Eur J Cancer Clin Oncol, Vol. 25, No. 3, pp. 429–437, 1989.
Printed in Great Britain

0277-5379/89$3.00 + 0.00

Mechanisms of Resistance to *Cis*-diamminedichloroplatinum (II) in a Rat Ovarian Carcinoma Cell Line

S. SEKIYA, T. OOSAKI, S. ANDOH, N. SUZUKI, M. AKABOSHI and H. TAKAMIZAWA

Department of Obstetrics and Gynceology, Second Department of Biochemistry, Chiba University School of Medicine, 1-8-1 *Inohana, Chiba* 280, *Japan and Research Reactor Institute, Kyoto University, Kumatori-cho, Sennan-gun, Osaka* 590-04, *Japan*

Abstract—*A* cis-*diamminedichloroplatinum(II) (cisplatin)-resistant subline (Cis-Pt*^{r}*) demonstated 20-fold greater resistance to the cytotoxic effects of cisplatin, compared with the parental cloned rat ovarian carcinoma cell line (ROT 68/C1). The uptake of cisplatin into the Cis-Pt*^{r} *cells was identical to that into the ROT68/C1 cells* in vitro *and* in vivo. *Glutathione activity in a cytoplasmic extract was 1.4-fold and 1.8-fold greater in the Cis-PT*^{r} *cells than in the ROT68/C1 cells* in vitro *and* in vivo, *respectively. There was no difference between the ROT68/C1 and Cis-PT*^{r} *cells in* ^{195m} *cisplatin binding per* μg *DNA. DNA repair of cisplatin DNA damage was increased in the Cis-PT*^{r} *cells but not in the ROT68/C1 cells. These results suggest that the mechanisms of resistance to cisplatin in rat ovarian carcinoma cells involve increased activity of the DNA repair system and increased cytosolic binding to thiols may also be involved.*

INTRODUCTION

DESPITE THE extremely high initial response rates of ovarian carcinoma to cisplatin, the 5-year survival rates have not improved primarily due to the frequent emergence of cisplatin resistance [1]. Although the mechanisms of cellular resistance to cisplatin are not completely understood, several possible mechanisms are proposed including decreased drug uptake, a high content of cisplatin-binding cytoplasmic thiols, reduction of DNA cross-linking and increased DNA repair [2]. Cisplatin resistance has been studied using several different cisplatin-resistant sublines derived from the cisplatin-sensitive parental cell lines *in vitro* [3]. The mechanisms of resistance to cisplatin are not unified and appear to vary among the cell lines. Therefore, we have recently developed a cisplatin-resistant subline (Cis-Pt^r) from a cisplatin-sensitive rat ovarian carcinoma cell line (ROT68/C1) using continuous exposure to increasing doses of cisplatin *in vitro* [3]. In this report, we examine the mechanisms of cisplatin resistance using these cell lines and present evidence which supports a multifactorial mechanism.

Accepted 28 September 1988.
Supported by a grant from the Ministry of Education, Science and Culture, Japan.

MATERIALS AND METHODS

Drugs

The solution of cisplatin in saline (0.5 mg/ml) was a gift from Nippon Kayaku Co. (Tokyo, Japan). ^{195m}Cisplatin with a specific activity of 203 μg/mg cisplatin and a half-life of 4.02 days was made at the Research Reactor Institute (Osaka, Japan). To produce ^{195m}platinum, a 10 mg sample of 95.06% enriched ^{194}platinum was irradiated in the hydraulic conveyor of KUR with a thermal neutron flux of approx. 8.15×10^{13} n.cm^{-2} for 75 h. ^{195m}Cisplatin was synthetized according to the method described in a previous paper [4]. Glutathione in a reduced form was purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Thymidine with a specific activity of 2 Ci/mole was purchased from the New England Nuclear Corp. (Boston, MA).

Cell line

A cisplatin-resistant subline (Cis-Pt^r) derived *in vitro* from a parental cloned rat ovarian adenocarcinoma cell line (ROT68/C1) was used [3]. The ROT68/C1 cells were tumorigenic to isologous Sprague–Dawley strain newborn rats [5] and showed sensitivity to the antitumor effect of cisplatin *in vitro* [6] and *in vivo* [7, 8]. Both cell lines grew as a monolayer in RPMI 1640 medium supplemented with antibiotics and 10% newborn bovine serum

(Flow Lab., New South Wales, Australia) in a humidified mixture of 5% carbon dioxide and 95% air at 37°C. The Cis-Pt^r cells were maintained with an intermittent exposure to 1 μg/ml of cisplatin and the 20-fold resistance of this subline to cisplatin remained stable for the period (data not shown).

Transplantation

Approximately 5×10^5 cells suspended in 0.2 ml of medium were inoculated subcutaneously into the interscapular region of newborn Sprague–Dawley strain rats (Clea Japan Inc., Tokyo) within 48 h after birth.

DNA histogram

Cells (10^6/25 cm^2 flask) were exposed to 1–10 μg/ml of cisplatin for 1 h, and harvested with 0.25% trypsin at 37°C. The cells were fixed in 50% ethanol and a DNA histogram was generated using the method previously described [9] with a SCS-1 flow cytometer (Nippon Bunkou, Tokyo).

