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The influence of tumor cell density on cellular accumulation of doxorubicin or cisplatin in vitro*

Yuzuru Takemura[1], Hiroyuki Kobayashi[2], Hayato Miyachi[3], Katsuji Hayashi[1], Susumu Sekiguchi[1], and Takao Ohnuma[4]

[1] Department of Laboratory Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359, [2] Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, [3] Department of Clinical Pathology, Tokai University School of Medicine, Bouseidai, Isehara, Kanagawa 259-11, Japan
[4] Department of Neoplastic Diseases and Cancer Center, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA

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Summary. The effect of tumor cell density on the cellular pharmacokinetics of doxorubicin (DXR) and cisplatin (CDDP) was studied using MOLT-3 human acute lymphoblastic leukemia cells. As determined by the MTT assay, the growth-inhibitory effect of DXR was approx. 40 times lower when cell density was increased from 10^6 to 10^8 cells/ml (positive inoculum effect), whereas little or no influence of cell density was observed in CDDP-induced cell-growth inhibition. As measured by high-performance liquid chromatography using a fluorescence detector, the cellular accumulation of DXR showed 6- and 18-fold decreases after 1 h incubation when the cells were concentrated from 10^6 to 10^7 and 10^8 cells/ml, respectively. Only at low cell density (10^6 cells/ml) did the amount of DXR in the cells increase with increasing exposure times of up to 6 h. The DXR concentration in the supernatant that was separated from a cell suspension showing a density of 10^8 cells/ml fell to 20% of that obtained at 10^6 cells/ml. The metabolites of DXR, including Adriamycinol and Adriamycinone, were not detectable in the cell extracts or supernatants at any cell density examined. In contrast, the cellular accumulation of CDDP calculated from the platinum concentration, which was measured with a flameless atomic absorption spectrophotometer, was essentially identical at all cell densities examined; moreover, extension of the exposure period resulted in a linear increase in the amount of CDDP in the cells. CDDP concentrations in the supernatants were equally retained, irrespective of cell densities. These observations indicate that the positive inoculum effect shown in DXR-induced cell-growth inhibition results from the decreased cellular accumulation of the drug at high cell densities. We found no influence for cell density on the cellular accumulation of CDDP that might be relevant to the therapeutic potentiation of this drug at high tumor-cell density.

Introduction

Certain chemotherapeutic agents such as doxorubicin (DXR) are progressively less efficacious in vitro when tumor-cell density is increased (positive inoculum effect), whereas the effects of platinum compounds are not influenced by cell density [19]. It has been shown that preincubation of DXR in the presence of cells at high density results in the loss of the drug's efficacy [19]. In addition, DXR produced lower lethality in cells cultured in multicellular tumor spheroids than in those raised in monolayer culture [12, 20]. Although several studies have indicated that DXR-induced cytotoxicity is related to the degree of intracellular uptake of the drug [2–5], the exact mechanism of the inoculum effect seen in DXR-induced cell killing remains unclear. In contrast to DXR, cisplatin (CDDP) proved to lack this inoculum effect [19, 21] and retained its therapeutic potentiation against tumor spheroids [12]. The pharmacokinetics of CDDP in cells at high densities has not yet been clearly demonstrated.

In the present study, we investigated the influence of cell density on the cellular pharmacokinetics of DXR and CDDP. An attempt was made to elucidate the mechanism of the inoculum effect by measuring not only DXR but also its major metabolites using HPLC with a fluorescence detector. We analyzed intra- and extracellular concentrations of these drugs after their incubation with cell suspensions at various densities so as to clarify their pharmacological disposition at high tumor-cell density.

