Diethylthiocarbamate Inhibition of Murine Bone Marrow Toxicity Caused by cis-Diaminedichloroplatinum(II) or Diammine-(1,1-cyclobutanedicarboxylato)platinum(II)

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ABSTRACT

We report here the effects of diethylthiocarbamate (DDTC) rescue on myelotoxicity caused by carboplatin (CBDCA) and cisplatin (DDP) in C57BL/6 × DBA/2 F1 mice. All drugs were administered by injection into the tail vein. Myelotoxicity was assessed by WBC, bone marrow cellularity, and assays for pluripotent bone marrow stem cells (spleen colony forming unit) and granulocyte/macrophage progenitor cells (granulocyte/macrophage colony forming unit in culture). The most significant protection occurred in stem cells, where a single dose of DDT (300 mg/kg) produced a platinum-dose modification factor of 3.3; i.e., the addition of DDT reduced stem cell toxicity to the level produced by approximately one-third the dose of platinum drug alone. On a molar basis, DDP was 2.4 times as toxic to stem cells as CBDCA. The response of the stem cells to CBDCA and DDP was linear both with and without rescue, and the dose modification factor remained constant for doses of CBDCA up to 120 mg/kg and doses of DDP up to 15 mg/kg. Moreover, stem cell rescue appeared to be independent of DDT dose (100–750 mg/kg) and time of administration (1.5 h before to 5 h after platinum drug). DDT protection was less impressive for more hematological cells (granulocyte/macrophage colony forming units in culture). In studies of bone marrow cellularity, addition of DDT (300 mg/kg) to DDP treatment (10 mg/kg) produced a 50% increase in the granulocyte/macrophage predominated cell population but had no effect on the lymphocyte population. Peripheral WBC showed no significant difference between rescued and unrescued groups and did not reflect the toxicity observed directly in the bone marrow.

INTRODUCTION

A major limitation to the clinical use of DDP and CBDCA is their toxicity to normal tissues. DDP adversely affects the kidney, gastrointestinal tract, peripheral nerves, and bone marrow (1–3), with myelosuppression becoming particularly severe in the high-dose DDP regimes developed in the last few years (4, 5). CBDCA, although relatively free of nephrotoxicity and neurotoxicity, is limited by profound myelosuppression (6). Clearly, any reduction of their myelosuppressive effects would permit further dose escalation of DDP and CBDCA and greater versatility in combination regimens based upon reduced toxicity.

In the last decade, we and others have attempted to improve the therapeutic ratios of the platinum drugs using chemoprotectors (7–11). Ideally, these compounds should inhibit platinum-mediated toxicity without reducing antitumor activity. Of the chemoprotectors investigated with DDP and CBDCA, DDTTC may show the greatest promise. At moderate doses, it causes no severe toxicity to any major organ system. Moreover, it inhibits DDP toxicity to the kidney, gastrointestinal tract, and bone marrow without reducing antitumor activity (11–15). DDT also inhibits CBDCA-induced myelosuppression (16, 17).

We report here our studies of the chemoprotective effects of DDTT against platinum drug myelosuppression, specifically the effects of DDTT on WBC, bone marrow cellularity, hematological stem cells (CFU-S), and granulocyte/macrophage progenitor cells (GM-CFC) after the administration of DDP or CBDCA. The possibility that DDTT exerts these chemoprotective effects through a mechanism other than simple chelation is also discussed.

