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Protection of bone-marrow granulocyte-macrophage colony-forming units in mice bearing in vivo alkylating-agent-resistant EMT-6 tumors

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Abstract. The survival of bone-marrow granulocyte-macrophage colony-forming units (CFU-GM), an alkylating-agent-sensitive normal tissue, was assessed in mice bearing the EMT-6 parental tumor or the in vivo resistant EMT-6/CDDP, EMT-6/CTX, EMT-6/Thio, and EMT-6/Carbo tumors. The survival pattern of the bone-marrow CFU-GM recapitulated the survival of the tumor cells, mimicking the development of resistance and reversion to sensitivity upon removal of the selection pressure for each of the four alkylating agents. When the EMT-6 parental tumor was implanted in the opposite hind limb of animals bearing the EMT-6/CDDP or EMT-6/CTX tumor, the survival of the parental tumor cells after treatment of the animals with the appropriate antitumor alkylating agent was enhanced. The EMT-6/CDDP tumor was cross-resistant to CTX and high-dose L-PAM, whereas the EMT-6/CTX tumor was somewhat resistant to CDDP and markedly sensitive to VP-16. In each case, the survival pattern of the bone-marrow CFU-GM reflected the survival of the tumor cells. These results indicate that the presence of an alkylating-agent-resistant tumor in an animal can affect the drug response of tissues distal to that tumor.

Introduction

The development of resistance to chemotherapeutic agents is a major problem in the clinical treatment of malignant disease [14, 15, 17]. Most preclinical studies on the development and characterization of resistance to chemotherapeutic agents have focused primarily on biochemical changes within tumor cells that allow isolated populations of tumor cells in culture to survive exposure to previously cytotoxic concentrations of anticancer drugs [12, 13, 19]. These changes include alterations in the plasma membrane such as increased expression of the multidrug-resistance efflux pump, resulting in inhibition of drug transport or uptake; alterations in the cytosol such as increased levels of enzyme or glutathione; and changes in the nucleus such as increases in lesion repair [12]. These changes are relevant in cell culture and in vivo, however, in situ the tumor is part of the host and, consequently, an additional array of alterations in the tumor cells that change the disposition of the drug in the host can result in resistance of the disease to treatment [12]. Although many fewer studies have addressed therapeutic resistance through mechanisms involving the tumor/host, it has long been recognized that the metabolic state of tumor-bearing animals is very different from that in normal animals and that the disposition of drugs in tumor-bearing animals can be different from that in normal animals [1, 2, 7, 11]. The EMT-6 mammary tumor lines in which resistance to antitumor alkylating agents was developed in vivo allowed the recognition that anticancer drug resistance could involve altered drug disposition in the tumor/host [18].

If alkylating agent resistance in the EMT-6 tumor system is due to systemic changes in the host, then this resistance might be detectable in alkylating-agent-sensitive normal tissues. A systemic change in the host could involve specific signaling non-specific metabolic interactions between the tumor and normal tissues, thus implicating secreted factors [protein(s) or small molecule(s)] that can alter the metabolic state of critical target tissues. In this article we report the survival of bone-marrow CFU-GM and tumor cells from animals bearing the EMT-6 parent and alkylating-agent-resistant tumors after treatment of the animals with various anticancer drugs.

Abbreviations: CDDP, *cis*-diamminedichloroplatinum(II); CTX, cyclophosphamide; L-PAM, melphalan (L-phenylalanine mustard); BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; mito C, mitomycin C; VIN, vincristine; Adria, doxorubicin (Adriamycin); VP-16, etoposide (VP-16-213); thiotepa, *N,N′,N″*-triethylenethiophosphoramide.

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Materials and methods

Drugs. cis-Diamminedichloroplatinum(II) (CDDP) and carboplatin were gifts from Dr. Alfred Crosswell, Bristol-Myers-Squibb Co. (Wallingford, Conn.). Cyclophosphamide (CTX), melphalan (L-PAM), mitomycin C (mito C), doxorubicin (Adriamycin Adria), vincristine (VIN), and etoposide (VP-16) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Thiotepa and BCNU (carmustine) were purchased from the Dana-Farber Cancer Institute pharmacy.

