The Number of in Vitro Stem Cells in AKR Leukemic Mice

By Christiane Chevalier, Nadine Gaillard, and Emilia Frindel

The total number of in vitro stem cells (CFUc) is lower in AKR leukemic mice than in nonleukemic AKR mice of the same age. This decrease is caused by a drop in the bone marrow CFUcs which is not compensated for by an increase in the number of these cells in the spleen. This decrease in CFUcs is not due to the presence of leukemic cells in the cultures, nor to the age of the mice, nor to a modified response to the stimulating factor (CSF) of CFUcs in leukemic mice.

In spontaneous AKR leukemia the kinetics of the lymphoma cell line have been extensively studied, but little work has been done on the normal components of hematopoiesis in these mice. Bruce compared the sensitivity to chemotherapy of lymphoma cells on one hand and of normal stem cells on the other. However, this comparison supposes that the kinetics of stem cells are the same in leukemic and normal mice. There is no data to show that the response to chemotherapy of stem cells in leukemic and normal mice is the same.

A rise in the number of stem cells has been reported by many authors in different viral murine leukemias. However, Lajtha reports a decrease in the stem cell number in a transplanted myeloid leukemia. Ideally, the stem cell compartment should be studied. However, in AKR leukemic mice, it is not possible to study the multipotent stem cell using the technique of Till and McCulloch because of the complication of invasion of the spleens of the irradiated recipients by the lymphoma cells. We have, therefore, studied a more committed compartment: the in vitro stem cell compartment, or CFUc, which is a cell committed to the granulocytic and monocytic series. Thus, with this technique, we are not studying the differentiation along the leukemic cell line, as would be the case for a myeloid leukemia. However, the influence of the presence of the leukemia on another cell line other than the leukemic one and in particular on the competition for differentiation at the stem cell level is also of major interest.

MATERIALS AND METHODS

Mice

The controls used were always AKR female mice, 2 3 mo of age. The leukemic mice (aged 8 10 mo) had advanced spontaneous leukemia with massive enlargement of the spleen and subcutaneous lymph nodes.

Cell Suspensions

The mice were killed by cervical dislocation. Marrow was flushed out from the bones of one leg with a syringe filled with MEM α medium (Eurobio-Paris), and spleen cells were dispersed...
with a homogenizer. Dispersed cell suspensions of bone marrow and spleen were counted in a hemocytometer and then diluted in MEM α medium.

Colony-stimulating Factor (CSF)—Fetal Calf Serum

The colony-stimulating factor used was pooled sera from C57Bl/6 mice bled 6 hr after intraperitoneal injection of 5 μg of endotoxin (lipopolysaccharide W. E. coli; Difco). Each batch of pooled sera was tested for its stimulating activity on bone marrow cells, diluted, and used at a concentration in the plateau region (giving maximum number of colonies for 10^4 cells plated).

All batches of fetal calf serum were tested before use.

Assay for in Vitro Colony-forming Cells (CFUs)

The culture technique was described by Worton and al. One milliliter of cell suspension containing 5 x 10^5 marrow cells or 5 x 10^6 spleen cells were mixed with 1 ml of diluted CSF, 1 ml of calf fetal serum, and 2 ml of methyl cellulose (2%, in MEM α medium). One milliliter of this final suspension was plated in 35-mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.). Four replicate plates of each cell suspension were incubated for 7 days in a humidified atmosphere of 5% CO₂ in air. The colonies containing more than 50 cells were scored with an inverted microscope (×25 magnification).

Because of the variability of the batches of CSF and fetal calf serum, control cultures of bone marrow and of spleen cells from 2-3-mo-old AKR mice were included for each assay (at least three mice). Colony counts on these control cultures were related to a standard value of 1 to determine the standardization ratio for individual assay runs.

RESULTS

Influence of the Presence of Leukemic Cells on the Growth of Nonleukemic Bone Marrow Cells in Vitro

We have never obtained colonies after plating 5.10^4, 10^5, 5.10^6 thymus or lymph node cells from leukemic mice. In one experiment, we obtained 15 colonies after plating 5 x 10^6 thymus cells.

We observed that the presence of thymus or lymph node leukemic cells did not modify the number of colonies found after plating control AKR marrow. Table 1 shows the results of three different experiments in which we plated either bone marrow cells alone or a mixture of bone marrow cells and leukemic lymph node cells (or leukemic thymus cells). One can see that for the same number of marrow cells plated the number of colonies obtained is the same in the presence or in the absence of leukemic cells.

Linearity of the Assay for Leukemic Bone Marrow Cells

The experiment shown in Fig. 1 was done with a pool of marrow cells from three young (2–3-mo-old) AKR mice and three leukemic mice. We wanted to be sure that the relationship between the number of colonies obtained and the number of cells plated was linear for the leukemic marrow cells.