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Abbreviations: DXR, doxorubicin; CDDP, cisplatin, *cis*-diamminedichloroplatinum(II); HPLC, high-performance liquid chromatography; D-PBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; ID_{50}, drug concentration that produces inhibition of cell growth to 50% of control values

Offprint requests to: Yuzuru Takemura, M. D. at Department of Laboratory Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359, Japan

Materials and methods

Drugs and chemicals. Doxorubicin hydrochloride was generously supplied by Kyowa Hakko Kogyo Co. (Tokyo, Japan). Daunorubicin hydrochloride, Adriamycinol and Adriamycinone were kindly provided by Farmitalia Carlo Erba (Milan, Italy). The anthracyclines were dissolved in 10 mM phosphate-buffered solution (PBS, pH 3) or methanol and were further diluted with D-PBS (Gibco, Grand Island, N.Y.) to the desired concentrations. The stock solutions of anthracyclines were stored at –20° C for a maximum of 1 month. CDDP was generously provided by Bristol-Myers, K. K. (Tokyo, Japan) as a reconstituted solution for injection. Solutions of CDDP diluted in D-PBS were prepared just before the experiments. Formic acid, methanol and chloroform were purchased from Wako Pure Chemical Industries (Tokyo, Japan) and were of analytical grade.

Exposure of cells to drugs. MOLT-3 cells, established from a patient with acute lymphoblastic leukemia [17], were maintained in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) and nourished with fresh medium twice a week. Cells in exponential growth with >90% viability were used for all experiments. Suspensions of MOLT-3 cells were diluted or concentrated to various cell densities ranging from 10^6 to 10^8 viable cells/ml RPMI-1640 medium in either 15-ml culture tubes (Falcon 3033, Becton-Dickinson, Oxnard, Calif.) or 50-ml centrifuge tubes (Falcon 2098). At different densities, cells were exposed to graded concentrations of DXR or CDDP for 1 h at 37° C in a humidified atmosphere comprising 5% CO_2/95% air. At the end of the incubation period, cells were washed twice with culture medium and their density was readjusted to 5×10^4 cells/ml RPMI-1640 medium supplemented with 10% FBS. Each 200-µl volume of cell suspension was placed in a 96-well flat-bottomed plate (Corning Glass Works, Corning, N.Y.) and then incubated for 3 days at 37° C in a humidified atmosphere consisting of 5% CO_2/95% air. Following incubation, viable cells were quantified using the MTT assay as described below. For the determination of DXR or CDDP concentrations in the cells or supernatants, $10^6 - 10^8$ cells/ml RPMI-1640 medium were incubated with DXR or CDDP for 30 min to 6 h at 37° C in an atmosphere containing 5% CO_2. For exposures lasting for >1 h, cell suspensions were shaken at 1-h intervals. After incubation, cell suspensions were centrifuged at 200 *g* for 10 min at room temperature for the separation of supernatants. The cell pellets were then washed twice with D-PBS and 1×10^7 or 5×10^7 cells were analyzed for cellular DXR or CDDP content, respectively.

MTT assay. The in vitro sensitivity of MOLT-3 cells at various densities to DXR and CDDP was determined using the MTT assay [7, 18]. This assay is dependent on the cellular reduction of MTT (Sigma Chemical Co., St. Louis, Mo.) by the mitochondrial dehydrogenases of viable cells, but not those of dead cells, to a blue formazan product that can be measured spectrophotometrically. After the cells had been incubated in the 96-well plate for 3 days, 10 µl MTT solution (4 mg/ml) and 10 µl 0.1 M sodium succinate were added to each well and the plate was further incubated at 37° C for 4 h. Next, the plate was centrifuged at 650 *g* for 5 min and supernatants were removed. The formazan crystals produced were solubilized by the addition of 150 µl dimethylsulfoxide (DMSO), following which the contents of the wells were thoroughly mixed on a plate shaker for 5 min and the absorbance of each well was measured at 540 nm using a multi-well spectrophotometer (Bio-Rad, Richmond, Calif.). Dose-response curves were drawn by plotting the absorbance at 540 nm, expressed as a percentage of the untreated control value, against drug concentrations.

Extraction procedure and HPLC analysis for DXR in cells and supernatants. After the cells had been incubated with DXR, supernatants were obtained by centrifugation at 200 *g* for 10 min. Cell pellets were washed twice with D-PBS and resuspended in 0.2 ml D-PBS. Cell suspensions containing 1×10^7 cells and supplemented with 1.8 ml 10 mM PBS (pH 7.8) and internal standard (daunorubicin) were extracted by shaking for 15 min with 6 ml chloroform/methanol (4 : 1, v/v). Following centrifugation at 1,250 *g* for 10 min, the lower organic phase was collected and evaporated under vacuum at 35° C. The dry residue was dissolved in 1 ml 100 mM PBS (pH 3)/methanol (1 : 1, v/v) by sonication for 30 s, and a 100-µl volume of clear supernatant was injected into the chromatograph using a 500-µl Hamilton syringe (Reno, Nev.). Samples of 0.5 ml supernatants were extracted in a similar way after the addition of 1.5 ml 10 mM PBS (pH 7.8) and internal standard.

