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High Proliferation of Granulocyte-Macrophage Progenitors in Tumor-bearing Mice[1]

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ABSTRACT

Cancer may affect hemopoiesis by altering the proliferative status of hemopoietic progenitor cells. In Lewis lung carcinoma (LLC), the proliferative rate of the granulocyte-macrophage colony-forming unit (culture) (GM-CFU_C) was studied using *in vivo* hydroxyurea techniques. The disposal of mature elements to the periphery was also monitored during tumor growth. Neutrophilia, anemia, and splenic hypertrophy developed during the course of the disease. By Day 6 post-tumor implant, myeloid hyperplasia of the marrow was evident, but the content of GM-CFU_C in LLC mice was similar to that of control. However, by Day 11, the marrow of LLC mice displayed an increased concentration of GM-CFU_C, which tripled by Day 19. There was an increased percentage of proliferating GM-CFU_C in LLC mice by Day 6 which was highest by Day 11 and thereafter declined. The level of colony-stimulating activity was higher in the serum of tumor bearers than in that of controls. The early increase in proliferative rate of these early hemopoietic precursors can account for the later accumulation of GM-CFU_C and myeloid elements in the marrow. Increased cycling of hemopoietic stem cells raises questions concerning the potential for early exhaustion of hemopoietic progenitor cells in these animals.

INTRODUCTION

Hemopoietic alterations in the presence of cancer have been well documented (12, 14, 16, 17, 21, 22, 26). These alterations can occur in the absence of marrow invasion by the tumor. In the area of chemotherapy where marrow function is the limiting factor in treatment, such hemopoietic changes may modify the susceptibility of patients to treatment.

The present study deals with hemopoietic alterations in LLC[3]-bearing mice. This tumor was used because, while it is a metastatic tumor, it does not directly involve the marrow. Using a clonal assay for the GM-CFU_C, we have confirmed an expansion of these hemopoietic progenitor cells in the marrow of LLC mice. We have further demonstrated that this expansion is due to an increase in their proliferative pool.

MATERIALS AND METHODS

Experimental Animals and Tumor System. Male C57BL/6 mice (18 to 20 g; Charles River Breeding Laboratories, Wilmington, Mass.) were used. They were fed Purina laboratory chow and water *ad libitum*.

LLC (Mason Research Institute, Worcester, Mass.) was serially transplanted in our laboratory every 14 days by s.c. implantation of tumor fragments. Every fifth passage was examined histologically for the typical LLC characteristics (32). Viral serology from tumor-bearing and non-tumor-bearing mice were done and showed that these animals were free of 10 common murine viruses (minute virus of mice, pneumonia virus of mice, reovirus type 3, mouse hepatitis virus, K virus, murine encephalomyelitis, Sendai virus, lymphocytic choriomeningitis, ectromelia, and polyoma). In all experiments, a predominantly single-cell suspension of tumor was prepared in cold HBSS (11). This procedure involved passage of tumor through 40 mesh wire cloth and then through a 100 mesh cytosieve. Each animal received 10^6 trypan blue-excluding LLC cells s.c. in 0.5 ml HBSS, and sham-injected mice received 0.5 ml HBSS.

A tumor growth curve was constructed (30, 31) following the implantation of 10^6 viable LLC cells, an inoculum producing 100% "takes."

Femoral bone marrow was studied on Day 17 PI of LLC for the presence of tumor, histologically and biologically by s.c. implantation of the femoral marrow (11). Marrow recipients were examined for 12 weeks for the presence of a s.c. tumor.

Hematological Studies. Heparinized whole blood was collected by cardiac puncture; hemoglobin, hematocrit, reticulocyte count, total WBC, and differential WBC were done. Femoral bone marrow was harvested and total cell number was determined. A myeloid:erythroid ratio was done on smears, counting 500 cells. The splenic weight of tumor-bearing and sham-injected control mice was recorded at the time of bone marrow harvest. Spleens were processed for histology.

