Corticosteroids exhibit extensive hematopoietic effects both in vitro and in vivo. Some of the previously studied effects suggested that corticosteroids may alter hematopoietic toxicity of chemotherapeutic agents. In this study, we examined (1) the optimum dose and schedule of cortisone acetate (CA) to reduce hematopoietic toxicity of carboplatin (CB) and (2) possible mechanisms involved in this protective effect. CA given subcutaneously at 0.5 mg/d per mouse for 7 days before CB reduced CB-induced mortality due to neutropenia from 88% in controls to 14% in CA-treated mice \( (P < .05) \). Lower CA doses were not effective. Three days of pretreatment (but not 1 day) was as effective as 7 days. CA given after CB had no effect on mortality. Pharmacokinetic studies of CA at 0.5 mg per mouse demonstrated blood levels of cortisol achievable in patients (peak level, 82 µg/dL). CA treatment markedly reduced spleen cell number and colony-forming units-granulocyte/macrophage (CFU-GM) as well as bone marrow CFU-GM. Bone marrow CFU-GM removed from CA-treated mice demonstrated increased resistance to platinum and increased resistance to high specific activity \( ^3\text{H}\)-thymidine. These findings suggest that treatment of mice with CA induces cellular resistance of hematopoietic precursors to platinum and, thus, reduces CB hematotoxicity. CA or other corticosteroids may be useful in reducing clinical toxicity of CB.

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clear cells were washed three times by centrifugation at 200g. Spleen mononuclear cells from treated animals were cultured in a methylcellulose hematopoietic progenitor cell assays for CFU-GM (see below). To produce LCM, washed spleen mononuclear cells from normal, untreated animals were resuspended in IDMEM plus 20% FCS plus 10 \(\mu\)g/mL phytohemagglutinin to a final concentration of 6 \(\times 10^5/mL\). The cell suspension was placed in 25-cm² tissue culture flasks and then incubated at 37°C for 5 days. After incubation, the spent media was centrifuged, and the resultant supernatant was passed through a 0.22-μm syringe filter (Gelman Sciences, Ann Arbor, MI) and then stored in 2-ml aliquots at −4°C.

Preparation and culture of bone marrow. Mice were killed by cervical dislocation, and tibias were placed in chilled HBSS (Gibco). Bone marrow cells were obtained by flushing cold HBSS through tibias with a 27-gauge needle. Cells were washed once in cold HBSS and then resuspended at 3 \(\times 10^5/mL\) in IDMEM supplemented with 20% FCS. Bone marrow mononuclear cells were incubated in methylcellulose at a final concentration of 3 \(\times 10^5/mL\) supplemented with 20% FCS, 1% antibiotic-antimycotic solution, and 10% LCM (or no LCM for negative controls). The cell cultures were plated in triplicate wells in six-well tissue culture dishes and incubated at 37°C in a fully humidified atmosphere of 5% CO₂ for 5 days. On the fifth day, the number of CFU-GM were determined by examination using an inverted microscope. Cultures that contained 10% LCM developed colonies of 50 to 150 cells at an incidence of 50 to 90 colonies per 3 \(\times 10^5\) bone marrow cells. Negative controls containing no LCM developed rare colonies (less than one to two per culture).

Administration of cortisone acetate (CA) and carboplatin (CB). CA (Cortone; Merck Sharp Dohme, West Point, PA) was dissolved in sterile 0.9% NaCl; injections (0.2 mL per mouse) were given subcutaneously. CB (Paraplatin; Bristol Laboratories, Evansville, IN) was dissolved in sterile 0.9% NaCl for injection according to the package directions. Individual animals in an experimental group were given an intravenous (IV) injection of CB (0.1 mL) in the lateral or dorsal tail vein.

Staining of bone marrow or spleen for flow cytometry. Bone marrow cells and spleen mononuclear cells were obtained as described above. Cells were washed three times in HBSS and resuspended to 2 \(\times 10^7/mL\) in HBSS without phenol red containing 1% bovine serum albumin (BSA; Fraction V, Sigma Chemical; staining buffer). Propidium iodide (50 \(\mu\)g/mL in 0.1 Na citrate with 0.1% Triton X100) was added to the cell suspension. After incubating for 20 minutes in an ice bath, the cells were washed by adding 2 mL of staining buffer and pelleting at 300g for 6 minutes at 4°C. Stained cells were resuspended in 400 μL cold staining buffer and held on ice until quantitation. A minimum of 10⁶ cell nuclei were counted with a Becton Dickinson (BD) FACScan without electronic gates using list mode analysis. The fluorescence intensity was determined with 1024 channel resolution, and the cell cycle analysis was performed using cellfit analysis software. Selected histograms were analyzed on Verity Modfit (Topsham, ME) software to verify the accuracy of the analysis.

