

# Splenic Granulopoiesis in Mice following Administration of Cyclophosphamide<sup>1</sup>

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## ABSTRACT

The adult mouse spleen retains granulocytic progenitor cells that are capable of proliferation and maturation. In mice given cyclophosphamide, spleen weight and cell content increased. *In vitro* granulocyte progenitor cells increased more than 300-fold in the spleen and remained elevated 14 days following cyclophosphamide. Proliferative and nonproliferative granulocytes were increased above controls until Days 21 and 28, respectively. In splenectomized mice, blood neutrophil recovery was delayed when compared to nonsplenectomized mice following cyclophosphamide. Although marrow cell number was similar in both groups, maximum marrow granulocyte progenitor cells were 2-fold greater in nonsplenectomized animals. These studies demonstrate that the murine spleen becomes a markedly granulopoietic organ and is a major contributor to recovery of granulocytes following cyclophosphamide. Splenic granulopoiesis should be taken into account in studies which measure toxicity of various agents in murine hematopoiesis.

## INTRODUCTION

The spleen is a major hematopoietic organ in the fetus (9). The adult mouse spleen retains small numbers of morphologically identifiable hematopoietic cells and is able to support increased hematopoiesis in response to greater demand such as after bleeding (1) or following endotoxin (3). Under these conditions, erythropoiesis is supported to a greater extent than is granulopoiesis (2). Although it is an organ rich in lymphocytes and phagocytic macrophages, the adult spleen contains small numbers of pluripotent hematopoietic cells (17) and also retains more differentiated CFU-C.<sup>3</sup> CFU-C in the spleen have been shown to proliferate and differentiate *in vitro* when stimulated by CSA (10).

After administration of cyclophosphamide to mice, neutropenia occurs and is followed by neutrophilia. These changes are associated with similar changes in marrow cellularity and CFU-C content. In a previous study, increases in both the weight and CFU-C concentration of the spleen were noted in mice following cyclophosphamide, and these changes persisted after neutropenia had been corrected (16). Few reports of the effects of cytotoxic

agents on hematopoiesis in the mouse have included studies of hematopoietic organs other than marrow (13, 16, 20, 25). The role of the adult mouse spleen as a granulopoietic organ, its influence on marrow recovery of granulopoiesis, and its effect on the duration and severity of neutropenia following cyclophosphamide form the basis of this report.

## MATERIALS AND METHODS

C57BL × DBA/2 F<sub>1</sub> mice bred in our laboratory from stock purchased from The Jackson Laboratory, Bar Harbor, Maine, were used in all experiments. Animals were between 8 and 15 weeks of age.

Cyclophosphamide (100 mg) with sodium chloride (45 mg) (Mead Johnson Laboratories, Evansville, Ind.) was dissolved in 5 ml preservative-free sterile water and administered (200 mg/kg) i.p. Control mice were given injections of a 0.9% NaCl solution.

Suspensions of murine spleen cells were prepared by gentle dispersion through stainless steel sieves into CMRL culture media. Marrow cell suspensions were obtained by flushing the isolated mouse humerus with CMRL. Peripheral blood was obtained by orbital sinus puncture. Cell concentrations were measured with an electronic particle counter (Model ZBI Coulter counter; Coulter Electronics, Inc., Hialeah, Fla.) and from 200 cell differential counts of Wright's-stained blood smears and 500 cell differential counts of cytocentrifuged spleen and marrow cell suspensions. The morphological features of granulocytes and their precursors in mice have been described previously (7). Briefly, proliferative cells included myeloblasts, promyelocytes, and myelocytes, in which the nucleus has a central opening the diameter of which was less than one-half the diameter of the doughnut nucleus. Granulocytes with a wider nuclear central opening or segmented nucleus were defined as nonproliferative cells.

*In vitro*, growth of CFU-C from mouse spleen and marrow was measured by using the soft gel system as described by Pluznik and Sachs (21) and Bradley and Metcalf (4). Methylcellulose (Dow Corning Corp., Midland, Mich.) was used in place of agar. Cells were suspended in 1.8% methylcellulose, containing culture medium CMRL, 15% horse serum, and 10% L-cell-conditioned medium. The combined mixture was then plated in triplicate in 1-ml volumes in culture dishes 35 × 10 mm and incubated at 37° in 7.5% CO<sub>2</sub> for 7 days. Colonies containing more than 50 cells were counted with an inverted microscope.