MATERIALS AND METHODS

Materials. DDP and CBDCA were kindly supplied as pure compounds by Bristol Laboratories, Wallingford, CT. DDTT, 2-mercaptoethanol, and bovine serum albumin (fraction V) were purchased from Sigma Chemical Company, St. Louis, MO. McCoy's Medium 5A, MEM-α, pokeweed mitogen, L-glutamine, sodium bicarbonate (7% solution), gentamicin, trypan blue, and fetal calf serum were purchased from Gibco, Grand Island, NY. Wright's stain, Giemsa stain, orcein, picric acid, sodium bicarbonate, and Falcon Petri dishes were purchased from Fisher Scientific Company, Springfield, NJ. Methylcellulose (A4M premium grade) was purchased through the courtesy of Dow Chemical Company, Midland, MI. Mixed bed resin AG501-X8(D) was purchased from Bio-Rad Laboratories, Richmond, CA. Fetal calf serum (colony forming unit/granulocyte/macrophage assay only) was obtained from HyClone Laboratories, Logan, UT. Formalin, 10% (v/v) solution, was purchased from J. T. Baker Chemical Company, Phillipsburg, NJ. Isoton Balanced Electrolyte Solution, Zap-Oglobin II Hi-Speed Stromatolysing Reagent, and Coulter 40-μl heparinized micropipets were purchased from Curtin Matheson Scientific, Houston, TX.

Animals. Male C57BL/6 × DBA/2 F1 (hereafter called B6D2F1) mice 6–8 weeks old and weighing 20–22 g were obtained from the Jackson Laboratory, Bar Harbor, ME. All mice were housed in plastic cages, 10 mice/cage, and provided with food and water ad libitum. Irradiated animals used in the CFU-S assay were housed in cages covered by filter bonnets.

Drug Treatment. DDP was dissolved in sterile 0.9% saline; CBDCA and DDTT were dissolved in sterile water. All drug solutions were prepared within 15 min of administration and stored on ice until use. The drugs were administered by injection into the tail vein after the mice had been gently warmed under a 100-W lamp and had their tails soaked briefly in a warm water bath. The mice received either a platinum drug alone, DDTT alone, or a platinum drug plus DDTT at doses and intervals specified; control animals received injections of sterile 0.9% saline. The volume of each injection was 0.2 ml.

WBC. Mice were bled under light ether anesthesia at approximately 10 a.m. on the days specified. A 40-μl sample of blood was drawn from the retroorbital venous plexus into a heparinized glass micropipet...
According to the method of Riley (18), each sample was added to 20 ml of Isoton, the RBCs were lysed by adding approximately 200 μl (6 drops) of Zap-Oglobin II, and the nucleated cells were counted with a Coulter Counter. Counts were made immediately before dosing, 24 h after dosing, and every 48 h thereafter until Day 11. The mean of three separate counts was recorded for each mouse in a group of five mice. Data are reported as the mean ± SE of each group.

Collection of Bone Marrow Samples. The mice were sacrificed by cervical dislocation, and their femurs were removed surgically, scraped free of tissue, and stored on ice in McCoy's Medium 5A containing 10% fetal calf serum until all femurs had been collected. The integrity of the bone and hip joints was maintained during the dissection. The marrow was harvested by snipping the tip of the femur and flushing it with 2 ml of ice-cold medium. Flushing was accomplished with a tuberculin syringe fitted with a 27-gauge needle. All cell suspensions were stored on ice and used in the various assays within 1 h of harvest.

Bone Marrow Cellularity. The bone marrow cell suspension was brought to a volume of 10 ml with ice-cold McCoy's Medium 5A, and a 0.25-ml aliquot was diluted 1:100 with Isoton. The cells in this solution were then counted with a Model ZM Coulter Counter equipped with a 100-μm high-resolution aperture and connected to a Model 256 Coulter Channelizer. The Channelizer provided a distribution of the marrow cells according to size. The number of nucleated cells in each femur was determined by counting each bone marrow sample again after the erythrocytes had been lysed with approximately 260 μl (8 drops) of Zap-Oglobin II. The mean of five separate counts was recorded for each femur; then the average of both femurs was calculated for each mouse in a group of five. Data are reported as the mean ± SE of each group.

A centrifugal elutriation system was used to identify the cell types shown in the Channelizer analysis of mouse bone marrow suspensions. The detailed centrifugal elutriation procedure has been described previously (19). Briefly, the Beckman JE6 elutriator was sterilized by autoclaving and washing with 70% ethanol the night before the experiment. The separation procedure, carried out at 4°C, required 1 × 10⁸ bone marrow cells to ensure sufficient (5 × 10⁴–1 × 10⁵) homogeneous cells in each fraction. The bone marrow cells were suspended in McCoy's 5A tissue culture medium, and sterile McCoy's Medium 5A served as the elutriation fluid.