Cell Culture. A single agar layer system for GM-CFU_C was used (5). Femoral marrow was harvested from tumor-bearing and sham-injected mice at different times by flushing the bone cavity with 5 ml of Hanks' minimal essential medium enriched with 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, sodium bicarbonate (1.25 ml of 5% solution per 100 ml), penicillin (10,000 units/ml), streptomycin (10,000 μg/ml), and 2% heat-inactivated fetal calf serum (Sterile Systems, Inc., Logan, Utah). Marrow cells were pelleted (400 × *g*, 8 min) and resuspended in enriched Hanks' minimal essential medium. Mononuclear cells were counted in a hemocytometer, and the cells were diluted to 7.5×10^6 cells/ml. Then, 1.5×10^5 cells were immediately plated in 35-mm tissue culture dishes in 1 ml final volume of α-minimal essential medium, 15% fetal calf serum, 0.3% agar, and 15% mouse lung-conditioned media (25) as the source of CSA. Four replicate plates were prepared for each experiment. The cultures were incubated at 37° in 5% CO_2 and were read at 6 days. Colonies composed of 40 or more cells were scored on an inverted microscope. To identify the colonies histologically, cytospin preparations of individual colonies were stained with Wright-Giemsa or the naphthol AS-D chloroacetate esterase (24).

The percentage of cycling GM-CFU_C was determined using *in vivo* HU techniques (28). The percentage of CFU_C killed by HU compared to 0.9% NaCl solution-treated controls was determined 4 times PI. Five mice were included in each of the HU- and 0.9% NaCl solution-treated control groups. Tumor-bearing and sham-injected mice received either HU (900 mg/kg; Sigma Chemical Co., St. Louis, Mo.) in 0.2 ml volume or 0.2 ml 0.9% NaCl solution i.v. by the tail vein. Two hr later, femoral marrow was harvested for CFU_C assay. These experiments were performed at Days 6, 11, 14, and 19 of tumor growth and were repeated twice.

The differences between the CFU_C concentration in tumor-bearing and sham-injected animals were analyzed by a 2-sample *t* test (13). This

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[3] The abbreviations used are: LLC, Lewis lung carcinoma; GM-CFU_C, granulocyte-macrophage colony-forming unit (culture); HBSS, Hanks' balanced salt solution; PI, post implant; CSA, colony-stimulating activity; HU, hydroxyurea; CFU_C, colony-forming unit (culture); CFU_S, colony-forming unit (spleen).

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same test was used to compare the percentage of CFU_C killed by HU in tumor-bearing and sham-injected mice.

CSA Studies. Serum was collected from LLC and sham-injected mice at Days 1, 7, and 19 of tumor growth and frozen. In a CFU_C assay against fresh murine marrow target cells, the individual sera were substituted as the source of CSA at 15% final concentration. The resulting number of colonies was expressed as a percentage of control with mouse lung-conditioned media as the standard CSA. These experiments were repeated twice. A 2-sample *t* test was used to compare the colony-stimulating activity of LLC and sham sera (13).

A predominantly single-cell suspension of tumor cells at 10^6 viable cells/ml was plated in 35-mm tissue culture dishes in Roswell Park Memorial Institute Medium 1640, penicillin-streptomycin, and 10% fetal calf serum and incubated for 7 days under standard conditions. Control dishes of complete media without cells were similarly treated. The conditioned and control media were harvested and concentrated 3-fold by dialysis against Carbowax polyethylene glycol 20,000. The samples were filter sterilized and substituted as the CSA in a CFU_C assay at 15% final concentration.

