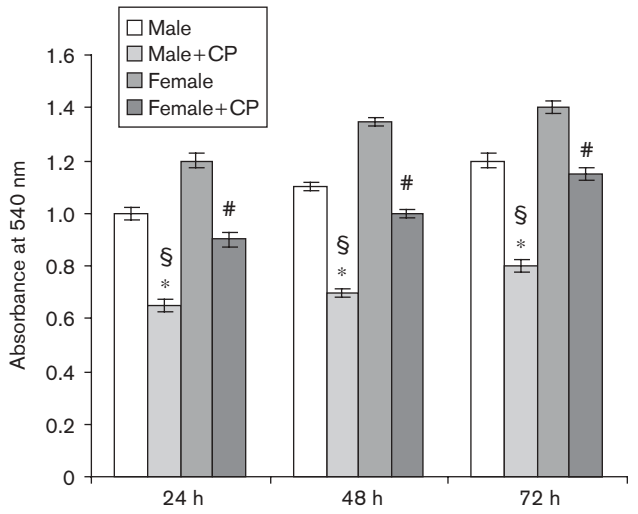
Viable DL cells ($1 \times 10^5$), obtained on the 10th day after tumor transplantation from male and female mice following in-vivo administration of PBS alone or containing cisplatin (5 mg/kg body weight), as described in Materials and methods, were incubated *in vitro* for the indicated time durations to estimate cell survival by MTT assay. Results are shown in Fig. 2. DL cells obtained from PBS-administrated or cisplatin-administrated male and female hosts showed a differential survival upon incubation *in vitro*. The survival of DL cells, however, obtained from male mice administered with cisplatin

**Table 1** **Sex dimorphism of cisplatin and doxorubicin in prolongation of survival of tumor-bearing hosts**[a]

| | Percentage of surviving mice ± SD[a] | | | | | |
|---|---|---|---|---|---|---|
| | Male tumor-bearing mice | | | Female tumor-bearing mice | | |
| Days following tumor transplantation | PBS | Cisplatin | Doxorubicin | PBS | Cisplatin | Doxorubicin |
| 24 | 50 ± 10 | 100 ± 00* | 100 ± 00* | 25 ± 05 | 100 ± 10* | 100 ± 10* |
| 27 | 25 ± 05 | 100 ± 05*# | 100 ± 05*# | 0 ± 0 | 75 ± 10* | 75 ± 10* |
| 29 | 0 ± 0 | 75 ± 10*# | 75 ± 10*# | 0 ± 0 | 50 ± 05* | 50 ± 05* |
| 33 | 0 ± 0 | 50 ± 05*# | 50 ± 05*# | 0 ± 0 | 15 ± 00* | 25 ± 00* |
| 36 | 0 ± 0 | 25 ± 00*# | 15 ± 00*# | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| 38 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |

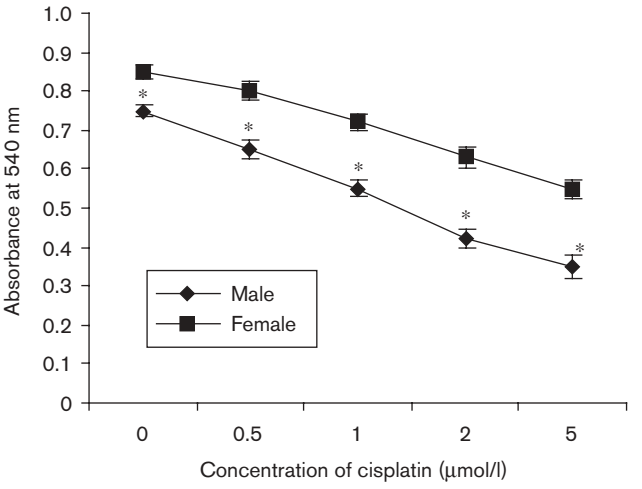
DL, Dalton's lymphoma; PBS, phosphate-buffered saline.
[a]DL-bearing male and female mice in a group of six each were administered PBS (0.5 ml), cisplatin, or doxorubicin (5 mg/kg body weight) into the peritoneal cavity on day 3 of DL transplantation as described in the legend of Fig. 1. The survival of DL-bearing mice was noted on a regular basis. The values are mean ± SD of triplicate experiments. *$P<0.05$ vs. values of respective sex without cisplatin treatment. #$P<0.05$ vs. values of cisplatin-administered male tumor bearing mice.