## RESULTS

There were marked changes in spleen weight and cell composition in mice following cyclophosphamide. Spleen

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<sup>3</sup> The abbreviations used are: CFU-C, granulocyte progenitor cells; CSA, colony-stimulating activity; CMRL, Connaught Medical Research Laboratories Medium 1066.

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weight decreased initially from a mean of  $83 \pm 12$  (S.E.) mg in controls to  $28 \pm 3$  mg on Day 4 (Chart 1A). This was followed by an increase to a peak value of  $242 \pm 56$  mg on Day 8. Thereafter, spleen weight gradually decreased to control levels by Day 21 after cyclophosphamide. Similar changes were observed in spleen cell number, as shown in Chart 1B. The total number of spleen cells decreased from  $10.0 \pm 2.4 \times 10^7$ /spleen in control mice to  $0.7 \pm 0.2 \times 10^7$ /spleen on Day 4 following cyclophosphamide. This was

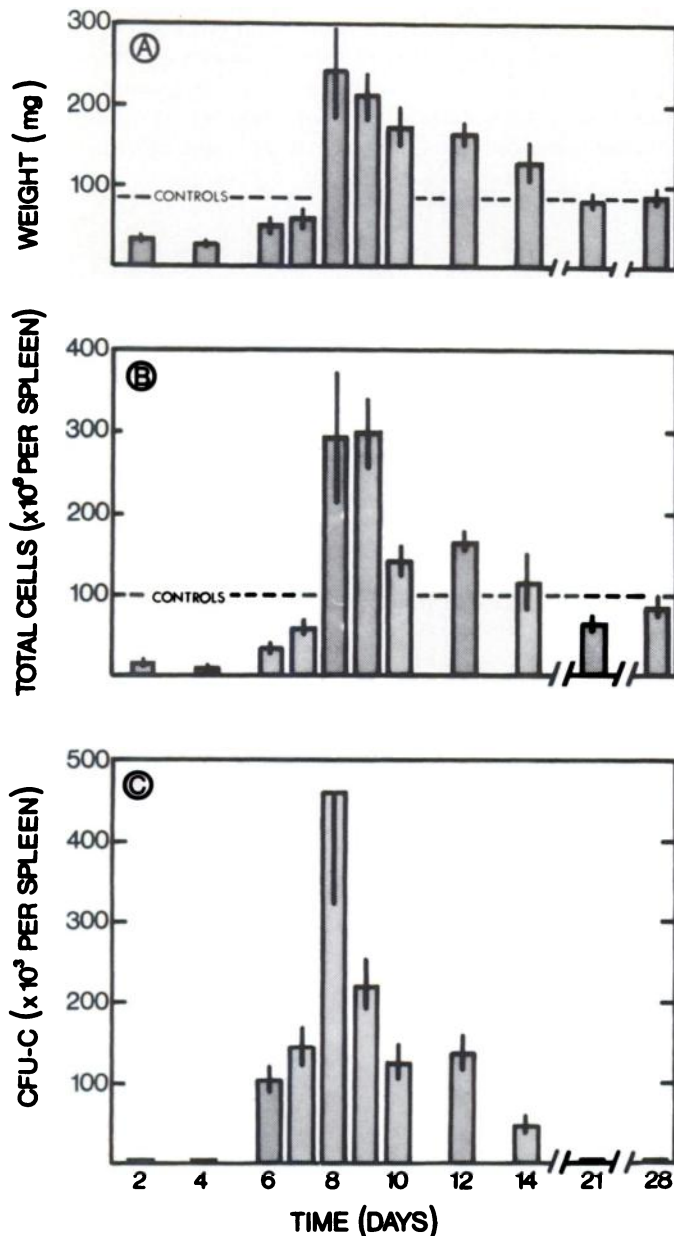


Chart 1. A, spleen weight after cyclophosphamide, 200 mg/kg, expressed as mean  $\pm$  S.E. (bars) of 3 to 8 mice. Spleen weight of untreated controls was  $83 \pm 12$  mg. B, spleen cell concentration after cyclophosphamide, 200 mg/kg. Values represent the number of cells per spleen from 3 to 8 mice, collected after passage through a stainless steel sieve and expressed as the mean  $\pm$  S.E. Untreated controls contained  $100 \pm 24 \times 10^6$  cells/spleen. C, spleen CFU-C after cyclophosphamide, 200 mg/kg. Values represent number of CFU-C per spleen, expressed as the mean  $\pm$  S.E. of spleen cell samples from groups of 3 mice in 2 separate experiments, each plated in triplicate. Untreated controls contained  $1.4 \pm 0.8 \times 10^3$  CFU-C/spleen.

