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Hematopoietic Recovery after Large Doses of Cyclophosphamide: Correlation of Proliferative State with Sensitivity[1]

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SUMMARY

Hematological recovery after a large dose of cyclophosphamide was characterized by early rapid recovery of marrow stem cells (colony-forming assay), followed successively by recovery of circulating stem cells (colony-forming assay), total marrow cellularity, and circulating blood cells. This recovery sequence provided a model in which marrow with different proliferative rates could be studied for sensitivity to cyclophosphamide by subjecting animals to a second treatment at various intervals after an initial treatment. The results of this type of study suggest that the proliferative state of a cell population is a determinant of sensitivity to cyclophosphamide.

INTRODUCTION

The alkylating agent cyclophosphamide (Cytoxan, NSC 26271) is an effective antineoplastic drug. Recent studies have emphasized the value of large intermittent doses of this drug (7, 8, 12, 24, 26). The hematological side effects of large doses of this drug have been studied in humans (2, 22, 23) and in experimental animals (11, 15, 16). These reports have described the effects on peripheral blood cells and on morphologically identifiable bone marrow cells. Many of the changes noted could be a consequence of depletion of hematopoietic stem cells (19), and therefore the present study was designed to correlate the effects of cyclophosphamide on stem cells (28) with the effects on bone marrow cells and peripheral blood cells.

Serial observations of bone marrow colony-forming cells and circulating colony-forming cells, total marrow cell counts and peripheral blood counts have been made in mice after treatment with a large dose of cyclophosphamide (275 mg/kg). The acute effects of a repeat treatment at various intervals after the initial treatment were studied to evaluate the status of recovery from the initial treatment and these results suggest that the proliferative state of a cell population may be a determinant of sensitivity to cyclophosphamide.

MATERIALS AND METHODS

Mice. Male CDF_1 mice, 10 to 12 weeks old, were the subjects of cyclophosphamide treatment. Male BALB/c mice, 10 weeks old, were the recipients for the colony-forming assay. The mice were kept 5 to 10/cage and fed Purina laboratory chow and water *ad libitum*.

Drug. Cyclophosphamide was dissolved in sterile 0.9% NaCl solution, and the appropriate concentrations were injected i.p. in a volume of 0.01 ml/g body weight.

Irradiation. BALB/c mice were given 450 R of whole-body irradiation with the following factors: 200 kV, 15 ma, 0.25 mm Cu and 0.55 mm Al filtration, target distance 54 cm, and dose rate 139 R/min.

Preparation of Bone Marrow Suspensions. Mice were killed by cervical dislocation and both femurs were dissected free. The ends of the bone were cut off, a 22-gauge needle was inserted into the marrow cavity and the marrow was flushed with 0.2 ml Eagle's medium (Microbiological Associates, Inc., Bethesda, Md.). The marrows of each group of 5 or more mice were pooled and the cells were separated and suspended in medium by flushing repeatedly through a 1-ml pipet. An aliquot of this suspension was then diluted in 3% acetic acid and the cells were counted in a hemacytometer.

Hematopoietic Stem Cell Assay (Colony-forming Units). Cell suspensions or blood specimens were assayed according to the technique of Till and McCulloch (28), which involves injection of cell suspensions into lethally irradiated mice. The number of hematopoietic colonies formed after 10 days in the spleens of recipients is considered proportional to the stem cells in the donor tissue.[3] Controls receiving irradiation but no injection had an average of less than 0.5 endogenous colony per spleen.

Other Techniques. White cells were counted with orbital sinus blood with standard techniques (9). All mononuclear cells are presented as lymphocytes in the results. Cell viability was tested by trypan blue exclusion.

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[3]The terms colony-forming cell and stem cell are used interchangeably in this paper.

Statistical Evaluation. Standard errors of each group average (*e.g.*, mean colony-forming units per femur) were obtained with 9 degrees of freedom (or 8 degrees of freedom if there were only 9 survivors/group at the time of assay) and reflect the mouse-to-mouse variation in the spleen colony end point. The coefficient of variation of each such average was then the ratio of the standard error to the average. When a particular average was expressed as a surviving fraction relative to an appropriate control average, the standard error of the surviving fraction was estimated as that fraction multiplied by the square root of the sum of the squares of the 2 coefficients of variation involved. For comparing 2 independent surviving fractions, the standard error of their difference was estimated by the square root of the sum of the squares of the 2 standard errors involved.