**Fig. 2**




Effect of in-vivo administration cisplatin (CP) to male and female tumor-bearing mice on the survival of DL cells *in vitro*. DL cells ($1 \times 10^5$ cells/well) obtained from cisplatin-administered (5 mg/kg body weight) male and female mice were incubated for the indicated time duration *in vitro* followed by estimation of cell survival by MTT assay, as described in Materials and methods. Cell survival was estimated by MTT assay, as described in Materials and methods. Values are mean ± SD of three independent experiments done in triplicate. *$P<0.05$ vs. values of DL cells obtained from PBS-administered male mice. #$P<0.05$ vs. values of DL cells obtained from PBS-administered female mice. §$P<0.05$ vs. values of DL cells obtained from female mice administered with cisplatin. PBS, phosphate-buffered saline; DL, Dalton's lymphoma.

**Fig. 3**




Effect of cisplatin treatment *in vitro* on the survival of DL cells obtained from male and female tumor-bearing mice. DL cells ($1 \times 10^5$ cells/well) obtained from male and female tumor-bearing mice following 10 days of tumor transplantation were cultured in medium alone or containing the indicated concentrations of cisplatin for 72 h. Cell proliferation was estimated by MTT assay, as described inMaterials and methods. Values are mean ± SD of three independent experiments done in triplicate. *$P<0.05$ vs. values of DL cells of female tumor-bearing mice.
DL, Dalton's lymphoma.

was significantly lower compared with that of DL cells obtained from cisplatin-administered female mice.

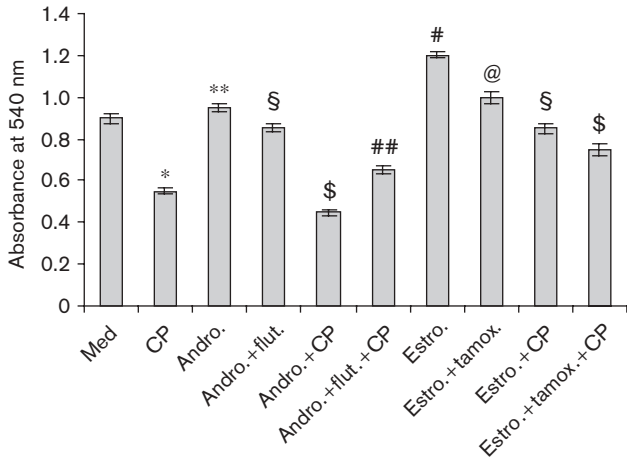
### Tumor cells obtained from male and female tumor-bearing mice showed a differential susceptibility to the cytotoxicity of cisplatin treatment *in vitro*

DL cells ($1 \times 10^5$) obtained from untreated male and female tumor-bearing mice were incubated *in vitro* in medium containing the indicated doses of cisplatin for 72 h followed by an estimation of the tumor cell survival by the MTT assay, as described in Materials and methods. Results are shown in Fig. 3. DL cells obtained from female tumor-bearing mice, following 10 days of DL transplantation, showed a significantly higher survival *in vitro* following incubation in medium alone or containing cisplatin. Treatment of the DL cells of male and female mice with cisplatin *in vitro* resulted in a dose-dependent inhibition of cell survival. The DL cells of female tumor-bearing mice showed a significantly higher survival at each of the doses of cisplatin treatment compared with that of the DL cells obtained from male mice. Nevertheless, the magnitude of inhibition between the no treatment group and the treatment group was observed to be higher in the DL cells of male mice (59.3%) compared with female mice (35.2%).

