Diethylthiocarbamate Modulation of Murine Bone Marrow Toxicity Induced by cis-Diammine(cyclobutanedicarboxylato)platinum(II)  

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ABSTRACT

Diethylthiocarbamate (DDTC) has been shown to ameliorate the myelosuppression induced by the platinum cancer chemotherapeutic drugs in mice. Optimal drug scheduling for DDTC and cis-diammine(cyclobutanedicarboxylato)platinum(II) (CBDCA) has been determined in C57BL/6×DBA/2 F1 mice, using the pluripotent stem cell assay to assess hematological toxicity. DDTC, at doses of 0.3 to 300 mg/kg given 3 h after 60 mg/kg CBDCA, tripled the number of proliferating spleen colony-forming units compared to treatment with CBDCA alone. No significant difference in efficacy was noted among these doses. DDTC, at the lowest myeloprotective dose (0.3 mg/kg), was most active when administered from 1 to 3 h after CBDCA. The combination of DDTC with CBDCA in vivo did not alter the clonogenic survival of L1210 cells compared to CBDCA alone. CBDCA depressed both bone marrow and tumor cell DNA synthesis. DDTC given 3 h after CBDCA hastened the recovery of DNA synthesis only in marrow cells; the addition of DDTC to CBDCA did not alter DNA synthesis in tumor cells. DDTC alone did not significantly affect DNA synthesis in either normal or tumor cells. These results suggest that the mechanism of DDTC myeloprotection involves stimulation of bone marrow cell proliferation and that the selectivity of DDTC is based upon the absence of stimulation in tumor cells.

INTRODUCTION

The platinum antitumor agents are among the most widely used drugs in the cancer chemotherapy armamentarium. These drugs, exemplified by cisplatin and carboplatin, are particularly effective in the treatment of testicular and ovarian cancer and have demonstrated therapeutic efficacy against a variety of other carcinomas (1). The cytotoxic activity of cisplatin is generally believed to be due to the binding of the aquated form of cisplatin to DNA. The bound platinum adducts subsequently form intra- and interstrand cross-links which block DNA replication (2, 3).

Cisplatin analogues such as CBDCA lack the dose-limiting side effects of cisplatin and can be administered at higher doses. CBDCA has a mechanism of action that is identical to that of cisplatin, differing only in a slower rate of formation of the cytotoxic metabolite (4). Myelosuppression, characterized primarily by neutropenia and thrombocytopenia, has emerged as the dose-limiting side effect of CBDCA (5–7). Amelioration of the mechanism of hematological protection by DDTC is unknown. The ability of DDTC to modulate hematological toxicity caused by 1,3-bis(2-chloroethyl)-1-nitrosourea and Adriamycin indicates that a mechanism of action independent of metal chelation is likely in the bone marrow (13). Elucidation of this mechanism is the focus of the data presented here.

MATERIALS AND METHODS

Materials. CBDCA was the generous gift of Johnson-Matthey, Inc. (Malvern, PA). DDTC, RNase A (bovine pancreas type I-AS), and propidium iodide were purchased from Sigma Chemical Company (St. Louis, MO). Fischer’s medium, L-glutamine, and antibiotics were purchased from BioWhittaker (Walkersville, MD). [3H]Thymidine and [3H]thymidine were purchased from ICN Biomedicals (Irvine, CA). [ methyl-3H]Thymidine was obtained from Amersham (Arlington Heights, IL). Other chemicals were purchased from J. T. Baker Chemical Company (Phillipsburg, NJ).

Experimental Animals. Male 6- to 8-week-old C57BL/6J×DBA/2J mice (hereafter called B6D2F1; mice) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed 10/cage in plastic cages and allowed food and water ad libitum, except that acidified water was given to irradiated mice. All mice were allowed a 7-day acclimatization period prior to experimentation.