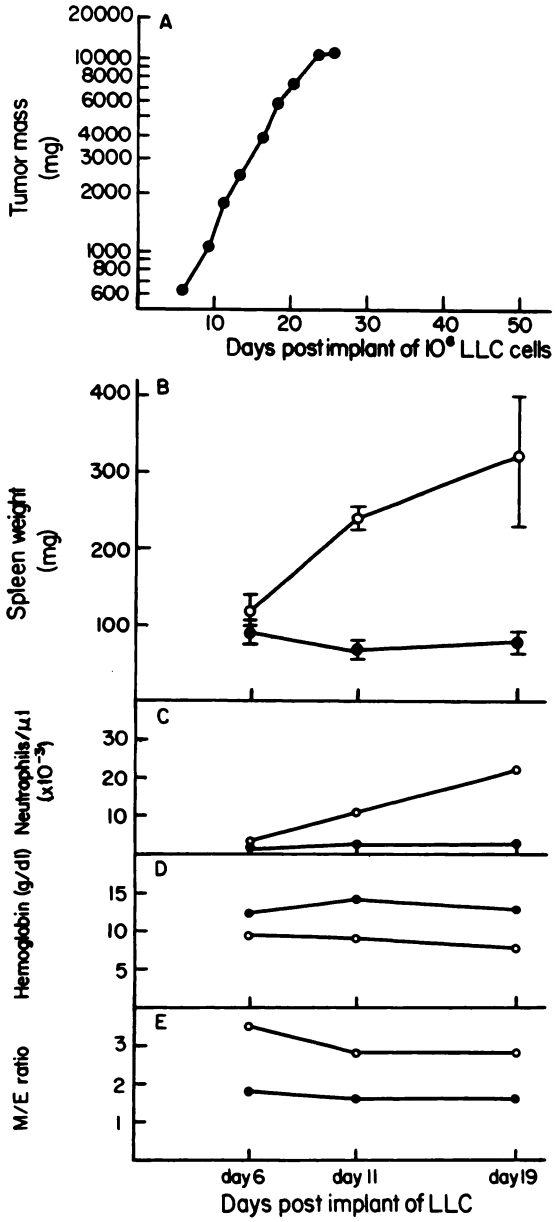

Chart 1. Relationship between tumor growth, hematological findings, and spleen weight in LLC-bearing mice and sham-injected controls. *A*, tumor growth curve (●) following s.c. implantation of 10^6 viable LLC cells. *B* to *E*, spleen weight and hematological parameters of LLC-bearing mice during the course of tumor growth compared to those of sham-injected controls. *B*, spleen weight; *bars*, S.D.; *C*, neutrophil count (neutrophils/μl × 1000); *D*, hemoglobin (g/dl); *E*, myeloid (*M*):erythroid (*E*) ratio in the marrow. *B* to *E*, O, tumor bearers; ●, sham controls.

RESULTS

Hematological Observations. LLC, palpable in the tumor-bearing mice by Day 3 PI, displayed a rapid Gompertzian growth pattern (Chart 1*A*). The median survival time was 22 days. LLC-bearing mice displayed hematological alterations during the course of tumor growth (Chart 1 *B* to *E*). A progressive neutrophilia, anemia, and splenic hypertrophy developed in these mice, the anemia being evident in the early phases of tumor growth (Chart 1, *B* to *D*). Histological examination of the spleen showed a marked increase in hemopoietic activity with myeloid hyperplasia by Day 14. By Day 6, myeloid hyperplasia of the marrow was already present, while the blood neutrophilia was not yet detectable (Chart 1, *C* and *E*). There was no difference in marrow cell counts in LLC and sham-injected mice [Day 6 PI: LLC, 1.45 ± 0.03 (S.D.) × 10^7 cells/femur *versus* sham, 1.32 ± 0.01 × 10^7 cells/femur; Day 19 PI: 1.48 ± 0.15 × 10^7 *versus* 1.40 ± 0.09 × 10^7]. Marrow metastases were not present either histologically or by bioassay for tumor growth (11).

GM-CFU_C Concentration. At Day 6 PI, when a myeloid hyperplasia was evident in the marrow (Chart 1*E*), the concentration of CFU_C was the same in LLC and control mice (Chart 2). However, by Day 11, LLC mice displayed an incremental change in the concentration of CFU_C (7,840 ± 300.8 *versus* 5,082.5 ± 353.3), which tripled by Day 19 PI as the disease advanced (21,088 ± 940.8 *versus* 8,317.5 ± 460.2). Cytospin preparations of representative colonies were identified histologically as either macrophage, granulocyte, or mixed. There was no change in distribution of these colony types in LLC or sham marrow and no evidence of abnormal-bearing colonies suggestive of tumor colonies in LLC marrow.

Stem Cell Kinetics. The percentage of CFU_C killed in LLC and sham-injected mice with respect to 0.9% NaCl solution-treated

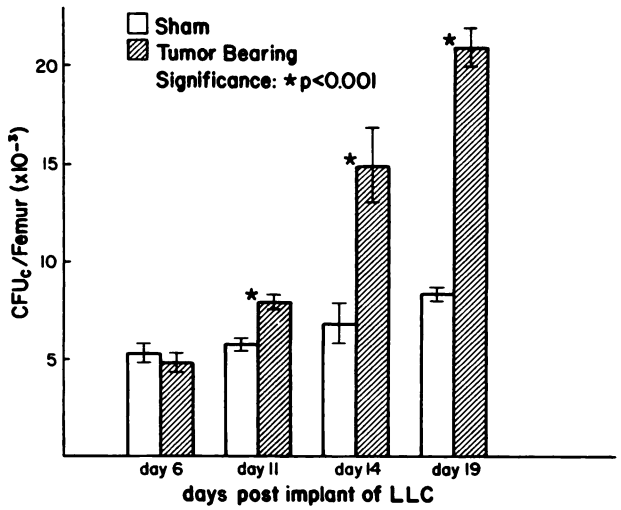

Chart 2. Marrow GM-CFU_C concentration per femur in LLC-bearing mice and sham-injected controls. Values should be ×1000. GM-CFU_C were assayed in the femoral marrow at Days 6, 11, 14, and 19 PI of 10^6 viable LLC cells. *Bars*, S.D.