The chromatographic system consisted of a Hitachi model L-6000 double-head pump (Hitachi, Ltd., Tokyo, Japan) and a sampling valve (Rheodyne, model 7125, Cotati, Calif.) with a sampling loop of 100 µl. The analytical column (250 × 4 mm, inside diameter) was packed with spherical silica gel Nucleosil 100-C18 (particle size, 10 µm; Macherey-Nagel, FRG). DXR fluorescence from the extracts was measured using a fluorescence detector (Hitachi, model F-1050) with an excitation wavelength of 475 nm and emission detection at 585 nm. The column was eluted with 1 *N* formic acid/methanol (40 : 60, v/v) at a flow rate of 1 ml/min. The integration of the peaks was performed using a Hitachi model D-2500 integrator. Quantification of drug concentration in the cells and supernatant was achieved by measuring the peak-area ratio of the drug and internal standard and was calculated from a calibration curve for peak-area vs concentration.

Measurement of platinum concentration in cells and supernatants. Pellets containing 5×10^7 cells were digested by the addition of a 0.5-ml volume of nitric acid (Wako Pure Chemicals) to yield homogeneous samples [13]. Platinum concentrations in acid-digested cells and supernatants were analyzed using a Varian Spectra AA-30 atomic absorption spectrophotometer (Varian Instruments, Victoria, Australia) fitted with a Varian GTA 96 microprocessor-controlled graphite-tube atomizer and a programmable auto-sampler (Varian). A constant lamp current of 10 mA was used and absorbance was monitored at 265.9 nm. Samples were atomized flamelessly at 2,700° C in a heated graphite atomizer. A three-stage heating program was used [16]: 95° C for 35 s (dry), 1,200° C for 20 s (ash) and 2,700° C for 1 s (atomize), with a ramp rate of 500° C/s. Depending on the drug concentration used, samples of 2–20 µl were injected onto the carbon-graphite atomizer by the auto-sampler. Platinum concentrations were quantified from a calibration curve for absorbance vs concentration obtained from the platinum standard solution (Wako Pure Chemicals). Total CDDP concentrations in the cells and supernatants were calculated from the platinum concentrations measured.

Results

The in vitro sensitivities of MOLT-3 cells at various densities to DXR and CDDP are shown in Fig. 1. Since there was a linear relationship between MTT-formazan production and cell number up to 1×10^5 cells/well (data not shown), 1×10^4 cells in 200 µl culture medium were initially seeded in each well following two washes in medium and were then incubated for 3 days. Under these conditions, the maximal growth of the untreated control cells was $< 1 \times 10^5$ cells/well at the end of the incubation period. An increase in cell density from 10^6 to 10^8 cells/ml resulted in an approx. 40-fold decrease in the growth-inhibitory effect of DXR on MOLT-3 cells as determined by comparison of ID_{50} values following incubation for 1 h, thus demonstrating the positive inoculum effect (Fig. 1 A). In contrast, dose-response curves for CDDP-induced cell-growth inhibition virtually overlapped each other, indicating that cell density had little, if any, influence on CDDP-induced inhibition of MOLT-3 cell growth (Fig. 1 B).