Drug Treatment. All drug solutions were prepared immediately prior to use and filter sterilized prior to administration. CBDCA was dissolved in D5W at a concentration of 4 mg/ml. DDTC was dissolved in sterile water at 5- and 50-mg/ml concentrations, respectively, for doses of 30 and 300 mg/kg. At lower doses sterile saline was used as the diluent, and the drug concentration was decreased proportionately. Mice were randomly assigned to different treatment groups (4/group). Each group was warmed 1–2 min under a heating lamp prior to drug administration, at the specified intervals, via the lateral tail vein. Control groups received injections of vehicle alone. The mean weight of the four mice per group was used to calculate the volume of the injected material. The total volume of the two injections was always less than 0.7 ml. When drug combinations were given, the time intervals were calculated from the time of CBDCA administration (or D5W in the case of control animals). The dose of CBDCA was 60 mg/kg in all experiments.

Pluripotent Stem Cell (CFU-S) Assay. This assay is a modification of the method of Till and McCulloch (16). Twenty-four h after drug treatment, each group of four mice was sacrificed by cervical dislocation. The femurs were dissected, cut into small pieces, and placed in ice cold Puck’s saline A. Bone marrow cells were harvested by flushing the cells from the femurs with ice-cold Puck’s saline A. A single cell suspension of the pooled marrow was made by gentle, repeated pipetting of the sample. The number of bone marrow cells was determined by counting the number of viable (dye-excluding) nucleated (determined via lysis with Turk’s solution) cells. The cell density was adjusted by dilution with additional Puck’s saline A. These cells were then injected i.v. into

Received 6/6/89; revised 8/28/89; accepted 9/1/89.

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1 This work was supported by Grants CA 34620 and CA 11198 from The National Cancer Institute and grants from the Wilmot and Wilson Foundations.

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The abbreviations used are: CBDCA, cis-diammine(cyclobutanedicarbonylato)platinum(II); DDTC, diethyldithiocarbamate; D5W, 5% dextrose in water; CFU-S, spleen colony-forming unit; PBS, phosphate-buffered saline.
syngeneic mice that had received 950 rad of total body irradiation from a cesium source 5–8 h earlier. The number of cells injected, 8 × 10⁴ to 15 × 10⁴ cells/0.2 ml, was adjusted to yield 5–20 colonies/spleen. Twelve days after marrow injection the spleens from the recipient mice were harvested and fixed in Bouin’s solution, and the macroscopic spleen colonies were counted with the aid of a dissecting microscope. The number of colonies was normalized to the number of cells injected, and the results were expressed as the fraction of CFU-S surviving compared to the vehicle-treated control group. Three experiments were performed for each drug dose.

L1210 Leukemia Cell Clonogenic Assay. L1210 tumor cell lines were maintained by weekly passage in DBA/2J mice. Experiments with L1210 leukemia cells were carried out by implanting 1 × 10⁶ cells i.p. in B6D2F₁ mice. Tumored mice were randomly divided into groups of four and were given i.v. injections, at the specified intervals prior to sacrifice, of CBDCA, DDTC, and/or vehicle as described above. Seven days after tumor implantation mice were sacrificed by cervical dislocation, and the ascitic fluid from each group was harvested, pooled, and maintained on ice. The number of viable nucleated cells was determined, and the cell density was adjusted to the desired concentration by further dilution with supplemented Fischer’s medium. Aliquots of these freshly harvested samples were used to assay DNA synthesis rates (see below) and clonogenicity.