Karyotypic analysis

A karyotype was generated using a standard method. Mitosis was arrested with colcemid and the cells underwent a KCl hypotonic treatment. Then they were fixed in acetic acid/methanol (1:3), dipped into 0.025% trypsin solution, rinsed in ethanol and stained with Giemsa.

Uptake of cisplatin

To evaluate *in vitro* cisplatin uptake, 10^8 cells grown in three 75 cm^2 flasks were incubated in a medium containing 10 μg/ml of cisplatin for appropriate exposure times at 37°C. The cells were then rinsed, harvested and centrifuged at 1000 rpm for 5 min to obtain a cell pellet. The cell pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) solution and disrupted in a sonicator, Handy Sonic Model UR-20P (Tomy Seiko Co., Ltd., Tokyo), at 20 kc for 1 min. After centrifugation at 10,000 rpm for 60 min the supernatant platinum content was determined using flameless atomic absorption spectrophotometry with a Hitachi Type 170-70 (Hitachi Ltd., Tokyo).

For *in vivo* cisplatin uptake, cisplatin at a dose of 4 mg/body was injected into tumor-bearing rats weighing approx. 200 g, at 8 weeks after intraperitoneal inoculation of the cells. At various intervals from 15 min to 24 h after drug injection, the tumors were resected and stored at −20°C. An assay of cisplatin levels in the tissues was done as previously described [7] using flameless atomic absorption spectophotometry.

Measurements of glutathione

Cells (10^8) in the exponential growth phase or tissues (0.5 g) were disrupted with a sonicator in 4 ml of ice-cold distilled water containing EDTA (pH 8). Protein was removed by cold centrifugation at 5000 ***g*** for 10 min after addition of 1 ml of 25% HPO_3. An aliquot of the supernatant fluid was then diluted with distilled water and the glutathione was measured according to the method of Cohn and Lyle [10] with a Shimazu Model RF 500 spectrophotofluorometer (Shimazu Corp., Tokyo). Fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm.

^{195m}Cisplatin DNA binding

Approximately 10^7 cells were suspended in 1 ml of medium and ^{195m}cisplatin was added to a final concentration of 1 μg/ml in a total volume of 2 ml. The culture tubes were promptly returned to the incubator (37°C). After various exposure times, the medium containing ^{195m}cisplatin was removed and washed with cold PBS following which the DNA was extracted by the method of Shibastani [11]. Radioactivity was determined with a Aloka TOC gamma counter (Aloka Co., Ltd., Tokyo). The concentration of DNA was determined by the method previously described [12].

Examination of unscheduled DNA synthesis

Aproximately 1.5×10^5 cells per 60 mm dish in the logarithmic phase of growth were washed in serum-free medium and incubated in medium containing various concentrations of cisplatin for 1 h at 37°C. Immediately after incubation, the cells were washed and re-incubated in pre-warmed medium containing [^{3}H]thymidine (2 μCi/ml) and 10 mM of hydroxyurea. After incubation for 2 h, the radioactive medium was removed. Levels of the unscheduled DNA synthesis were measured by the method previously reported [13] using a liquid scintillation spectrometry with a Beckman LS-235 scintillation spectrometer (Beckman, Fullerton, CA).

RESULTS

We previously reported that Cis-Pt^r were 20-fold more resistant to cisplatin cytotoxicity than the parental cell line (ROT68/C1), using drug concentrations required for 50% colony inhibition *in vitro* [3]. In the present study, we further confirmed the Cis-Pt^r cell cisplatin resistance by changes in the DNA histogram after exposure to cisplatin *in vitro*. As shown in Fig. 1, cell accumulation in the S phase was first observed in the ROT68/C1 cells 48 h after treatment with 1 μg/ml for 1 h. Cell accumulation in the G2-M phase and cell debris which suggested intense cytotoxicity of cisplatin [14, 15] was prominent in the ROT68/C1 cells after treatment with concentrations of 5 μg/ml or more, resulting in complete arrest of growth [3]. In contrast, changes in the DNA histogram were not prominent or long

