Corticosteroids have the ability to suppress the production of growth factors and cytokines and are thus implicated in the negative regulation of hematopoiesis. We have shown that the corticosteroids, prednisolone and dexamethasone, were able to effectively protect progenitor cells in four strains of mice against cell-cycle–specific antimetabolic chemotherapy agents. The highest levels of protection against 5-fluorouracil (FU; 200 mg/kg) were achieved when two or three intraperitoneal injections of dexamethasone were administered between 7 and 3 hours at a dose of 7.5 mg/kg/injection (optimal dose) or by continuous infusion between 4 and 20 hours. This protective effect is manifested as an increase in the number of high proliferative potential colony-forming cells that survive in the bone marrow 3 days after treatment with FU from between 0.5% and 11% to between 10% and 34% of normal. The bone marrow progenitors and blood cell numbers return to normal from 3 to 5 days and 1 to 2 days earlier, respectively. Less dexamethasone than prednisolone is required to give an equivalent protective effect, which is consistent with their anti-inflammatory potency. These findings are further evidence of the negative regulatory role played by corticosteroids, and indicate that the treatment schedules of corticosteroids during cancer therapy need to be reexamined to obtain the maximum benefit from their use.

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Protection of Murine Bone Marrow by Dexamethasone During Cytotoxic Chemotherapy

By A.B. Kriegler, D. Bernardo, and S.M. Verschoor

The hematopoietic system consists of a hierarchical system in which multipotential stem cells continuously produce functional blood cells via a series of progressively maturing progenitor cell populations. Three distinct progenitor populations that can be assayed in agar cultures have been identified over the last few years. The most primitive of these are high proliferative potential colony-forming cells (HPP-CFC) that form large colonies (diameter, ≥0.5 mm) in agar cultures in the presence of the cytokine combination of macrophage colony-stimulating factor (CSF-1) plus interleukin-3 (IL-3) plus IL-1 (HPP-CFC-1). These HPP-CFC-1 give rise to a second more mature HPP-CFC population that forms similar large colonies in the presence of CSF-1 + IL-3 (HPP-CFC-2) and that, in turn, gives rise to the granulocyte-macrophage colony-forming cells (GM-CFC). When mice are treated with a high dose (200 mg/kg) of the cell-cycle–specific cytotoxin, 5-fluorouracil (FU), the more mature cycling GM-CFC and HPP-CFC-2 populations are almost completely killed off. A significant proportion of the more primitive HPP-CFC-1 survive, suggesting that they are not all in cycle, a property that distinguishes them from the more mature HPP-CFC-2.

One of the major factors limiting the effective use of cytotoxic chemotherapy agents is their toxic effects on normal tissue, particularly the hematopoietic system. These toxic effects arise from the ability of cytotoxic drugs to kill off the cycling progenitors in the BM, resulting in a reduction in the peripheral blood cell count, which remains low until quiescent progenitors and stem cells replenish these compartments. However, the use of autologous bone marrow (BM) and peripheral blood stem cell transplant and the clinical application of recombinant (r) hematopoietic growth factors have proved to be effective in shortening the posttreatment periods of neutropenia.

It has been suggested that when cell-cycle–specific drugs are used repeatedly, it may be possible to use agents such as hematopoietic inhibitors to arrest the cycling of BM progenitors and hence protect them from these cytotoxic agents. Although several inhibitors have been described, only the monococyte-derived pentapeptide (pEEDCK), the fetal calf BM-derived tetrapeptide (AcSDKP), macrophage-inflammatory factor (MIP-1α), and tumor necrosis factor-α (TNF-α) have been shown to be hematoprotective in mice when these cytotoxic agents are used. The anti-inflammatory and immunosuppressive effects of corticosteroids are well known. Recent work suggests that these actions may be mediated by their ability to suppress the production of the cytokines, IL-1 and TNF-α, the levels of which have been shown to be closely correlated with the inflammatory process. Corticosteroids are also able to suppress the production of other cytokines, which are associated with the inflammatory process, including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and IL-3, but not macrophage colony-stimulating factor (M-CSF). Because all these cytokines are involved in the regulation of hematopoiesis, these findings suggest that corticosteroids may have the ability to act as negative regulators of hematopoiesis by suppressing the production of cytokines. In fact, corticosteroids have been shown to inhibit GM-CFC in agar cultures, although the mechanism of this action has not yet been determined.

