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Hemotoxicity by Prolonged Etoposide Administration to Mice Can Be Prevented by Simultaneous Growth Factor Therapy[1]

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ABSTRACT

In this study, we determined *in vivo* interactions between hemopoietic growth factors and etoposide (VP-16) to assess whether normal blood cell production could be maintained during chemotherapy if hemopoietic growth factors were simultaneously administered. Groups of mice were treated for 7 consecutive days with four different doses of VP-16 in combination with three different doses of erythropoietin (EPO) or granulocyte colony-stimulating factor (G-CSF). In total, 12 combinations of VP-16 plus EPO and 12 combinations of VP-16 plus G-CSF were thus evaluated. Intricate dose-response surfaces of the effects of the different treatments on colony-forming units-erythroid, reticulocytes, hematocrit, colony-forming units-granulocyte/macrophage, and absolute neutrophil count were obtained, which revealed that: (*a*) simultaneous EPO administration was able to maintain reticulocyte production and to protect mice from VP-16 induced anemia; (*b*) simultaneous G-CSF administration was able to maintain granulocyte production and to protect mice from VP-16 induced neutropenia; (*c*) VP-16 dose escalation was feasible when EPO or G-CSF were simultaneously administered; and (*d*) no increased myelotoxicity on erythroid or granuloid progenitors was observed when EPO or G-CSF was simultaneously administered with VP-16. These results suggest that *in vivo* either individual hemopoietic progenitors can become resistant against VP-16-induced cell death by appropriate simultaneous growth factor administration or that the loss of overall cell amplification, induced by VP-16, can be compensated by extra amplification of surviving progenitors. Furthermore, these data indicate that a strict separation in time of cytostatic drug and growth factor treatment is not necessarily the optimal schedule with respect to the reduction of hemotoxicity.

INTRODUCTION

Hemopoietic growth factors are currently being intensively investigated to assess their role in hastening the restoration of reduced peripheral blood cell numbers in chemo- or radiotherapeutic settings. These growth factors are, in the vast majority of cases, administered to patients after they have been treated with anti-neoplastic agents, thus separating the cytotoxic and the cytokinetic therapy in time. This strategy is pursued because very little is known about the *in vivo* interactions of growth factors and cytostatic drugs. Concern exists about possible enhanced hemotoxicity when growth factors and cytostatic drugs are administered simultaneously, because growth factors may increase the sensitivity of hemopoietic progenitors for cytostatic drugs. Up to now, however, this approach has not been investigated satisfactorily, although there are reasons to believe that the simultaneous administration of specific hemopoietic growth factors and certain cytostatic drugs may well be beneficial in terms of normal blood cell formation, thus allowing chemotherapeutic dose escalation.

Theoretically, it can be argued that a certain dose of a cytostatic drug kills a certain percentage of a specific hemopoietic progenitor cell pool. If a growth factor is administered simultaneously, amplification of the fraction of progenitors which was not killed is increased, which may result in increased peripheral blood cell production. This argument assumes that the sensitivity of the remaining progenitors for the cytostatic drug is not increased by the growth factor.

A second indication for a possible beneficial effect of simultaneous treatment with growth factors and cytostatic drugs comes from the fact that many cytotoxic agents, acting with different molecular mechanisms, induce the appearance of apoptotic characteristics in target cells (1, 2). In addition, it has also been shown that many growth factors prevent dividing cells from apoptosis (3–5). The question arises whether *in vivo* toxicity, resulting from apoptosis-inducing cytostatic agents, can be prevented by appropriate, apoptosis-preventing growth factors. There are indications from several groups that *in vitro* this is indeed possible. It has been demonstrated that the induction of apoptosis in leukemic cells, treated with several cytotoxic agents (Adriamycin, 1-β-D-arabinofuranosylcytosine, and vincristine), could be prevented by GM-CSF,[3] G-CSF and interleukin 6 (6). Collins *et al.* (7) analyzed the *in vitro* effect of a prolonged simultaneous coincubation of BAF-3 cells with VP-16 or cisplatin and interleukin 3. Interleukin 3 was able to protect cells from apoptotic cell death induced by these drugs (7). If these *in vitro* results can be extrapolated to *in vivo* situations, it would open the possibility to protect normal hemopoietic progenitors during chemotherapy.