The effect of cell density on the cellular accumulation of DXR is illustrated in Fig. 2 A. Because the viability of highly concentrated cells (10^8 cells/ml) fell to 50%–70% of that measured at a density of 10^6 or 10^7 cells/ml when the cells had been incubated at 37° C for >6 h (even in the

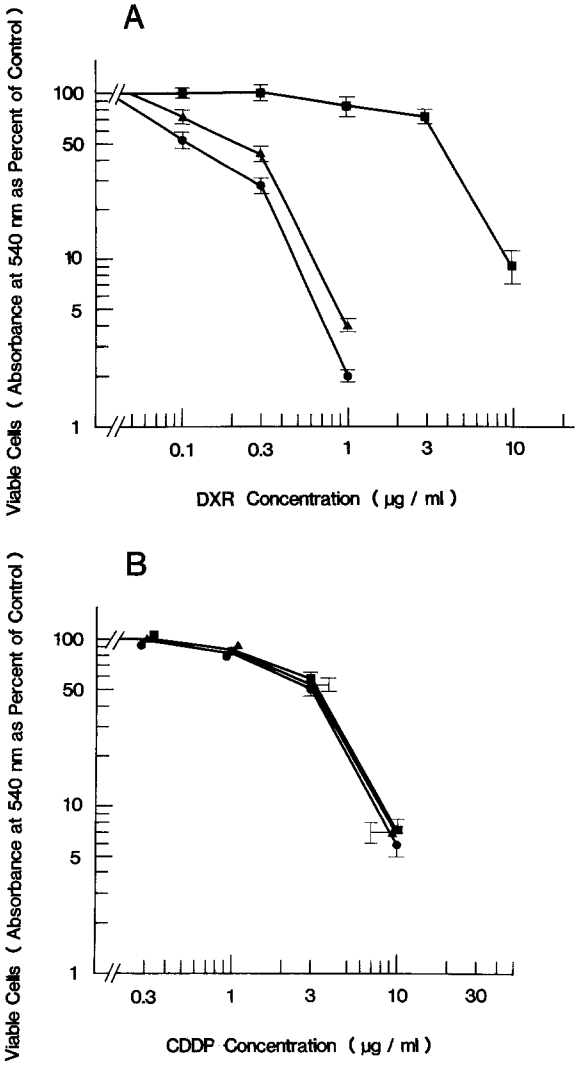

Fig. 1 A, B. Growth inhibition of MOLT-3 cells at densities of 10^6 (●), 10^7 (▲) and 10^8 cells/ml (■) induced by **A** DXR and **B** CDDP. Cells at different densities were exposed to graded concentrations of drug for 1 h at 37° C in a humidified atmosphere comprising 5% CO_2/95% air. After cells had been washed twice with culture medium, 1×10^4 cells in 200 μl medium supplemented with 10% FBS were placed in each well of a 96-well plate and then incubated for 3 days. Viable cell number was determined by MTT assay and defined as the absorbance at 540 nm, expressed as a percentage of the control value. Data points and bars represent the means ± SD

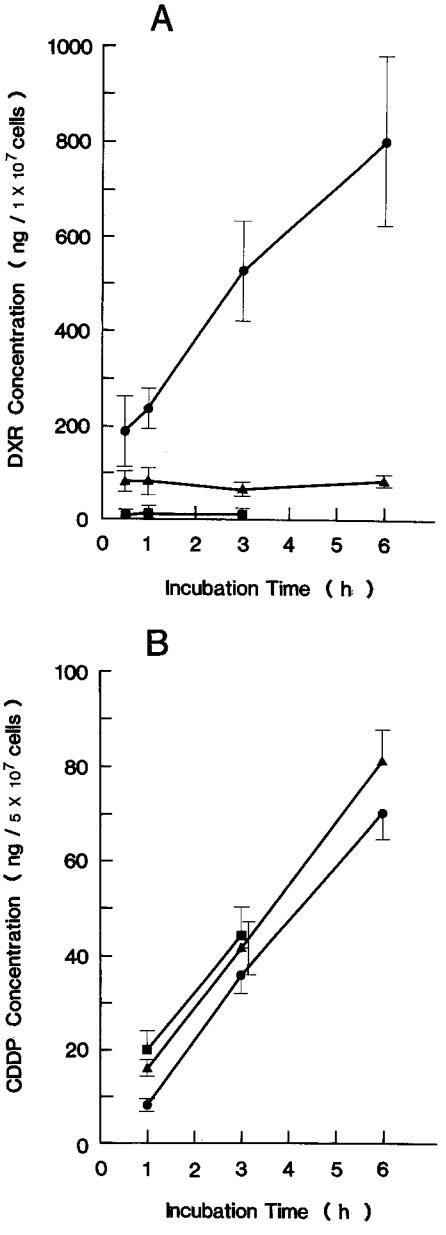

Fig. 2 A, B. Cellular contents of **A** DXR and **B** CDDP after exposure of MOLT-3 cells at densities of 10^6 (●), 10^7 (▲) and 10^8 cells/ml (■) to 1 μg/ml DXR or CDDP. The amounts of cellular DXR or CDDP were measured using HPLC with a fluorescence detector or flameless atomic absorption spectrophotometer, respectively. Data points and bars represent the means ± SD of 3–6 measurements

