Corticosteroid Alteration of Carboplatin-Induced Hematopoietic Toxicity in a Murine Model

By John Rinehart, Lisa Keville, John Measel, A. Michael Spiekerman, and Kimberly Burke

Corticosteroids exhibit extensive hematopoietic effects both in vitro and in vivo. Some of the previously studied effects suggested that corticosteroids may affect 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 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 pg/dL). CA 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 \(^3\)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 assay 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 μg/mL phytohemagglutinin to a final concentration of 6 x 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 x 10^6/mL in IDMEM supplemented with 20% FCS. Bone marrow mononuclear cells were incubated in methylcellulose at a final concentration of 3 x 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%.

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 manufacturer's instructions. Propidium iodide (50 μg/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 10^5 cell nuclei were counted with a Beckman Coulter (Becton Dickinson) 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 cell fit analysis supplied by BD. 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, then resuspended to 4 x 10^5/mL in IDMEM containing 20% FCS and 1% antibiotic-antimycotic solution. Cells (4 x 10^6, 100 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) was 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 μg/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 x 10^6/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:

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

Cisplatin inhibition assay. Bone marrow and spleen cells were collected and prepared as previously described, then resuspended to 4 x 10^5/mL in IDMEM containing 20% FCS and 1% antibiotic-antimycotic solution. Cells (16 x 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) was added to achieve final cisplatin concentrations of (1) 0 μg/mL (control), (2) 0.62 μg/mL, (3) 1.2 μg/mL, (4) 2.5 μg/mL, or (5) 5.0 μg/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 x 10^6/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:

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

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.

Fig 1. Effect of CA dose on CB-induced mortality. Normal mice were treated for 7 days with subcutaneous CA at 0.10, 0.25, and 0.50 mg per mouse and on day 8, received a single tail-vein injection of CB 600 mg/m². The data shown indicate the percent survival of animals from four experiments (controls, n = 81; CA 0.1 mg, n = 10; CA 0.25 mg, n = 26; CA 0.50 mg, n = 51). Only CA at 0.5 mg improved survival post-CB (P < .05).
**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 v 8.2 ± 1.2 μg/dL. P < .057). Blood cortisol levels were also examined in mice that had received six daily injections of CA 0.5 mg SC: at 24 hours after the sixth CA dose, cortisol levels were 5 μg/dL, and at 3, 6, and 12 hours after the seventh CA dose, cortisol levels were 31, 22, and 15 μg/dL.

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-

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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.

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**Fig 2.** CA pharmacokinetics in normal mice. Normal mice were given subcutaneous CA 0.5 mg per mouse and were killed at indicated time intervals after the injection (n = 6 at all time points). Heparinized plasma was obtained from animals and assayed for cortisol levels as described in Materials and Methods. Data are presented as means ± standard errors (SE).

**Fig 3.** Effect of CA schedule on CB-induced mortality. Normal mice were treated with various schedules of CA at 0.50 mg per mouse by subcutaneous injection before receiving CB 600 mg/m² on day 0. Mice were then observed for survival daily. (A) The combined results of two separate experiments are shown: control, n = 28; day -1, n = 15; days -3 to -1, n = 16; days -7 to -1, n = 25; day 0/-3 hour, n = 20. (B) Control, n = 10; days -7 to -1, n = 10; days +1 to +7, n = 10.
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/m² (day 0, Fig 4A through C). A lower dose of CB was used in these experiments so that peripheral blood cell counts could be observed through recovery in control as well as CA-pretreated mice. Treatment with CA induced, as expected, a granulocytosis and lymphopenia (day 0, pre-CB counts, Fig 4A and B). Mice pretreated with CA demonstrated less severe granulocyte and platelet nadirs and more rapid recovery times. Interestingly, CA-treated mice remained relatively lymphopenic for 20 days post-administration of CB (Fig 4B). Treatment of mice for 7 days with CA 0.5 mg markedly reduced the number of CFU-GM in both bone marrow and spleen, as well as the total cell number of spleen cells (Tables 1 and