### Effect of androgen and estrogen antagonists on the survival of tumor cells treated *in vitro* with cisplatin in presence of gonadal hormones

DL cells ($1 \times 10^5$ cells/well) obtained from *in vitro* serially passaged stock were incubated in medium alone or containing the indicated concentration of androgen or estrogen in the presence or absence of cisplatin with or without antagonists of androgen and estrogen for 72 h. Results are shown in Fig. 4. Incubation of DL cells in medium containing cisplatin resulted in a significant inhibition in cell survival compared with those incubated in medium without cisplatin. Presence of androgen and estrogen in the culture medium resulted in a significant augmentation of cell survival, with estrogen showing a significantly higher augmentation in cell survival compared with that of the DL cells incubated in medium containing androgen. Incubation of DL cells in the presence of androgen and its antagonist flutamide resulted in a significant inhibition of cell survival compared with the cells incubated in medium containing androgen in the absence of flutamide. Similarly, incubation of DL cells in medium containing estrogen and its antagonist tamoxifen resulted in a significant inhibition of cell survival compared with the DL cells incubated in medium without antagonist containing estrogen without tamoxifen. The presence of cisplatin in the culture medium along with androgen or estrogen inhibited cell survival. The magnitude of inhibition was, however, more in case of cells incubated in medium containing cisplatin and androgen compared with that observed in medium containing estrogen and cisplatin, which was found to be significantly upregulated when the DL cells were incubated in medium containing androgen or estrogen and their respective antagonists along with cisplatin (Fig. 4).

**Fig. 4**




Effect of androgen (Andro.) and estrogen (Estro.) antagonist on the in-vitro survival of DL cells treated with cisplatin in the presence of gonadal hormones. DL cells ($1 \times 10^5$ cells/well) were incubated for 72 h in medium alone or containing cisplatin (5 μ/ml), androgen (1 μmol/l) in presence or absence of androgen antagonist flutamide (flut.;1 μmol/l), and estrogen (1 μmol/l) in the presence or absence of estrogen antagonist tamoxifen (tamox.; 1 μmol/l) for 72 h followed by estimation of cell survival by MTT assay, as described in the Materials and methods. Values shown are mean ± SD of three independent experiments done in triplicate. *$P<0.05$ vs. values of DL cells incubated in medium alone. **$P<0.05$ vs. values of DL cells incubated in medium alone or containing cisplatin. #$P<0.05$ vs. values of DL cells incubated in medium alone or containing cisplatin or androgen alone. §$P<0.05$ vs. values of DL cells incubated in medium containing androgen/estrogen or cisplatin alone or cisplatin and androgen or estrogen. $$P<0.05$ vs. values of DL cells incubated in medium containing androgen and cisplatin or estrogen and cisplatin. @$P<0.05$ vs. values of DL cells incubated in medium containing estrogen alone or estrogen and cisplatin. DL, Dalton's lymphoma.

### Sex dimorphism in the induction of apoptosis in tumor cells following in-vivo administration of cisplatin

DL cells obtained from male and female tumor-bearing hosts 10 days after tumor transplantation with or with out in-vivo administration of PBS alone or containing cisplatin, as described in Materials and methods, were enumerated under a microscope for the percentage of cells exhibiting of apoptotic morphology or processed for quantitative estimation of percent specific DNA fragmentation (Table 2). The percentage of apoptotic tumor cells was significantly higher in the tumor cell population obtained from male tumor-bearing mice administered with cisplatin compared with those obtained from the female tumor-bearing mice with cisplatin administration. Cisplatin administration to male tumor-bearing mice resulted in a maximum augmentation in the count of cells with apoptotic morphology. Similarly, DNA fragmentation was also found to be significantly higher in the tumor cell samples obtained from cisplatin-treated male tumor-bearing mice compared with those from cisplatin-treated female mice (Table 2).