absence of drug), the accumulation of drug was not measured in cells at a density of 10^8 cells/ml that had been incubated for >3 h. The cellular accumulation of DXR showed 6- and 18-fold decreases following 1 h incubation with 1 μg/ml DXR when the cells were concentrated from 10^6 to 10^7 or 10^8 cells/ml, respectively. Only at low cell density (10^6 cells/ml) did the amount of DXR in the cells increase with increasing exposure periods of up to 6 h. In contrast, the cellular accumulation of CDDP at high cell densities was, if not identical, virtually the same as that observed at low cell density; moreover, extension of the exposure period resulted in a linear increase in the cellular content of CDDP, irrespective of cell density (Fig. 2B).

Following the exposure of cells to either drug, supernatants were separated from cell pellets by centrifugation. The DXR concentration in the supernatant obtained from a suspension containing 10^8 cells/ml that had been exposed to 1 μg/ml DXR for 1 h fell to 20% of that measured at 10^6 cells/ml (Fig. 3A). Stepwise decreases in the DXR concentration in supernatants were more clearly demonstrated at 10^6 cells/ml than at 10^7 or 10^8 cells/ml with increases in the incubation time, corresponding with increases in the cellular content of DXR. Figure 3B shows that CDDP concentrations in supernatants after exposure of the cells to 1 μg/ml drug were equally retained at all cell densities examined. Only slight decreases in CDDP con-