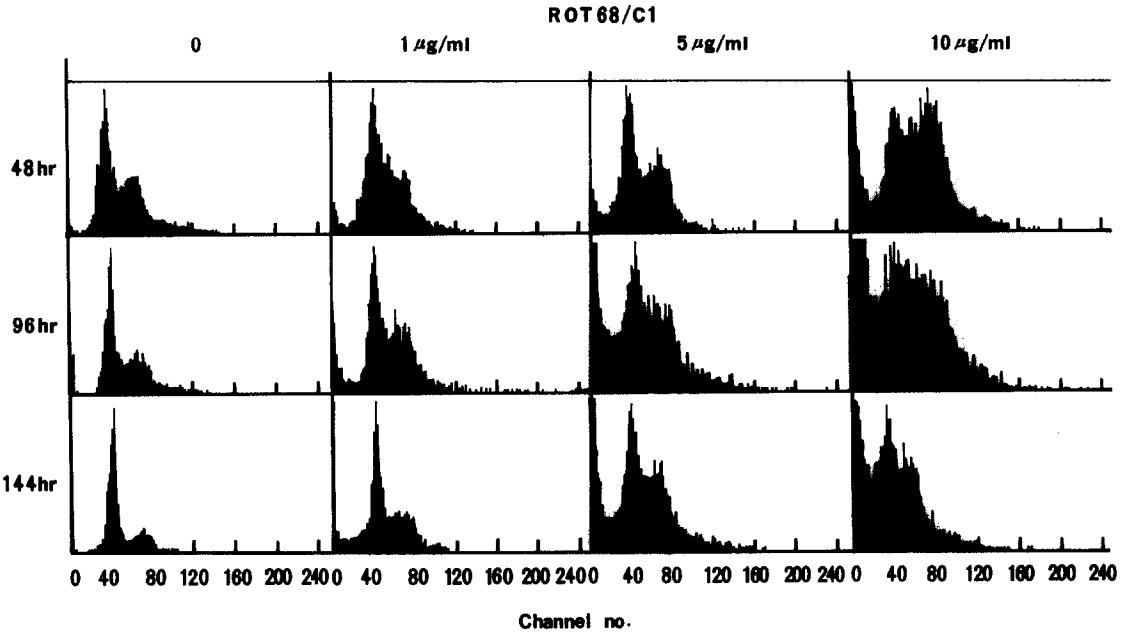

Fig. 1. DNA histogram of ROT68/C1 cells after treatment with various concentrations of cisplatin for 1 h *in vitro. The hours indicated along the left hand margin refer to the hours of incubation following cisplatin exposure. The primary peak position of diploid lymphocytes derived from a normal volunteer was adjusted to channel number* 40.

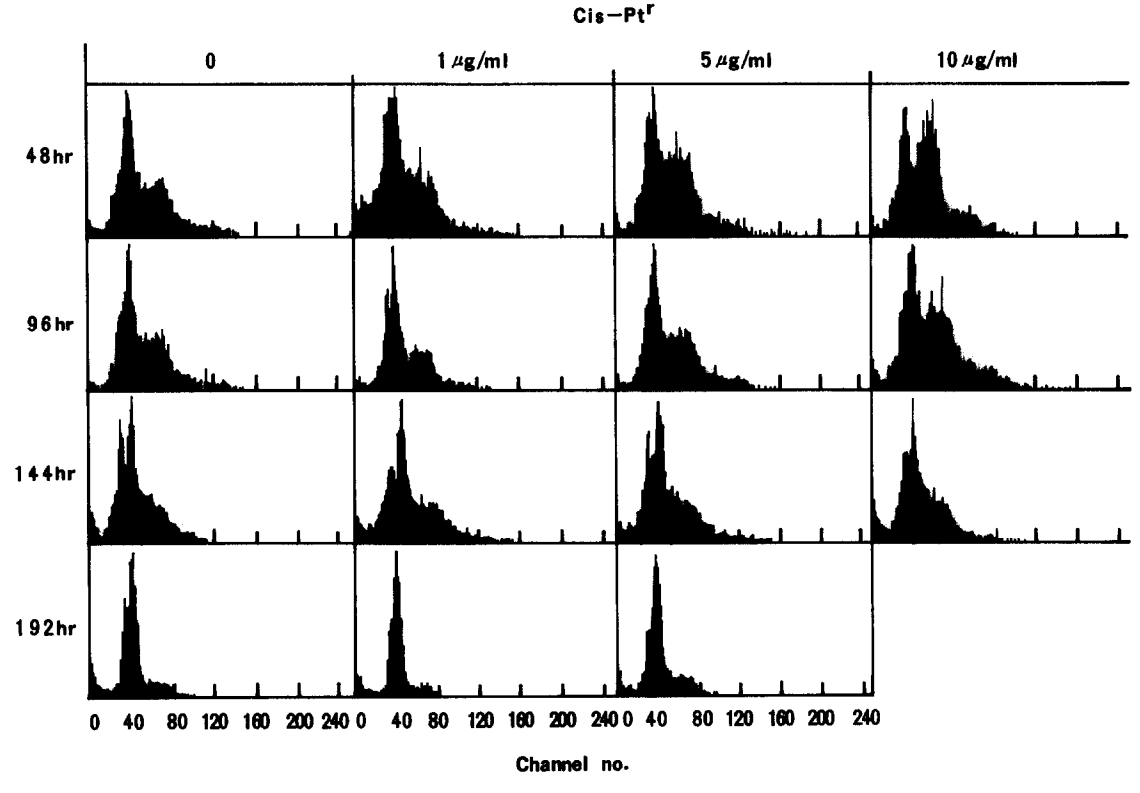

Fig. 2. DNA histogram of Cis-Pt^r cells after treatment with various concentrations of cisplatin for 1 h *in vitro.*

lasting in the Cis-Pt^r cells even when treated with 10 μg/ml for 1 h (Fig. 2), which resulted in only slight inhibition of growth [3].

The ROT68/C1 and Cis-Pt^r cells had modal chromosome numbers of 93–95 and 90–92 respectively, and many marker chromosomes were shared by both cell lines (Figs. 3 and 4). Abnormalities noted specifically in the Cis-Pt^r cells included the addition of a long arm of chromosome 13 (13q+) observed in all Cis-Pt^r cells (Fig. 4). Behrens *et al.* [16] also reported a characteristic 13p+ chromosome in a cisplatin-resistant human ovarian cancer cell subline. Double minute chromosomes were absent in both cell lines, as reported in other cisplatin-resistant cell lines [16, 17].