<table>
<thead>
<tr>
<th>Table 1. Growth of 2.5 x 10^4 Cells From Control 2-mo-old AKR Mice Marrow (BC) Alone or in Presence of Leukemic Thymus Cells (TC) or Leukemic Lymph Nodes (LNC): Three Different Experiments</th>
</tr>
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<tr>
<td>#</td>
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<tr>
<td>---</td>
</tr>
<tr>
<td>Number of colonies per plate</td>
</tr>
<tr>
<td>2</td>
</tr>
</tbody>
</table>
It can also be seen in this figure that the marrow pooled from three AKR leukemic mice contained much less CFUc per $10^6$ cells plated than the control marrow. From then on, unless stated, we studied each AKR leukemic mouse individually.

*Number of in Vitro Stem Cells in the Marrow and Spleen of Leukemic AKR Mice*

The individual values found for nine different leukemic mice are shown in Table 2. The number of colonies has been measured for $10^6$ marrow cells and $10^6$ spleen cells plated and calculated for the total marrow and total spleen knowing the number of cells per leg and per spleen. We have considered that one leg represents 10% of the total marrow.

Figure 2 is another way of presenting the same results. The points represent the ratios of the mean value for a leukemic mouse on the mean value for the corresponding controls. We can see in this figure that, except for one mouse (No. 9), the total number of in vitro stem cells is lower in leukemic mice than in 2-mo-old AKR mice. This is essentially due to a drop in the number and density of bone marrow stem cells. This drop is not compensated for by the rise in the spleen in vitro stem cells.

*Number of in Vitro Stem Cells in Old Nonleukemic Mice*

The controls were always 2–3-mo-old AKR mice. The drop in the number of CFUc in old leukemic AKR mice could be due to their age and not to the fact that they were leukemic. We studied the number of marrow and spleen in vitro stem cells in seven old nonleukemic AKR mice (9 mo or more) not presenting our clinical criteria of leukemia.
Table 2. In Vitro Stem Cell in the Marrow and Spleen of Leukemic AKR Mice

<table>
<thead>
<tr>
<th>Leukemic Mouse Number</th>
<th>Marrow</th>
<th></th>
<th></th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Marrow Colonies per $10^5$ Cells</td>
<td>Total Marrow Colonies</td>
<td>Spleen Colonies per $10^5$ Cells</td>
<td>Total Spleen Colonies</td>
</tr>
<tr>
<td>1 C</td>
<td>49 ± 8</td>
<td>83,500 ± 14,600</td>
<td>5.5 ± 0.3</td>
<td>685 ± 37</td>
</tr>
<tr>
<td>L</td>
<td>2.7 ± 3.3</td>
<td>5,600 ± 480</td>
<td>3.5 ± 0.8</td>
<td>1,920 ± 470</td>
</tr>
<tr>
<td>2 C</td>
<td>182 ± 22</td>
<td>328,000 ± 40,000</td>
<td>121 ± 24</td>
<td>21,500 ± 4,000</td>
</tr>
<tr>
<td>L</td>
<td>105 ± 16</td>
<td>22,900 ± 4,000</td>
<td>110 ± 35</td>
<td>38,200 ± 12,000</td>
</tr>
<tr>
<td>3 C</td>
<td>176 ± 32</td>
<td>208,000 ± 40,000</td>
<td>106 ± 9</td>
<td>10,400 ± 2,000</td>
</tr>
<tr>
<td>L</td>
<td>104 ± 32</td>
<td>154,000 ± 47,000</td>
<td>99 ± 15</td>
<td>14,900 ± 2,300</td>
</tr>
<tr>
<td>4 C</td>
<td>176 ± 17</td>
<td>318,000 ± 30,800</td>
<td>53 ± 17</td>
<td>4,600 ± 1,560</td>
</tr>
<tr>
<td>L</td>
<td>133 ± 17</td>
<td>141,000 ± 13,500</td>
<td>222 ± 74</td>
<td>75,500 ± 23,000</td>
</tr>
<tr>
<td>5*</td>
<td>142 ± 28</td>
<td>260,100 ± 40,000</td>
<td>42 ± 15</td>
<td>4,300 ± 1,600</td>
</tr>
<tr>
<td>C</td>
<td>54 ± 4</td>
<td>134,000 ± 10,000</td>
<td>30 ± 24</td>
<td>22,400 ± 18,000</td>
</tr>
<tr>
<td>6*</td>
<td>142 ± 28</td>
<td>260,000 ± 40,000</td>
<td>42 ± 15</td>
<td>4,300 ± 1,600</td>
</tr>
<tr>
<td>L</td>
<td>2.6 ± 3.9</td>
<td>4,600 ± 3,400</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td>7 C</td>
<td>100 ± 5</td>
<td>203,000 ± 10,000</td>
<td>27 ± 3</td>
<td>3,500 ± 390</td>
</tr>
<tr>
<td>L</td>
<td>46.5 ± 2</td>
<td>67,000 ± 2,800</td>
<td>21 ± 8</td>
<td>9,800 ± 1,700</td>
</tr>
<tr>
<td>8 C</td>
<td>44 ± 1</td>
<td>116,000 ± 2,900</td>
<td>29 ± 3</td>
<td>4,100 ± 430</td>
</tr>
<tr>
<td>L</td>
<td>0.66 ± 0.01</td>
<td>3,140 ± 42</td>
<td>70 ± 10</td>
<td>38,800 ± 5,500</td>
</tr>
<tr>
<td>9 C</td>
<td>214 ± 32</td>
<td>308,000 ± 46,000</td>
<td>43 ± 4</td>
<td>4,050 ± 380</td>
</tr>
<tr>
<td>L</td>
<td>220 ± 26</td>
<td>443,000 ± 52,000</td>
<td>23 ± 12</td>
<td>6,900 ± 3,600</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SE of four replicate culture dishes. C, controls (three mice), L, leukemic mouse.