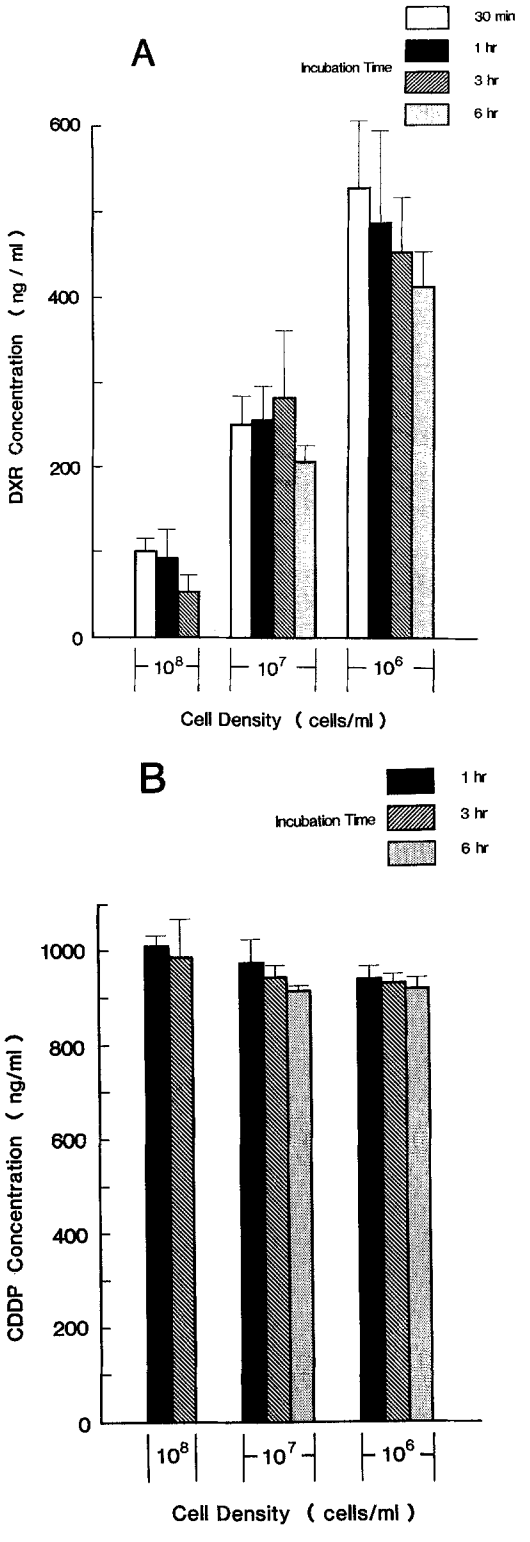

Fig. 3 A, B. Concentrations of **A** DXR and **B** CDDP in the supernatants separated from cell suspensions at different cell densities following exposure to 1 μg/ml DXR or CDDP. Columns and bars indicate the means ± SD of 3–6 measurements

centrations in supernatants were observed with increasing incubation time.

In addition to the measurement of DXR concentrations in the cells and supernatants, we analyzed the metabolism of DXR by tumor cells. Figure 4 shows typical chromato-

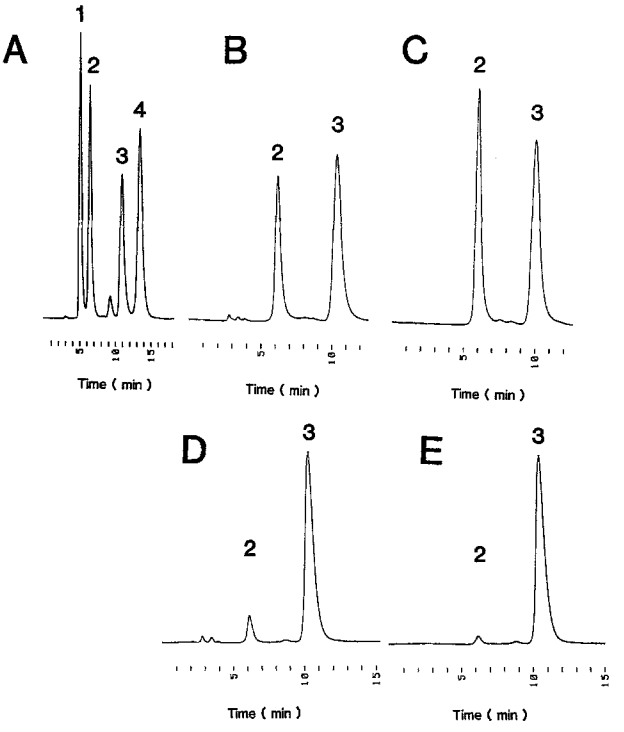

Fig. 4 A–E. Typical chromatograms of **A** a mixture of the authentic samples containing 1 μg/ml each of Adriamycinol (*1*), DXR (*2*), daunorubicin (*3*) and Adriamycinone (*4*), **B, D** the extracts of supernatans and **C, E** 1×10^7 cells following the exposure of **B, C** 10^6 or **D, E** 10^8 cells/ml to 1 μg/ml DXR for 3 h. Daunorubicin served as the internal standard

graphic profiles for a mixture of the authentic samples containing DXR, Adriamycinol, Adriamycinone and daunorubicin (A) and for supernatant (B, D) and cell extracts (C, E). As demonstrated in Fig. 4 A, DXR, its major metabolites, and daunorubicin which served as an internal standard were eluted with sufficient separation under the chromatographic condition used. We could not detect peaks for DXR metabolites, including Adriamycinol and Adriamycinone, in supernatant or cell extracts at any cell density or exposure period tested (Fig. 4 B–E).

Discussion

In the present study, we demonstrated differences in the disposition of DXR and CDDP at high tumor-cell density. The higher the cell density, the lower the antitumor effect and cellular accumulation of DXR. Since no detectable peaks for DXR metabolites were observed, this result clearly indicated that the positive inoculum effect seen in DXR-induced cell-kill kinetics [19, 21] resulted from the decreased cellular accumulation of the drug at high cell density rather than from the inactivation of DXR by tumor cells. In contrast, even when the cell density had been increased from 10^6 to 10^8 cells/ml, the cellular accumulation of CDDP remained essentially identical, in correspondence with previous findings of the similar antitumor efficacy of CDDP irrespective of cell densities [19, 21].