The first step in the exploration of cisplatin resistance was an examination of cisplatin uptake *in vitro* and *in vivo*. The uptake of concentrations of 10 μg/ml in the Cis-Pt^r cells and the ROT68/C1 cells was identical and there was a linear increase in cellular platinum content with increasing exposure times from 20 min to 4 h *in vitro* (Fig. 5). On the other hand, tissue platinum content after a single intraperitoneal injection of cisplatin (4 mg/body) *in vivo* was also similar in both Cis-Pt^r and ROT68/C1 cells (Fig. 6). There was a linear increase in tissue platinum content during the first hour and the content decreased gradually thereafter.

Endogenous glutathione interacts with cisplatin and binds such electrophiles to nucleophilic SH-containing compounds prior to the interaction with the DNA, and seems to reduce the cytotoxicity of cisplatin *in vitro* and *in vivo* [18]. Table 1 shows that the cytosolic glutathione content of the Cis-Pt^r cells was greater than that of the ROT68/C1 cells (1.4-fold *in vitro* and 1.8-fold *in vivo*).

Considerable evidence suggests that cisplatin exerts its cytotoxic effect via an interaction with the DNA [19]. Figure 7 illustrates the binding of ^{195m}cisplatin per μg DNA. The binding was similar in both cell lines.

Altered DNA repair activity after cytotoxic DNA cross linkage may also be related to the development of acquired resistance to cisplatin. Figure 8 illustrates cisplatin-induced unscheduled DNA synthesis in ROT68/C1 and Cis-Pt^r cells. Under the condition of inhibition of scheduled DNA synthesis by hydroxyurea, specific activities of cisplatin-induced tritium incorporation per μg DNA in Cis-Pt^r cells were evidently larger than those in ROT68/C1 cells, as well as the activities shown by the incorporation per 10^4 cells. Thus, the cisplatin-resistant Cis-Pt^r cells acquire resistance to cisplatin partly due to an increase in the activity of DNA repair systems.

DISCUSSION

Several studies using cisplatin-resistant sublines derived from a murine leukemia cell line L1210 *in vitro* demonstrated that a decrease in cisplatin uptake contributes to an acquired resistance to cisplatin [20, 21]. The absence of a transport defect in cisplatin resistance has been reported using human bladder carcinoma cell lines [22, 23], as well as in the present study using a rat ovarian carcinoma cell line. Thus, although a transport defect is a possibility, experimental discrepancies exist among the cisplatin-resistance cell lines and there is no simple relationship between cisplatin uptake and differential cytotoxicity.

Alterations in the intracellular metabolism of cisplatin can explain the development of cisplatin resistance. Elevation in cellular glutathione, a major nonprotein thiol, is a known characteristic of some cisplatin-resistant cell lines [22–26]. Our present study also supports this possibility. However further studies are necessary for confirmation, i.e. reversion of resistance to cisplatin by addition of buthionine sulfoximine [25].

Endogenously synthesized metallothioneins, a major source of protein thiols, are also increased in cisplatin-resistant cells [27, 28]. One role of these thiols in the modification of the cellular response to cisplatin is a possible component of resistance.

Cisplatin binds to DNA in a manner analogous to bifunctional alkylating agents and produces interstrand and intrastrand DNA cross-linkage. DNA cross-linkage correlates with cytotoxicity and interference with this process may be a mechanism of resistance [22–24, 29, 30]. Recently, it has been proposed than an adduct not detected by alkaline elution such as an intrastrand cross-linkage is responsible for the cytotoxicity of cisplatin [19, 30, 31]. The binding of ^{194m}cisplatin per μg DNA was similar in the cisplatin-sensitive parental ROT68/C1 cells and in the cisplatin-resistant Cis-Pt^r cells.

Increased activity of DNA repair systems in cell lines with acquired cisplatin resistance has been reported [23, 32]. In our study, unscheduled DNA synthesis increased in the cisplatin-resistant Cis-Pt^r cells but the parent cells showed no increased capacity to repair DNA damage.

In conclusion, mechanisms of resistance in our rat ovarian carcinoma cell line involve increased DNA repair activity. In addition, an increased endogenous thiol content and other undetermined factors appear to be involved.