To perform the separation, cells were loaded into the separation chamber of the elutriator at a rotor speed of 4050 rpm and a fluid flow rate of 6 ml/min. After loading was completed, 40-ml fractions were collected by decreasing the rotor speed in increments of 50–100 rpm while holding the fluid flow rate constant at 9 ml/min. The cell numbers and cell size distributions of each fraction were measured with a Coulter Counter and Channelizer, and the cell types were identified histologically with a Cytospin centrifuge and Giemsa staining.

Pluripotent Stem Cell (CFU-S) Assay. This method is based on that of Till and McCulloch (20). Twenty-four h after drug treatment, bone marrow cells from five mice were collected, pooled, and maintained on ice. The number of nucleated cells was determined using a Coulter Counter, and their viability was assessed using a hemacytometer and trypan blue exclusion. The concentration of viable nucleated cells was adjusted to 4 × 10⁵/ml with McCoy's Medium 5A containing 10% fetal calf serum. These cells were then injected into the tail veins of 15 mice per group earlier that day. The standard injection contained 8 × 10⁴ cells, while holding the fluid flow rate constant at 9 ml/min. The cell numbers and cell size distributions of each fraction were measured with a Coulter Counter and Channelizer, and the cell types were identified histologically with a Cytospin centrifuge and Giemsa staining.

Results

Peripheral WBC. A single dose of DDP (10 mg/kg) caused the WBC to fall by approximately 40% to a nadir on Day 4 of 5400 ± 730 cells/μl. By Day 5, however, the WBC returned to within 10% of saline-treated control values. A single dose of DDTC (300 mg/kg) given 2 h after DDP gave a nadir of 6215 ± 540 cells/μl, indicating that DDTC does not prevent this DDP-mediated decrease in WBC. In mice treated with DDTC alone (300 mg/kg), mean WBC did not differ significantly from saline-treated control values. However, the variability of each mean value is quite large regardless of drug treatment, even though each mean is calculated from five samples.

Bone Marrow Cellularity. The Channelizer tracing of normal bone marrow was trismodal (Fig. 1). The cells of Region I had diameters of 3.8–5.7 μm with a mode at 4.5 μm; histological identification showed that >90% of these cells were erythrocytes. The cells of Region II had diameters of 5.7–7.7 μm with a mode at 6.9 μm; >90% of these cells were lymphocytes. The cells of Region III had diameters of 7.7–11.5 μm with a mode at 8.5 μm. This region contained granulocytes, monocytes, megakaryocytes, and progenitor cells. These values agree with marrow cell sizes of 5.5 μm (erythrocytes), 6–9 μm (lymphocytes), 10–12 μm (granulocytes), 9–11 μm (GM-CFC), and 11.2
survival was linear across the range of doses tested for both platinum drugs, decreasing as the dose of platinum drug increased. Although survival was greater than expected with DDP alone at a dose of 32 mg/kg, the concentration of injected drug exceeded the usual solubility limit, and heating and sonication were required to maintain the drug in solution. Thus reduced bioavailability may have contributed to the diminished cytotoxicity at this dose. If a single dose of DDTC (300 mg/kg) was added to the regimen 2 h after DDP or 3 h after CBDCA, stem cell survival remained linear and showed impressive protection. The ratio of the slopes of the two regression lines was 14/4.2, and the slopes were significantly different (P < 0.001). Thus DDTC provides a dose modification factor of 3.3; i.e., the combination of DDTC and platinum drug produced toxicity equivalent to that produced by approximately one-third the dose of platinum drug alone. When given by itself, DDTC had no effect on stem cell number; at DDTC doses from 300 to 750 mg/kg, CFU-S counts did not vary by more than 5% from saline-treated control values. Stem cell protection appeared to be independent of DDTC dose over the range from 100 to 750 mg/kg (Fig. 4A) and was essentially independent of DDTC administration time from 1.5 h before to 5 h after CBDCA (Fig. 4B).