Several investigations have demonstrated that the ratio between cell number and total available drug is important in cell lethality induced by DXR in single cells [2, 4, 5,

19, 21] and in tumor spheroids [3, 12, 15, 20] in vitro. In the latter culture, cells became progressively more resistant to DXR when the size of tumor spheroids increased and DXR was poorly taken up by cells in the core of spheroids [12]. The present study showed that cells in suspension culture also exhibited prominent DXR resistance, concomitant with a decrease in the cellular content of DXR with increasing cell density. Vaupel et al. [23] reported that tumor masses measuring 1 cm in diameter could exhibit extracellular pH as low as 6–6.5; when cells had been concentrated at 10^8 cells/ml, extracellular pH would fall with increasing incubation time, interfering with drug uptake across the cell membrane [1, 10, 22]. In fact, Hindenburg et al. [11] demonstrated that the decrease in the cytostatic effect of daunorubicin on HL-60 cells at lower pH (pH 5) occurred parallel to a decrease in the amount of drug retained by the cells and to the sequestration of drug within the membrane of the cells. These data suggest that the anthracyclines failed to pass through membranes and bind to cytoplasmic and nuclear structures at lower pH; instead, most of the drug was taken up by the Golgi apparatus, by which it was then transported out of the cells [11].

Our finding that cellular accumulation of DXR did not increase at higher cell densities (10^7 and 10^8 cells/ml), even with increases in incubation time, may be attributable to the acidification of extracellular fluid. Furthermore, extracellular concentrations of DXR showed marked decreases when cell density was increased from 10^6 to 10^7 and 10^8 cells/ml. This is consistent with the previous observation that preincubation of DXR in the presence of cells at high density resulted in a decrease in the cytocidal activity of the drug [19]. Because the metabolites of DXR, including Adriamycinol and Adriamycinone, could not be detected in the present study, the inoculum effect seen in DXR-induced cell lethality seems to result from the absorption of the drug into the cells rather than from drug inactivation by the cells. The reduction in the extracellular concentration of DXR that occurred after incubation with cells at high density without a concomitant increase in cellular drug content might have been caused by DXR's high affinity to cell-membrane components, especially glycoprotein [14] and phospholipids [6]. At high cell density and under acidotic conditions, the DXR that was absorbed loosely into the cell surface but did not pass through the membrane was likely diffused out of the cells during the washing procedure following incubation.

On the other hand, CDDP did not exhibit the inoculum effect; indeed, after preincubation of the drug with tumor cells at high density, it showed antitumor efficacy similar to that previously observed without preincubation [19]. This phenomenon can be clearly explained by our observations that intracellular accumulation of CDDP at high cell densities was essentially identical to that measured at low cell density and that CDDP concentrations in supernatants were equally retained at all cell densities tested. In contrast with DXR, it has been shown that low pH has little influence on the cellular accumulation of CDDP [9] or on its cytotoxicity to hypoxic EMT6 mouse mammary tumor cells at 37° C [8]. Therefore, it appears that uncharged CDDP species, including dichloro and chlorohydroxy complexes, enter cells according to the extra- and intracellular gradient of the drug concentration, irrespective of the cell density or pH in extracellular fluid. This pharmacological disposition of CDDP might have an important clinical implication for chemotherapy of solid tumors and mass-formed hematological malignancies such as lymphoma. Indeed, in multicellular tumor spheroids, the cytotoxic effect of CDDP on tumor cells was superior to that of DXR with increasing size of the spheroids [12].

Whereas in vitro data on tumor cells alone are insufficient to enable extrapolation to the biological behavior of a drug in vivo, the inoculum effect is an additional factor indicating drug resistance for certain chemotherapeutic agents such as DXR. In contrast, we found no influence for cell density on the cellular accumulation of or the cell lethality induced by CDDP that might be relevant to the high therapeutic potentiation of this drug in various kinds of solid tumors.