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controls showed that there was an increased proliferative rate of CFU_C early in tumor growth, which declined by Day 19 PI (Table 1). At Day 6 PI, the percentage of CFU_C killed in LLC mice was 52.2 ± 2.5% *versus* 29.8 ± 9.8% in sham-injected mice. This difference became greatest at Day 11 PI (56.2 ± 4.4% *versus* 22.2 ± 14%) and persisted through Day 14. By Day 19 PI, there was no difference in the percentage of CFU_C killed in tumor-bearing and sham-injected mice.

CSA. To investigate one mechanism of increased myelopoiesis, sera collected at different times PI were assayed for their ability to stimulate CFU_C. At Days 1, 7, and 19 of tumor growth, sera from LLC mice showed a higher level of CSA than did control sera (Table 2). In all serum samples (both LLC and sham), a qualitatively similar pattern of stimulation was observed, producing chiefly mixed granulocyte-macrophage colonies. In further experiments, granulocyte-macrophage CSA could be demonstrated in media conditioned by a preparation of cells from the tumor (Table 3). This activity could be detected after the media were concentrated 3-fold.

DISCUSSION

These data indicate an expansion of early myeloid precursors in the marrow of tumor-bearing mice. Such an expansion occurred in the absence of marrow metastases. This increase in GM-CFU_C was attributable to accelerated cycling of these cells, detectable at Day 6 PI. CFU_C began to accumulate in the marrow of LLC mice by Day 11 PI and progressed to 3 times normal concentration by Day 19 (Chart 2). The fraction of CFU_C which was cycling in tumor-bearing mice was increased by Day 6, was highest by Day 11, and thereafter declined (Table 1). These data are quite compatible by assuming that there must be an increase in the proliferative rate of the CFU_C before an accumulation of marrow CFU_C and granulocytes is seen. Also, the Day 19 accumulation of the CFU_C is a consequence of the earlier increased rate of production. A reason why these precursors accumulate at all despite the simultaneous occurrence of peripheral neutrophilia may be that they cannot be disposed of to the periphery as quickly as they are produced.

A possible mechanism through which the stem cells are triggered into cycle is by the direct (or indirect) action of the tumor through a CSA-like activity (26). If not elaborated by the tumor, such activity may originate in the tumor-stimulated immune system (26). The serum levels of such an activity were elevated at Days 1, 7, and 19 of tumor growth (Table 2). CSA was qualitatively similar at all 3 times assayed, inducing chiefly mixed GM-CFU_C. Further experiments demonstrating CSA in the media from LLC cell preparations suggested that the tumor cells were a source of CSA. Obviously, our data do not rule out the possibility of additional sources of CSA originating in the immune system, nor do they address which cell type(s) in the increasing tumor load is responsible.

Increased serum CSA is associated with human and animal tumors (1, 7, 36). However, it is unlikely that CSA alone can account for all the observed hemopoietic changes in LLC. In the present work, it was not possible to correlate strictly the variations in CFU_C proliferative status with the level of serum CSA. Also, we reported previously that CFU_S are increased in LLC (2), and experiments to prove that CSA can induce the proliferation of the CFU_S have not been reported.

The decline in the growth fraction at Day 19 cannot be explained on the basis of a decline in CSA, because our data indicate a continued elevation of this activity in the serum (Table 2). One could postulate the abatement of other undefined myelopoietins by Day 19. Also, local feedback inhibition may be operative within the hemopoietic tissue such that the CFU_C accumulation becomes inhibitory to their further replication (15, 18).

A discussion of potential stimuli for stem cell proliferation must include the possibility of tumor-induced alterations of the hemopoietic microenvironment. The hemopoietic stroma has been well characterized in its effects on hemopoiesis (35). The suggestion that cancer alters the microenvironment is made by the results of DeGowin *et al.* (9) in which clonal growth of marrow fibroblasts is decreased in tumor-bearing animals.