In the present study we have shown that corticosteroids, particularly dexamethasone, are effective hematopoietic agents against antimetabolic cytotoxic agents. This is a novel finding and, because these drugs are often administered to patients undergoing chemotherapy, it is possible that increased clinical benefits could be obtained by expedient scheduling of these drugs.

MATERIALS AND METHODS

Growth factors. Recombinant human IL-1α was supplied by Hoffman-La-Roche (Nutley, NJ). Conditioned medium from the bone marrow 3 days after treatment with FU from between 0.5% and 11% to between 10% and 34% of normal. The bone marrow progenitors and blood cell numbers return to normal from 3 to 5 days and 1 to 2 days earlier, respectively. Less dexamethasone than prednisolone is required to give an equivalent protective effect, which is consistent with their anti-inflammatory potency. These findings are further evidence of the negative regulatory role played by corticosteroids, and indicate that the treatment schedules of corticosteroids during cancer therapy need to be reexamined to obtain the maximum benefit from their use.
genetically altered mouse mammary cell line (C127) was used as a source of recombinant murine IL-3.31 The murine CSF-1 source, pregnant mouse uterus extract (PMUE), was prepared in this laboratory32 and purified by immunoaffinity chromatography using an antibody supplied by Dr E.R. Stanley (Albert Einstein College of Medicine, New York, NY). IL-1α was used at 4,000 U/culture. PMUE and the IL-3 preparations were used at the minimum concentrations required for maximal colony growth as predetermined using 3-day post-FU-treated murine BM (FU3BM).

Treatment of mice with dexamethasone and prednisolone. In most experiments, 3-month-old specific pathogen-free (C57BL/6J x DBA/2)F1 mice, obtained from the Animal Resources Centre (Perth, Western Australia), were treated with FU (200 mg/kg) by intravenous (IV) injection. In all experiments, 3 mice per treatment were used, except in the experiment in Fig 7, in which 4 mice per time point were used. Their BM was collected and pooled at set times by flushing femoral shafts with cold HEPES-buffered balanced salt solution containing 2% newborn calf serum (NBCS) after anesthesia (Fluothane; ICI Australia) and cervical dislocation. The experiments were performed with either all male or all female mice.

Dexamethasone (John Bull Laboratories Pty Ltd, Australia) was supplied as a 24 mg/mL solution and was diluted with saline as required. For a 30 mg/kg IP dose, a 3 mg/mL solution was used and twofold serial dilutions of this were made for dose response studies (see Fig 2). Continuous infusions were performed using 1-day mini-osmotic pumps (Alza Corp, Palo Alto, CA) that had a start up time of 3 hours and were found to have a mean pumping rate of 3.3 μL/h. When filled with a 24 mg/mL solution and implanted subcutaneously, they thus had a pumping rate of 6.6 mg/kg/h. To obtain the treatment schedules outlined in Fig 3 the pumps were filled and preincubated in saline at 37°C for different lengths of time before implantation. For dose response experiments (see Fig 4) twofold serial dilutions of the 24 mg/mL solution were used. The 12 mg/kg/h dose rate was obtained by implanting two pumps filled with the 24 mg/mL solution (see Fig 4). Prednisolone (Upjohn Pty Ltd, Australia) was dissolved in saline to give a 24 mg/mL solution. This was serially diluted twofold for dose response experiments (see Fig 5).

Assays for BM progenitors and peripheral blood cells. Murine BM was assayed for various progenitors in the double-layer nutrient assay system using 35-mm plastic petri dishes (Bunzl, Camden Park, South Australia). The medium used was α-minimal essential medium (α-MEM) supplemented with vitamins (2X), glutamine (2X), and 20% NBCS. Growth factors were included in a 1-mL untargeted cell plating per dish were 30,000 for HPP-CFC-2 and HPP-CFC-1, whereas the development of CSF-1 alone and grew colonies with diameters less than 0.5 mm. HPP-CFC-2 and HPP-CFC-1 were assayed in the presence of CSF-1 and IL-3 and CSF-1 + IL-3 + IL-1, respectively, and developed colonies with diameters ≤ 0.5 mm.