In the present study, therefore, we assessed normal hemopoietic cell production in mice treated with several doses of VP-16 simultaneously with two widely used growth factors, EPO and G-CSF. It has been shown that continuous administration of VP-16 results in superior responses in patients (8). In the murine system, the VP-16 dose killing 10% of the mice after a 5-day treatment has been shown to be approximately 0.2 mg/day (9). These reports led us to design a multifactorial experiment in which mice were treated with 4 doses of VP-16 (0, 0.05, 0.1, and 0.2 mg/day) for 7 days. Concomitantly, rhEPO or rhG-CSF, each in three doses, was infused. With this approach, we were able to prevent anemia and neutropenia and could increase VP-16 doses significantly without inducing hemotoxicity. By a detailed statistical analysis of the data, we were able to construct equi-response curves of each cellular parameter. These curves illustrate the intricate behavior of the different parameters.

MATERIALS AND METHODS

Mice. Female C57Bl/6 mice, aged 12–16 weeks were used in all experiments.

Growth Factors. Recombinant human EPO, a gift from Boehringer Mannheim (Almere, the Netherlands) was administered at doses of 10 and 25 units/day/mouse. Recombinant human G-CSF, a gift from Amgen (Thousand Oaks, CA) was administered at doses of 0.25 and 2.5 μg/day/mouse.

VP-16. VP-16 (etoposide; Vepesid) was obtained from Bristol-Myers Squibb and was administered at doses of 0.05, 0.1, and 0.2 mg/day/mouse.

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[3] The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte-CSF; EPO, erythropoietin; rh, recombinant human; CFU-E, colony-forming units-erythroid; VP-16, etoposide.

Table 1 *Summary of the statistical analysis of the EPO + VP-16 experiment*

This is a summary of the significance (NS, not significant) and the sign of all coefficient estimates of the regression model which was used for the statistical analysis. Thus it indicates whether a certain cell stage was significantly affected by low EPO or VP-16 doses alone (b and d term), whether this effect was saturated at high EPO or VP-16 doses alone (c and e term), and whether in the combination of EPO and VP-16 significant interaction occurred. As an example, the hematocrit was increased by low EPO doses. At high EPO doses no saturation was observed. VP-16 reduced the hematocrit at low doses, at higher VP-16 doses this linear inhibitory effect was saturated (*i.e.*, had reached its maximum). Furthermore VP-16 had specifically inhibitory effects on EPO-stimulated erythropoiesis.

	b (EPO)	c (EPO^2)	d (VP-16)	e (VP-16^2)	f (EPO × VP-16)
Hematocrit	+	NS	−	+	−
Reticulocytes	NS	+	−	+	−
CFU-E/femur	NS	NS	NS	NS	NS
CFU-E/spleen	+	NS	NS	NS	−

Osmotic Pumps. In order to ensure constant serum levels, growth factors and VP-16 were appropriately diluted and administered by s.c. implanted osmotic pumps (Alza, Palo Alto, CA). EPO and VP-16 were administered by the same pump. G-CSF, however, appeared to be inactivated when mixed with VP-16; therefore, two pumps were implanted in the G-CSF/VP-16 mice.

Cell Suspensions and Assays. Femoral and splenic single cell suspensions were made using standard procedures. Hemopoietic progenitors were cultured with the methylcellulose method of Iscove and Sieber (10). CFU-GM/burst-forming unit-erythroid cultures were supplemented with 100 ng/ml recombinant rat stem cell factor (a gift from Amgen), 10 ng/ml recombinant murine GM-CSF (a gift from Behringwerke, Marburg, Germany) and 2 units/ml rhEPO. This resulted in optimal colony formation in our assay. CFU-E cultures were supplemented with 500 milliunits/ml EPO. Reticulocytes and neutrophils were counted on, respectively, brilliant crysol blue or May-Grünwald-Giemsa-stained blood smears using light microscopy. The WBC was determined with a Coulter cell analyzer.

Statistical Analysis. Data as given in the figures are the means of 2–4 mice/datapoint. The EPO/VP-16 and G-CSF/VP-16 experiments were designed in a multifactorial way (3 EPO/G-CSF doses × 4 VP-16 doses). The statistical evaluation of this type of experiment involves a regression analysis to determine whether and how much a certain parameter significantly affects the shape of the three-dimensional dose-response surface. This type of analysis is far more powerful than a pairwise comparison of selected datapoints and thereby reduces the number of experimental animals needed to detect significant effects.

We have used this strategy previously in experiments investigating the effects of stem cell factor plus EPO (11) and G-CSF plus EPO (12). In the present study, we could not expect simple linear growth factor dose-response relationships. Therefore the regression model had to be specified in a meaningful way. For both EPO and G-CSF, we have published information showing dose-response saturation characteristics on a logarithmic scale (13, 14). As the regression model analyzes the biological effect (response) of a given G-CSF or EPO dose, these given EPO and G-CSF doses were logarithmically transformed. Thus, EPO doses were coded 0 units = 0, 10 units = 1, and 25 units = 1.4. Similarly, G-CSF doses were coded as 0 μg = 0, 0.25 μg = 1, and 2.5 μg = 2. This coding expresses that the biological "distance" (response) from 0 to 0.25 μg G-CSF is similar as from 0.25 to 2.5 μg G-CSF. The EPO coding analogously acknowledges that the effect from 0 to 10 units EPO is stronger than from 10 to 25 units EPO. This coding protocol is similar as previously published analyses (11, 12).