³H-thymidine suicide assay. Bone marrow and spleen cells were collected and prepared as previously described and then resuspended to 4 \(\times 10^7/mL\) in IDMEM containing 20% FCS and 1% antibiotic-antimycotic solution. Cells (4 \(\times 10^6\), 100 \(\mu\)L) were transferred to four 15-mL conical tubes with the following additions: (1) control, no additions; (2) 1.2 mg of thymidine (Sigma); (3) 40 μCi \(^3\)H-thymidine, 70 to 85 Ci/mmol (Amersham Life Sciences, Arlington Heights, IL) or (4) 1.2 mg thymidine and 40 μCi \(^3\)H-thymidine. The cell suspension in each tube was adjusted to 1.0 mL volume with IDMEM containing 20% FCS. Cells were then incubated in a 37°C water bath for 20 minutes. After incubation, cells were washed twice in media containing 20% FCS. Cells were cultured after the methylcellulose hematopoietic progenitor cell assay previously described. Each triplicate culture contained 10% LCM. After 5 days of incubation, the number of CFU-GM was determined. Results were calculated as percent inhibition of the control:

\[
1 - \left(\frac{\text{No. of Colonies With Thymidine}}{\text{No. of Colonies Without Thymidine}}\right) \times 100
\]

Cisplatin inhibition assay. Bone marrow and spleen cells were collected and prepared as previously described, then resuspended at 4 \(\times 10^7/mL\) in IDMEM containing 20% FCS and 1% antibiotic-antimycotic solution. Cells (16 \(\times 10^5/mL\); 400 μL) were transferred to five 15-mL conical tubes, and 100 μL of IDMEM either containing cisplatin (Platinol; Bristol Meyers) or no cisplatin (in case of control) were added to achieve final cisplatin concentrations of (1) 0 pg/mL (control), (2) 0.62 pg/mL, (3) 1.2 pg/mL, (4) 2.5 pg/mL, or (5) 5.0 pg/mL. The cell suspension in each tube was adjusted to a 4-mL volume with IDMEM containing 20% FCS. Cells were then incubated in a 37°C water bath for 30 minutes. After incubation, cells were washed twice with IDMEM and resuspended at 3 \(\times 10^7/mL\) in IDMEM containing 20% FCS. Cells were cultured using the methylcellulose hematopoietic progenitor cell assay previously described. Each triplicate culture contained 10% LCM. After 5 days of incubation, the number of CFU-GM was determined. Results were calculated as percent inhibition of the control:

\[
1 - \left(\frac{\text{No. of Colonies With Cisplatin}}{\text{No. of Colonies Without Cisplatin}}\right) \times 100
\]

Plasma cortisol levels. Cortisol plasma levels were determined using the Abbott TDx sys (Arlington, TX), which uses a fluorescence polarization immunoassay; 50 μL of each sample was run in duplicate, simultaneously with cortisol controls. Plasma samples exceeding 60 μg/dL of cortisol were diluted until levels were within the range of the system.
CORTICOSTEROID ALTERATION OF HEMATOPOIESIS

We examined the schedule dependency of the activity of CA (at 0.5 mg per mouse per dose) in reducing the mortality induced by high-dose CB (Fig 3): pretreatment of mice daily with CA for 3 days was as effective as pretreatment for 7 days. Single doses of CA at 3 or 24 hours before CB did not statistically improve survival, although some delay and reduction in mortality is suggested by inspection of the sur-

Statistical methods. Statistical analysis was performed using the Student's t test and one-way analysis of variance (ANOVA). Differences in survival between various treatment groups were analyzed using Scheffe's test.

