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The importance of schedule on diethyldithiocarbamate modulation of drug-induced myelosuppression*

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Summary. Sodium diethyldithiocarbamate (DDTC) has been investigated as a biochemical modulator of the toxicity associated with clinically used cancer chemotherapeutic agents. In the present study, we assessed the ability of DDTC to accelerate recovery of the granulocyte/macrophage progenitor cel (GM-CFC) population following treatment with the myelosuppressive drugs carboplatin (CBDCA), tetrachloro(d,l-*trans*)1,2-diaminocyclohexane platinum(IV) (tetraplatin), 5-fluorouracil (5-FU), and etoposide (VP-16) in B6D2F$_1$ mice. Myelotoxicity was assessed 24 h after the injection of the anticancer drug using a GM-CFC clonogenic assay. In the case of all four anticancer drugs, the timing of DDTC administration appeared to be a critical parameter with regard to protection. A delay time of 1 h between the injection of the myelotoxic drug and treatment with DDTC (30 mg/kg) resulted in a significant reduction in cytotoxicity to GM-CFC, whereas a longer delay time did not. These results suggest that the timing of DDTC administration may be essential in modulating the myelosuppression associated with many chemotherapeutic regimens used in the clinic.

Introduction

The biochemical modulation of clinically used cancer chemotherapeutic drugs is a strategy that has received some attention over the course of the past decade. This "chemoprotection" involves the administration of a second drug in addition to the anticancer drug; the ideal result would be to inhibit toxicity to normal tissues without interfering with the efficacy of the antitumor agent. Myelosuppression is the most common of the dose-limiting toxicities associated with anticancer drugs in man [3, 4]. We and other investigators have focused attention on the use of the chemoprotector sodium diethyldithiocarbamate (DDTC) to ameliorate drug-induced myelosuppression [5]. Although initially explored as a chelating agent to reduce platinum-induced nephrotoxicity, DDTC also protects against the myelotoxic effects of a variety of different anticancer drugs [2, 6, 7, 9, 11, 12]. In the present study, we investigated the ability of DDTC to accelerate the recovery of the granulocyte/macrophage progenitor cell (GM-CFC) population from the myelosuppressive drugs carboplatin (CBDCA), 5-fluorouracil, tetraplatin, and etoposide (VP-16).

Materials and methods

Animals. Male B6D2F$_1$ mice aged 6–8 weeks were obtained from the Jackson Laboratory (Bar Harbor, Me.). All mice were housed in plastic cages in groups of nine and were provided with food and water ad libitum.

Materials. CBDCA (carboplatin, JM-8) was generously provided by Bristol-Myers (Syracuse, N.Y.). 5-Fluorouracil was purchased from Solopak Laboratories (Franklin Park, Ill.). Tetraplatin was kindly supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Etoposide (VP-16) was obtained from Bristol-Myers Squibb (Evansville, Ind.). DDTC, 2-mercaptoethanol, and bovine serum albumin (BSA, Cohn fraction V) were purchased from Sigma Chemical Co. (St. Louis, Mo.). α-Minimum essential medium (α-MEM), sodium bicarbonate (7.5% solution), pokeweed mitogen, L-glutamine, and gentamicin were obtained from Gibco (Grand Island, N.Y.). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, Utah). Methylcellulose (A4M premium grade) was provided by Dow Chemical Co. (Midland, Mich.). Mixed bed resin AG501-X8(D) was acquired from Bio-Rad Laboratories (Richmond, Calif.). Falcon petri dishes were purchased from VWR Scientific Co. (Rochester, N.Y.).