Tumor system. The EMT-6/parent mouse mammary carcinoma grown as a solid tumor subcutaneously in the flanks of female BALB/c mice (Taconic Farms, Germantown, N.Y.) has been used widely in radiobiology and chemotherapy studies. We have established four alkylating-agent-resistant EMT-6 tumor lines by repeated treatment of tumor-bearing animals with CDDP (20 mg/kg), CTX (300 mg/kg), *N,N′, N″-triethylenethiophosphoramide (thiotepa, 15 mg/kg), or carboplatin (100 mg/kg) injected intraperitoneally (i.p.) 24 h before passage of each tumor line into fresh host animals. The parent tumor line was passaged in the same manner in the absence of drug treatment. The alkylating-agent-resistant sublines designated EMT-6/CDDP (resistant to CDDP), EMT-6/CTX (resistant to CTX), EMT-6/Thio (resistant to thiotepa), and EMT-6/carbo (resistant to carboplatin) were maintained as frozen tumor brei in liquid nitrogen and used for experiments during the second and third tumor passages [18].*

Tumor-cell survival assay. The EMT-6 murine mammary carcinoma is an in vivo-in vitro tumor system [18]. The EMT-6/parent and alkylating-agent-resistant tumors were grown in BALB/c mice (Taconic Farms, Germantown, N.Y.). For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted i.m. into the hind legs of BALB/c mice aged 8–10 weeks. When the tumors had reached a volume of approximately 100 mm^3 (day 8 after tumor implantation), the animals were given i.p. injections of the drugs being tested or X-rays (Gamma Cell 40, Atomic Energy of Canada Ltd.). A 24-h interval was incorporated before the mice were killed so as to allow for the full expression of drug cytotoxicity and repair of potentially lethal damage. Mice were immersed briefly in 95% ethanol, and the tumors were excised under sterile conditions in a laminar flow hood and minced to a fine brei with two scalpels. Four tumors were pooled to make each treatment group. Approximately 300 mg tumor brei was used to make each single-cell suspension. All reagents were sterilized with 0.22-μm Millipore filters and were added aseptically to the tumor cells.

Each sample was washed in 20 ml Waymouth's media (I.S.I. Corp., Chicago, Ill.), after which the liquid was gently decanted and discarded. The samples were resuspended in 450 units collagenase/ml (Sigma) and 0.1 mg DNase/ml (Sigma) and incubated for 10 min at 37° C in a shaking water bath. The samples were resuspended as described above and incubated for another 15 min at 37° C. Next, 1 ml of 1-mg/ml DNase was added and incubation was continued for 5 min at 37° C. The samples were then filtered through two layers of sterile gauze. The samples were washed twice, then resuspended in Waymouth's media supplemented with 15% newborn calf serum. These single-cell suspensions were counted and plated at three different cell concentrations in duplicate for the colony-forming assay. No significant difference was observed in the total cell yield from the pooled tumors in any treatment group. After 1 week, the plates were stained with crystal violet and colonies of more than 50 cells were counted. The untreated tumor-cell suspensions had a plating efficiency of 10%–16%. The results were expressed as the surviving fraction (± SE) of cells from the treated groups as compared with untreated controls.

Bone marrow toxicity. Bone marrow was taken from the same animals used for the tumor-excision assay. A pool of marrow from the femurs of two animals was obtained by gently flushing the marrow through a 23-gauge needle, and the CFU-GM assay was carried out as described previously [16]. Colonies of at least 50 cells were scored on an Acculite colony counter (Fisher Scientific, Springfield, N.J.). The results of three experiments, in which each group was measured at three cell concentrations in duplicate, were averaged. The results were expressed as the surviving fraction of treated groups as compared with untreated controls.