followed by an increase to peak levels on Day 9 ( $29.6 \pm 4.4 \times 10^7$ ) after which the cell number remained elevated until Day 14. A marked increase in spleen CFU-C was also observed during this time period, as shown in Chart 1C. Few CFU-C's were detected in spleens of untreated controls ( $1.4 \pm 0.8 \times 10^3$ /spleen). By Day 6, CFU-C were increased 100-fold to  $105 \pm 17 \times 10^3$ /spleen, reached a maximum value of  $460 \pm 140 \times 10^3$  on Day 8, and remained significantly above control levels ( $p < 0.001$ ) 14 days after cyclophosphamide. Changes in the number of morphologically identifiable spleen cells after cyclophosphamide are shown in Table 1. Proliferative granulocytes were reduced until Day 6 after cyclophosphamide; then they increased 100-fold to a maximum of  $86.7 \pm 13.8 \times 10^6$ /spleen on Day 8 and remained above control values ( $0.9 \pm 0.1 \times 10^6$ /spleen) until Day 21. Nonproliferative granulocytes were also increased by Day 6, reached peak concentrations ( $124.7 \pm 19.8 \times 10^6$ /spleen) on Day 8, and did not return to control levels until 28 days following cyclophosphamide. Spleen lymphocytes decreased initially to  $6.5 \pm 0.7 \times 10^6$ /spleen on Day 3 and gradually increased thereafter.

To determine the effect of splenic granulopoiesis on neutrophil recovery following cyclophosphamide, blood neutrophil concentrations were measured in mice splenectomized 4 weeks earlier and in nonsplenectomized mice following cyclophosphamide. The results are shown in Table 2. In both groups, similar levels of neutropenia were reached by Day 4 after cyclophosphamide. An earlier recovery of blood neutrophil concentration was seen in nonsple-

Table 1  
Spleenic cellular composition after cyclophosphamide

Time (days)	Granulocytes ( $\times 10^6$ /Spleen)		Lymphocytes ( $\times 10^6$ /Spleen)
	Proliferative	Nonproliferative	
3	$0.07 \pm 0.001^a$	$0.43 \pm 0.03$	$6.5 \pm 0.7$
6	$4.1 \pm 0.4$	$10.3 \pm 0.9$	$14.7 \pm 1.2$
8	$87.7 \pm 13.8$	$124.7 \pm 19.8$	$66.7 \pm 10.8$
10	$30.3 \pm 2.7$	$50.0 \pm 4.6$	$54.0 \pm 4.6$
14	$7.8 \pm 0.5$	$37.9 \pm 2.1$	$83.0 \pm 6.6$
21	$1.2 \pm 0.2$	$5.0 \pm 0.2$	$57.8 \pm 2.4$
28	$0.43 \pm 0.10$	$2.5 \pm 0.3$	$81.0 \pm 5.7$
Controls	$0.9 \pm 0.1$	$3.6 \pm 0.5$	$100.3 \pm 3.8$

<sup>a</sup> Mean  $\pm$  S.E. of spleens from 3 to 8 mice given cyclophosphamide, 200 mg/kg i.p.

Table 2  
Blood neutrophils after cyclophosphamide

Time (days)	Blood neutrophils/ $\mu$ l	
	Splenectomized	Nonsplenectomized
2	$2000 \pm 100^a$	$1000 \pm 100$
4	$20 \pm 10$	$100 \pm 90$
5	$200 \pm 50$	$800 \pm 300$
6	$1000 \pm 100$	$1700 \pm 200$
7	$4900 \pm 900$	$6800 \pm 700$
8	$9200 \pm 900$	$9000 \pm 700$
10	$6700 \pm 700$	$3400 \pm 600$
12	$4500 \pm 750$	$2300 \pm 300$
Controls	$1600 \pm 400$	$1600 \pm 200$

<sup>a</sup> Mean  $\pm$  S.E. of blood neutrophils from 6 mice.

nectomized mice which differed significantly ( $p < 0.01$ ) from that of splenectomized animals. By Day 6, neutrophil concentration in nonsplenectomized animals returned to normal levels, whereas splenectomized mice remained neutropenic. Blood neutrophils peaked in both groups by Day 8 and then gradually decreased but remained above normal for 12 days.