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Abbreviations: DDTC, sodium diethyldithiocarbamate; cDDP, *cis*-diamminedichloroplatinum; CBDCA, diammine(1,1-cyclobutanedicarboxylato)platinum(II); tetraplatin, tetrachloro(d,l-*trans*)1,2-diaminocyclohexane platinum(IV); GM-CFC, granulocyte/macrophage colony-forming unit; α-MEM, α-minimum essential medium; PWM-SCCM, pokeweed mitogen-stimulated spleen-cell conditioned medium

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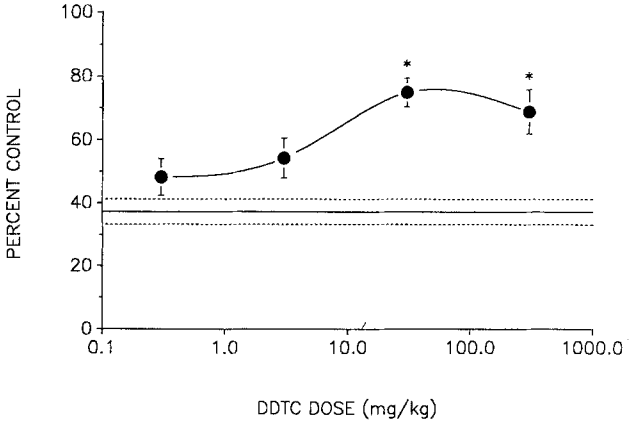

Fig. 1. DDTC dose response for rescue of GM-CFC following i. v. administration of 60 mg/kg CBDCA. Data are expressed as the percentage of survival as compared with saline-treated controls (bars, ± SE; $n = 7$). Control values were 287 ± 26 colonies/10^5 cells. * Significantly different from values obtained using CBDCA alone

Drug treatment. All drug solutions were prepared fresh just prior to injection. CBDCA and DDTC were dissolved in sterile deionized water; tetraplatin, 5-FU, and VP-16 were prepared in 0.9% saline. Drug solutions were sterilized by passage through a 0.22-μm filter. Mice were randomized in groups of three, and each mouse was warmed under a heating lamp to facilitate drug injection via the tail vein. Each mouse received two injections at the indicated interval. The volume of drug solution injected was dictated by the mean weight of the group; the total volume for the two injections never exceeded 0.7 ml. Control mice were injected with 0.9% saline.

Preparation of pokeweed mitogen-stimulated spleen-cell conditioned medium for the GM-CFC assay. Pokeweed mitogen-stimulated spleen-cell conditioned medium (PWM-SCCM) was prepared as described previously [7, 11]. Briefly, spleens were removed aseptically from four male $B6D2F_1$ mice and forced through a wire-mesh screen, and a single-cell suspension was prepared. The splenocyte suspension (10 ml, $2-4 \times 10^7$ cells/ml) was added to 90 ml α-MEM supplemented with 1% deionized BSA, 0.3% freshly reconstituted pokeweed mitogen, 10 μM 2-mercaptoethanol, and 50 μg gentamicin/ml. This mixture was incubated for 5 days at 37° C in a fully humidified incubator containing 5% CO_2 and 95% air. The conditioned medium was centrifuged (800 *g*, 10 min) and filtered successively through 0.45- and 0.22-μm filters. Aliquots of 10 ml were kept frozen at –20° C until use. Maximal stimulating activity was determined by titration.

GM-CFC assay. Bone marrow cells were harvested at 24 h after drug treatment (generally the time of maximal toxicity) and added to α-MEM supplemented with methylcellulose, fetal bovine serum, BSA, gentamicin, and PWM-SCCM. Aliquots were plated and incubated for 7 days (37° C, 5% CO_2) [7, 11]. A dissecting microscope was used to count the granulocyte/macrophage colonies (>50 cells/colony). Verification of cell morphology was determined by removing representative colonies with a fine-tipped Pasteur pipet, spinning them onto a slide using a Cytospin centrifuge, and staining them with Wright-Giemsa. The mean (± SE) number of colonies in each drug-treatment group was expressed as a percentage of the control value.

Statistical analysis. Significant differences were determined by Student's two-tailed *t*-test using the mean value ± SE for at least three separate experiments ($P \leq 0.05$).