RESULTS

Hematopoietic Recovery. A dose of 275 mg/kg of cyclophosphamide was selected for study because preliminary testing had shown this to be the highest nonlethal dose in the mice used for this study. The serial changes in the parameters studied are presented in Charts 1 to 3. The marrow stem cell population showed a drop to about 10% of control values 24 hr after treatment (Chart 1). The marrow stem cell population then increased, with a population doubling time of 18 hr, with a return to pretreatment values by Day 3 and an overshoot on Days 4 and 5 (Chart 1).

The total marrow cell counts (Chart 1) fell progressively for 3 days after cyclophosphamide treatment. Marrow suspensions on Days 1 and 2 showed viability by trypan blue exclusion of greater than 95%. This progressive drop in cell counts for 3 days, although the cells appeared viable by dye exclusion, suggests delayed cell death after cyclophosphamide exposure. The marrow count then increased exponentially with a population doubling time of 18 hr. Pretreatment values were reached by Day 7.

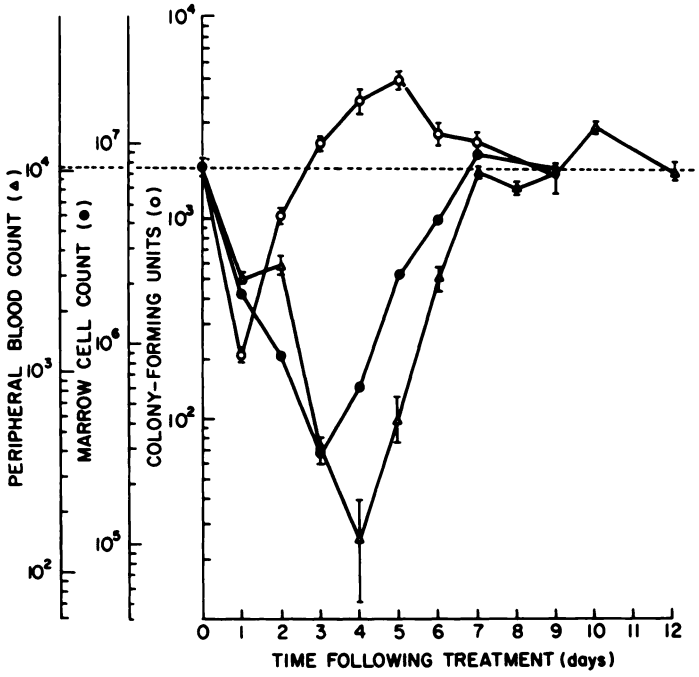

Chart 1. Effect of treatment with 275 mg/kg cyclophosphamide i.p. on marrow CFU (○), marrow cell counts (●), and peripheral leukocyte counts (△). The scales have been adjusted to superimpose pretreatment values, – – –. *Vertical bars*, ±1 S.E.