Marrow granulopoiesis was also studied in splenectomized and nonsplenectomized mice following cyclophosphamide, and the results are shown in Table 3. No significant difference in total marrow cell counts was detected. In nonsplenectomized mice, there was a more rapid decrease in marrow proliferative granulocytes. A nadir was reached in both groups on Day 2 following cyclophosphamide. The marrow concentration of proliferative and nonproliferative granulocytes was not significantly different in splenectomized and nonsplenectomized mice during marrow granulopoietic recovery following cyclophosphamide. However, marrow CFU-C differed considerably. In splenectomized mice, marrow CFU-C decreased to  $2100 \pm 200$ /humerus on Day 1 and increased 3-fold above controls to a maximum of  $19,200 \pm 4,100$  on Day 4. In nonsplenectomized mice, a greater decrease in CFU-C was observed on Days 1 through 4. Thereafter, CFU-C were increased greater than 5-fold above controls to a maximum of  $51,000 \pm 7,500$  on Day 5. Sera from splenectomized and nonsplenectomized mice given cyclophosphamide were tested for CSA in cultures of normal mouse marrow cells. Increased CSA was noted by Day 2 and remained elevated until Day 7 with no significant difference between the 2 groups.

Since peak marrow CFU-C in nonsplenectomized mice was noted at a time when spleen CFU-C rapidly increased, the influence of cellular migration on CFU-C content was studied. Following cyclophosphamide, circulating CFU-C were measured in blood collected from the inferior vena cava of splenectomized and nonsplenectomized mice. Blood CFU-C in splenectomized mice increased from  $500 \pm 200$ /ml blood on Day 4 to  $6600$ /ml on Day 6 following cyclophosphamide. A similar increase was seen in nonsplenectomized mice, from  $600 \pm 100$ /ml on Day 4 to  $5000 \pm 600$ /ml on Day 6.

## DISCUSSION

These studies demonstrate that the spleen becomes markedly granulopoietic and contributes to the recovery of blood neutrophils in mice following cyclophosphamide. Spleen weight, cell content, and number of CFU-C increased following cyclophosphamide and remained elevated for 14 days. Proliferative and nonproliferative granulocytes were increased above controls until Days 21 and 28, respectively. In splenectomized mice, the level of neutropenia following cyclophosphamide was similar to that in nonsplenectomized mice. However, neutropenia was of shorter duration, and neutrophil concentrations were significantly greater on Days 5, 6, and 7 in nonsplenectomized mice. Marrow cell number was similar in both groups, but maximum marrow CFU-C was 2-fold greater in nonsplenectomized animals.

The murine spleen is an active hematopoietic organ in fetal and neonatal life and may become hematopoietic in adult mice following a variety of stresses which increase demand for blood cell production (3). The adult spleen contains a number of different cells capable of influencing granulopoiesis. Macrophages are a major source of CSA that is necessary for the growth of granulocytes and mononuclear cells *in vitro* (8, 11, 15). Thymus-derived splenic lymphocytes have also been demonstrated to release CSA (24). In the present study, serum CSA was increased to a similar degree in both splenectomized and nonsplenectomized mice following cyclophosphamide, suggesting that the splenic contribution to CSA release did not affect neutrophil recovery or marrow CFU-C content. Previous studies have suggested that the adult murine spleen retains stem cells, both pluripotent hematopoietic stem cells (CFU-S) and more differentiated CFU-C, in an apparent noncycling state (18). Recent studies using liquid cultures have demonstrated that the spleen CFU-C can be induced to proliferate and mature in the presence of CSA (10). In addition, CFU-C have been demonstrated to circulate in the blood of normal animals and humans (6, 22) and are increased during recovery from myelosuppressive therapy (5, 23). Since CFU-C were found to be increased in the

Table 3

### Marrow granulopoiesis after cyclophosphamide

Each value represents marrow samples from 3 to 5 SPX<sup>a</sup> and NSPX mice in 2 separate experiments. Mice were SPX 4 weeks before cyclophosphamide.