Another possibility that may account for the decline in the growth rate of the CFU_C by Day 19, and of more concern to the chemotherapist, is that the self-renewal capacity of the stem cells has been taxed to its maximum, and the decline in proliferative rate heralds imminent marrow failure. This study does not offer any data in support of this hypothesis; however, there is a growing body of data to support the concept of the finite nature of the hemopoietic stem cells (4, 20, 27). A progressive exhaustion of the CFU_S self-renewal capacity was suggested by serial passages of marrow in lethally irradiated mice where the yield of CFU_S decreased at each successive passage (27). On

Table 1

Proliferation of marrow GM-CFU_C in LLC-bearing mice and sham-injected controls following in vivo HU treatment, compared to 0.9% NaCl solution-treated mice

Days PI of 10^6 LLC cells	% of GM-CFU_C killed		p[a]
	LLC bearing	Sham	
6	52.2 ± 2.5[b]	29.8 ± 9.8	<0.005
11	56.2 ± 4.4	22.2 ± 14	<0.002
14	36.3 ± 9.5	12.5 ± 5.6	<0.005
19	16.6 ± 9.0	10.25 ± 12.2	NS[c]

[a] Two-sample t test (all p values are 2-tailed).
[b] Mean ± S.D.
[c] NS, not significant.

Table 2

CSA levels in the serum of LLC mice and sham-injected controls at different times PI of LLC

The sera were substituted as the source of CSA against normal mouse target cells, and the results were expressed as a percentage of control (one batch of mouse lung-conditioned media).

Days PI of LLC	Tumor sera[a] (%)	Sham (control) sera (%)	p[b]
1	68.6 ± 6.8[c]	19.0 ± 3.9	<0.001
7	70.7 ± 3.8	38.1 ± 4.0	<0.001
19	73.8 ± 9.8	17.9 ± 4.2	<0.001

[a] Tumor and control sera added at 15% final concentration in cultures.
[b] Two-sample t test (all p values are 2-tailed).
[c] Mean ± S.D.

Table 3

Stimulation of marrow GM-CFU_C by different sources of CSA

LLC cells were incubated in dishes under conditions described in "Materials and Methods." Media without cells were similarly treated. Mouse lung-conditioned media were the standard CSAs.

CSA source	GM-CFU_C/1.5 × 10^5 mononuclear cells
Conditioned media from LLC cells (15%)[a]	76 ± 9[b]
Control media (15%)	3 ± 1
Mouse lung-conditioned media (15%)	150 ± 13
None	1

[a] Final concentration in cultures.
[b] Mean ± S.D.

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the other side, when the seeding factor of marrow-derived CFU_S was measured (the ratio between CFU_S obtained and CFU_S injected), an unlimited self-renewal potential of these elements was suggested (34). Therefore, in normal animals, the question of stem cell self-renewal remains unclear but, following certain hemopoietic perturbations including stem cell proliferation in chronic phenylhydrazine-induced anemia, even seeding experiments support this concept (19, 29). As increased stem cell proliferation is associated with the tumor state, one can postulate exhaustion of these progenitors in cancer.

Whenever the proliferative activity of hemopoietic progenitors is increased over their normally low level of activity, a higher fraction of these progenitors will be killed by cycle-active agents, and delayed hematological recovery can be expected (33). DeWys and Mansky (12) found that cyclophosphamide treatment produced a deeper and more prolonged depression of CFU_S in LLC-bearing mice, and the present work explains how increased cycling of these precursors can produce such injury.

Our laboratory (2) and Ledney *et al.* (16) have reported increased marrow and splenic CFU_S and CFU_C in LLC-bearing mice. Other authors similarly have reported increased concentrations of CFU_S and CFU_C in animal tumors (14, 17, 21, 26). Mizoguchi *et al.* (23) have described an increased growth fraction of CFU_S and CFU_C to account for the increased number of these elements in nude mice bearing a transplantable human lung tumor.

Although this selected literature suggests the association of the tumor-bearing condition with increased hemopoietic progenitor proliferation, there is also literature contradicting such generalization. Reincke *et al.* (26) have addressed a question similar to ours in studying the rate of cycling of CFU_S and CFU_C in a murine granulocytosis-inducing mammary tumor. These precursors remained low in the marrow as tumor growth progressed while the splenic compartment accumulated CFU_S and CFU_C. They were, however, unable to detect an increased growth fraction of the CFU_S. In other experimental tumor systems, depressed erythropoietic activity, decreased marrow CFU_C, and decreased stromal cell growth were suggested (3, 6, 8–10, 37).

We have shown that the GM-CFU_C proliferative rate is increased in mice bearing a metastatic tumor. Although this finding cannot be considered a generalized effect of all cancers, new questions have been raised concerning the stimulus for increased myeloid production in tumor-bearing animals, the role of splenic hemopoiesis, and the potential for early exhaustion of hemopoietic stem cells in these animals.

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