### Immunodetection of tumor growth regulatory cytokines in the ascitic fluid of male and female tumor-bearing mice upon in-vivo treatment with cisplatin

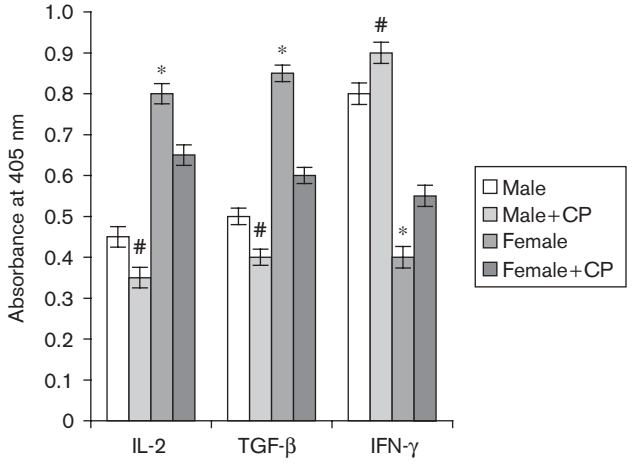
Ascitic fluid collected on day 10 after tumor transplantation from PBS or cisplatin-treated male and female tumor-bearing mice was check by ELISA for the presence of IL-2, TGF-β, and IFN-γ. Results are shown in Fig. 5. Cisplatin administration resulted in a significant augmentation in the ascitic fluid level of IFN-γ in both male and female mice, whereas cisplatin administration resulted in an inhibition in the level of TGF-β and IL-2

**Table 2 Sex dimorphism of tumor cell apoptosis in response in-vivo administration of cisplatin (CP)**

| Properties | Male | Male + CP | Female | Female + CP |
|---|---|---|---|---|
| Percent apoptotic cell count/mouse ± SD | 34.5 ± 0.1 | 38.4 ± 02* | 22.5 ± 0.15 | 28.5 ± 02 |
| Percent DNA fragmentation/ mouse ± SD | 47.5 ± 0.2 | 52.5 ± 0.2** | 32.2 ± 0.1 | 39.0 ± 0.2 |

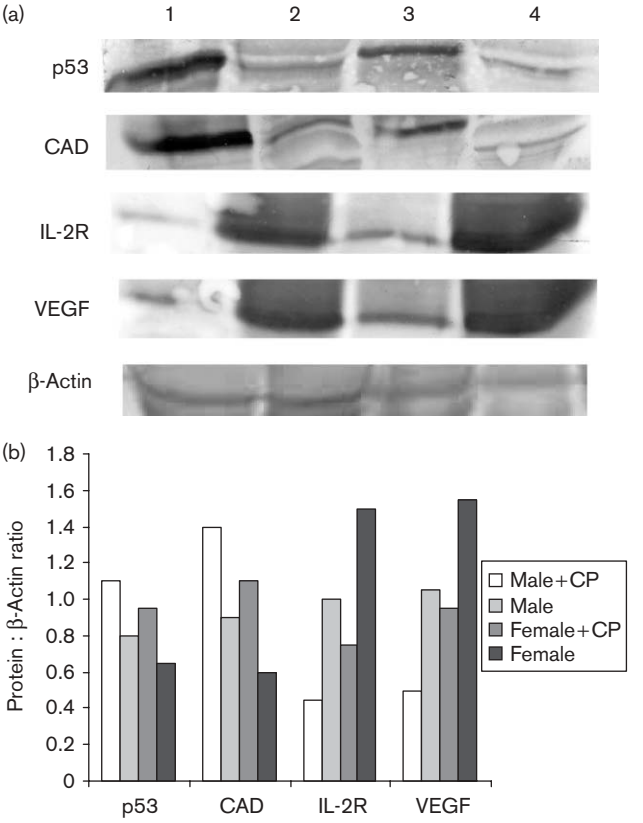
Values shown are mean ± SD of three independent experiments done in triplicate. *, **$P<0.05$ vs. values of cisplatin-treated female tumor-bearing mice.