Erythrocytes, leukocytes, and platelets in peripheral blood were determined using a Sysmex K100 haemolyser, whereas reticulocytes were determined with a Retic-Count kit and a FACStar Plus fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA). Differential counts were performed on blood cells after staining with the Diff-Quick staining set, a modification of the Wright stain technique (Lab-Aids; Narrabeen, New South Wales, Australia).

Fig 1. Effect of dexamethasone treatment administered by different injection schedules on the survival of BM progenitor cells 3 days after FU treatment. Mice were treated with FU at time 0 and with dexamethasone administered as single IP injections (30 mg/kg) at various times in the treatment schedule (A through J): A, −2, −1.5, −0.5, +1, +2, and +4 hours; B, −2 hours; C, −1 hours; D, 0 hours; E, +1 hour; F, +1.5 hours; G, +3 hours; H, +4 hours; I, −2, and +4 hours; and J, no dexamethasone treatment. (a) GM-CFC; (b) HPP-CFC-2; (c) HPP-CFC-1.

Statistics. Each experiment was performed at least three times and shown to be reproducible, except for the experiment in Fig 7, which was performed twice. Assays for BM progenitors were performed in triplicate and are presented as the mean ± SEM. Levels of significance were determined using the Student's t distribution.

RESULTS

Treatment schedule for dexamethasone using IP injections. For hematoprotection against a cell-cycle-specific chemotherapy agent to be effective, the BM progenitors should be cell-cycle-arrested before administration of the chemotherapy agent and remain in this state while the agent is present at toxic levels. In most experiments, mice were treated with FU (200 mg/kg) and their BM assayed for HPP-CFC-1, HPP-CFC-2, and GM-CFC in agar cultures 3 days later (FU3BM), when the numbers of these progenitors usually reached a nadir. The level of BM protection achieved with dexamethasone was assessed by the increase in the number of these progenitors as compared with mice receiving FU alone.

Attempts to establish the optimal treatment schedule were performed by administering to mice single and multiple treatments of dexamethasone (IV injections) at a dose of 30 mg/kg, both before and after treatment with a high dose of FU. This dose of dexamethasone was chosen for these initial experiments because it has been reported to be able to protect mice from the lethal effects of endotoxin.1,2 The results in Fig 1 indicate that the multiple injection schedule A ranging from −2 to +4 hours showed a highly significant increase (P < .001) in the number of HPP-
CFC-1 and HPP-CFC-2 progenitors surviving in FU-treated BM but a less significant increase ($P < 0.05$) in the number of GM-CFC surviving. However, the 2-injection schedule (Fig 1) covering the same time span appeared to be more effective than the multi-injection schedule A, suggesting that the level of protection achievable may be limited and is in fact reduced when the overall dose is further increased. Single injections over the time span −2 to +4 hours indicate that they are significantly more effective when administered before the FU injection rather than after the FU injection (Fig 1). Alteration of the −2 and +4 hours 2-injection treatment schedule for dexamethasone to −2 and +2 hours schedule or extending the treatment schedule to −4 or +6 hours had no significant effect on the level of protection obtained ($P > 0.05$ in all cases, Table 1). Using the two injection schedule (−2 and +4 hours), the level of protection appeared to reach a maximum with dexamethasone concentrations ≥7.5 mg/kg (Fig 2).

**Treatment schedules for dexamethasone using continuous infusions.** The effect of dexamethasone on BM protection when it was continuously infused was studied using a number of different schedules ranging from −16 to +20 hours (Fig 3). One-day mini-osmotic pumps, which had a pumping rate of 6.6 mg/kg/h when they were filled with the most concentrated dexamethasone preparation supplied (24 mg/mL), were used in these experiments. The results indicate that, apart from the number of HPP-CFC-2 surviving after treatment with the −16 to +8 hours schedule, the numbers of HPP-CFC-1 and HPP-CFC-2 surviving after treatments with these schedules were not significantly different from that obtained with the 2-injection control schedule. Continuous infusion over the period of −4 to +20 hours using different concentrations of dexamethasone suggested, despite the large SEMs in some cases (Fig 4), that maximal levels of protection are reached at quite low infusion rates (0.8 mg/kg/h).