Fig 4. CA alteration of CB-induced cytopenias. Normal mice or mice pretreated with 0.5 mg/d CA SC on days -7 to -1 were given 450 mg/m² CB 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 × 7 days)*</th>
<th>CA 0.5 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td># Leukocytes/bone marrow</td>
<td>9.9 ± 0.9 × 10⁶</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>2264.2 ± 308.4</td>
</tr>
<tr>
<td>% Bone marrow leukocytes in S phase</td>
<td>19.5 ± 1.2</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.
CORTICOSTEROID ALTERATION OF HEMATOPOIESIS

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 ± 6.0</td>
<td>61.0 ± 4.01</td>
</tr>
<tr>
<td>No. of leukocytes/spleen</td>
<td>20.4 × 10^6 ± 1.2 × 10^6</td>
<td>2.3 × 10^6 ± 0.3 × 10^6</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>520.3 ± 70.3</td>
<td>122.8 ± 42.61</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.

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/m² CB. In patients, the maximum tolerated dose of CB is 400 mg/m² 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 recovery.

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, [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 [H]-thymidine, suggesting a decreased fraction of CFU-GM from CA-treated mice were in S-phase.

Fig 5. Effect of treatment of mice (in vivo) with CA versus carrier-treated controls on in vitro resistance to platinum of bone marrow hematopoietic precursors (CFU-GM). Mice were treated with CA 0.5 mg per mouse or carrier (six separate experiments) for 7 days. Mice were killed on day 8, and bone marrow (A) and spleen (B) cells were removed as described in Materials and Methods. Bone marrow (A) or spleen (B) cells were incubated with cisplatin for 30 minutes and then cultured for assay of CFU-GM. Data are presented as percent inhibition of CFU-GM in bone marrow or spleen cells not exposed to cisplatin. Bone marrow cells from mice treated with CA were more resistant to cisplatin than controls at cisplatin concentrations of 0.62 and 1.2 µg/mL (P < .05). Data are presented as means ± SE.
controls (no exposure to thymidine in vitro). The percent inhibition of fraction of CFU-GM in S-phase. CFU-GM from mice treated with CA to cold thymidine, ¹H-thymidine, both, or no thymidine (control) before assay for CFU-GM (see Materials and Methods). Data are expressed as a percentage of the number of CFU-GM observed in controls (no exposure to thymidine in vitro). The percent inhibition of CFU-GM in the presence of ¹H-thymidine alone is a measure of the fraction of CFU-GM in S-phase. CFU-GM from mice treated with CA exhibited a significant decrease in the fraction of cells in S-phase (n = seven experiments; P < .05). Data are presented as means ± SE.

Two general mechanisms may be involved in CA reduction in CB hematotoxicity: alteration in pharmacokinetics and induction of CB resistance on a cellular level. We examined this latter possibility and found that, paradoxically, bone marrow but not splenic CFU-GM from CA-treated mice compared with control mice demonstrated greater resistance to in vitro concentrations of platinum obtained with standard doses of CB in patients. The mechanism of this resistance was not elucidated by our studies, but as reviewed by Los and Muggia, possible mechanisms include (1) decreased protein kinase-C, (2) decreased cyclic adenosine monophosphate (AMP), (3) increased glutathione and metallothionein, and (4) enhancement of DNA repair mechanisms. Corticosteroids are known to alter cyclic AMP in some cells. Decreased levels of protein kinase-C are associated with decreased proliferative rate. Decreased number of CFU-GM in CA-treated mice and the resistance of CFU-GM to high specific activity ¹H-thymidine suggest but do not prove that during CA treatment, CFU-GM have a lower proliferative rate and that fewer CFU-GM are in S-phase. However, reduction in the fraction of CFU-GM in S-phase 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 ¹H-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 ¹H-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.

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