**Fig. 5**




Immunodetection of IFN-γ, IL-2, and TGF-β in the ascitic fluid of cisplatin (CP)-administered male and female tumor-bearing mice by ELISA. Dalton's lymphoma ascitic fluid (DLAF) obtained from male and female tumor-bearing mice 10 days after tumor transplantation was plated in 96-well ELISA plate at a protein concentration of 10 μg/well and the presence of the indicated cytokines was detected by ELISA, as described in Materials and methods. The values shown are mean ± SD of three independent experiments done in triplicate. *$P<0.05$ vs. values of DLAF obtained from male tumor-bearing mice. #$P<0.05$ vs. values of DLAF obtained from cisplatin-treated female tumor-bearing mice. ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL-2, interleukin-2; TGF, tumor growth factor.

**Fig. 6**




Western blot analysis of DL cells obtained from cisplatin (CP)-administered male and female mice for expression of p53, CAD, IL-2R, VEGF, and β-Actin. (a) DL cell lysate ($1 \times 10^8$) obtained from male and female tumor-bearing mice 10 days after tumor transplantation with or without cisplatin treatment were resolved on SDS–PAGE and subjected to western blot analysis, as described in Materials and methods, for detecting expression of p53, CAD, IL-2R, VEGF, and β-Actin protein. Lane 1: DL cells of cisplatin-administered male mice; lane 2: DL cells of male mice; lane 3: DL cells of cisplatin-administered female; and lane 4: DL cells of female mice. The blot shown in (a) was scanned by a gel documentation system and the band size was analyzed by Quantity One software (Bio-Rad), as described in Materials and methods. Results are representative of three independent experiments with similar results. CAD, caspase-activated DNase; DL, Dalton's lymphoma; IL-2R, interleukin-2 receptor; VEGF, vascular endothelial growth factor.

that found in the ascitic fluid of female/male tumor-bearing mice without cisplatin administration.

### Sex-dependent modulation in the expression of tumor growth regulating proteins in Dalton's lymphoma cells following in-vivo administration of cisplatin

Lysate of DL cells ($1 \times 10^8$ cells) obtained from male and female tumor-bearing mice administered with PBS or containing cisplatin were immunoblotted for detecting the expression of p53, CAD, IL-2R, and VEGF proteins. The results are shown in Fig. 6a and b. The expression of p53 and CAD was found to be increased in the DL cell lysates of cisplatin-treated male mice compared with that in the DL cells of cisplatin-treated female mice. In contrast, the expression of IL-2R and VEGF was observed to be inhibited in DL cells obtained from cisplatin-administered both male and female mice. The inhibition was, however, more prominent in the case of DL cells of cisplatin-treated male mice compared with that observed in case of female mice.

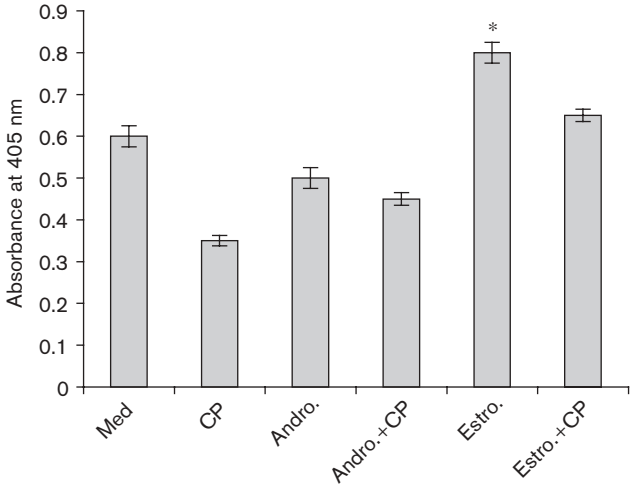
### Detection of interleukin-2 in the culture supernatant of Dalton's lymphoma cells treated with androgen, estrogen, and cisplatin *in vitro*