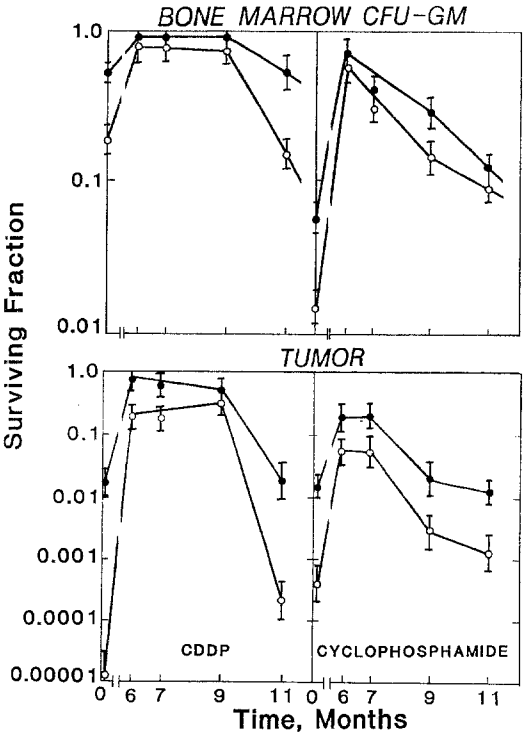

Fig. 1. Survival of bone-marrow CFU-GM and EMT-6 tumor cells from tumors treated in vivo with: CDDP (20 mg/kg, ●) or CDDP (50 mg/kg, ○) prior to (0 time) or after 10 passages, with CDDP (20 mg/kg) being given i.p. 24 h prior to each passage over 6 months and then stopped; cyclophosphamide (200 mg/kg, ●) or cyclophosphamide (500 mg/kg, ○) prior to (0 time) or after 10 passages, with cyclophosphamide (300 mg/kg) being given i.p. 24 h prior to each passage over 6 months and then stopped. *Points* represent means values for four independent experiments; *bars* represent SEM

Results

Resistance to the antitumor alkylating agents CDDP, CTX, thiotepa, and carboplatin was developed in the EMT-6 mouse mammary carcinoma using selection pressure by serial treatment and passage [18]. Tumors were used for study after ten rounds of treatment passage over 6 months. The survival of the parental tumor and the alkylating-resistant-tumors after treatment of tumor-bearing animals with two doses of the agents to which resistance was developed is shown in Figs. 1 and 2. Bone marrow was removed from the femurs of the same animals at the time of tumor excision, and the survival of the bone-marrow CFU-GM from these animals was determined. The bone-marrow CFU-GM in animals bearing tumors that were resistant to the antitumor alkylating agents were less affected by the cytotoxic actions of these drugs. The tumors continued to be maintained animal-to-animal without further treatment from 6 months through 11 months after the initiation of the study. Resistance in the tumor sublines made resistant to CTX and carboplatin was stable for about 1 month, whereas that of the tumor subline made resistant