With the data obtained in the EPO/VP-16 experiment for the number of each cell stage (Y), a regression was performed based upon the model: $Y = a + b \times \text{EPO} + c \times \text{EPO}^2 + d \times \text{VP-16} + e \times \text{VP-16}^2 + f \times (\text{EPO} \times \text{VP-16})$. With the data obtained in the G-CSF/VP-16 experiment, a similar regression was performed, based upon the model $Y = a + b \times \text{G-CSF} + c \times \text{G-CSF}^2 + d \times \text{VP-16} + e \times \text{VP-16}^2 + f \times (\text{G-CSF} \times \text{VP-16})$. These models are identical as the ones we have previously published (11, 12). They assess whether: (*a*) there is a significant linear effect of low EPO/G-CSF doses ("b" term); (*b*) this linear D/R effect is saturated at higher EPO/G-CSF doses ("c" term); (*c*) there is a significant effect of low VP-16 doses ("d" term); (*d*) this linear VP-16 D/R effect reaches saturation at high doses ("e" term); and (*e*) there is an interaction between EPO and VP-16 ("f" term).

Estimates for the regression coefficients and the 95% confidence intervals were determined. A summary of the results of the regression analysis is given in Tables 1 and 2. For each cell stage, it is indicated whether the regression coefficient of a term differed significantly from 0 ($P < 0.05$). Positive values of the linear terms (EPO/G-CSF and VP-16) should be interpreted as stimulatory activity; negative values of these terms indicate inhibitory effects. A negative or positive value of a quadratic term (EPO^2/G-CSF^2 and VP-16^2) indicates flattening of a linear effect, which is saturation of the linear response. A negative value of the interaction term (EPO/G-CSF × VP-16) indicates that VP-16 has stronger inhibitory activity in stimulated than in nonstimulated hemopoiesis. All analyses were performed using SAS PROC REG.

Visualizing the Regression Analysis. The regression analysis evaluated the shape of the complete three-dimensional dose-response surface mathematically. The dose-response surface, as described by the regression model, was graphically depicted in a two-dimensional fashion by filling in all possible coded EPO/G-CSF and VP-16 combinations in the regression model and by calculating the predicted Y value. The contour plots thus obtained give a quantitative description of the behavior of each cell stage in response to EPO, G-CSF, and VP-16. They evaluate at which dose ranges VP-16 dose-escalation was feasible and facilitate interpretation of Table 1. These contour plots show so-called "equi-response curves," dose combinations which resulted in similar cell counts.

RESULTS

Simultaneous EPO and VP-16 Administration for 7 Days. VP-16 administration during 7 days induced a dose-dependent reticulocytopenia and anemia (Fig. 1). Reticulocytes were undetectable at a dose of 0.1 mg/day or more. This was also reflected in the hematocrit which was maximally reduced to about 37% at a dose of 0.1 mg/day or more. Simultaneous EPO administration dose-dependently increased both the number of reticulocytes and the hematocrit at VP-16 doses of 0.05 and 0.1 mg/day. At a VP-16 dose of 0.2 mg/day, however, no protective effect of EPO was observed anymore. The corresponding contour plots, which were created by the regression analysis, illustrate the dose ranges of combined EPO and VP-16 administration which are able to maintain normal values (Fig. 2). It is evident from the slopes of the graphs in Fig. 1 that, although EPO was

Table 2 *Summary of the statistical analysis of the G-CSF + VP-16 experiment*

This is a summary of the significance (NS, not significant) and the sign of all coefficient estimates of the regression model which was used for the statistical analysis. Thus it indicates whether a certain cell stage was significantly affected by low G-CSF or VP-16 doses alone (b and c term), whether this effect was saturated at high G-CSF or VP-16 doses alone (d and e term), and whether in the combination of G-CSF and VP-16 significant interaction occurred. For an example see legend to Table 1.

	b (G-CSF)	c (G-CSF^2)	d (VP-16)	e (VP-16^2)	f (G-CSF × VP-16)
Granulocytes/ml	NS	+	−	+	−
CFU-GM/femur	+	−	NS	+	NS
CFU-GM/spleen	NS	+	+	−	−