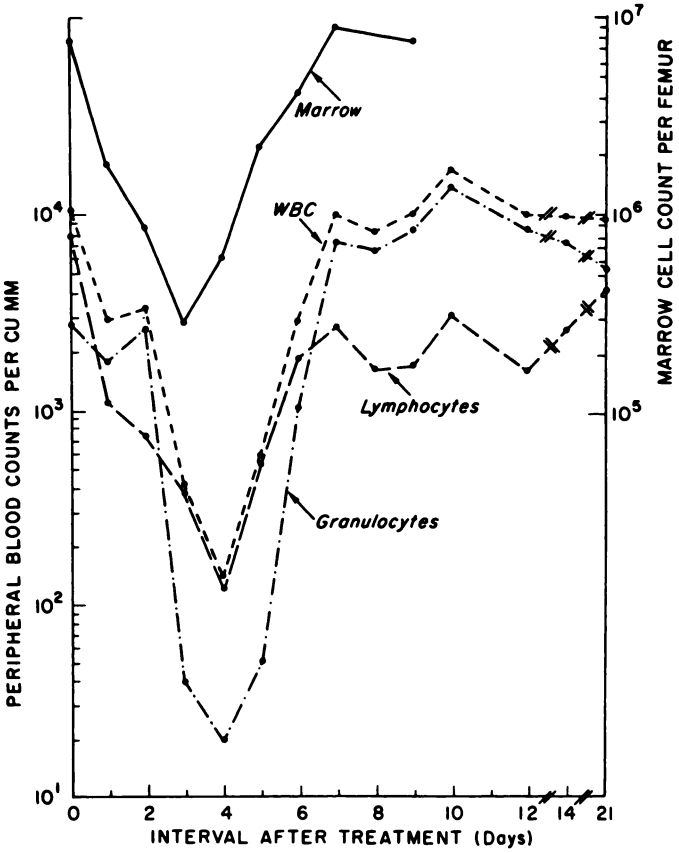

Chart 2. Serial changes in marrow cell count and peripheral blood leukocyte, lymphocyte, and granulocyte values after 275 mg/kg cyclophosphamide.

The recovery of the peripheral blood leukocyte count lagged about 1 day behind the recovery of the total marrow cell count (Chart 1). The total leukocyte count returned to pretreatment values by Day 7. Granulocytes were the cell type most numerous in this recovery, in contrast to the normal mouse differential count, in which lymphocytes are the predominant cell type (Chart 2). The absolute lymphocyte count increased more slowly and had not returned to pretreatment values by Day 21.

Circulating Colony-forming Cells. The possible role of circulating colony-forming cells in hematopoietic recovery was studied by serial determinations of blood and bone marrow CFU[4] after treatment with cyclophosphamide (Chart 3). The CFU in the blood dropped to below 10% of pretreatment values and remained in that range for 2 days.

[4]The abbreviation used is: CFU, colony-forming units.

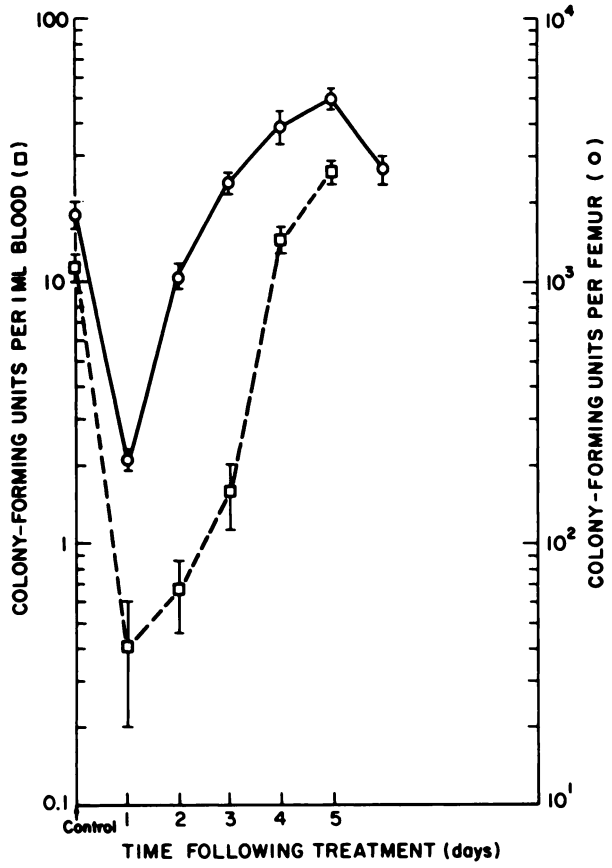

Chart 3. Serial changes in blood CFU and marrow CFU after 275 mg/kg cyclophosphamide. *Vertical bars*, ± 1 S.E.

The slight increase on Day 2 is not significantly different from the Day 1 value. A rise in circulating colony-forming cells was noted on Day 3 and pretreatment values were reached on Day 4.

Response of Marrow to Repeat Cyclophosphamide Treatment. After the above system was established, an experiment was designed to study the effects of a 2nd dose of cyclophosphamide on recovering marrow. Mice were treated with cyclophosphamide, 275 mg/kg, on Day 0; and on Day 3 or 5 groups received 300 mg/kg, 150 mg/kg, or no drug. (See Tables 1 and 2 for details of experimental design.) Previously untreated control mice were treated with 300 mg/kg or 150 mg/kg. Marrow cells counts and CFU were determined at the intervals shown in the tables. The data are presented as families of curves showing the relationship between the value at the time of treatment and the value at the time of assay 24 hr after treatment (Charts 4 and 5). Inspection of these charts suggests a particularly great sensitivity to repeat treatment on Day 3. Where repeat treatment was delayed until Day 5, the effect was less than that elicited by the initial treatment relative to stem cells, but comparable to the initial treatment effect relative to total marrow cells counts.