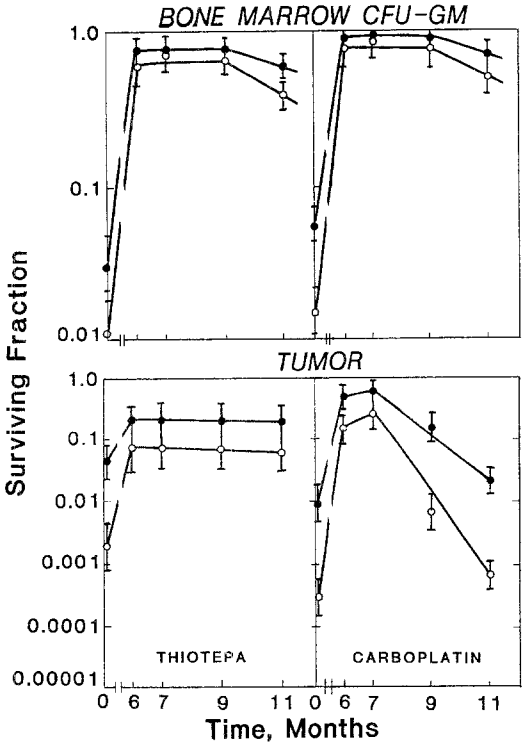

Fig. 2. Survival of bone-marrow CFU-GM and EMT-6 tumor cells from tumors treated in vivo with: thiotepa (15 mg/kg, ●) or thiotepa (30 mg/kg, ○) prior to (0 time) or after 10 passages, with thiotepa (15 mg/kg) being given i.p. 24 h prior to each passage over 6 months and then stopped; carboplatin (100 mg/kg, ●) or carboplatin (500 mg/kg, ○) prior to (0 time) or after 10 passages, with carboplatin (100 mg/kg) being given i.p. 24 h prior to each passage over 6 months and then stopped. *Points* represent mean values for four independent experiments; *bars* represent SEM

to CDDP was stable for about 3 months, and the tumor subline made resistant to thiotepa appeared to be stably resistant over the entire period examined. Over the period from 6 to 11 months when selection pressure was removed, as the tumors lost resistance to the antitumor alkylating agents, the bone-marrow CFU-GM were no longer spared from the toxicity of these drugs.

To examine the effect of the presence in the same host of the EMT-6/parent and EMT-6/CDDP or EMT-6/CTX tumors on the tumor response to the drugs to which resistance had been developed, animals were implanted with the EMT-6/parent tumor in one hind leg and the EMT-6/CDDP or EMT-6/CTX tumor in the other hind leg. In all cases the tumors grew at a normal rate. When the tumors had reached a volume of about 100 mm^3, the animals were treated with the drugs to which resistance had been developed and tumor-cell survival was determined (Fig. 3). The survival of the EMT-6/CDDP tumor cells was the same in animals bearing either the EMT-6/CDDP tumor in both legs or the EMT-6/parent tumor in one leg and the EMT-6/CDDP tumor in the other leg. The survival of the EMT-6/parent tumor cells was increased in the animals also bearing the EMT-6/CDDP tumor such that at a dose of 20 mg/kg CDDP there was about a 3.5-fold sparing of EMT-6/parent cells and at a dose of 50 mg/kg CDDP there was about a 300-fold sparing of EMT-6/parent cells in these animals as compared with those carrying only the EMT-6/parent tumor. The presence of the EMT-6/parent tumor in the host did not alter the response of the EMT-6/CTX tumor to CTX as compared with the response of the EMT-6/CTX tumor in animals bearing only that tumor. However, there was about an 8- to 10-fold increase in the survival of EMT-6/parent tumor cells after treatment with 300 or 500 mg/kg CTX in animals also bearing the EMT-6/CTX tumor as compared with those carrying only the EMT-6/parent tumor.

Bone marrow was removed from the femurs of the same animals at the time of tumor excision, and the survival of the bone-marrow CFU-GM from these animals was determined (Fig. 3). The bone-marrow CFU-GM in animals bearing the EMT-6/parent tumor line and the EMT-6/CDDP tumor line were protected from the cytotoxic actions of CDDP to the same level as in animals bearing the EMT-6/CDDP in both legs. The bone-marrow CFU-GM in animals bearing the EMT-6/parent tumor line and the EMT-6/CTX tumor line were protected from the cytotoxic actions of CTX to a level intermediate between that observed in animals bearing the EMT-6/parent tumor in both legs or the EMT-6/CTX tumor in both legs.