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References

1. Born R, Eicholtz-Wirth H (1981) Effect of different physiological conditions on the action of Adriamycin on Chinese hamster cells in vitro. Br J Cancer 44: 241
2. Chambers SH, Bleehen NM, Watson JV (1984) Effect of cell density on intracellular Adriamycin concentration and cytotoxicity in exponential and plateau phase EMT6 cells. Br J Cancer 49: 301
3. Durand RE (1981) Flow cytometry studies of intracellular Adriamycin in multicell spheroids in vitro. Cancer Res 41: 3495
4. Durand RE, Olive PL (1981) Flow cytometry studies of intracellular Adriamycin in single cells in vitro. Cancer Res 41: 3489
5. Ganapathi R, Reiter W, Krishan A (1982) Intracellular Adriamycin levels and cytotoxicity in Adriamycin-sensitive and Adriamycin-resistant P388 mouse leukemia cells. J Natl Cancer Inst 68: 1027
6. Goormaghtigh E, Chatelain P, Caspers J, Ruysschaert JM (1980) Evidence of a specific complex between Adriamycin and negatively-charged phospholipids. Biochim Biophys Acta 597: 1
7. Harker WG, Slade DL, Dalton WS, Meltzer PS, Trent JM (1989) Multidrug resistance in mitoxantrone-selected HL-60 leukemia cells in the absence of P-glycoprotein overexpression. Cancer Res 49: 4542
8. Herman TS, Teicher BA, Collins LS (1988) Effect of hypoxia and acidosis on the cytotoxicity of four platinum complexes at normal and hyperthermic temperatures. Cancer Res 48: 2342
9. Herman TS, Teicher BA, Pfeffer MR, Khandekar VS, Sotomayor EA (1990) Interaction of platinum complexes of thiazin and xanthene dyes with hyperthermia. Cancer Chemother Pharmacol 26: 127
10. Hindenburg AA, Stewart VJ, Baker MA, Taub RN (1987) Effects of pH on cellular accumulation of daunorubicin. Proc Am Assoc Cancer Res 28: 261
11. Hindenburg AA, Gervasoni JE Jr, Krishna S, Stewart VJ, Rosado M, Lutzky J, Bhalla K, Baker MA, Taub RN (1989) Intracellular distribution and pharmacokinetics of daunorubicin in anthracycline-sensitive and -resistant HL-60 cells. Cancer Res 49: 4607
12. Inoue S, Ohnuma T, Takaoka K, Suzuki Y, Kaneko M, Safirstein R, Holland JF (1987) Effect of doxorubicin and cisplatin on multicellular tumor spheroids from human lung cancer. Cancer Drug Deliv 4: 213
13. Ishida H, Kohmoto O, Bridge JHB, Barry WH (1988) Alterations in cation homeostasis in cultured chick ventricular cells during and after

recovery from adenosine triphosphate depletion. J Clin Invest 81: 1173
14. Kessel D (1979) Enhanced glycosylation induced by Adriamycin. Mol Pharmacol 16: 306
15. Kohno N, Ohnuma T, Kaneko M, Holland JF (1988) Interactions of doxorubicin and cisplatin in squamous carcinoma cells in culture. Br J Cancer 58: 330
16. LeRoy AF, Wehling ML, Sponseller HL, Friauf WS, Solomon RE, Dedrick RL (1977) Analysis of platinum in biological materials by flameless atomic absorption spectrophotometry. Biochem Med 18: 184
17. Minowada J, Ohnuma T, Moore GE (1972) Rosette-forming human lymphoid cell lines: I. Establishment and evidence for origin of thymus-derived lymphocyte. J Natl Cancer Inst 49: 891
18. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55
19. Ohnuma T, Arkin H, Holland JF (1986) Effect of cell density on drug-induced cell kill kinetics in vitro (inoculum effect). Br J Cancer 54: 415
20. Sutherland RM, Eddy HA, Bareham B, Reich K, Vanantwerp D (1979) Resistance to Adriamycin in multicellular spheroids. Int J Radiat Oncol Biol Phys 5: 1225
21. Takemura Y, Ohnuma T, Norton L, Holland JF (1986) Combination chemotherapy in vitro based on inoculum effect. Proc Am Assoc Cancer Res 27: 373
22. Tannock IF, Rotin D (1989) Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res 49: 4373
23. Vaupel PW, Frinak S, Bicher HI (1981) Heterogenous oxygen partial pressure and pH distribution in C3H mouse mammary adenocarcinoma. Cancer Res 41: 2008