Results

The DDTC dose response for modulation of CBDCA toxicity to GM-CFC was determined (Fig. 1). An approximately 2-fold increase in GM-CFC was observed at DDTC doses of 30 or 300 mg/kg, whereas a modest but statistically nonsignificant increase in GM-CFC was noted at a dose of 0.3 or 3 mg/kg. A dose of 30 mg/kg DDTC was used in all subsequent experiments. The ability of DDTC to modu-

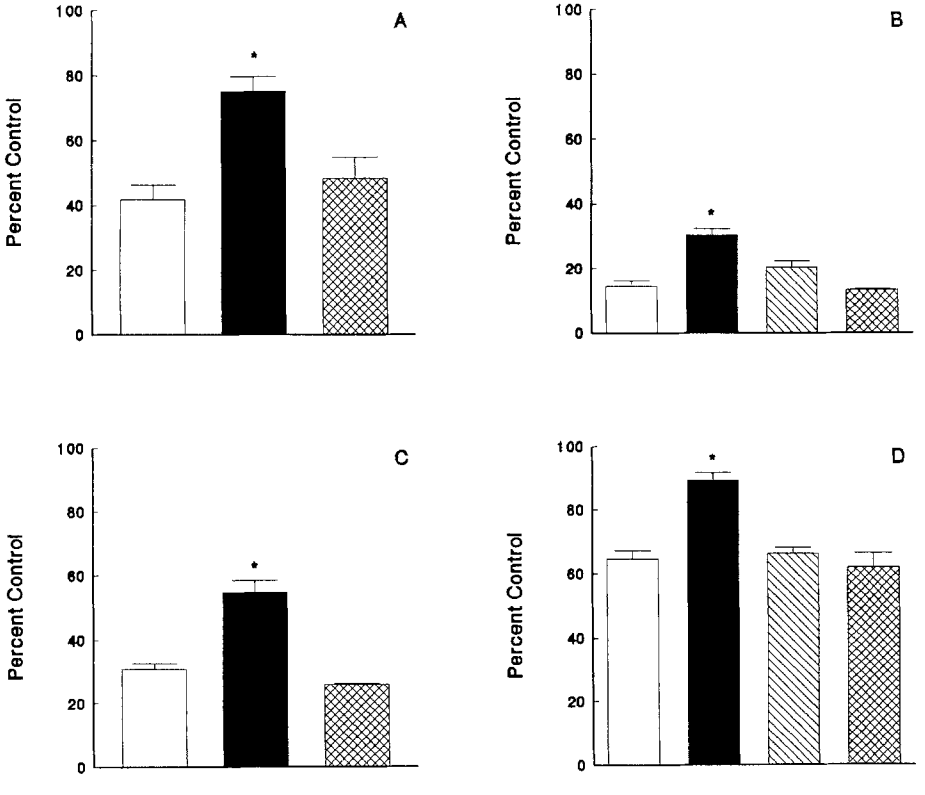

Fig. 2 A – D. Survival of GM-CFC after i. v. administration of myelosuppressive drug followed by saline (□) or 30 mg/kg DDTC either 1 (■), 2 (▨), or 3 (▩) h later. **A** 60 mg/kg CBDCA. **B** 5 mg/kg tetraplatin. **C** 50 mg/kg 5-FU. **D** 32 mg/kg VP-16. Data are expressed as the percentage of survival as compared with saline-treated controls (bars, ± SE). * Significantly different from values obtained using myelosuppressive drug + saline

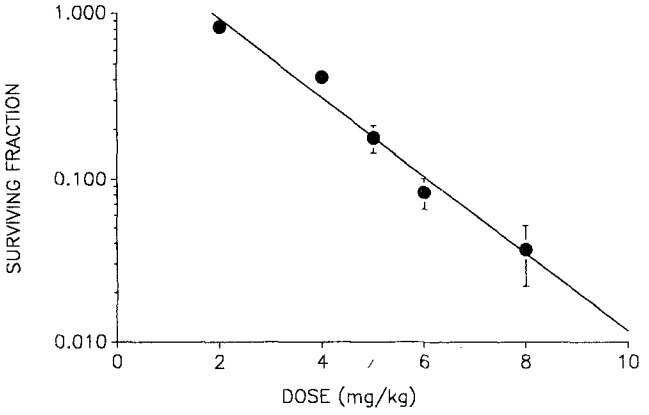

Fig. 3. Survival of GM-CFC following i. v. administration of tetraplatin. Data represent the percentage of survival as compared with saline-treated controls (bars, ± SE). Control values were 256 ± 16 colonies/10^5 cells