The cross-resistance of the EMT-6/CDDP and the EMT-6/CTX tumors as compared with the EMT-6/parent tumor toward several anticancer drugs is shown in Fig. 4. The EMT-6/CTX tumor was somewhat resistant to CDDP, especially at high doses of the drug, as compared with the EMT-6/parent tumor line. The EMT-6/CDDP tumor line was as resistant to CTX as was the EMT-6/CTX tumor line. The response of the EMT-6/parent tumor, the EMT-6/CDDP tumor, and the EMT-6/CTX tumor to BCNU, mito C, Adria, and VIN was the same. The EMT-6/CDDP tumor was somewhat resistant to high-dose L-PAM as compared with the EMT-6/parent and EMT-6/CTX tumor lines, and the EMT-6/CTX tumor was markedly more sensitive to VP-16 than were the EMT-6/parent and EMT-6/CDDP tumors. In each case, the survival of the bone-marrow CFU-GM paralleled the survival of the tumor cells following their exposure to each agent.

Discussion

Tumor masses are made up of subpopulations of cells of differing drug sensitivities [3, 4, 6]. In 1981, Miller et al. [9] showed that clonal subpopulations with differing drug sensitivities derived from a mouse mammary tumor could influence each other's response in vivo. In one set of experiments, syngeneic mice were given injections of CTX in opposite flanks. CTX administration was begun 2 days later. The sensitivity of line 168 tumors was not affected by line 410 tumors, but the sensitivity of line 410 tumors was increased by the presence of line 168 tumors. Mice bearing line 168 tumors only or both line 168 and line 410 tumors were more sensitive to the acute toxic effects of single, high doses of CTX than were the line 410 tumor-bearing mice, suggesting that effects on CTX activation are responsible for the drug-sensitivity interaction [9]. Similar experiments were carried out with methotrexate.

Rosenberg et al. [10] established an L-PAM-resistant human rhabdomyosarcoma xenograft, TE-671 MR, in