To test this impression, we converted the data to survival fraction form and the colony assay data were analyzed for statistical significance (Tables 1 and 2). Marrow cell counts were not subjected to statistical testing because the data were single counts of pooled specimens. Surviving fractions for groups receiving repeat treatment were derived as a fraction of the value for the control group assayed concurrently with the repeat treatment group (Tables 1 and 2). Comparison of surviving fractions shows a greater sensitivity to both doses tested for early repeat treatment (Day 3) for both the colony assay and the total marrow cell count when compared with groups not receiving prior treatment (Groups 6 and 7 *versus* 2 and 3, Tables 1 and 2). Comparison of the response of marrow colony-forming cells receiving repeat treatment on Day 5 shows a decreased sensitivity compared with the response of groups not receiving prior treatment (Groups 10 and 11 *versus* 2 and 3, Chart 4 and Table 1). The response of total marrow cellularity to repeat treatment on Day 5 (Groups 10 and 11) shows a sensitivity essentially similar to that of the groups not receiving prior treatment

Table 1

Response of marrow colony-forming cells to repeat treatment with cyclophosphamide

Group	Treatment (mg/kg)	Day of assay	CFU/femur[a]	Surviving fraction[b]
1	None	1	$2.0 \pm 0.15 \times 10^3$	As a fraction of Group 1 value:
2	150 on Day 0	1	$1.0 \pm 0.05 \times 10^3$	0.50 ± 0.045
3	300 on Day 0	1	$2.9 \pm 0.23 \times 10^2$	0.14 ± 0.015
4	275 on Day 0	3	$2.3 \pm 0.21 \times 10^3$	
5	275 on Day 0	4	$2.9 \pm 0.21 \times 10^3$	As a fraction of Group 5 value:
6	275 on Day 0, 150 on Day 3	4	$3.4 \pm 0.40 \times 10^2$	0.12 ± 0.016[c]
7	275 on Day 0, 300 on Day 3	4	$4.9 \pm 0.48 \times 10^1$	0.017 ± 0.002[d]
8	275 on Day 0	5	$3.8 \pm 0.50 \times 10^3$	
9	275 on Day 0	6	$2.5 \pm 0.27 \times 10^3$	As a fraction of Group 9 value:
10	275 on Day 0, 150 on Day 5	6	$2.2 \pm 0.26 \times 10^3$	0.88 ± 0.14[e]
11	275 on Day 0, 300 on Day 5	6	$9.8 \pm 0.52 \times 10^2$	0.39 ± 0.047[d]

[a] Mean ± S.E.
[b] Observed ratio ± S.E. of ratio.
[c] Statistically different from corresponding value of 0.50 for Group 2 at $p \leq 0.001$.
[d] Statistically different from corresponding value of 0.14 for Group 3 at $p \leq 0.001$.
[e] Statistically different from corresponding value of 0.50 for Group 2 at $p \leq 0.02$.

Table 2

Response of marrow cell count to repeat treatment with cyclophosphamide

Group	Treatment (mg/kg)	Day of count	Cell count/femur	Surviving fraction[a]
1	None	1	7.6×10^6	As a fraction of Group 1 value:
2	150 on Day 0	1	4.1×10^6	0.54
3	300 on Day 0	1	2.0×10^6	0.26
4	275 on Day 0	3	2.8×10^5	
5	275 on Day 0	4	1.1×10^6	As a fraction of Group 5 value:
6	275 on Day 0, 150 on Day 3	4	8.2×10^4	0.07
7	275 on Day 0, 300 on Day 3	4	4.4×10^4	0.04
8	275 on Day 0	5	2.3×10^6	
9	275 on Day 0	6	5.5×10^6	As a fraction of Group 9 value:
10	275 on Day 0, 150 on Day 5	6	2.3×10^6	0.42
11	275 on Day 0, 300 on Day 5	6	1.5×10^6	0.27

[a]Values for Groups 6 and 7 are strikingly smaller than corresponding values for Groups 2 and 3 and values for Groups 10 and 11 are essentially similar to corresponding values for Groups 2 and 3.