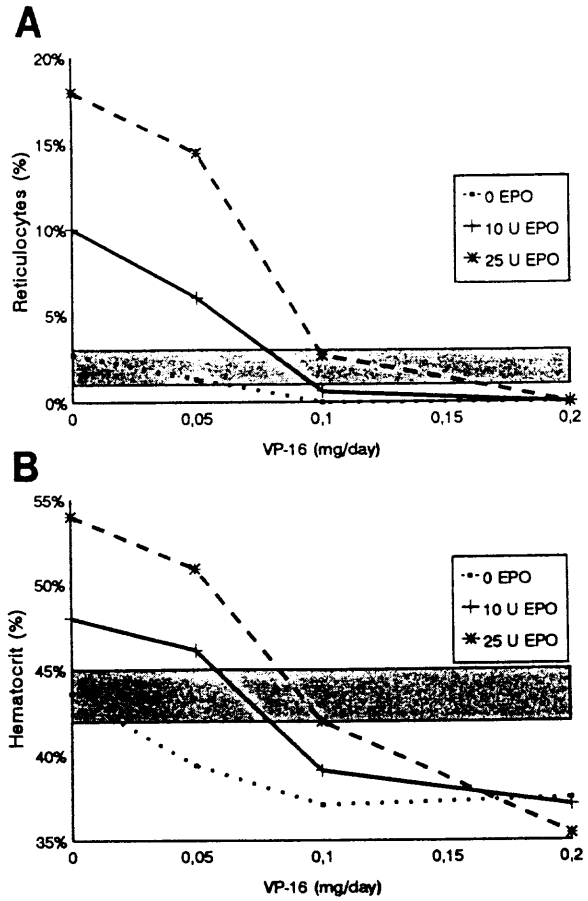

Fig. 1. Effect of a simultaneous administration of EPO and VP-16 for 7 days on reticulocytes (*A*) and hematocrit (*B*). *Shaded area* represents normal range.

able to prevent anemia, VP-16 relatively had a greater effect in reducing RBC production in EPO-stimulated than in nonstimulated hemopoiesis (indicated by the negative EPO × VP-16 interaction term; Table 1).

To assess whether simultaneous administration of EPO and VP-16 increased hemotoxicity at an earlier level, erythroid progenitors were cultured. Fig. 3 demonstrate that VP-16 alone did not inhibit CFU-E numbers, neither in bone marrow nor in the spleen. On the contrary, at moderate VP-16 doses, increased CFU-E numbers were found, although this did not reach significant levels (Table 1). Additional treatment with EPO augmented CFU-E numbers, especially in the spleen. Whereas VP-16 alone did not affect CFU-E numbers, it was very potent in reducing EPO-stimulated splenic CFU-E numbers (negative EPO × VP-16 interaction term; Table 1). This effect was reflected in the reticulocyte count and hematocrit, as discussed above. Fig. 4 shows the contourplots of the behavior of marrow and spleen CFU-E. These graphs more clearly illustrate the responses in the CFU-E compartment. Most importantly for this study, CFU-E numbers lower than the normal range were never observed. This was also the case for burst-forming unit-erythroid numbers (data not shown).

Simultaneous G-CSF and VP-16 Administration for 7 Days. Similar to the effects observed in the erythroid lineage, VP-16 induced a profound neutropenia in 7 days. No dose-dependence in the tested dose range was observed; however, the lowest VP-16 dose of 0.05 mg/day already resulted in peripheral neutrophil numbers reduced to 25% of normal. Increasing the VP-16 dose did not result in enhanced neutropenia. Simultaneous G-CSF administration dose-dependently prevented the neutropenia completel G-CSF at a dose of 0.25 μg/day, which by itself only had a we stimulatory effect, was sufficient to normalize neutrophil numbe G-CSF (2.5 μg/day), which by itself strongly increased neutrop numbers, induced a mild neutrophilia in combination with VP-16 doses tested (Fig. 5). Analogous to the erythroid lineage, is evident that, although G-CSF could protect mice from develo ing a neutropenia, VP-16 relatively had a stronger inhibiting effe on G-CSF-stimulated than on normal, nonstimulated hemopoie (negative interaction term of G-CSF × VP-16; Table 2). Fig. shows at which VP-16 and G-CSF dose ranges normal absolu neutrophil count can be maintained.

To test whether the simultaneous administration of G-CSF a VP-16 was detrimental for myeloid progenitors, CFU-GM numbers bone marrow and spleen were determined. Fig. 7*A* demonstrates th CFU-GM numbers in the femur were not decreased by VP-16, neith alone nor in combination with G-CSF. G-CSF itself had oppos effects at different doses; CFU-GM numbers were increased at lo G-CSF doses but slightly decreased at high doses. As expected, hi doses of G-CSF markedly stimulated splenic granulopoiesis (Fig. 7*E* VP-16 alone did not inhibit CFU-GM numbers in the spleen.