The L1210 clonogenic assay used is a modification of the dilute agar colony method described by Chu and Fischer (18). Feeder cells, used to improve the plating efficiency (average plating efficiency, 61%), were harvested from CBDCA-treated animals as described previously. These cells were irradiated with 10,000 rad to prevent clonogenic growth. Cells from treated animals were used to eliminate any indirect effect CBDCA might have on clonogenicity. Both experimental and control cells were therefore plated in the presence of cells which had been exposed to CBDCA. Varying numbers of cells harvested from experimental or control groups were combined with the appropriate concentration of feeder cells to bring the total density of cells to 5 × 10⁴ cells/ml. The number of experimental or control cells plated was adjusted to yield 10 to 40 colonies/well. Nine ml of 46°C supplemented Fischer’s media, containing 0.2% Noble agar, were added to the 3 ml of cell suspension. This was mixed well, and 1 ml was placed in each of 10 wells in a 24-well tissue culture plate. Feeder cells alone were also plated to verify that the radiation dose was sufficient to eliminate colony formation. Unused wells in the plate were filled with 1 ml of sterile water. The plates were placed in a 37°C fully humidified 5% CO₂ incubator for 10 days. Spherical colonies (>50 cells/wells in a 24-well tissue culture plate. Feeder cells alone were also plated, and the number of viable nucleated cells was determined, and the cell density was adjusted to the desired concentration by further dilution with supplemented Fischer’s medium. The number of colonies in the 10 wells was normalized to the number of cells injected, and the results were expressed as the fraction of CFU-S surviving compared to the vehicle-treated control group. Three experiments were performed for each drug dose tested.

DNA Synthesis Assay. The effects of CBDCA and DDTC, alone and in combination, on DNA synthesis occurring in normal bone marrow and in L1210 tumor cells were investigated. Bone marrow DNA synthesis rates were determined via the method of Schein et al. (19). Normal B6D2F₁ mice were divided into groups of four, and in this study, syngeneic mice that had received 950 rad of total body irradiation from a cesium source 5–8 h earlier. The number of cells injected, 8 × 10⁴ to 15 × 10⁴ cells/0.2 ml, was adjusted to yield 5–20 colonies/spleen. Twelve days after marrow injection the spleens from the recipient mice were harvested and fixed in Bouin’s solution, and the macroscopic spleen colonies were counted with the aid of a dissecting microscope. The number of colonies was normalized to the number of cells injected, and the results were expressed as the fraction of CFU-S surviving compared to the vehicle-treated control group. Three experiments were performed for each dose and time.

A modification of this procedure was used to determine DNA synthesis rates in L1210 cells. The tissue ascites the tumors cells were harvested and used to determine DNA synthesis rates (Fig. 1, closed circles). The addition of DDTC at doses ranging from 0.3 to 300 mg/kg resulted in a 2.9–3.5-fold increase in the fraction of proliferating CFU-S compared to CBDCA alone. Interestingly, no significant differences were seen among the effective DDTC doses used. At DDTC doses < 0.3 mg/kg, no significant increase in CFU-S surviving fraction was observed. As reported previously, administration of DDTC alone did not significantly alter the number of CFU-S compared to control [21 ± 2 versus 20 ± 2 CFU-S per 10⁵ cells injected for DDTC and control groups, respectively (13)]. Fig. 2 depicts the surviving fraction of CFU-S following administration of DDTC, 0.3 mg/kg, at times preceding or following CBDCA. DDTC was most effective when given 1 to 3 h after CBDCA, inducing a 4-fold increase in the CFU-S proliferation compared to CBDCA alone.

Bone Marrow DNA Synthesis Rates. Administration of CBDCA caused a rapid decrease in bone marrow DNA synthesis rates (Fig. 3). DNA synthesis decreased as early as 8 h after CBDCA administration, reaching a nadir by 24 h. Recovery was evident at 48 h, and a large overshoot to levels up to 4 times control was consistently observed 72 h following CBDCA injection. The addition of DDTC (3 or 300 mg/kg 3 h after
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Fig. 1. Survival of pluripotent stem cells (CFU-S) (•) and rate of bone marrow DNA synthesis (○) following injection of CBDCA (60 mg/kg i.v.) followed 3 h later by the indicated dose of DDTC. CFU-S data represent the surviving fraction of colonies from drug-treated animals compared to vehicle-treated control values ± SEM (bars) from 4 drug-treated and 6–10 recipient mice per group in 3 experiments. DNA synthesis results, measured in dpm/µg DNA, are expressed as a percentage of the vehicle-treated DNA synthesis ± SEM from 3 experiments. The mean ³H incorporation in untreated control marrow cells was 5.87 x 10⁴ dpm/µg DNA. See “Materials and Methods” for details. *, CFU-S and DNA synthesis significantly different from CBDCA alone.