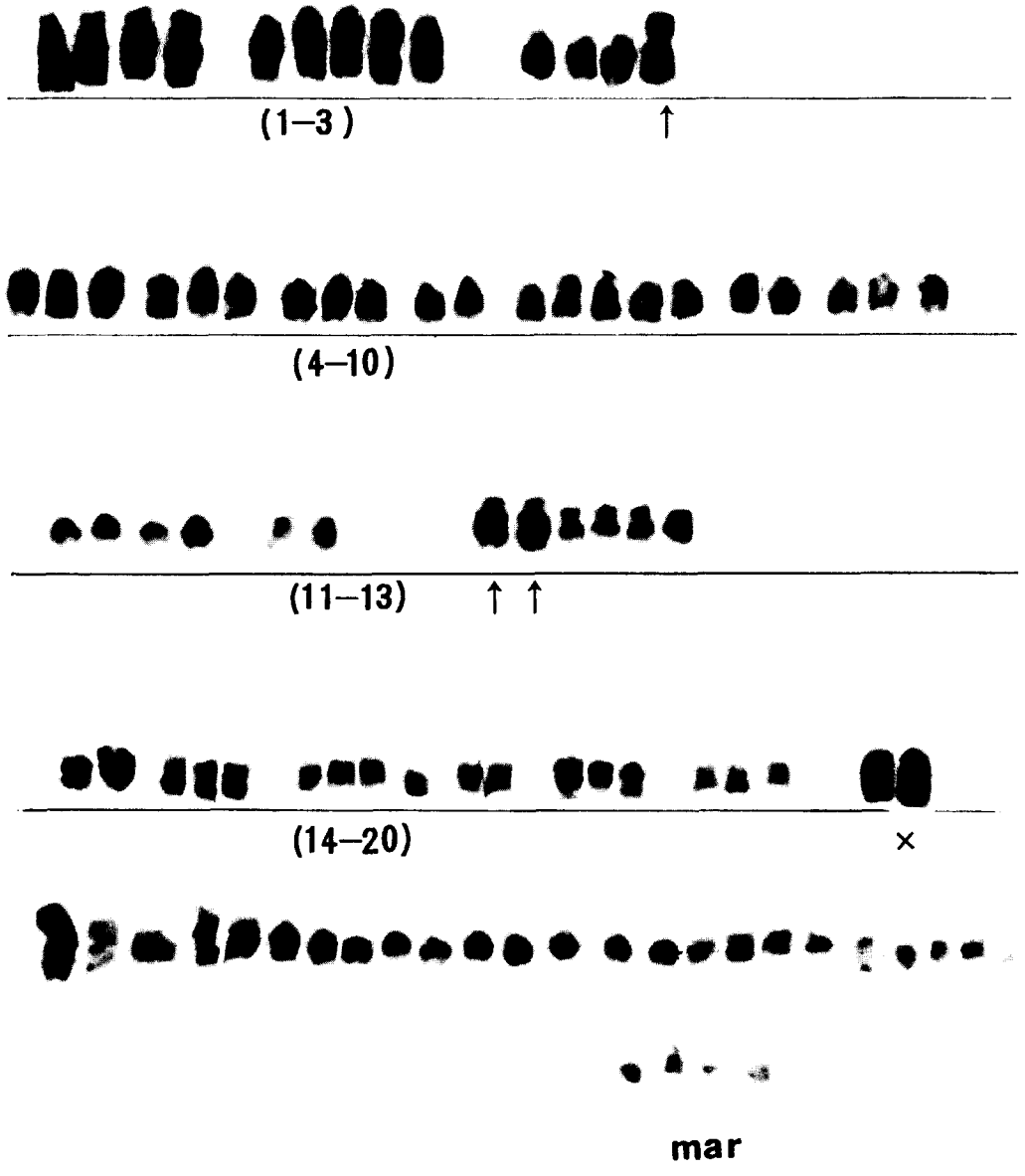

Fig. 3. G-banding pattern of metaphase chromosomes from ROT68/C1 cells. Arrows indicate abnormal chromosomes.