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**Table 1. Protection of BM Progenitors From FU by Dexamethasone Using Different Treatment Schedules**

<table>
<thead>
<tr>
<th>Treatment Schedule</th>
<th>GM-CFC (CSF-1)</th>
<th>HPP-CFC-2 (CSF-1 + IL-3)</th>
<th>HPP-CFC-1 (CSF-1 + IL-3 + IL-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−2, +4</td>
<td>584 ± 206</td>
<td>1,964 ± 308</td>
<td>705 ± 113</td>
</tr>
<tr>
<td>−2, +2</td>
<td>420 ± 170</td>
<td>1,634 ± 123</td>
<td>549 ± 24</td>
</tr>
<tr>
<td>−4, −2, +4</td>
<td>429 ± 116</td>
<td>1,411 ± 116</td>
<td>549 ± 149</td>
</tr>
<tr>
<td>−2, +4, +6</td>
<td>475 ± 87</td>
<td>1,448 ± 352</td>
<td>611 ± 68</td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 0</td>
<td>100 ± 24</td>
<td>100 ± 40</td>
</tr>
</tbody>
</table>

* Mice were treated with dexamethasone (7.5 mg/kg) at the times shown and with FU (200 mg/kg) at time 0 and their BM assayed 3 days later.
† Calculated from the means ± SEM of triplicate dishes.

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**Fig 2.** Effect of different doses of dexamethasone administered by a 2-injection treatment schedule (−2 and +4 hours) on the survival of BM progenitors 3 days after FU treatment. (−−−) GM-CFC; (−−−) HPP-CFC-2; (−−−) HPP-CFC-1.

**Fig 3.** Effect of dexamethasone treatment administered by continuous infusion (6.4 mg/kg/h) on the survival of BM progenitors 3 days after FU treatment. For the continuous infusion, the treatment schedules A through E were used: A, −16 to +8 hours; B, −4 to +4 hours; C, −4 to +14 hours; D, −4 to +14 hours; and E, −4 to +20 hours. Control mice were treated with dexamethasone (schedule F) using the 2-injection schedule (−2 and +4 hours, 7.5 mg/kg/injection) or with FU alone (schedule G). (■) GM-CFC; (□) HPP-CFC-2; (■) HPP-CFC-1.

**Fig 4.** Effect of different doses of dexamethasone administered as continuous infusions from −4 to +20 hours on the survival of BM progenitors 3 days after FU treatment. (−−−) GM-CFC; (−−−) HPP-CFC-2; (−−−) HPP-CFC-1.
Level of protection of different BM progenitor cells. The data in Table 2, obtained by combining the results from 14 different experiments, indicates that the number of HPP-CFC-2 and HPP-CFC-1 that survive in FU3BM (approximately 0.5% and 11% of normal, respectively) increased to 10% and 34%, respectively, when dexamethasone was coadministered. Although the use of dexamethasone resulted in a significant increase ($P < .05$) in the number of both types of HPP-CFC surviving in all 14 experiments, there were relatively large variations in the magnitudes of these increases. In individual experiments (data not shown) the number of HPP-CFC-2 surviving after treatment with FU plus dexamethasone thus varied between 3% and 36%, whereas the number of HPP-CFC-1 varied between 14% and 38%, indicating that there is a significant variation between different experiments. However, survival of GM-CFC in FU3BM remains minimal even after dexamethasone treatment (Table 2) and in some experiments no protection of these cells was observed (data not shown).

Protective effects of prednisolone. To determine whether other corticosteroids were also able to protect BM progenitors against FU treatment, mice were injected with different doses of prednisolone using a $-2$, $+1$, and $+4$ hours 3-injection schedule. As can be seen from the results in Fig 5, prednisolone was able to significantly increase the survival of BM progenitors in a dose-related manner. Although the maximum number of HPP-CFC surviving after prednisolone treatment was not significantly different ($P > .05$) from the number surviving after dexamethasone treatment, the dose of prednisolone required (120 mg/kg) is approximately 16-fold greater than the optimal dose (7.5 mg/kg) of dexamethasone (Fig 5).