RESULTS

CB was chosen for these studies because at the doses of 300 to 600 mg/m² (ie, 100 mg/kg to 200 mg/kg) used, no nonhematologic toxicity was detected biochemically (including serum urea nitrogen and creatinine, bilirubin, and hepatic transaminase levels) 4 days after administration of the drug. Death in mice after administration of CB occurred at the granulocyte nadir and was associated with bacterial peritonitis as previously described. Thus, death in mice that received CB was due to hematopoietic toxicity. Furthermore, CB induces thrombocytopenia and neutropenia compared with other alkylators such as cyclophosphamide, which at lethal doses in mice, does not alter platelet counts. We previously observed in a small number of animals that CB at 600 mg/m² intravenously is fatal to 80% to 90% of mice and that pretreatment of mice with IL-1, CA, or both similarly reduced this mortality to 0 to 20%. In verifying these findings, we treated mice subcutaneously (SC) with CA at 0.1, 0.25, or 0.5 mg/d per mouse for 7 days, and on day 8, with CB at 600 mg/m² intravenously (Fig 1). CA at 0.5 mg per mouse reduced mortality from 88% to 14% (P < .05).

We examined blood levels of cortisol (the principal active hepatic metabolite of the biologically inactive CA) induced by administration of 0.5 mg SC CA (Fig 2): peak levels of 82 μg/dL were observed at 1 hour, and blood levels remained above baseline at 24 hours (4.5 ± 1.1 μg/dL vs 8.2 ± 1.2 μg/
vival curves (Fig 3A). Administration of CA post-CB was ineffective (Fig 3B).

We examined the effect of treatment of mice with CA at 0.5 mg SC for 7 days (days -7 to -1) on peripheral blood cell counts before and after CB 450 mg/ml on day 0. Peripheral blood was obtained by retroorbital bleeding of anesthetized mice, and blood was analyzed on a Coulter S counter and by 100-cell white blood cell differentials of Giemsa-stained blood smears (see Materials and Methods). Data are means ± SE; n = 4 at each time period. (A) Absolute granulocyte count. (B) Absolute lymphocyte count. (C) Platelet count.

Table 1. Effect of CA on Hematopoietic Parameters in C3H/HeJ Mouse Bone Marrow

<table>
<thead>
<tr>
<th>Dose (mg/mouse/d x 7 days)</th>
<th>0 (control)</th>
<th>CA 0.5 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td># Leukocytes/bone marrow</td>
<td>9.9 x 10^6 ± 0.9 x 10^6</td>
<td>8.0 x 10^6 ± 0.6 x 10^6</td>
</tr>
<tr>
<td>CFU-GM Bone marrow</td>
<td>2264.2 ± 308.4</td>
<td>735.4 ± 82.4</td>
</tr>
<tr>
<td>% Bone marrow leukocytes in S phase</td>
<td>19.5 ± 1.2</td>
<td>20.7 ± 1.7</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE of two separate experiments (n = 10).
* Mice were killed on day 8 after 7 days of subcutaneous CA at dose indicated.
† P < .001, statistical analysis by one-way ANOVA.
CORTICOSTEROID ALTERATION

Table 2. Effect of CA on Hematopoietic Parameters in C3H/HeJ Mouse Spleen Leukocytes

<table>
<thead>
<tr>
<th>Dose (mg/mouse/d × 7 days)*</th>
<th>0 (control)</th>
<th>CA 0.5 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen weight (mg)</td>
<td>134.0 ± 8.0</td>
<td>61.0 ± 4.9</td>
</tr>
<tr>
<td>No. of leukocytes/ spleen</td>
<td>20.4 x 10^6 ± 1.2 x 10^6</td>
<td>2.3 x 10^6 ± 0.3 x 10^6</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>526.3 ± 70.3</td>
<td>122.8 ± 42.81</td>
</tr>
<tr>
<td>% Spleen leukocytes in S phase</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE of two separate experiments (n = 10).
* Mice were killed on day 8 after 7 days of subcutaneous CA at dose indicated.
† P < .001, statistical analysis by one-way ANOVA.

2). The fraction of cells in S-phase in total bone marrow and spleen as estimated by flow cytometry was unchanged by treatment with CA (Tables 1 and 2).