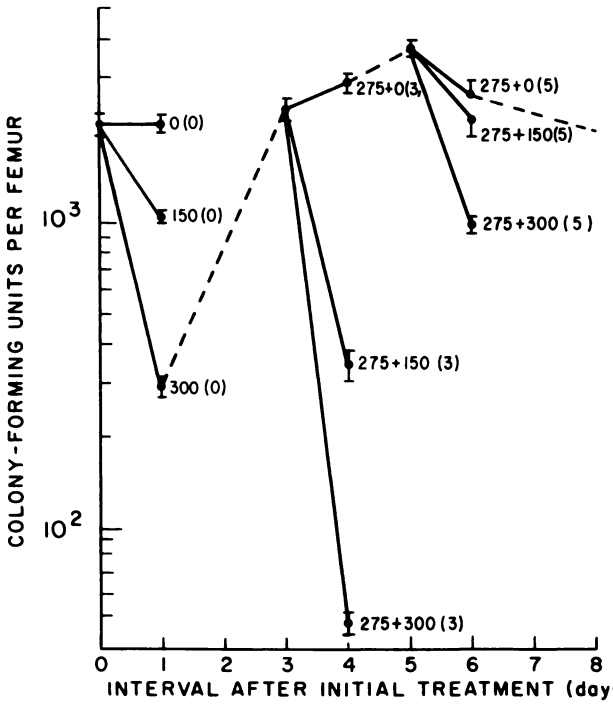

Chart 4. Response of CFU to repeat treatment with cyclophosphamide on Day 3 or Day 5 after initial treatment. All assays from Day 3 onward were made on mice receiving 275 mg/kg on Day 0. In addition, they received 0, 150, or 300 mg/kg cyclophosphamide, respectively, on the day shown in parentheses. Day 0 value was assumed to have been the same as the control value assayed on Day 1, based on the known constancy of marrow values in untreated controls (17). *Vertical bars*, ±1 S.E.

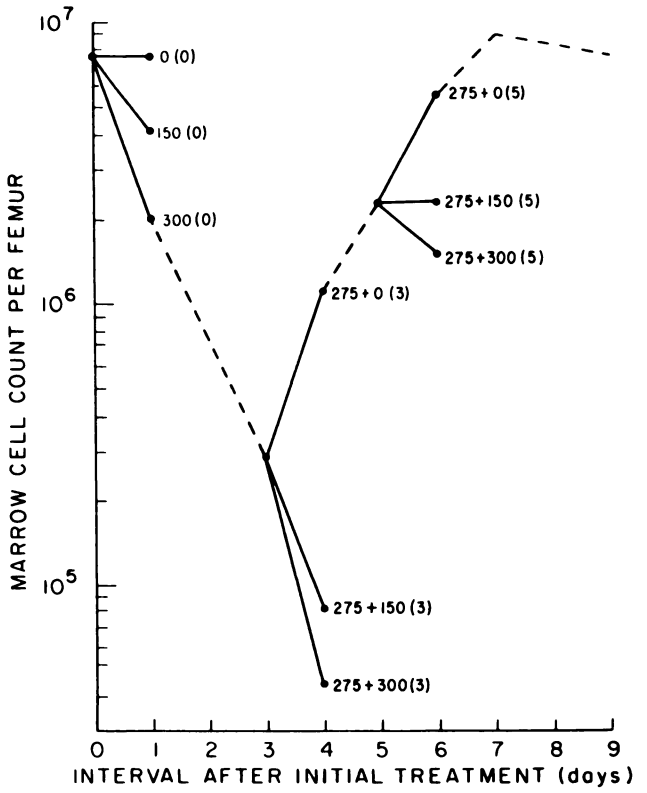

Chart 5. Response of marrow cell counts to repeat treatment with cyclophosphamide. Legend is the same as Chart 4.

(Groups 2 and 3, Table 2). This leads to the conclusion that on Day 5 the stem cell population is less sensitive to cyclophosphamide than in previously untreated marrow, while the sensitivity of the total marrow population is relatively unchanged.

These hematological data can be correlated with survival after a repeat treatment. In an experiment done in parallel with the above experiment, 40 CDF_1 mice are given i.p. injections of cyclophosphamide, 275 mg/kg. Groups of 10 each received a 2nd dose of 275 mg/kg on Days 4, 5, 6, and 7 after the 1st dose. Mortality was 60% for the Day 4 group and 0% for the other groups during 45 days of observation. Thus, although hematological recovery was not complete on

Day 5, the status of the marrow was such that a repeat dose of drug could be given without producing death.