Time (days)	Total cells ( $\times 10^6$ /humerus)		Granulocytes ( $\times 10^6$ /humerus)				CFU-C ( $\times 10^3$ /humerus)	
	NSPX <sup>a</sup>	SPX	Proliferative		Nonproliferative		NSPX	SPX
			NSPX	SPX	NSPX	SPX		
1	$3.2 \pm 0.2^b$	$2.9 \pm 0.3$	$0.36 \pm 0.02$	$0.71 \pm 0.07$	$2.12 \pm 0.13$	$1.51 \pm 0.16$	$0.4 \pm 0.1$	$2.1 \pm 0.2$
2	$0.9 \pm 0.1$	$1.2 \pm 0.2$	$0.04 \pm 0.01$	$0.39 \pm 0.06$	$0.83 \pm 0.09$	$0.70 \pm 0.12$	$1.7 \pm 0.2$	$3.2 \pm 0.5$
3	$0.5 \pm 0.1$	$0.6 \pm 0.1$	$0.46 \pm 0.09$	$0.56 \pm 0.09$	$0.02 \pm 0.01$	$0.01 \pm 0.01$	$4.7 \pm 0.7$	$6.8 \pm 1.3$
4	$1.0 \pm 0.1$	$1.6 \pm 0.2$	$0.99 \pm 0.09$	$1.54 \pm 0.19$	$0.01 \pm 0.01$	$0.04 \pm 0.01$	$8.3 \pm 0.7$	$19.2 \pm 4.1$
5	$2.9 \pm 0.3$	$3.5 \pm 0.3$	$2.23 \pm 0.23$	$2.69 \pm 0.23$	$0.51 \pm 0.05$	$0.56 \pm 0.05$	$51.0 \pm 7.5$	$9.0 \pm 0.5$
8	$7.9 \pm 0.9$	$8.9 \pm 1.4$	$1.41 \pm 0.16$	$2.63 \pm 0.41$	$6.43 \pm 0.73$	$6.23 \pm 0.98$	$4.7 \pm 1.7$	$3.3 \pm 0.2$
10	$8.3 \pm 0.8$	$6.8 \pm 0.3$	$0.88 \pm 0.08$	$0.63 \pm 0.03$	$7.14 \pm 0.69$	$6.00 \pm 0.26$	$4.0 \pm 0.4$	$6.9 \pm 0.4$
Controls	$7.1 \pm 0.6$	$7.1 \pm 0.5$	$1.80 \pm 0.15$	$2.24 \pm 0.16$	$3.32 \pm 0.28$	$2.91 \pm 0.20$	$9.0 \pm 0.5$	$7.3 \pm 0.8$

<sup>a</sup> SPX, splenectomized; NSPX, nonsplenectomized.

<sup>b</sup> Mean  $\pm$  S.E.

blood of both splenectomized and nonsplenectomized mice following cyclophosphamide, it is unclear to what extent splenic granulopoiesis is due to activation of preexisting noncycling progenitor cells in the spleen or migration of cells to the spleen. Splenic CFU-C may also enter the blood, and this could account for the greater peak marrow CFU-C content observed in nonsplenectomized mice following cyclophosphamide.

Previous studies in humans (14, 19) and the mouse (12) have provided conflicting data of the effect of splenectomy on marrow tolerance to myelosuppressive therapy. The present study indicates that the mouse spleen is an important granulopoietic organ following cyclophosphamide, that it influences blood neutrophil recovery, and that granulopoiesis persists for a time after both marrow and blood have appeared to recover. These observations demonstrate that splenic granulopoiesis should be taken into account in studies which measure toxicity of various agents on murine hematopoiesis.