To determine if treatment of mice with CA induced resistance of CFU-GM to CB on cellular level, bone marrow and spleen cells were harvested from mice after treatment with CA or carrier for 7 days (Fig 5A and B). Whole cell populations were incubated with no cisplatin or four concentrations of cisplatin for 30 minutes, washed, and assayed for CFU-GM. Bone marrow (Fig 5A) but not spleen (Fig 5B) CFU-GM from mice treated with CA exhibited significant resistance to platinum. To determine if this resistance was related to alteration in the number of CFU-GM in S-phase, ^3^H-thymidine suicide assays were undertaken in identically treated mice (Fig 6). Bone marrow and splenic CFU-GM from mice treated with CA exhibited marked reduction in sensitivity to high specific activity ^3^H-thymidine, suggesting a decreased fraction of CFU-GM from CA-treated mice were in S-phase.

DISCUSSION

Previous investigators have demonstrated that corticosteroids given to mice before sublethal doses of hematotoxic chemotherapeutic agents increased the number of residual, postchemotherapy hematopoietic precursors in bone marrow. We have expanded these studies to (1) determine if, at optimal dose and schedule of corticosteroids, the hematopoietic effects were clinically relevant in reducing hematopoietic toxicity of carboplatin, and (2) examine relevant biologic effects of corticosteroids that may explain induction of resistance to CB.

Pretreatment of mice for 3 or 7 days with CA significantly reduced mortality (from 88% to 14%) induced by 600 mg/m^2^ CB. In patients, the maximum tolerated dose of CB is 400 mg/m^2^ without supportive measures, and hematotoxicity is dose-limiting; our findings in mice are similar. CA in this schedule (3 or 7 days pre-CB) ameliorated CB-induced granulocyte and platelet nadirs and enhanced recovery times. The dose of CA used induced high (82 μg/dL) levels of cortisol, the active metabolite of CA. However, these levels are routinely achieved in clinical practice using similar corticosteroids. Thus, at a clinically achievable dose and practical schedule, CA markedly reduced hematotoxicity of carboplatin administered at a dose similarly toxic in humans.

The mechanisms by which CA reduces CB hematotoxicity are very likely much different from cytokines such as IL-1. Pretreatment of mice with IL-1 reduces CB hematotoxicity as effectively as does CA. However, IL-1 increases splenic cellularity, CFU-GM, and the number of spleen cells in S-phase. In contrast, CA reduced splenic cellularity and CFU-GM in spleen and bone marrow. Thus, IL-1 may act by increasing the number of hematopoietic precursors at risk for damage by CB; ie, given a similar fraction of hematopoietic precursors killed by a given CB dose, IL-1-pretreated mice exhibit higher postchemotherapy residual hematopoietic precursor (eg, CFU-GM) levels and more rapid hematopoietic
was not sufficient alone for induction of cellular resistance to cisplatin, because in CA-treated mice, both bone marrow and spleen CFU-GM demonstrated increased in vitro resistance to high specific activity \(^{3}H\)-thymidine, but only bone marrow CFU-GM were resistant to platinum in vitro. Therefore, of the mechanisms of platinum resistance discussed, increased glutathione or metallothionein levels and increased capacity for DNA repair may be more likely to explain CA induction of relative platinum resistance. Evaluation of the mechanisms of CA-increased platinum resistance will be difficult in vivo, and therefore, we are attempting to reproduce the phenomenon in vitro using expanded populations of purified normal hematopoietic cells. We have not explained the difference in response to CA treatment of bone marrow and spleen in regard to cellularity and induction of resistance to platinum in vitro. However, these differences were consistently present through multiple experiments. Although we only examined in vitro resistance of CFU-GM from CA-treated and normal mice to platinum and high-dose \(^{3}H\)-thymidine, other hematopoietic precursors may be similarly affected, as CA-treated mice demonstrated protection from CB-induced thrombocytopenia as well as neutropenia.

The data presented here suggest that at a clinically achievable dose and schedule, corticosteroids reduce the hematotoxicity of moderate to high-dose CB. The extent of reduction in hematotoxicity is similar to that observed with the use of hematopoietic growth factors in clinical situations and experimental models. One mechanism of CA reduction in hematotoxicity may be induction of resistance to platinum at the cellular level. These findings raise the possibility that corticosteroids may be useful in reducing hematopoietic toxicity of CB in patients.

**REFERENCES**

9. Joyce RA, Chervenick PA: Corticosteroid effect on granulo-