As noted above, the stem cell population on Day 5 was less sensitive to cyclophosphamide than the stem cell population in previously untreated marrow. An experiment was designed to test whether mice with this less sensitive stem cell population could survive a larger dose of cyclophosphamide than could previously untreated controls. Groups of 10 CDF_1 mice received various doses of cyclophosphamide 5 days after an initial dose of 275 mg/kg. A 2nd dose of 365 mg/kg on Day 5 resulted on no mortality. In concurrent previously untreated controls, this dose resulted in 40% mortality.

DISCUSSION

As summarized in Charts 1 to 3, hematopoietic recovery after a large dose of cyclophosphamide was characterized by early recovery of the marrow stem cell pool, followed by recovery of the total marrow pool and the circulating blood cells. Reappearance of circulating stem cells followed recovery of the marrow stem cell pool. This suggests that colonization by circulating stem cells does not have a major role in the early phases of recovery after cyclophosphamide. In this regard, recovery after cyclophosphamide is similar to recovery after irradiation (1), in which circulating colony-forming cells increased late in the recovery sequence.

Further comparison with recovery after irradiation is of interest. McCulloch and Till have reported the serial changes in bone marrow CFU after exposure to 155 rad (20, 27). One day after treatment, CFU dropped to 15% of control values. A slow rise toward normal was noted, with subnormal values persisting until 18 days after exposure. In the present study, 1 day after treatment with 275 mg/kg of cyclophosphamide, CFU dropped to 10% of controls. The dose of 275 mg/kg of cyclophosphamide thus causes a comparable degree of acute damage to marrow CFU. This was followed by a rapid recovery, with normal levels of CFU being reached by Day 3. This difference in the rate of recovery of CFU after radiation compared with recovery after cyclophosphamide has also been noted by other investigators (30). As discussed by others (21, 25), recovery of bone marrow must be considered, not only in terms of the direct damage to marrow, but also in terms of effects on marrow-stimulating and marrow-inhibiting factors. The importance of humoral marrow-stimulating factors in recovery after irradiation is suggested by studies in which primitive cells in 1 area respond when similar cells in another area are damaged (13). Further study of marrow-stimulating factors in recovery after radiation and cyclophosphamide is needed for more complete understanding of the role of these factors in recovery.

The response to repeat treatment may be taken as evidence that the proliferative state of a cell population is a determinant of sensitivity to cyclophosphamide. In the untreated animal, the marrow stem cell population remains constant (17) and 20% of the stem cells are nonproliferating (5). Three days after a large dose of cyclophosphamide the stem cell population is proliferating actively with a population doubling time of 18 hr. Repeat treatment during this active proliferative state was characterized by a more marked effect than treatment of previously untreated controls. Five days after a large dose of cyclophosphamide, the stem cells were decreasing in number and thus may have been in a less active proliferative state than in an untreated animal. Repeat treatment on Day 5 was characterized by a lesser effect than treatment of previously untreated controls. A correlation between sensitivity and proliferative state has also been found for vinblastine (29) and 5-fluorouracil (6).

This study of response to repeat treatment has several implications which may be applicable to use of cyclophosphamide in man (4). If the interval between treatments with large doses of cyclophosphamide is too brief, evidence of greater hematopoietic toxicity would be expected; this has been reported in a clinical study of this drug (2). One need not, however, wait for complete hematopoietic recovery before giving a repeat treatment, but treatment should be delayed until the stem cell population has recovered and is no longer in an active proliferative state. In the present study, this coincided with the earliest stages of recovery of the circulating blood cells. Clinical study has also shown that repeat treatment can be given before the leukocyte count has returned to the pretreatment value (2). The importance of the interval between repeat treatment with cyclophosphamide has been reported previously (11, 18, 31). The present study presents data on the kinetics of marrow recovery which corroborate the previous empirical studies. Also, the present study indicates that an optimally timed repeat treatment may cause less hematopoietic damage than did the initial treatment. This is suggested by the response of the stem cells to repeat treatment on Day 5 and is supported by the survival study in which previously treated animals all survived a dose of cyclophosphamide which was lethal to a fraction of previously untreated controls. Optimally timed repeat treatment with cyclophosphamide may be added to the list of experimental manipulations aimed at reducing the hematopoietic toxicity of irradiation or anticancer drugs (3, 14, 25).

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