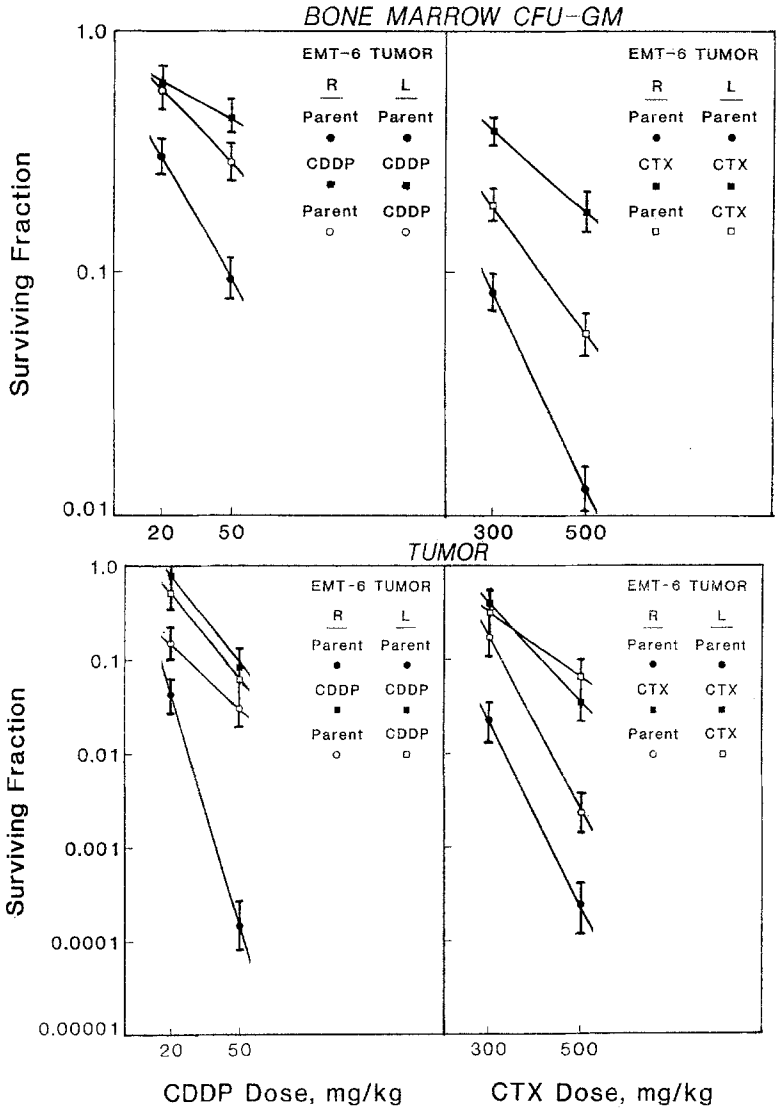

Fig. 3. Survival of bone-marrow CFU-GM and parental or alkylating-agent-resistant EMT-6 tumor cells after the treatment of mice with two different doses of CDDP or CTX. ●, surviving fractions from animals bearing EMT-6 parent tumors in both legs; ■, surviving fractions from animals harboring EMT-6/CDDP or EMT-6/CTX tumors in both legs; ○, surviving fractions of EMT-6 parent tumors from animals bearing EMT-6 parent tumors in one leg and either EMT-6/CDDP or EMT-6/CTX tumors in the other leg; □, surviving fractions of EMT-6/CDDP or EMT-6/CTX tumors from animals bearing EMT-6 parent tumors in one leg and either EMT-6/CDDP or EMT-6/CTX tumors in the other leg. *Points* represent mean values for three independent experiments; *bars* represent SEM

athymic mice by serial L-PAM treatment of the parent xenograft, TE-671, at the 10% lethal dose (LD_{10}); significant resistance was evident after ten passages of the tumor. TE-671 MR demonstrated variable degrees of cross-resistance to chlorambucil, CTX, ifosfamide, thiotepa, mito C, CDDP, VIN, and bleomycin [8]. Measurement of tumor-to-plasma drug ratios at 180 min following the i.p. administration of L-PAM at half of the 10% lethal dose showed mean values of 3.81 in TE-671 MR and 7.38 in TE-671, respectively. The lower drug levels in TE-671 MR may be contributing to the resistance to L-PAM [8]. When TE-671 MR cells were exposed to L-PAM in monolayer cell culture, they were as sensitive to the drug as were the parent TE-671 cells, thus indicating that interaction with the host environment is critical to the expression of resistance in this tumor line (Henry S. Friedman, personal communication).

In rats bearing the Walker 256 carcinoma, there was a decrease in the microsomal cytochrome P-450 content of the liver, which resulted in increased sleeping times after pentobarbital administration. Beck et al. [2] found that the activity of δ-aminolevulinic acid synthetase was only 16% of normal control values in animals bearing 7-day Walker 256 tumors, whereas the activity of hepatic microsomal heme oxygenase in the tumor-bearing rats was nearly 8 times that measured in the normal control animals. These data indicate that the presence of a neoplasm in the host not only impaired the metabolism of a drug by hepatic microsomes but also perturbed the synthesis of the microsomal P-450 cytochromes in those animals [2].

The conclusion that must be drawn from these mixed-tumor studies and the current tumor and bone-marrow survival experiments is that the presence of an alkylating-agent-resistant tumor in an animal can have effects on the drug response of tissue distal to the tumor. It is becoming clear that growth factors that are secreted by tumor cells or whose secretion by other cells is induced by the presence of a neoplasm may affect the metabolism of tissues throughout a host organism [5]. The implications of this type of resistance to the treatment of clinical disease are major. The signal-transduction pathways that control cellular responses to these proteins and peptides provide new targets for the medicinal chemist. Therapeutic strategies that focus on enhanced drug activation (such as CTX in the

