Proliferative State of Normal In Vitro Colony-forming Cells During Development of L5222 Rat Leukemia and Their Reaction to Chemotherapy

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In the experimental rat leukemia, L5222, the decrease of normal in vitro colony-forming cells (CFU-C) after chemotherapy with daunomycin is much less than in non-leukemic controls. The leukemia is therefore used here to test the hypothesis that in leukemia the CFU-C are expelled from the active cell cycle to a resting state and are thereby less sensitive to cycle-dependent chemotherapeutic agents. The L5222 leukemia has the advantage that the leukemic blast cells do not form colonies in agar culture so that normal CFU-C can be assessed under leukemic conditions. To compare the proportions of CFU-C in the S-phase in normal and leukemic rats, two S-phase-specific agents, $^3$H-thymidine and hydroxyurea, were used to kill proliferating bone marrow cells. Following treatment with $^3$H-thymidine in vitro, about 41% of the CFU-C were killed in normal and about 25% in leukemic bone marrow. Hydroxyurea administered in vivo resulted in the death of about 33% and 26%, respectively. The results indicate that fewer normal CFU-C are in S-phase in the L5222 leukemia, which might help to explain how enough normal stem cells survive chemotherapy to regenerate the bone marrow.

CHEMOTHERAPEUTIC DRUGS used in the treatment of human acute leukemia are known to act mainly on proliferating cells, even specifically on cells in certain phases of the cell cycle. Since it is generally agreed from $^3$H-thymidine ($^3$H-TdR) labeling studies that the proliferating fraction of the leukemic cell population is far lower than that of normal bone marrow, one might expect that such chemotherapeutic agents would destroy more normal than leukemic cells and thus be an unsatisfactory form of treatment. The apparent paradox of the successful induction of remission, therefore, might be due to the faster regeneration of the more rapidly proliferating, normal hemopoiesis in comparison with leukemic cells. Another explanation might be that during the development of leukemia normal hemopoietic cells, especially the hemopoietic stem cells, are not only reduced in number but also move out of the proliferative cell cycle into a resting state so that they are no longer vulnerable to cycle-specific agents. Studies that are possibly pertinent to this point have been made by Moore et al. on the proliferative state of agar colony or cluster-forming cells (CFU-C) in bone marrow or blood of patients with acute or chronic leukemia using high doses of $^3$H-TdR to kill the cells in vitro. They have found a reduced proliferative fraction of CFU-C in leukemic tissues in comparison with normal, but since they assume that the colonies or clusters are derived...
from leukemic cells, no conclusion can be drawn about the proliferative state of the remaining normal stem cells.

In the present study, the experimental rat leukemia L5222 has been used to test the hypothesis that in this leukemia fewer normal stem cells are proliferating. Previous studies with this leukemia have suggested that in the presence of leukemic cells the dormant small lymphocyte population, labeled by continuous infusion of $^{3}H$-TdR and presumably including the pluripotent hemopoietic stem cells, is inhibited from proliferating and from subsequently replenishing a markedly reduced normal hemopoiesis.$^{2,3}$ In addition, in pilot experiments on the effect of chemotherapy of this leukemia with daunomycin, it has been found that normal CFU-C are less affected by the drug in leukemic rats than in normal rats. The present experiments were undertaken to investigate the proliferative state of normal CFU-C during development of the leukemia, using the phase-specific killing agents $^{3}H$-TdR and hydroxyurea (HU). Furthermore, the effect of daunomycin on normal CFU-C has been studied in more detail.

**MATERIALS AND METHODS**

**Animals and Leukemia**

Female rats of the BDIX strain,$^{4,5}$ 5–7 mo old, weighing 240–260 g, and the leukemia L5222$^{5,6}$ were used. Leukemic cells, separated from the peripheral blood of leukemic donors and stored under liquid nitrogen, were transferred to experimental rats intravenously. Following the administration of $1 \times 10^{7}$ cells, survival time was about 7 days.

**Chemotherapy of Leukemic Rats**

Daunomycin (Ondena, Bayer, Leverkusen) was dissolved in saline at a concentration of 3 mg/ml and a single dose of 7.5 mg/kg body weight was injected intravenously 5 days after transfer of $10^7$ leukemic cells when the blood leukocyte count was 100,000–200,000 cells/cu mm. Following this dose of daunomycin, survival of leukemic rats was increased from 7 to about 17 days. Pairs of treated normal and leukemic rats were sacrificed at daily intervals after chemotherapy.