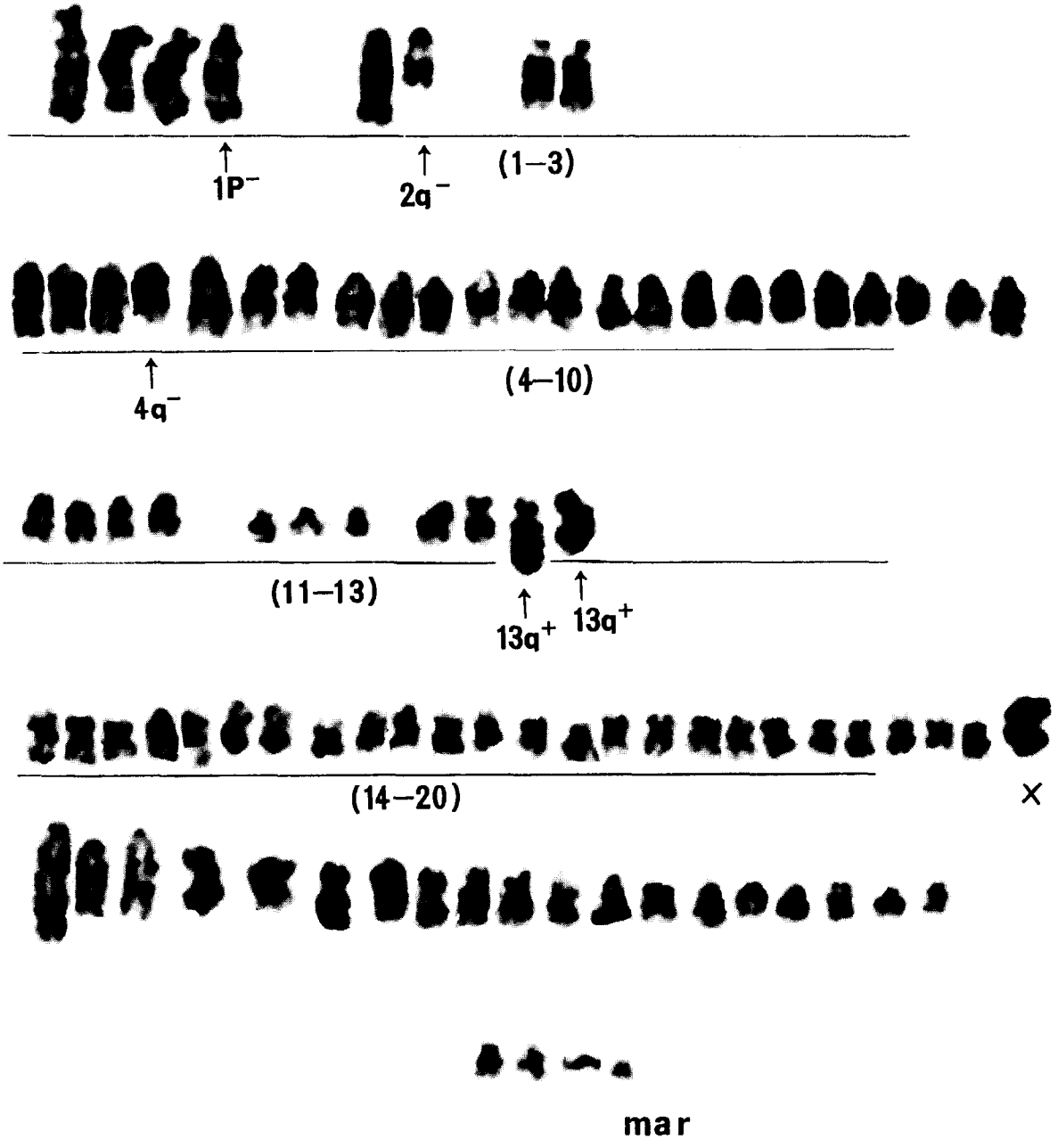

Fig. 4. G-banding pattern of metaphase chromosomes from Cis-Pt' cells.

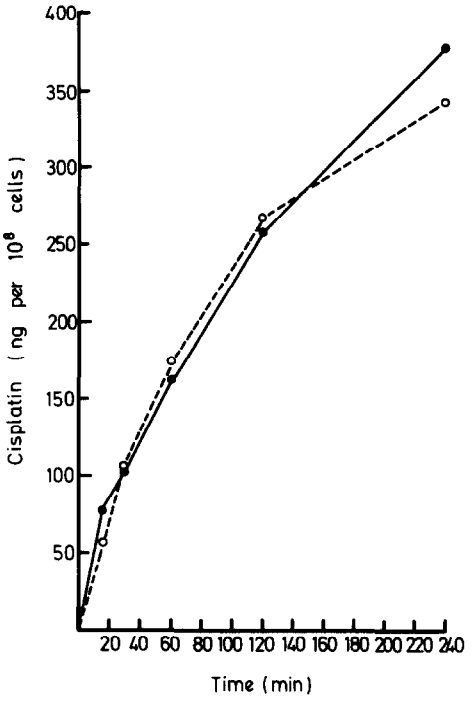

Fig. 5. Uptake of cisplatin into ROT68/C1 cells (●) and Cis-Pt^r cells (○) grown in medium containing 10 μg/ml *of cisplatin* in vitro. *Values are the mean of duplicates.*

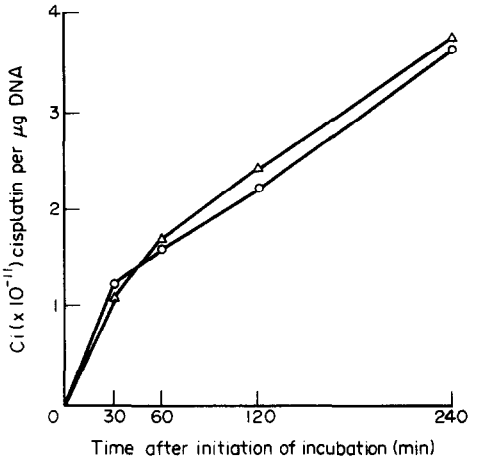

Fig. 7. Binding of ^{195m}cisplatin per μg *DNA (ROT68/C1, ●; Cis-P^r cells, ▲) after various exposure times to* 1 μg/ml. *See Materials and Methods for details. Values are the mean of duplicates.*

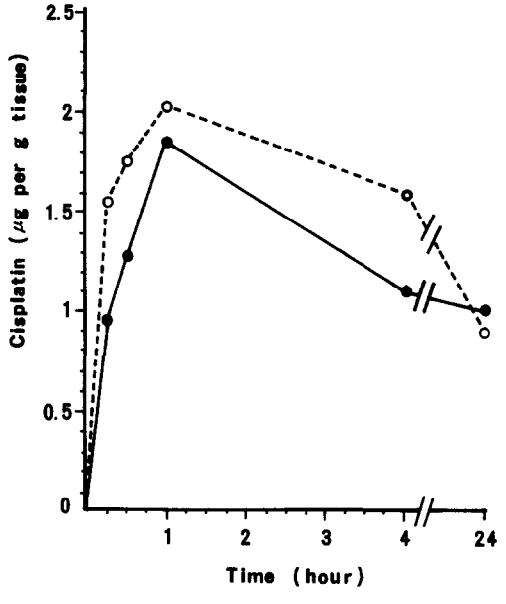

Fig. 6. Uptake of cisplatin into ROT68/C1 tissues (●) and Cis-Pt^r tissues (○) after a single intraperitoneal injection of 4 mg/*body cisplatin* in vivo. *Values are the mean of two or three samples.*

Table 1. Glutathione levels in ROT68/C1 and Cis-Pt^r cells in vitro *and* in vivo

Cell line	*In vitro* (μg/mg protein)	*In vivo* (μg/mg protein)
ROT68/C1	11.4*	16.8
Cis-Pt^r	15.6	30.6

*Values are the mean of duplicates.