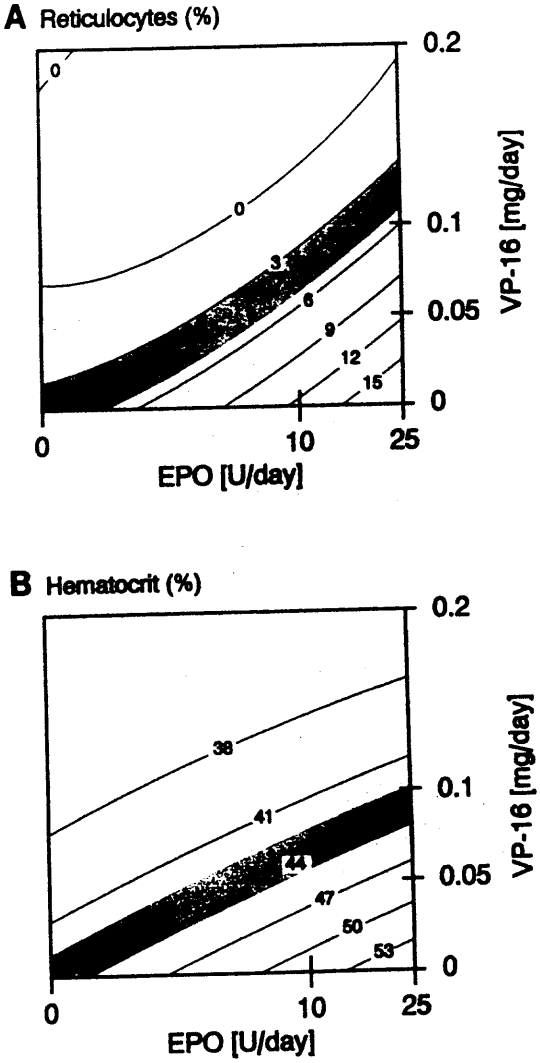

Fig. 2. Contour plot, resulting from the regression analysis of the data of Fi showing equi-response curves of the reticulocytes (*A*) and hematocrit (*B*) during bined administration of EPO and VP-16. *Shaded area* indicates dose ranges at w normal values (± 1 SEM) can be maintained.

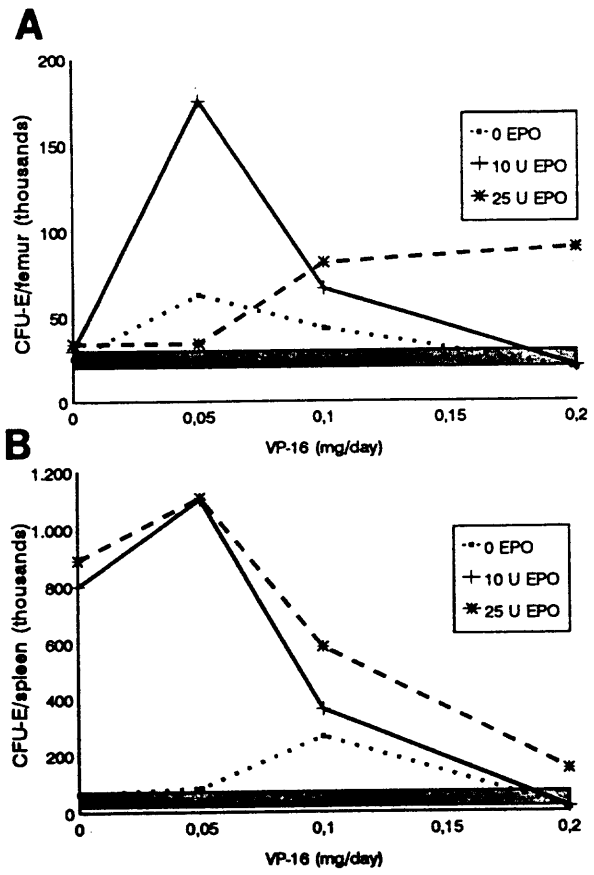

Fig. 3. Effect of simultaneous administration of EPO and VP-16 for 7 days on CFU-E numbers/femur (*A*) and spleen (*B*). *Shaded area* represents normal range.

combination with high-dose G-CSF, however, CFU-GM numbers were affected but only at the highest VP-16 dose. Importantly however, CFU-GM numbers were never found to be below normal values.

Fig. 8 further illustrates the general interactions between G-CSF and VP-16 on CFU-GM numbers in marrow and spleen. It is clear that the behavior of CFU-GM is primarily regulated by G-CSF. With varying VP-16 doses, CFU-GM numbers change only slightly.