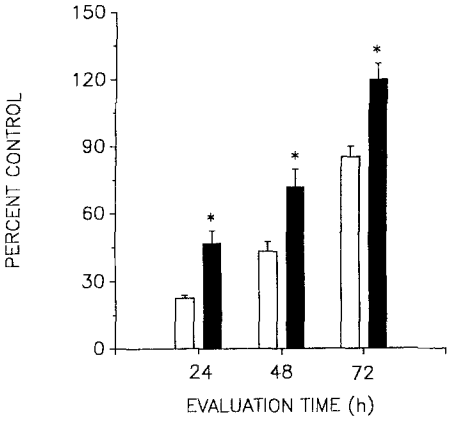

Fig. 4. Acceleration of recovery of GM-CFC after exposure to tetraplatin. Mice (3/group) were injected i. v. with 5 mg/kg tetraplatin followed by saline (□) or 30 mg/kg DDTC (■) given i. v. 1 h later. Marrow cells were harvested at 24, 48, or 72 h thereafter. Data represent the percentage of survival as compared with saline-treated controls (bars, ± SE; $n = 6$). Control values were 265 ± 11 colonies/10^5 cells. * Significantly different from values obtained using tetraplatin alone

late CBDCA-induced toxicity to the GM-CFC population is shown in Fig. 2a. A delay time of 1 h between the two treatments resulted in a 2-fold increase in GM-CFC, whereas a delay time of 3 h was without effect. The absence of protection noted when the interval was greater than 1 h is consistent with previous results [7]. DDTC has also been shown to rescue CFU-S from CBDCA-induced toxicity over a 4-log dose range (0.3 – 300 mg/kg) [12].

The effect of tetraplatin on the survival of GM-CFC according to dose is shown in Fig. 3. The tetraplatin dose lethal to 90% of the GM-CFC progenitors (LD_{90}) was determined to be 6.1 mg/kg (13.6 μmol/kg). In previous studies by Gringeri et al. [7], the LD_{90} values obtained for cDDP and CBDCA were 66.6 and 339 μmol/kg, respectively. Thus, tetraplatin's toxicity to GM-CFC on a molar basis is approximately 25 times that of CBDCA and 5 times that of cDDP. The effect of the delay time between the injection of tetraplatin and treatment with DDTC is shown in Fig. 2b. A 2-fold increase in the GM-CFC population was observed at a 1-h drug interval. Significant modulation of tetraplatin-induced toxicity was not observed when the delay time was increased to 2 or 3 h; when an interval of 15 or 30 min was used, modulation was equivalent to that observed following a 1-h interval (data not shown). DDTC-induced acceleration of GM-CFC recovery following tetraplatin treatment is illustrated in Fig. 4. Interestingly, it appears as though DDTC has shifted the proliferation of the GM-CFC population by 24 h.

DDTC was also evaluated for its ability to reduce the toxicity of non-platinum-based drugs to GM-CFC. 5-FU at a dose of 50 mg/kg was cytotoxic to approximately 70% of the GM-CFC population. When a delay time of 1 h between the administration of 5-FU and the injection of DDTC was used, a 1.8-fold increase in the number of GM-CFC was observed; however, DDTC injected 2 h after 5-FU had no effect (Fig. 2c). VP-16 is a highly myelosuppressive drug that is often used in association with cDDP in the clinic [8, 15]; the ability of DDTC to reduce VP-16-induced toxicity to GM-CFC was also assessed (Fig. 2d). The timing of DDTC administration was again critical in accelerating the recovery of GM-CFC; the progenitors increased 1.4-fold when a 1-h drug interval was used but were not enhanced at longer intervals.