Granulocyte/Macrophage Progenitor Cell (GMP-CFC) Assay. In contrast to the marked protection provided by DDTC to stem cells, protection by DDTC of platinum drug toxicity to granulocyte/macrophage progenitor cells was minimal. Fig. 5 shows the effect of CBDCA on GM-CFC. The GM-CFC depression was roughly linear across the range of doses tested, although at no dose was it as great as the CFU-S depression (slope, $9 \times 10^{-3}$ versus $14 \times 10^{-3}$ for CFU-S). A single dose of DDTC (300 mg/kg) given 3 h after CBDCA did not ameliorate this toxicity. DDTC alone had no effect on GM-CFC; at doses from 100 to 1000 mg/kg, GM-CFC counts varied less than 5% from saline-treated control values.

DDP toxicity to GM-CFC was less than to CFU-S, and the protection afforded by DDTC was greater than observed for CBDCA. A DDP dose of 10 mg/kg gave GM-CFC counts of 54 ± 6% and 78 ± 8% of control counts in the absence and presence of 300 mg/kg DDTC, respectively. The corresponding values for 15 mg/kg DDP were 22 ± 2% and 35 ± 4%,
control values ± SE (ears); 5 drug-treated and 15 recipient mice per group. All drug. Spleen colonies were evaluated 12 days after the treated marrow cells were harvested 24 h after treatment with platinum drug. A 3-h interval was chosen in both figures, marrow cells were harvested 24 h after treatment with platinum drug. Spleen colonies were evaluated 12 days after the treated marrow cells were injected into lethally irradiated hosts. Data are plotted as surviving fraction of control values ± SE (bars); 5 drug-treated and 15 recipient mice per group. All CBDA + DDTC treatment groups were significantly different from CBDA treatment alone (P < 0.01). * significantly different from the 30-min before and 1-3-h after CBDA groups.

respectively. The same values were obtained when the DDTC dose was increased to 500 mg/kg.

DISCUSSION

The most important effect of DDTC on platinum drug-mediated myelotoxicity is its dramatic rescue of bone marrow stem cells, with a dose modification factor in excess of 3 for cisplatin and carboplatin. Stem cells, the ultimate ancestors of all the marrow cell lineages, are responsible for repopulating the blood and bone marrow after a pharmacological insult. They represent only a fraction of the total bone marrow, perhaps as few as 1 per 10^3-10^4 cells (24, 25), yet successful recovery depends completely upon their surviving in adequate numbers. DDTC rescue can maintain stem cell toxicity within acceptable limits at doses of platinum drug that would be intolerable otherwise.

The time interval between platinum drug and DDTC was selected on the basis of platinum drug pharmacokinetics. The 2-h interval for cisplatin has been used extensively in our previous studies (11-14) and represents approximately 4 plasma half-lives for the parent drug. A 3-h interval was chosen for carboplatin because of the slower plasma clearance for this drug. The data in Fig. 4 suggests that reduction of carboplatin hematological toxicity at this DDTC dose is essentially independent of administration time from 30 min before to 3 h after carboplatin. However, Dible et al. (17) reported that single-dose DDTC inhibited the carboplatin-induced decrease in hematoctit in mice only when given simultaneously; no benefit was noted with intervals of 2, 4, or 24 h. In contrast, single-dose simultaneous DDTC had a minimal effect on the carboplatin-induced leukopenia in rats. The basis for these discrepancies is not clear; optimum timing for DDTC rescue of CBDCA hematological toxicity has not been definitively established.

The stem cell survival data obtained after treatment with DDP became colinear with those obtained after treatment with CBDCA if the DDP dose was corrected by a factor of 3. This demonstrates that both DDP and CBDCA can produce significant stem cell toxicity and that, on a molar basis, DDP is 2.4 times as toxic to stem cells as CBDCA. Under ordinary circumstances, however, the severe toxicity of DDP to other tissues precludes its administration at the doses routinely used in CBDCA chemotherapy. Marrow toxicity becomes problematic for DDP only in high-dose regimens. The colinearity of the stem cell survival data was maintained after DDTC rescue, showing that the magnitude of stem cell protection is equivalent for equitoxic doses of the two analogues. This suggests that these platinum drugs cause stem cell toxicity by the same mechanism.