Dexamethasone protection against other cytotoxic agents. The ability of dexamethasone to protect the BM from damage by the cytotoxic agents, cyclophosphamide, busulphan, daunorubicin, and methotrexate, was examined. As can be seen, significant protection occurred when the cell-cycle-specific agent methotrexate was used, whereas no significant protection of early progenitors occurred with the remaining 3 cell-cycle–nonspecific drugs (Fig 6).

Effect of mouse strain and gender. To show that the effect of dexamethasone is not restricted to a single mouse strain, its effect on other strains was also examined. When female (C57Bl/6J × DBA/2) F1, Balb/c, C3H/HeJ, and DBA/2 mice were treated with IP injections of dexamethasone (7.5 mg/kg) at $-2$ and $+4$ hours and with FU at time 0, there was a significant increase in the number of HPP-CFC-1 and HPP-CFC-2 surviving in FU3BM in all strains compared with FU-treated control mice (data not shown). Similar treatments of male and female (C57Bl/6J × DBA/2) F1 mice indicated that there was no significant difference between the increases in the number of BM progenitors surviving in FU3BM (data not shown), indicating that there is no difference between the sexes.

Effect of dexamethasone on BM and blood cell recovery rates. As can be seen in Fig 7, treatment with FU together with dexamethasone results in a significant increase in the recovery rate of total nucleated cells, GM-CFC, HPP-CFC-2, and HPP-CFC-1 in the BM, with values returning to normal between 3 and 5 days earlier. Reticulocytes, erythrocytes, neutrophils, and platelets in the blood also appear to show an increased rate of recovery, although these increases are smaller than those observed for the BM parameters (Fig 7). In addition, it should be noted that dexamethasone-treated mice have significantly more platelets and neutrophils surviving at the nadir than the FU-treated controls (Fig 7). In contrast to this, dexamethasone treatment results in

![Graph](image-url)
PROTECTION OF BONE MARROW BY CORTICOSTEROIDS

Fig 6. Effect of dexamethasone (DEX) administered in a 2-injection schedule (−2 and +4 hours, 7.5 mg/kg/injection) on the survival of BM progenitors after treatment with cyclophosphamide (CPM; 250 mg/kg, IV), busulphan (BUS; 30 mg/kg, IP), daunorubicin (DNR; 30 mg/kg, IP), or methotrexate (MTX; 400 mg/kg, IV). BM was collected and assayed at the nadir, which was predetermined to be on days 2, 5, 2, and 3 after treatment and was plated at 10,000, 7,000, 10,000, and 5,000 cells per dish for cyclophosphamide, busulphan, daunorubicin, and methotrexate, respectively. Control mice treated with the chemotherapy agent only were included for each agent. (□) HPP-CFC-2; (▣) HPP-CFC-1.

Fig 7. Effect of dexamethasone treatment administered in a 2-injection schedule (−2 and +4 hours, 7.5 mg/kg/injection) on the recovery rate of BM and peripheral blood parameters after treatment with (●) FU compared with (○) FU-treated controls. The recovery rates of total nucleated cells, GM-CFC, HPP-CFC-2, and HPP-CFC-1 in the BM are shown in (A), (B), (C), and (D), respectively. Peripheral blood parameters are shown in (E) through (H) and are the means ± SEM of data from four individual mice.

because corticosteroids are known to be cytotoxic to lymphoid cells.34 Although precise optimal treatment schedules for dexamethasone have not been established, the highest levels of protection against FU toxicity were achieved in this study with 2 or 3 single IP injections (7.5 mg/kg/injection) administered between −7 and +3 hours (Figs 1, 3, and 4) or with continuous infusion (0.8 mg/kg/h) over a period −4 to +20 hours (Figs 4 and 5).