DISCUSSION

In this study, we wanted to investigate the *in vivo* interactions between hemopoietic growth factors and cytostatic drugs. We demonstrate that the anemia and neutropenia induced by a prolonged VP-16 treatment of mice could be prevented by concomitant administration of EPO or G-CSF. This prophylactic use of growth factors did not result in increased myelotoxicity on immature progenitors and allowed dose escalation. VP-16 inhibited erythropoiesis between the CFU-E and the reticulocytes; CFU-E were unaffected, but reticulocytes were absent. Simultaneous EPO administration could normalize the production of reticulocytes from CFU-E at several VP-16 doses. Granulopoiesis was also affected by VP-16, which became apparent in a severely reduced absolute neutrophil count. This was not due, however, to reduced CFU-GM numbers, which remained unaffected. It appears that VP-16 at the doses used inhibits granulopoiesis at some stage between the CFU-GM and the mature granulocytes. Simultaneous G-CSF administration could restore this defective production and was able to prevent neutropenia. Granulopoiesis appeared to be more sensitive to VP-16 than erythropoiesis because, at the lowest VP-16 dose, maximal inhibitory effects were already observed. No effects on platelet count were detected (data not shown).

There are several possibilities which may explain the protection from VP-16-induced hemotoxicity by EPO and G-CSF. The first option takes into account only populations of cells. A certain dose of VP-16 may eliminate a fixed percentage of a specific hemopoietic compartment. If the surviving cells are stimulated by simultaneous EPO or G-CSF therapy, this will increase their amplification and may compensate for the loss of cells induced by VP-16. This implies that the sensitivity for VP-16 of the surviving fraction of cells is not or not strongly increased. In this respect, it is important to consider the *in vivo* effect of EPO and G-CSF on their target cells. It is unknown to what extent apoptosis regulates *in vivo* blood cell production (15, 16). If EPO and G-CSF would act primarily by preventing premature cell death, saving these cells by exogenous growth factor administration will rapidly increase their number without necessarily affecting their cycling activity, *i.e.*, their sensitivity for a cell cycle-specific drug. Another mechanism of growth factor activity, however, could be that *in vivo* they act by increasing the cycling activity of (dormant) cells (17, 18). This would increase the sensitivity of target cells for cell cycle-specific drugs but not necessarily for less S-phase-specific drugs, like VP-16, which can induce apoptosis in quiescent thymocytes (19).

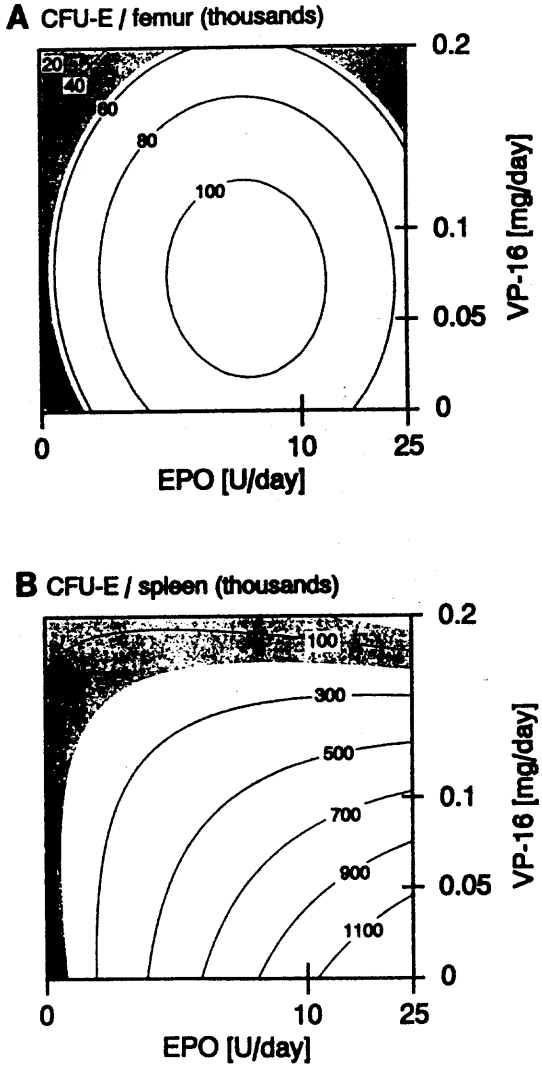

Fig. 4. Contour plot, resulting from the regression analysis of the data of Fig. 3, showing equi-response curves of the marrow (*A*) and spleen (*B*) CFU-E during combined administration of EPO and VP-16. *Shaded area* indicates dose ranges at which normal values (± 1 SEM) can be maintained.