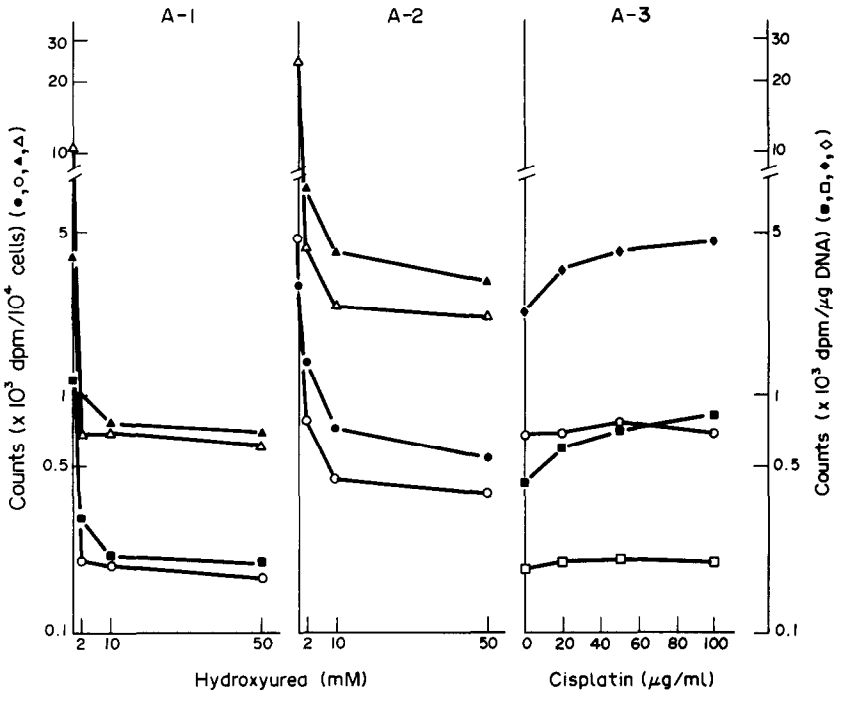

Fig. 8. Effect of hydroxyurea concentration on the incorporation of [^{3}H]*thymidine into* 50 μg/ml *cisplatin-treated* (●, ▲) *and non-treated* (○, △) *ROT68/C1 cells* (*A*-1) *and Cis-Pt^r cells* (*A*-2), *and effect of cisplatin concentration on the incorporation into* 10 mM *hydroxyurea-treated ROT/68C1* (□, ◇) *and Cis-Pt^r* (■, ◆) *cells* (*A*-3). *Cisplatin-treated and mock-treated cells were incubated for* 2 h *in the presence of* [^{3}H]*thymidine and hydroxyurea, and radioactivity counting was done as described in Materials and Methods. Values are the mean of four separate experiments.*

REFERENCES

1. Ozols RF, Young RC. Ovarian cancer. *Curr Probl Cancer* 1987, **11**, 57–122.
2. Eastman A, Richon VM. Mechanisms of cellular resistance to platinum coordination complexes. In: DCH McBrien, TF Slater, eds. *Biochemical Mechanisms of the Platinum Antitumor Drugs*. Oxford, IRL Press, 1986, 91–119.
3. Sekiya S, Oosaki T, Andoh S, Suzuki N, Nagao K, Takamizawa H. The isolation and characterization of cisplatin resistant rat ovarian cancer cells. *Eur J Cancer Clin Oncol* 1988, **24**, 851–859.
4. Akaboshi M, Kawai K, Maki H, Nakano Y. Improved method for the synthesis of radioactive *cis*-diamminedichloroplatinum(II) using high-performance liquid chromatography. *Ann Rep Reactor Inst,* Kyoto University, 1987, **20**, 150–154.
5. Sekiya S, Endoh N, Kikuchi Y *et al. In vivo* and *in vitro* studies of experimental ovarian adenocarcinoma in rats. *Cancer Res* 1979, **39**, 1108–1112.
6. Sekiya S, Iwasawa H, Yamauchi K, Kubota K, Takamizawa H. Effect of *cis*-dichlorodiammineplatinum(II) on cloned ovarian adenocarcinoma cells of the rat *in vitro*. *Gynecol Oncol* 1981, **12**, 14–22.
7. Sekiya S, Iwasawa H, Takamizawa H. *In vivo* studies on the antitumor effect of *cis*-dichlorodiammineplatinum(II) against transplantable ovarian adenocarcinoma cells of the rat. *Gynecol Oncol* 1983, **16**, 85–94.
8. Sekiya S, Iwasawa H, Takamizawa H. Comparison of the intraperitoneal and intravenous routes of cisplatin administration in an advanced ovarian cancer model of the rat. *Am J Obstet Gynecol* 1985, **153**, 106–111.
9. Sekiya S, Kawata M, Iwasawa H *et al.* Charaterization of human embryonal carcinoma cell lines derived from testicular germ-cell tumors. *Differentiation* 1985, **29**, 259–267.
10. Cohn VH, Lyle J. A fluorometric assay for glutathione. *Anal Biochem* 1966, **14**, 434–440.
11. Shibatani A. *In vitro* incorporation of ^{32}P into nucleic acids of lymphatic cells. Effects of X-irradiation and some other agents. *Exp Cell Res* 1959, **17**, 131–143.
12. Tomita KY, Kubota K, Kasai T, Sekiya S, Takamizawa H, Shimizu B. Detection of human papillomavirus DNA in genital warts, cervical dysplasias and neoplasias. *Intervirology* 1986, **25**, 151–157.
13. Suzuki N, Fuse A. A UV-sensitive human clonal cell line, RSa, which has low repair activity. *Mutation Res* 1981, **84**, 133–145.
14. Bergerat JP, Barlogie B, Gohde W, Johnston DA, Drewinko B. *In vitro* cytokinetic response of human colon cancer cells to *cis*-dichlorodiammineplatinum(II). *Cancer Res* 1979, **39**, 4356–4363.
15. Fujii M, Inuyama Y, Tanaka J, Takaoka T, Hosoda H. The synergistic effect mechanism of cisplatin and peplomycin therapy with special reference to the effect on cell cycle progression. *Keio J Med* 1986, **35**, 188–197.