Fig 6. Effect of dexamethasone (DEX) administered in a 2-injection schedule (−2 and +4 hours, 7.5 mg/kg/injection) on the survival of BM progenitors after treatment with cyclophosphamide (CPM; 250 mg/kg, IV), busulphan (BUS; 30 mg/kg, IP), daunorubicin (DNR; 30 mg/kg, IP), or methotrexate (MTX; 400 mg/kg, IV). BM was collected and assayed at the nadir, which was predetermined to be on days 2, 5, 2, and 3 after treatment and was plated at 10,000, 7,000, 10,000, and 5,000 cells per dish for cyclophosphamide, busulphan, daunorubicin, and methotrexate, respectively. Control mice treated with the chemotherapy agent only were included for each agent. (□) HPP-CFC-2; (▣) HPP-CFC-1.

an increase of approximately 40% (data from the experiment described in Fig 7) in the number of lymphocytes killed by FU, although it appears to have no significant effect on the recovery rate (data not shown). Although the nadirs for GM-CFC, HPP-CFC-2, and HPP-CFC-1 were not all on day 3 in Fig 7, when taken together with data from two other experiments (data not shown), day-3 post-FU-treatment was found to be the mean time for these minima and did not change with dexamethasone treatment.

DISCUSSION

In this study, it has been shown that the corticosteroids dexamethasone and prednisolone have the ability to protect the BM of various strains of mice that have been treated with cell-cycle-specific antimetabolic cytotoxic agents. Less dexamethasone than prednisolone is required to give an equivalent protective effect, which is to be expected from their relative anti-inflammatory potency.16 With dexamethasone, this protective effect is manifested as a substantial increase in the survival of the HPP-CFC-2 and HPP-CFC-1 to 10% and 34% of normal, respectively, in FU3BM (Table 2). This results in the BM progenitor and blood cell numbers returning to normal from 3 to 5 days and from 1 to 2 days earlier, respectively (Fig 7). Although the protective effects on GM-CFC, as defined by the number surviving in FU3BM, are often not detectable (Fig 7B), there is a significant increase in their rate of recovery with dexamethasone treatment, which is probably caused by an increase in the survival of their precursors, the HPP-CFC-2 (Table 2 and Fig 7C). The fact that dexamethasone treatment increases the number of lymphocytes killed by FU is to be expected because corticosteroids are known to be cytotoxic to lymphoid cells.34 Although precise optimal treatment schedules for dexamethasone have not been established, the highest levels of protection against FU toxicity were achieved in this study with 2 or 3 single IP injections (7.5 mg/kg/injection) administered between −7 and +3 hours (Figs 1, 3, and 4) or with continuous infusion (0.8 mg/kg/h) over a period −4 to +20 hours (Figs 4 and 5).
Corticosteroids are known to suppress the production of various cytokines, including IL-1, IL-3, IL-6, GM-CSF, TNFα, and but not M-CSF. In agar cultures, corticosteroids have been found to inhibit colony formation by GM-CFC. However, it is not clear from these experiments whether they act directly on these progenitors to inhibit their growth or whether they act indirectly through other cell populations that produce inhibitors. Protection of BM progenitors from cell-cycle-specific cytotoxicity is thought to require the cell-cycle-arrest of cycling progenitors while the cytotoxic agent is present in toxic amounts. Whether cell-cycle-arrest occurs, and if it does whether it is brought about indirectly through the reduction of cytokine levels or the increase of inhibitors levels or through a direct inhibitory effect of corticosteroids on BM progenitors or through a combination of these mechanisms, is not clear and requires further study. The lack of protection against cyclophosphamide and daunorubicin (Fig 6), despite the fact that they are also dependent on proliferation for their cytotoxic actions, is unexpected and indicates that more comprehensive studies with these agents should be undertaken.

Corticosteroids are often used clinically to control edema, pain, and nausea and as antiproliferative agents against tumor cells in conjunction with chemotherapy and radiotherapy. They have been shown to inhibit growth factor production by human fibroblasts and endothelial cells and, if it can be shown that they are able to induce cell-cycle-arrest of murine BM progenitors, this is likely to also occur in humans. Apart from the potential BM protective effects of corticosteroids during chemotherapy, their clinical use when the BM is in the recovery phase after chemotherapy or radiotherapy could retard recovery if cell-cycle-arrest occurs. Furthermore, because cells are resistant to irradiation in the S-phase, the use of corticosteroids during radiotherapy may result in cell-cycle-arrest and possibly increase damage to the BM. These considerations indicate that the mechanisms by which corticosteroids exert this chemoprotective effect should be determined so that the treatment schedules of corticosteroids during cancer therapy can be adjusted if required.

REFERENCES


Protection of murine bone marrow by dexamethasone during cytotoxic chemotherapy

AB Kriegler, D Bernardo and SM Verschoor