Our data demonstrate clearly that stem cell rescue can be achieved over a wide range of DDTC doses and administration times. The mechanism by which DDTC effects stem cell rescue cannot be deduced from these data, but it is unlikely to involve chelation of platinum species before they reach their biological targets. Chelation involves a simple bimolecular reaction; the amount of chelate formed would be expected to decrease as the reactant concentrations decrease. Yet in these experiments, rescue remains relatively constant although the DDTC concentration varies by a factor of almost 8. Moreover, rescue never restores stem cell survival completely to control levels, indicating that some degree of irreversible damage has occurred independent of the amount of DDTC administered. Also, DDTC is relatively unreactive toward CBDCA and DDP; the half-lives of the substitution reactions (1 mM DDTC, 37°C) are 25 and 3 min, respectively (26). These substitution reactions are even slower at the plasma concentrations of DDTC typically achieved in vivo. The plasma half-life of DDTC is 10-15 min in the mouse (13). For the platinum drugs, the plasma half-life in the α phase is 2-3 min for both, while the half-lives in the β phase are 25-26 min for CBDCA and 10-15 min for DDP (27). Thus, high plasma concentrations of DDTC and the platinum drugs were not found simultaneously under the schedules of drug administration used in our experiments. Yet similar rescues were achieved when DDTC was administered between 0.5 h before and 5 h after CBDCA. It is unlikely, therefore, that DDTC acts by intercepting and inactivating appreciable quantities of the platinum drugs before they undergo tissue distribution.

In contrast to stem cells, granulocyte/macrophage progenitor cells react differently both to the platinum drugs and to DDTC rescue. Granulocyte/macrophage progenitors, the bipotent progeny of pluripotent stem cells, act predominantly as an amplification compartment, producing large numbers of mature, fully differentiated granulocytes and macrophages. Severe toxicity to GM-CFC is likely to manifest itself as granulocytopenia. Our data show that these cells are markedly less sensitive than stem cells to the toxicity of the platinum drugs; the reason for this discrepancy is not clear; optimum timing for DDTC rescue of CBDCA hematological toxicity has not been definitively established.
for this diminished sensitivity is unclear. Furthermore, DDTC offers little or no protection to these cells. This, too, makes it difficult to argue that simple chelation is the mechanism by which DDTC reverses platinum-mediated bone marrow toxicity, for both stem cells and granulocyte/macrophage progenitors, as components of the same tissue, are subjected to the same concentrations of DDTC and the platinum drugs.

The assays of bone marrow cellularity and WBC did not provide reliable estimates of myelosuppression. Our data showed that the platinum drugs were more toxic to the earliest cells examined (stem cells), and less toxic to more differentiated cells (GM-CFC, bone marrow cellularity, WBC). The magnitude of DDTC rescue followed the same pattern. Differential toxicities in hematological cell populations have been noted before for DDP (28) and for other drugs as well (29, 30). It has also been reported that peripheral WBC in mice do not accurately reflect the bone marrow toxicity of the platinum drugs, although they are reasonably accurate for other marrow toxins (31).

We suggest that DDTC may exert its protective effects in bone marrow cells through some unidentified stimulatory mechanism and that this effect is most pronounced in stem cells. Furthermore, the absence of DDTC-mediated stimulation in saline-treated controls indicates that DDTC triggers proliferation only in the presence of a toxic insult. DDTC provides this chemoprotection without causing any measurable hematological toxicity of its own. Our data do not permit us to make conclusions about the exact nature of this mechanism, but further experiments delineating its scope are under way in our laboratory. Our findings support a large body of evidence that DDTC is a generalized chemoprotector against most platinum-laboratory. Our findings support a large body of evidence that DDTC is a generalized chemoprotector against most platinum-

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