Fig. 2. Survival of pluripotent stem cells (CFU-S) after administration of CBDCA (60 mg/kg i.v.) followed or preceded (indicated by – times) by DDTC treatment (0.3 mg/kg). ---, mean survival of CFU-S following treatment with CBDCA alone. See “Materials and Methods” for details. *, significantly different from CBDCA alone. Bars, SEM.

Fig. 3. DNA synthesis rates in bone marrow cells following i.v. injection of 60 mg/kg CBDCA (○) or CBDCA followed 3 h later by 300 mg/kg DDTC (●). *, significantly different from CBDCA alone. Bars, SEM.

CBDCA did not alter the general pattern of DNA synthesis inhibition but caused both the nadir and recovery to occur earlier. DDTC (0.3 to 300 mg/kg 3 h after CBDCA) significantly elevated 48-h DNA synthesis compared to CBDCA alone (Fig. 1, open circles); no effect was noted for DDTC doses < 0.3 mg/kg. DNA synthesis rates following administration of DDTC alone (300 mg/kg) ranged from 40 to 161% of control values over the 8- to 96-h time period.

Cell Cycle Status of Treated Bone Marrow Cells. Cell cycle distributions were essentially identical at all time points for marrow cells taken from drug-treated and vehicle-treated mice. Approximately 70% of bone marrow cells were in the G₀-G₁ phase, ~15% were in S phase, and the remaining ~15% were in G₂-M. This distribution was not altered by the administration of CBDCA, DDTC, or the combination of the two drugs.

L1210 Clonogenicity. The response of L1210 cells growing i.p. to a single i.v. injection of 60 mg/kg CBDCA is depicted in Fig. 4. The ascitic fluid isolated from treated animals at 4, 8, and 24 h following injection contained a relatively large fraction of viable cells that were incapable of forming colonies in vitro. CBDCA, administered alone or followed by DDTC, resulted in a 3-log reduction of clonogenicity. Following the nadir at 24 h, rapid regrowth occurred; by 96 h, the ascitic fluid contained nearly the same fraction of clonogenic cells as did vehicle-treated controls. Similar results were obtained using a low dose of DDTC (3 x 10⁻³ mg/kg). Either dose of DDTC alone had no significant effect on L1210 clonogenicity (the number of clonogenic cells ranged from 75 to 110% of control).

L1210 DNA Synthesis Rates. DNA synthesis in L1210 cells was inhibited by CBDCA (Fig. 5). Initially synthetic rates remained equivalent to control levels before declining to a nadir at 48 h. Recovery was slow, and DNA synthesis remained depressed even after 96 h. The addition of DDTC (3 or 300 mg/kg 3 h after CBDCA) did not significantly change DNA synthesis compared to CBDCA alone. Both doses of DDTC alone had no effect on DNA synthesis compared to control values.

DISCUSSION

Myelosuppression is monitored clinically by measuring peripheral WBC, but murine peripheral counts do not accurately
reflected the hematological toxicity associated with CBDDCA (22). CFU-S are the ultimate precursor cells that divide and differentiate to yield all the lineages of blood cells (23) and thus are crucial for regeneration and maintenance of the hematopoietic system following a toxic insult. The survival and proliferation of these cells are indicative of bone marrow integrity.