Discussion

The data presented herein suggest that the timing of DDTC administration may be critical in protecting the GM-CFC population from the cytotoxic effects of cancer chemotherapeutic agents. This result is quite different from that observed for CFU-S; in those studies, DDTC protection was effective at drug intervals of 0–5 h [7, 11, 12]. The reason for this difference is not clear; the CFU-S assay measures an early pluripotent progenitor cell whose proliferation is regulated differently from that of the committed granulocyte/macrophage progenitor assessed in the GM-CFC assay.

DDTC reduces the myelotoxicity of four different chemotherapeutic drugs: two platinum-based alkylating agents, an antimetabolite, and a topoisomerase inhibitor. Thus, the protection of GM-CFC appears to be independent of the mechanism of action of the anticancer agent. This generalized protection of hematopoietic progenitor cells is consistent with the results of previous studies in which DDTC was shown to reduce toxicity to CFU-S induced by both platinum- (CBDCA, cDDP) and non-platinum-based drugs (Adriamycin, carmustine) [7, 11, 12].

The importance of the timing of DDTC administration in the protection of GM-CFC may explain the controversial results obtained using this drug in clinical trials. In one study, DDTC did not ameliorate the myelotoxicity of high-dose CBDCA in women with relapsed ovarian cancer [10]. In this case, DDTC was given 3 h after CBDCA. Although it is possible that the extensive prior chemotherapy received by these patients could have attenuated any myeloprotective effect of DDTC, the timing of DDTC administration may have been suboptimal for the protection of bone-marrow progenitor-cell populations (i.e., GM-CFC) other than CFU-S. DDTC has also been evaluated in two

separate studies in which patients received high-dose cDDP after the failure of conventional treatment [1, 6]. Although modulation of nephrotoxicity was the primary focus of these studies, the hematologic toxicity normally associated with high-dose cDDP was not observed. It is noteworthy that DDTC administration was initiated 45 min after cisplatin treatment in these trials.

A recent report suggests that DDTC may accelerate bone marrow recovery following chemotherapy by inducing the release of colony-stimulating factors that are known to be essential for the proliferation and differentiation of many hematopoietic cells [13]. The difference in the timing of DDTC administration with regard to protection of the CFU-S and/or GM-CFC populations may be a function of this observation. It is possible that protection is dependent on the kinetics of the cytotoxic insult and DDTC-induced growth-factor release and subsequent interaction with the progenitor cells. In the case of the CFU-S population, the timing of DDTC administration may not be as critical because normally only 10% of these cells are cycling at the time of cytotoxic insult [14]. The cytotoxicity of the anticancer drug to the hematopoietic microenvironment that gives rise to many of these growth factors may also be a factor. If the kinetics are such that the anticancer agent causes cell death within the microenvironment before DDTC can stimulate the growth-factor release necessary for GM-CFC proliferation, then this may explain why the timing of administration is so important. Furthermore, DDTC cannot stimulate myelopoiesis in vivo if it is given in the absence of a cytotoxic insult; this is presumably due to the strict regulation of the hematopoietic system [7]. Thus, there may be a critical window for DDTC administration that will determine whether or not certain hematopoietic progenitor cells such as GM-CFC will respond to DDTC stimulation. Although the optimal drug-DDTC interval is approximately 1 h in this murine model, temporal differences in the hematopoietic response between animals and humans may result in a different optimal interval in the clinic.

In conclusion, DDTC enhances proliferation of the surviving GM-CFC population following chemotherapy-induced toxicity. This protection may be a function of the kinetics involved between the recognition of a toxic insult by the bone marrow, chemotherapy-induced cell death in the microenvironment, and the ability of DDTC to stimulate growth-factor production. Further studies to delineate the mechanism of action of DDTC are in progress in our laboratory.

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