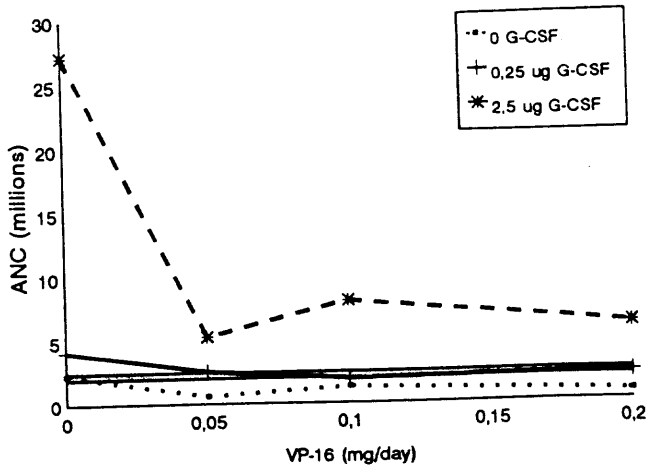

Fig. 5. Effect of simultaneous administration of G-CSF and VP-16 for 7 days on absolute neutrophil count/ml. *Shaded area* represents normal range.

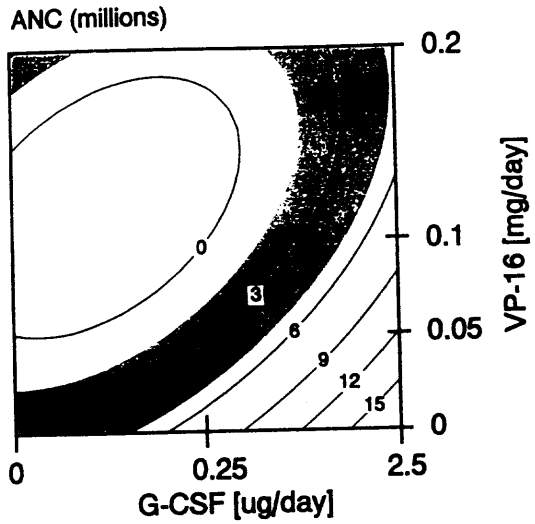

Fig. 6. Contour plot, resulting from the regression analysis of the data of Fig. 5, showing equi-response curves of the absolute neutrophil count (in millions/ml) during combined administration of EPO and VP-16. *Shaded area* indicates dose ranges at which normal values (± 1 SEM) can be maintained.

A second option which may explain our findings is that EPO and G-CSF in fact prevent the VP-16-induced apoptotic death of an individual cell. This implies that the sensitivity of a single cell for certain cytostatic drugs can be decreased. There have been several reports showing that this phenomenon exists in myeloid cell lines (6, 7, 20). Several cytostatic drugs which induce apoptosis, like VP-16, are generally thought to specifically inhibit the activity of topoisomerase II. This leads to a stabilization of a drug-topoisomerase II-DNA complex, the so-called cleavable complex (21). It is unknown how this cleavable complex formation ultimately results in apoptotic cell death but is has been suggested that DNA fragmentation is not a direct consequence of VP-16 activity (19). It would be of interest to determine whether growth factors like EPO or G-CSF influence the formation of cleavable complexes or whether these growth factors prevent VP-16 activity via a different molecular mechanism.

The first observations which showed that cells could be protected from DNA damaging agents by hemopoietic growth factors were made with leukemic cells (6, 7). We show in this study that these effects can be extrapolated to normal *in vivo* hemopoiesis. Thus, it appears that the current, strict separation of chemo- and growth factor therapy of cancer patients is not necessarily the optimal way to reduce hemotoxicity. An exception, however, may be leukemic patients; there malignant cells may be protected against chemotherapy (22). The priming of leukemic blast cells with GM-CSF pretreatment before therapy with cytostatic drugs has been studied by several groups, which have reported conflicting results (23–25). The discrepancies, varying from increased complete remission rates (23) to reduced remission rates (25) may reflect the effect of GM-CSF on leukemic cells of different patients (increasing cycling activity *versus* preventing apoptosis) and the different cytostatic drugs used (6).