DL cells ($1 \times 10^6$/ml) from the serially passaged stock were treated *in vitro* in medium alone or containing androgen (1 μmol/l), estrogen (1 μmol/l), or cisplatin (5 μmol/l) for 72 h and cell-free culture supernatant was harvested and checked for presence of IL-2 by ELISA, as described in Materials and methods. Results are shown in Fig. 7. Treatment of DL cells *in vitro* with androgen had little effect on the production of IL-2 compared with the DL cells incubated in medium alone. In contrast, treatment of DL cells with estrogen resulted in a significant augmentation in the production of IL-2 compared with those incubated in medium containing estrogen and cisplatin.

## Discussion

The results of this study suggest that the progressive growth of a T-cell lymphoma in mice shows a differential

**Fig. 7**




Immunodetection of IL-2 in the DL-cell culture supernant. DL cells ($1 \times 10^6$ cells/well) were incubated in the medium alone or containing androgen (Andro; 1 μmol/l), estrogen (Estro; 1 μmol/l), or cisplatin (CP; 5 μmol/l) for 72 h, and the culture supernant was immunodetected by ELISA for the presence of IL-2, as described in Materials and methods. Values are mean ± SD of three independent experiments done in triplicate. $*P < 0.05$ vs. values for IL-2 in the culture supernant of untreated DL cells or those treated with cisplatin, androgen, cisplatin and androgen, and cisplatin and estrogen. ELISA, enzyme-linked immunosorbent assay; DL, Dalton's lymphoma; IL, interleukin.

susceptibility to the tumor response of the chemotherapeutic drug cisplatin, depending upon the sex of the tumor-bearing host. Cisplatin-administered male tumor-bearing mice showed a longer survival period compared with the cisplatin-administered female tumor-bearing mice. The precise mechanism(s) underlying the observed sex-dependent differential antitumor response of cisplatin on tumor growth remains unclear. Some possibilities were, however, considered. The possibility of a sex-dependent differential conditioning of tumor cells *in vivo* with respect to cell survival was investigated. Indeed, our results demonstrate that the tumor cells obtained from cisplatin-administered male and female mice showed a differential rate of survival and cell death *in vitro*.

The differential behavior of the DL cells obtained from male and female tumor-bearing mice subjected to in-vivo administration of cisplatin could possibly also be attributed to a sex-specific 'internal milieu' of cytokines and hormones, which in turn could condition the tumor cells to display a sex-dependent dimorphism in their susceptibility to the cytotoxicity of cisplatin. This hypothesis was corroborated by the observation that the ascitic fluid of cisplatin-administered male and female tumor-bearing mice showed a sex-specific differential level of tumor-growth regulating cytokines: IL-2, TGF-β, and IFN-γ with a higher level of IFN-γ along with low levels of IL-2 as compared with that in the ascitic fluid of female tumor-bearing mice. Previously we have demonstrated that IFN-γ and IL-2 play a key regulatory and antagonizing roles in the growth of DL [23,24]. Moreover, IFN-γ has been shown to augment tumoricidal action of macrophage [34] in addition to its direct antitumor action [35]. Nevertheless, TGF-β, which was found to be higher in the ascitic fluid of female tumor-bearing mice, has been reported to have upregulatory effect on the survival of lymphocytic cells [24]. These cytokines have also been demonstrated to differentially modulate the expression of cell cycle growth controlling proteins like p53, caspases, and VEGF [19,24]. This is also supported by the results of this study demonstrating that the DL cells obtained from cisplatin-treated male and female tumor-bearing mice showed a sex dimorphism in the expression of p53, CAD, IL-2R, and VEGF proteins. p53 and CAD were found to be higher in the DL cells of cisplatin-treated male mice, which could in turn be correlated to the increased level of apoptosis in these DL cells (Table 2) as compared with that in the DL cells of cisplatin-treated female mice. Thus, a sex-dependent differential expression of tumor growth regulatory proteins in DL cells may lead to a differential survival of DL cells in cisplatin-treated male and female mice as is also displayed by sex dimorphism of tumor-growth upon cisplatin administration. p53 and CAD proteins have been demonstrated to have inhibitory effects on the growth of tumor cells of T-lymphocyte origin [23,36–38]. IL-2R and VEGF indeed have tumor-growth-promoting action in a variety of tumor cells including several T-cell lymphomas and non-T-cell lymphomas [24,39,40]. VEGF and IL-2R have been reported to regulate the cell division and apoptosis in a variety of cells. Nevertheless, IL-2 and IL-2R are considered to be key regulators of T-lymphocyte proliferation [39,40]. Moreover, VEGF has been reported to augment the growth of several types of tumor cells and antagonize induction of apoptosis [41].