**Agar Colony Technique**

**Bone marrow cell suspensions.** These were prepared by flushing out the femur, attached by silicon rubber tubing to a 5-ml syringe, with buffered Hanks’ balanced salt solution. A single cell suspension was achieved by repeatedly drawing the suspension in and out of the syringe through the femur. Large particles were allowed to settle into a serum underlayer, the supernatant cell suspension was centrifuged into serum, and the cells were resuspended in buffered Hanks’ solution with 10% fetal calf serum. The cell concentration was determined in a hemocytometer.

**Culture medium.** This consisted of McCoy’s 5A medium containing antibiotics, supplemented with pyruvate, vitamins, and amino acids.$^{7}$ For colony growth from rat bone marrow it was found essential to have rat serum in the complete medium. Tests of sera from various rat strains showed that Sprague-Dawley serum gave the best colony yield for BDIX rat bone marrow. A combination of 15%, Sprague-Dawley rat serum and 5%; horse serum gave only slightly lower colony yields than 20%; rat serum. In the interests of economy, this mixture was adopted routinely. All batches of media and sera were tested before use. Bacto-agar was added to a final concentration of 0.3%.

**Colony-stimulating factor (CSF).** CSF was prepared by incubating whole rat lungs in 10 ml supplemented McCoy’s medium, without serum, for 2 days in a humidified 3%, CO$_2$ atmosphere. The supernatant medium was pooled, centrifuged, and dialyzed against three 100-volume changes of distilled water for 3 days. The supernatant was inactivated for 30 min at 56°C and, after further centrifugation, sterilized by Millipore filtration and stored at −20°C.$^{8}$ For use, the CSF was diluted with three parts water, and 0.05 ml was pipetted to 35-mm Petri dishes.

**Cultures.** The cultures were set up in quintuplicate with 1 ml agar medium containing a number of bone marrow cells (10,000–100,000) estimated to give a colony count of between 20 and 50.
per plate. Two cell doses were plated for each cell suspension. This procedure was deemed necessary because the colonies were about 75% macrophagic and loosely structured so that counting of more than 50 colonies per plate became difficult and unreliable.

The plates were incubated for 6 days in a humidified 3% CO₂ atmosphere in closed glass containers. Aggregates of more than 50 cells were counted as colonies; clusters of less than 50 cells were not evaluated. Colony counts were converted to absolute values of colonies per femur taking an average of 45 x 10⁶ nucleated cells per normal femur. This figure was corrected for changes in the cellularity of bone marrow following chemotherapy as determined from counts of nucleated cells per unit area in histologic sections of tibiae.

**Effect of Leukemic Cells on Colony Growth**

To test whether the leukemic cells had an inhibitory or stimulatory influence on normal colony growth in agar culture, leukocytes from the peripheral blood of leukemic rats taken 6 days after transfer of leukemia, when 70%–80% of the nucleated cells were leukemic blast cells, were added to normal bone marrow cells before plating.

**Treatment of Bone Marrow Cell Suspensions With ³H-TdR In Vitro**

To determine the fraction of CFU-C in the S-phase of the cell cycle, the ³H-TdR suicide method of Iscove et al. ¹⁰ was used. Cell suspensions of 5 x 10⁶ cells/ml were prepared in buffered Hanks’ solution without serum and three aliquots of 1 ml were pipetted to fresh tubes. To one was added 1 ml ³H-TdR (Amersham-Buchler, Frankfurt) in Hanks’ solution, 200 μCi/ml (specific activity 18 Ci/mM, final concentration 100 μCi/ml). To another was added thymidine in Hanks’ solution, 200 μg/ml (final concentration 100 μg/ml) to test whether thymidine itself had a toxic effect. The third was left at 4°C (untreated). With ³H-TdR of specific activity 18 Ci/mM no "overkill" due to uptake by cells not in the S-phase was to be expected.¹¹ The tubes with added thymidine were held at 37°C on a water bath for 20 min, washed three times with ice-cold Hanks’ solution containing 20% fetal calf serum and 100 μg/ml thymidine, and finally resuspended in 1 ml complete culture medium for agar colony culture. The culture medium was supplemented with 10 μg/ml thymidine to mitigate possible effects of reutilization in the growing culture of ³H-la beled products from dying cells.¹²

**Treatment of Rats With HU in vivo**

HU (Nutritional Biochemicals, Cleveland, Ohio) was dissolved in saline immediately before use at a concentration of 125 mg/ml. A dose of 1000 mg/kg body weight in 2 ml was injected intraperitoneally into normal rats and leukemic rats at 5 or 6 days after transfer of leukemia. Rats were sacrificed 2 hr after treatment and bone marrow cell suspensions were prepared as usual for agar colony culture.