The *in vivo* protection of normal hemopoiesis as we have observed in this study will depend upon the type of growth factors and, more importantly, the type of cytostatic drugs used. Most of the apoptosis-inducing cytostatic drugs, like VP-16, are not very cell cycle specific. Different results may be obtained when growth factors are simultaneously administered with cell cycle-specific drugs, like hydroxyurea or 5-fluorouracil. In fact, it has already been reported that, in humans, the simultaneous use of G-CSF and 5-fluorouracil severely increases myelosuppression (26). Shaffer *et al.* (27) reported a Phase 1 trial in which the simultaneous use of GM-CSF and VP-16 appeared to result in increased myelotoxicity, compared to VP-16 alone (27). Treatment of mice with stem cell factor, followed by a single injection of 5-fluorouracil, recently has been reported to result in total marrow failure and death (28). Also, different effects may be observed when combinations of growth factors are administered. Recently, we have demonstrated how erythropoiesis and granulopoiesis mutually inhibit each other in splenectomized mice (12). Thus, the present results may differ

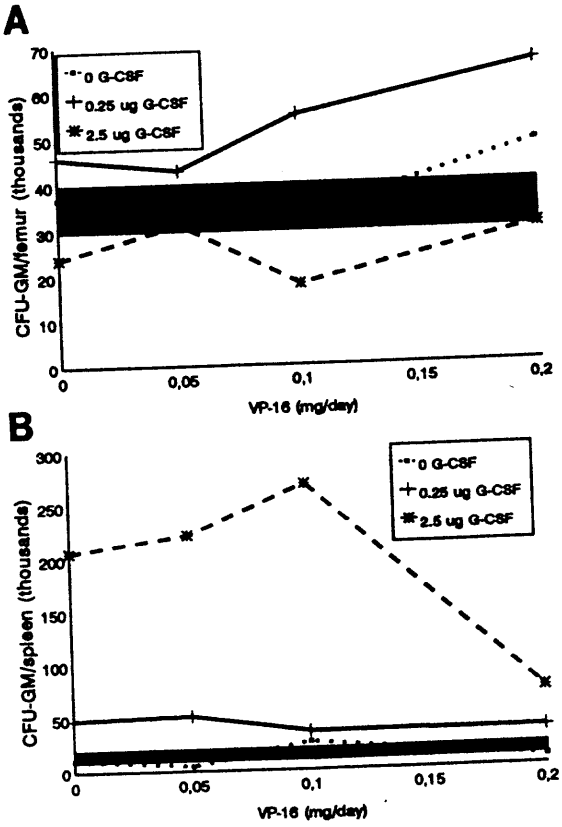

Fig. 7. Effect of simultaneous administration of G-CSF and VP-16 for 7 days on CFU-GM numbers/femur (*A*) and spleen (*B*). *Shaded area* represents normal range.

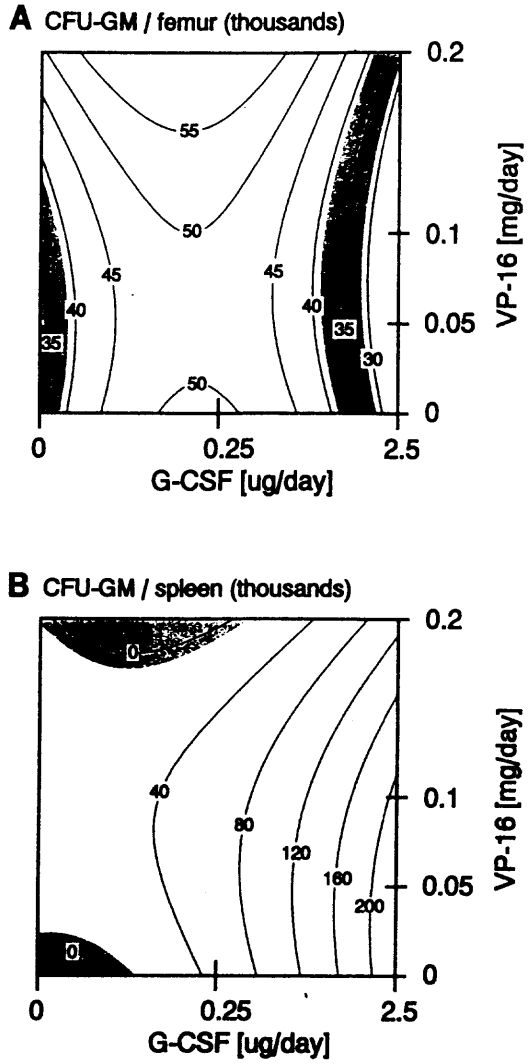

Fig. 8. Contour plot, resulting from the regression analysis of the data of Fig. 7, showing equi-response curves of CFU-GM/femur (*A*) and spleen (*B*) during combined administration of EPO and VP-16. *Shaded area* indicates dose ranges at which normal values (± 1 SEM) can be maintained.

quantitatively when EPO plus G-CSF are simultaneously administered. These data illustrate the importance of clarifying the underlying mechanisms of the protective effects in order to select beneficial combinations and timing schedules of growth factors and cytostatic drugs.

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