We also considered the role of gonadal hormones in the exhibited sex dimorphism with respect to the susceptibility of DL cells to the cytotoxic action of cisplatin. DL cells obtained from an in-vitro serially passaged stock (to remove the in-vivo influences of hormones on DL cells) when treated *in vitro* with cisplatin in the presence or absence of the male hormone androgen or female hormone estrogen showed a hormone-dependent differential susceptibility to the cytotoxic action of cisplatin. Androgen was observed to promote the cytotoxic action of cisplatin on DL cells, whereas estrogen did not alter the same to a similar magnitude. The direct tumor-growth-modulating action of these hormones was also supported by the experiments using hormone-specific antagonists, which led to a reversal in the promoting action of androgen in enhancing the cytotoxicity of cisplatin. Recent reports showed that gonadal hormones can modulate the cytotoxicity of chemotherapeutic agents on the testicular and breast cancer [42,43]. Moreover,

cisplatin itself can also upregulate or downregulate the expression of hormone receptors on cancer cells [44,45]. Some studies have also reported that antihormone drugs like tamoxifen influence the therapeutic efficacy of cisplatin [46]. Some studies have also shown that the anticancer action of cisplatin could involve the modulation of the expression of p53, TRAIL, and caspase proteins, whose expression can also be regulated by gonadal hormones [47–49]. Interestingly, we also observed that treatment of DL cells with cisplatin and androgen could downregulate the production of IL-2 by these cells (Fig. 7). Thus, the observed sex dimorphisms of DL growth could possibly result from a cooperation of cytokines and sex-specific hormones in differentially 'priming' the tumor cells for growth leading to a differential susceptibility to the antitumor action of cisplatin.

A recent preliminary study of Huang *et al.* [28] has shown that there exist population and sex differences toward cytotoxic action of chemotherapeutic drugs against Epstein–Barr virus-transformed B lymphoblastoid cell lines obtained from different races of the human population. The underlying mechanism, however, remained unclear. Our study, in contrast, provides novel information with respect to the molecular mechanism(s) of differential susceptibility of a T-cell lymphoma to the chemotherapeutic action of cisplatin indicating the roles of p53, CAD, VEGF, IL-2, IL-2R, IFN-γ, and TGF-β proteins along with the gonadal hormones androgen and estrogen. Nevertheless, the present investigation also indicates that sex dimorphism of the antitumor response is not only restricted to cisplatin but is also seen in case of doxorubicin, which has a distinct mode of antitumor action [50]. This suggests that the influence of the gonadal hormones and cytokine environment in the tumor-bearing host may cause a generalized sex dimorphism in the antitumor response of chemotherapeutic drugs irrespective of their modes of action. Thus, the results of the present investigation will be of immense clinical significance in designing sex-specific chemotherapeutic strategies for the treatment of T-cell malignancies.

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