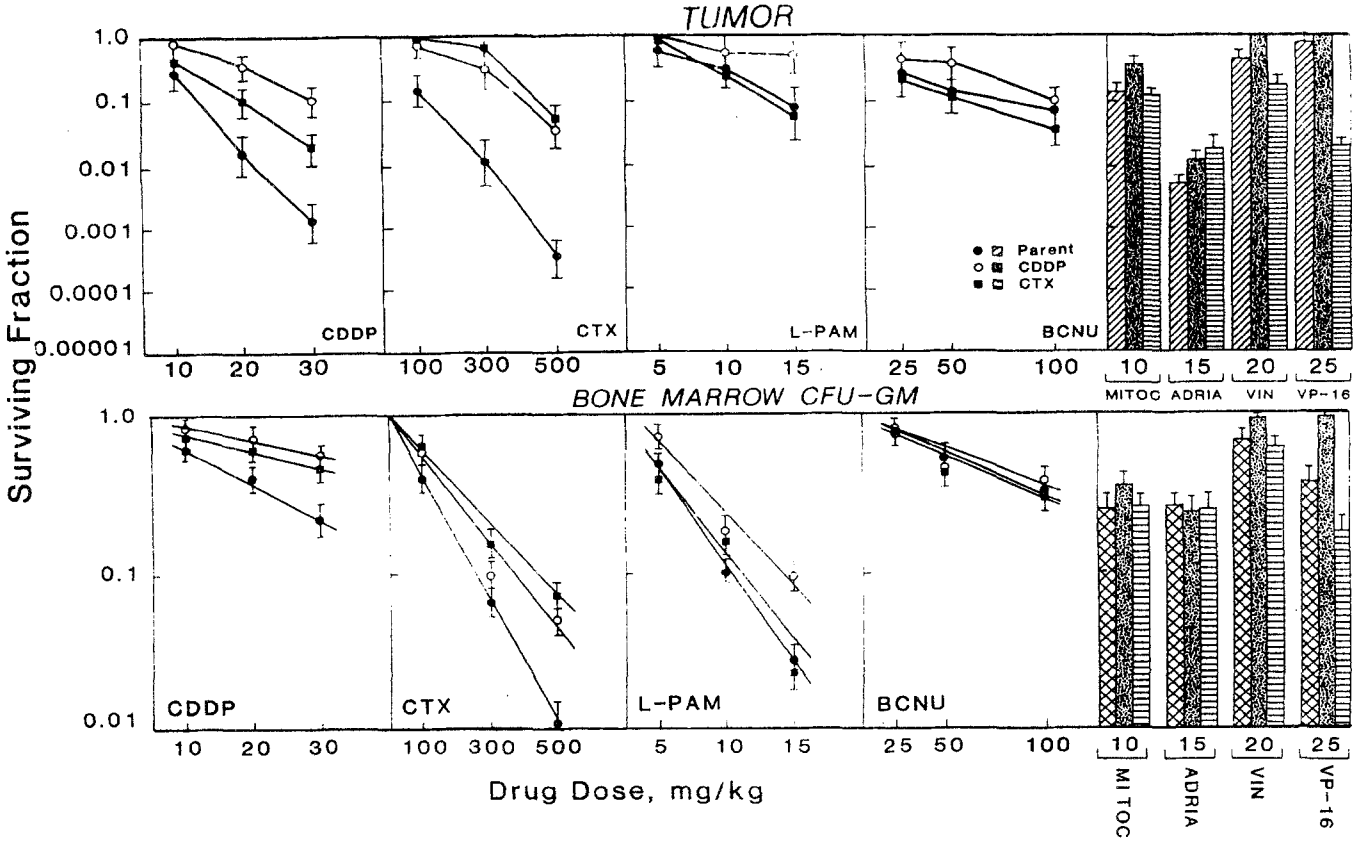

Fig. 4. Survival of bone-marrow CFU-GM and EMT-6/parent (●, ▨), EMT-6/CDDP (○ , ■), and EMT-6/CTX (■, ▤) tumor cells from tumors treated in vivo with various doses of CDDP, CTX, L-PAM, or BCNU or with single doses of mito C, Adria, VIN, or VP-16. *Points* represent mean values for three independent experiments; *bars* represent SEM

liver) or decreased drug catabolism may provide new ways of improving the efficacy of chemotherapeutic agents.