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## REFERENCES

1. Boggs, D. R., Geist, A., and Chervenick, P. A. Contribution of the Mouse Spleen to Post-hemorrhagic Erythropoiesis. *Life Sci.*, **8**: 587-599, 1969.
2. Boggs, D. R., Marsh, J. C., Chervenick, P. A., Bishop, C. R., Cartwright, G. E., and Wintrobe, M. M. Factors Influencing Hematopoietic Spleen Colony Formation in Irradiated Mice. II. The Effect of Foreign Materials. *J. Exptl. Med.*, **126**: 851-870, 1967.
3. Boggs, D. R., Marsh, J. C., Chervenick, P. A., Cartwright, G. E., and Wintrobe, M. M. Factors Influencing Hematopoietic Spleen Colony Formation in Irradiated Mice. VI. The Different Effects of Foreign Plasma, Endotoxin, and Bleeding on Colony Forming Cell Kinetics. *Radiation Res.*, **35**: 68-77, 1968.
4. Bradley, T. R., and Metcalf, D. The Growth of Mouse Bone Marrow Cells *in Vitro*. *Australian J. Exptl. Biol. Med. Sci.*, **44**: 287-299, 1966.
5. Chervenick, P. A. Interrelationship of Colony-stimulating Factor (CSF) and Colony-forming Cells (CFC) in Blood and Marrow during Marrow Regeneration. *J. Clin. Invest.*, **52**: 18a, 1973.
6. Chervenick, P. A., and Boggs, D. R. *In Vitro* Growth of Granulocytic and Mononuclear Cell Colonies from Blood of Normal Individuals. *Blood*, **37**: 131-135, 1971.
7. Chervenick, P. A., Boggs, D. R., Marsh, J. C., Cartwright, G. E., and Wintrobe, M. M. Quantitative Studies of Blood and Bone Marrow Neutrophils in Normal Mice. *Am. J. Physiol.*, **215**: 353-360, 1968.
8. Chervenick, P. A., and LoBuglio, A. F. Human Blood Monocytes: Stimulators of Granulocytes and Mononuclear Colony Formation *in Vitro*. *Science*, **178**: 164-166, 1972.
9. Crosby, W. H. Normal Functions of the Spleen Relative to Red Blood Cells: A Review. *Blood*, **14**: 399-408, 1959.
10. Golde, D. W., Failla, A., and Cline, M. J. Induction of Splenic Granulopoiesis *in Vitro*. *Proc. Soc. Exptl. Biol. Med.*, **152**: 544-548, 1976.
11. Golde, D. W., Finley, T. N., and Cline, M. J. Production of Colony-stimulating Factor by Human Macrophages. *Lancet*, **2**: 1397-1399, 1972.
12. Gunale, S., Hardiman, P., Stott, P., Huang, A., Zelkowitz, L., Kurkirka, M., and Tucker, W. Effects of Nitrogen Mustard and Splenectomy on Mouse Bone Marrow Colony Formation *in Vitro*. *Cancer*, **38**: 1266-1271, 1976.
13. Hodgson, G. S., Bradley, T. R., Martin, R. F., Sumner, M., and Fry, P. Recovery of Proliferating Haemopoietic Progenitor Cells after Killing by Hydroxyurea. *Cell Tissue Kinet.*, **8**: 51-60, 1975.
14. Ihde, D. C., DeVita, V. T., Canellos, G. P., and Young, R. C. Effect of Splenectomy on Tolerance to Combination Chemotherapy in Patients with Lymphoma. *Blood*, **47**: 211-222, 1976.
15. Joyce, R. A., and Chervenick, P. A. Stimulation of Granulopoiesis by Liver Macrophages. *J. Lab. Clin. Med.*, **86**: 112-117, 1975.
16. Joyce, R. A., and Chervenick, P. A. Corticosteroid Effect on Granulopoiesis in Mice after Cyclophosphamide. *J. Clin. Invest.*, **60**: 277-283, 1977.
17. McCulloch, E. A., Till, J. E., and Siminovitch, L. The Role of Independent and Dependent Stem Cells in the Control of Hemopoietic and Immunologic Responses. *Wistar Inst. Symp. Monograph*, **4**: 61-66, 1965.
18. Metcalf, D., and Stevens, S. Influence of Age and Antigenic Stimulation on Granulocyte and Macrophage Progenitor Cells in the Mouse Spleen. *Cell Tissue Kinet.*, **5**: 433-466, 1972.
19. Panetiere, F., and Coltman, C. A., Jr. Splenectomy Effects on Chemotherapy in Hodgkin's Disease. *Arch. Internal Med.*, **131**: 362-366, 1973.
20. Pannacciulli, I. M., Massa, G., Saviane, A. G., Bianchi, G., Bogliolo, G. V., and Ghio, R. The Effects of Chronic Administration of Cyclophosphamide on Haemopoietic Stem Cells. *Scand. J. Haematol.*, **19**: 217-223, 1977.
21. Pluznik, D. H., and Sachs, L. The Cloning of Normal "Mast" Cells in Tissue Culture. *J. Cellular Physiol.*, **66**: 319-324, 1965.
22. Quesenberry, P. J., Morley, A., Ryan, M., Howard, D., and Stohman, F. The Effect of Endotoxin on Murine Stem Cells. *J. Cellular Physiol.*, **82**: 239-244, 1973.
23. Richman, C. M., Weiner, R. S., and Yankee, R. A. Increase in Circulating Stem Cells following Chemotherapy in Man. *Blood*, **47**: 1031-1039, 1976.
24. Ruscetti, F. W., and Chervenick, P. A. Release of Colony-stimulating Activity from Thymus-derived Lymphocytes. *J. Clin. Invest.*, **55**: 520-527, 1975.
25. Silini, G., Andreozzi, U., and Pozzi, L. V. The Role of the Spleen in the Repopulation of the Haemopoietic System of Heavily-irradiated Mice. *Cell Tissue Kinet.*, **9**: 341-350, 1976.