**RESULTS**

**Effect of Leukemic Cells on Growth of Normal CFU-C**

When peripheral blood cells from leukemic rats were plated alone (50,000 cells per plate), some degree of growth of very small aggregates was observed in agar culture. The growth of aggregates of 50 cells or more, which could be classified as colonies, however, was negligible, with only one colony in each of two of five plates. This growth might have been due to residual normal CFU-C in the peripheral blood. To confirm that colonies arose from normal CFU-C and not from leukemic cells, the agar medium containing colonies grown from leukemic bone marrow was collected and injected into recipient BDIX rats. None developed leukemia, although transfer of single leukemic cells is sufficient to induce leukemia and eventual death after 16–17 days.¹³ Injection of fresh leukemic cells set in agar medium resulted in death from leukemia.
When increasing numbers of leukemic cells were added to a constant number of normal bone marrow cells before plating, no statistical difference in the colony yield was observed, even when leukemic cells exceeded normal cells five-fold. It was therefore assumed that the leukemic cells have no stimulatory or inhibitory effect on the growth of normal CFU-C in vitro.

**CFU-C in Normal and Leukemic Rats Before and After Chemotherapy**

Reduced numbers of CFU-C were found on the fifth day of the leukemia before chemotherapy, in comparison to normal controls assayed on the same day (Fig. 1), confirming previous evidence of their disappearance during the development of the leukemia.²

The depression and regeneration of bone marrow CFU-C in normal and leukemic rats after treatment with 7.5 mg/kg daunomycin is also shown in Fig. 1. In two experiments in which two normal and two leukemic animals were studied daily following chemotherapy, the immediate decrease on the first day after treatment was less in leukemic than in normal rats (by analysis of variance, \( p < 0.001 \)). The regeneration at 3 days after treatment was similar for the two groups.

**Killing Effect of \(^3\)H-TdR In Vitro**

To test whether the difference in response of CFU-C in normal and leukemic rats might be due to a difference in their proliferative state, the killing effect of a high dose of \(^3\)H-TdR was measured. Bone marrow cell suspensions from normal and leukemic rats, 5 or 6 days after transfer of leukemia, were incubated with 100 μCi/ml high-specific activity \(^3\)H-TdR before being placed in agar culture. As controls, cells from the same suspensions were incubated with unlabeled thymidine or were left untreated. The colony counts for cells treated with unlabeled thymidine were essentially similar to those for untreated cells, showing that the incubation procedure had no effect on CFU-C viability. The average of these two values for each suspension was therefore taken as the 100% value to calculate the percentage of CFU-C killed by treatment with \(^3\)H-TdR.

In Table 1, the results of three experiments with \(^3\)H-TdR are summarized. In
Table 1. Proportion of Normal CFU-C Killed by S-Phase–specific Agents

<table>
<thead>
<tr>
<th>Day, Exp. No.</th>
<th>Percentage of Normal CFU-C Killed by $^{3}$H-TdR in Vitro* (100$\mu$Ci/ml)</th>
<th>Percentage of Normal CFU-C Killed by HU In Vivo† (100 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Leukemic</td>
</tr>
<tr>
<td>Day 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>44.4</td>
<td>20.7</td>
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<tr>
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<td>3</td>
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</tr>
<tr>
<td>Day 6</td>
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<td></td>
</tr>
<tr>
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<td>25.0</td>
</tr>
<tr>
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<tr>
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</table>

*Mean values for bone marrow suspensions from two normal and two leukemic rats in each experiment.
†Average colony count for two treated rats expressed as a percentage of the average colony count for two untreated rats in each experiment.

normal bone marrow an average of about 41% CFU-C were killed, whereas in leukemic bone marrow at days 5 and 6 of the leukemic an average of about 25% CFU-C were killed.