References

1. Beck WT, Mandel G, Fabro S (1975) Physiological disposition of pentobarbital in tumor-bearing mice. Cancer Res 35:1333–1340
2. Beck WT, Dedmon ML, Ouellette MA (1982) Biochemical basis for impaired drug metabolism in tumor-bearing rats. Biochem Pharmacol 31: 1535–1543
3. Fidler IJ (1990) Host and tumor factors in cancer metastasis. Eur J Clin Invest 20: 481–486
4. Fidler IJ, Balch CM (1987) The biology of cancer metastasis and implications for therapy. Curr Probl Surg 24: 131–209
5. Goustin AS, Leof EB, Shipley GD, Moses HL (1986) Growth factors and cancer. Cancer Res 46: 1015–1029
6. Heppner G (1984) Tumor heterogeneity. Cancer Res 44: 2259–2265
7. Kato R, Takanaka A, Oshima T (1968) Drug metabolism in tumor-bearing rats: II. In vivo metabolisms and effects of drugs in tumor-bearing rats. Jpn J Pharmacol 18: 245–254
8. Lilley ER, Elion GB, Dewhirst MW, Schold SC Jr, Blum MR, Savina PM, Laskowitz DT, Bigner DD, Friedman HS (1991) Therapeutic analysis of the melphalan-resistant human rhabdomyosarcoma xenograft TE-671 MR. Cancer Res 51: 3906–3909
9. Miller BE, Miller FR, Heppner GH (1981) Interactions between tumor subpopulations affecting their sensitivity to the antineoplastic agents cyclophosphamide and methotrexate. Cancer Res 41: 4378–4381
10. Rosenberg MC, Colvin OM, Griffith OW, Bigner SH, Elion GB, Horton JK, Lilley E, Bigner DD, Friedman HS (1989) Establishment of a melphalan-resistant rhabdomyo-sarcoma xenograft with cross-resistance to vincristine and enhanced sensitivity following buthionine sulfoximine-mediated glutathione depletion. Cancer Res 49: 6917–6922
11. Rosso R, Donelli MG, Franchi G, Garattini S (1971) Impairment of drug metabolism in tumor-bearing animals. Eur J Cancer 7: 565–577
12. Teicher BA (ed) (1993) Mechanisms of resistance in oncology. Marcel Dekker, New York
13. Teicher BA, Frei E III (1988) Development of alkylating agent resistant human tumor cell lines. Cancer Chemother Pharmacol 21: 292–298
14. Teicher BA, Cucchi CA, Lee JB, Flatow JL, Rosowsky A, Frei E III (1986) Alkylating agents: in vitro studies of cross-resistance patterns. Cancer Res 46: 4379–4383
15. Teicher BA, Holden SA, Kelley MJ, Shea TC, Cucchi CA, Rosowsky A, Henner WD, Frei E III (1987) Characterization of a human squamous carcinoma cell line resistant to *cis*-diamminedichloroplatinum(II). Cancer Res 47: 388–393
16. Teicher BA, Holden SA, Jacobs JL (1987) Approaches to defining the mechanism of Fluosol-DA 20%/carbogen enhancement of melphalan antitumor activity. Cancer Res 47: 513–518
17. Teicher BA, Holden SA, Cucchi CA, Cathcart KNS, Korbut TT, Flatow JL, Frei E III (1988) Combination of *N,N′,N″*-triethylenethiophosphoramide and cyclophosphamide in vitro and in vivo. Cancer Res 48: 94–100
18. Teicher BA, Herman TS, Holden SA, Wang Y, Pfeffer MR, Crawford JM, Frei E III (1990) Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. Science 247: 1457–1461
19. Teicher BA, Holden SA, Herman TS, Alvarez Sotomayor E, Khandekar V, Rosbe KW, Brann TW, Korbut TT (1991) Characteristics of five human tumor cell lines and sublines resistant to *cis*-diamminedichloroplatinum(II). Int J Cancer 47: 252–260