The data presented here delineate the dose range and timing of DDTC administration for optimal protection of stem cells. It is particularly noteworthy that DDTC reduces hematological toxicity at doses that are 2–3 orders of magnitude below the minimum effective dose required for reduction of cisplatin nephrotoxicity (9, 10). The effective dose range of DDTC has been extended from that previously reported (12) to one covering almost 4 orders of magnitude. The fraction of proliferating CFU-S does not significantly differ over this dose range, and higher doses of DDTC do not restore stem cells to control levels. Thus, a degree of irreversible myelosuppression occurs that is not modulated by DDTC administration.

The lowest effective dose of DDTC was used to assess the timing of stem cell chemoprotection. Since DDTC has such a broad myeloprotective dose range, the amount of drug injected can critically affect measurement of the optimal time of administration. For example, given the plasma half-life of DDTC (10), administration of DDTC at a dose of 300 mg/kg would still provide myeloprotective plasma levels almost 2 h after DDTC treatment. The optimal time of low-dose DDTC administration for stem cell protection was found to be 1–3 h after CBDDCA injection. This observation is consistent with data reported by Dible et al. in which multiple doses of DDTC were given (24).

The marrow cell cycle data did not provide evidence of a greater proportion of DDTC-treated cells in S phase. However, these studies were performed on unfractionated bone marrow which contains all the various types of hematopoietic cells, each of which has a different replicative ability. The frequency of clonogenic marrow cells, the hematopoietic cells with the greatest replicative activity, and thus the cells most likely to account for the increased DNA synthesis is only 1.5% (25). A dramatic increase in cell cycle progression in this small fraction may thus have been obscured.

To be clinically applicable a chemoprotective agent must provide protection for, or ameliorate toxicity of, a cancer chemotherapeutic drug without interfering with the tumoricidal efficacy. DDTC has been shown to fulfill both these criteria; it is devoid of inhibitory effect on the antitumor activity of platinum drugs against L1210 (10, 26) and P388 leukemias (11); B16 melanoma, Lewis lung, and colon 26 tumors (10); and TA3 Ha mammary tumor (27). The clonogenic assays of L1210 cells treated with CBDDCA and DDTC in vivo provide further evidence of this selectivity, in that DDTC had no effect on inhibition of clonogenicity or DNA synthesis resulting from CBDDCA treatment. The comparative effects of DDTC on DNA synthesis in L1210 and normal bone marrow cells are also of interest. Both cell types exhibited dramatic reductions in DNA synthesis following CBDDCA treatment. This was not unexpected, since the postulated mechanism of cytotoxicity of CBDDCA involves formation of DNA intra- and interstrand adducts with subsequent inhibition of DNA replication (2, 3).

An excellent correlation was observed between marrow DNA synthesis and stimulation of CFU-S proliferation at different DDTC doses following CBDDCA administration (Fig. 1). Surprisingly, DDTC had no effect on marrow DNA synthesis of CFU-S proliferation in the absence of cytotoxic drug treatment. This suggests that marrow damage may be a prerequisite for hematopoietic protection by DDTC. Alternatively, DDTC may not initiate accelerated bone marrow proliferation but may enhance proliferation after induction by some other event. In either case, DDTC appears to be operating within the limits of normal hematopoietic regulation, where an increase in hematopoiesis is produced by DDTC only if it represents an appropriate physiological response.

In summary, DDTC does not inhibit the direct toxicity of CBDDCA to hematopoietic progenitor cells; its mechanism of bone marrow protection is based instead upon DDTC stimulation of marrow proliferation, thus accelerating recovery from hematological injury. This stimulation is likely to be the basis for the DDTC bone marrow protection observed with other myelosuppressive drugs. Although the effect is similar to that observed following administration of bone marrow colony-stimulating factors, DDTC is unique in that a proliferative response occurs only when it is given shortly after a myelosuppressive drug. The precise mediators and targets involved in this proliferative response are currently under investigation.

ACKNOWLEDGMENTS

The authors wish to thank Peter C. Keng for his expert assistance with the bone marrow cell cycle experiments.

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