In spite of some variation in the individual values for leukemic cells, the average values were significantly different from the normal average (by Student’s t test, $p < 0.01$).

**Killing Effect of HU In Vivo**

Since the results with $^{3}$H-TdR were variable and might possibly have been affected by reutilization of $^{3}$H-labeled breakdown products in the subsequent culture, it seemed desirable to have confirmation from an independent method. Accordingly, another S-phase–specific agent, HU, was used in vivo. Bone marrow was taken from femurs of HU-treated rats 2 hr after injection, or from control untreated rats, and put into agar culture as usual. The results of three experiments for leukemic (5 and 6 days after transfer of leukemia) and non-leukemic rats are summarized in Table 1. Again, a significantly smaller proportion of CFU-C were killed in leukemic bone marrow than in normal bone marrow. An analysis of variance for individual animals gave $p < 0.05$.

**DISCUSSION**

The investigations described here indicate that during the development of the L5222 leukemia, there is an absolute decrease in the number of normal CFU-C in the bone marrow. Using high doses of $^{3}$H-TdR in vitro or HU in vivo to kill cells in S-phase, it has been found that a smaller proportion of normal CFU-C are in DNA synthesis in leukemic than in normal rats. When daunomycin was given on the fifth day of the leukemia, the immediate decrease of normal CFU-C was less in leukemic than in similarly treated normal rats.

Regarding the experiments with $^{3}$H-TdR, the degree of variation in the percentage of CFU-C killed in leukemic bone marrow was somewhat disturbing. It might have been due to an inherent inaccuracy in the method since Metcalf.
found values varying from 33% to 77% for normal C57B1 mouse bone marrow. The average value of about 41% CFU-C killed in normal rat bone marrow found here is in general agreement with published average data for various mouse strains (43%, 35%, 35%, 48%, 46%). In the experiments reported here the variation for leukemic bone marrow was greater than for normal, thus the main reason for the variation might be genuine differences in the proportion of normal CFU-C in the S-phase in individual leukemic rats.

The finding of a smaller percentage kill with 3H-TdR of normal CFU-C in leukemic compared with normal bone marrow is confirmed by the experiments with high doses of HU administered in vivo. The lower killing rate with S-phase-specific agents of normal CFU-C in L5222 leukemic bone marrow might have been due to a lower growth fraction with more out-of-cycle cells, or to a lengthening of the total cell cycle in relation to the S-phase. From the present results, no decision can be made between these two alternatives. However, for a given number of cells, both of the above possibilities would result in a reduced flow into the recognizable bone marrow cell population and could thereby contribute to the failure of normal hemopoiesis in the L5222 leukemia. The mechanism by which CFU-C are expelled from the proliferation cycle in vivo is not clear, and in experiments mixing leukemic cells with normal bone marrow no such effect could be reproduced in vitro.

The observation that daunomycin has a less immediate effect on normal CFU-C in leukemic than in normal rats might be explained on the basis of the smaller proliferating fraction in leukemic rats. Although from the exponential nature of the dose–response curve for the action of daunomycin on normal spleen colony-forming cells (CFU-S), lymphoma cells, and erythropoietic cells it must be assumed that cells in all stages of the division cycle are affected, the cells most sensitive to the drug are those in late S-, G2-, and M-phase. The greater effect of daunomycin on AKR lymphoma cells or L1210 cells than on normal CFU-S shows that a rapidly proliferating cell population is more sensitive to the drug than a population known to have a low growth fraction. It therefore seems reasonable to consider as a reason for the reduced response to daunomycin of normal CFU-C in leukemic rats that a smaller proportion are actively participating in the cell cycle, i.e., that they are pushed into an out-of-cycle, resting state.

The L5222 is a rapidly proliferating experimental leukemia with a high growth fraction, and the relevance of the above findings to human acute leukemia is therefore limited. However, if such a reduction in the proportion of stem cells in the S-phase also occurs here, it might contribute to the insufficiency of normal hemopoiesis and also help to explain how enough stem cells survive to regenerate the bone marrow after chemotherapy.

ACKNOWLEDGMENT

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Proliferative state of normal in vitro colony-forming cells during development of L5222 rat leukemia and their reaction to chemotherapy

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