16. Behrens BC, Hamilton TC, Masuda H *et al.* Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res* 1987, **47**, 414–418.
17. Frei III E, Cucchi CA, Rosowsky A *et al.* Alkylating agent resistance: *in vitro* studies with human cell lines. *Proc Natl Acad Sci USA* 1985, **82**, 2158–2162.
18. Mitchell JB, Russo A. The role of glutathione in radiation and drug induced cytotoxicity. *Br J Cancer* 1987, **55**, Suppl VIII, 96–104.
19. Pinto AL, Lippard SJ. Binding of the antitumor drug *cis*-diamminedichloroplatinum(II) (cisplatin) to DNA. *Biochim Biophys Acta* 1985, **780**, 167–180.
20. Kraker AJ, Steinkampf RW, Moore CW. Transport of cis-pt and cis-pt analogs in sensitive and resistant murine leukemic cell lines. *Proc Am Assoc Cancer Res* 1986, **27**, 286.
21. Hromas RA, North JA, Burns CP. *Cis*-platinum uptake in L1210 leukemia cells resistant and sensitive to the drug. *Proc Am Assoc Cancer Res* 1986, **27**, 263.
22. Bedford P, Shellard SA, Walker MC, Whelan RDH, Masters JRW, Hill BT. Differential expression of collateral sensitivity or resistance to cisplatin in human bladder carcinoma cell lines pre-exposed *in vitro* to either X-irradiation or cisplatin. *Int J Cancer* 1987, **40**, 681–686.
23. Bedford P, Walker MC, Sharma HL *et al.* Factors influencing the sensitivity of two human bladder carcinoma cell lines to *cis*-diamminedichloroplatinum(II). *Chem-Biol Interact* 1987, **61**, 1–15.
24. Teicher BA, Holden SA, Kelley MJ *et al.* Characterization of a human squamous carcinoma cell line resistant to *cis*-diamminedichloroplatinum(II). *Cancer Res* 1987, **47**, 388–393.
25. Hamilton TC, Winker MA, Louie KG *et al.* Augmentation of adriamycin, melphalan, cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem Pharmacol* 1985, **34**, 2583–2586.
26. Richon VM, Schulte N, Eastman A. Multiple mechanisms of resistance to *cis*-diamminedichloroplatinum(II) in murine leukemia L1210 cells. *Cancer Res* 1987, **47**, 2056–2061.
27. Bakka A, Endresen L, Johnsen ABS, Edminson PD, Rugstad HE. Resistance against *cis*-dichlorodiammineplatinum in cultured cells with a high content of metallothionein. *Toxicol Appl Pharmacol* 1981, **61**, 215–226.
28. Andrews PA, Murphy MP, Howell SB. Metallothionein-mediated cisplatin resistance in human ovarian carcinoma cells. *Cancer Chemother Pharmacol* 1987, **19**, 149–154.
29. Zwelling LA, Michaels S, Schwartz H, Dobson PP, Kohn KW. DNA cross-linking as an indicator of sensitivity and resistance of mouse L1210 leukemia to *cis*-diamminedichloroplatinum(II) and L-phenylalanine mustard. *Cancer Res* 1981, **41**, 640–649.
30. Strandberg MC, Bresnick E, Eastman A. The significance of DNA cross-linking to *cis*-diamminedichloroplatinum(II)-induced cytotoxicity in sensitive and resistant lines of murine leukemia L1210 cells. *Chem-Biol Interact* 1982, **39**, 169–180.
31. Reed E, Behrens B, Yuspa SH, Poirier MC, Hamilton T, Ozols RF. Differences in cisplatin–DNA adduct formation in sensitive and resistant sublines of human ovarian cancer cells. *Proc Am Assoc Cancer Res* 1986, **27**, 285.
32. Masuda H, Hamilton T, Young RC, Ozols RF. Increased DNA repair in human ovarian cancer (OC) cell line (CL) with induced resistance (R) to cisplatin (P) or melphalan (M). *Proc Am Assoc Cancer Res* 1986, **27**, 264.