*In these two mice, cells have been plated the same day and with the same controls.

Fig. 2. In vitro stem cells in the marrow and spleen of leukemic AKR mice; each point represents the ratio of mean number of colonies for the leukemic mouse to the mean number of colonies for the corresponding controls (mean value for four replicate dishes). The difference between these two means has been tested by the Student’s t test. •, not statistically different; *, statistically different.
STEM CELLS IN AKR LEUKEMIC MICE

Figure 3 shows the comparison between old leukemic and old nonleukemic AKR mice. We have not reproduced the results for the spleen because, although there is a big variation from one mouse to another, in the mean the number of CFUc in the spleen of old nonleukemic mice is not different from that of young AKR mice.

In conclusion, if we had taken, for the controls, old nonleukemic mice instead of young AKR, the drop in the number of CFUc in leukemic mice would have been amplified.

Mean Number of Colonies per Petri Dishes as a Function of the Concentration of CSF

Another reason which might explain this drop is that a higher concentration of CSF is necessary for CFUc in leukemic mice.

Figure 4 shows the number of colonies in leukemic and control marrow per 10^6 cells plated with different dilutions of pure CSF. The plateau, in both cases, starts for a CSF dilution of 1/4.

Figure 5 is another experiment where CSF was prepared by injecting endotoxin into 20 young AKR (noted C-CSF) and 20 leukemic AKR (L-CSF). Cells plated were control marrow or leukemic marrow cells. We can see that the mean CSF concentration is the same in leukemic and normal sera. The number of colonies depends on the type of marrow cells plated but not on the source of CSF.

DISCUSSION

We have found a decrease of about 50%, in the total number of in vitro stem cells (CFUc) in leukemic AKR mice as compared to 2-3 mo-old AKR mice. This drop is caused by a constant decrease in the total number of these cells in

Fig. 3. In vitro stem cells in the marrow and spleen of old nonleukemic AKR mice: ratio of mean number of colonies for the old mouse to the mean number of colonies for the controls. The ratios of leukemic mice have been plotted also for comparison (•).
Mean number of colonies for $10^9$ marrow cells plated

- Controls
- Leukemic mouse

Fig. 4. Response to different doses of stimulating factor (CSF) of normal and leukemic bone marrow cells. Each point represents the mean ± SE of pooled individual values.

the marrow not compensated for by a rise of their total number in the spleen (Fig. 2). In the marrow, the total cellularity is not different from the controls, but the density of the CFUcs is lower. On the contrary, the increase in the number of CFUcs in the spleen is essentially due to a rise in the number of cells in the spleen. To have a compensation in the total number of CFUcs, the number of cells in the spleen should be multiplied by at least 20, which we have
never found. This and the fact that there is no correlation between the rise in splenic CFUCs and the drop in marrow CFUCs seem to indicate that it is not only a migration from the invaded marrow to the spleen. This drop in the number of CFUCs in leukemic mice does not seem to be an in vitro artifact because: (1) The presence of leukemic cells in the culture does not influence the number of colonies found after plating bone marrow cells of 2-mo-old mice (Table 1). (2) The changes in the number of CFUCs in the marrow and spleen of leukemic mice would have been even greater if we could have taken old non-leukemic AKR mice for the controls instead of 2-3-mo-old mice (Fig. 3). We have shown that the mean number of spleen CFUC is the same in old and young AKR mice as Metcalf has also found in C57BL, but the number of marrow CFUC is higher in old mice. (3) The CFUCs in leukemic and in young mouse marrow respond in vitro to the same doses of CSF.

A striking fact is the similarity between our results in leukemic mice and those reported by Metcalf after bleeding in normal mice: "Bled mice developed spleen enlargement with some increase in the total number of in vitro colony-forming cells in the spleen but this did not compensate for the reduced number of such cells in the bone marrow." Shadduck et al. reported similar modifications at the stem cell level after stimulation of the erythroid system. A hypothesis to explain the low level of CFUCs in leukemic mice could be the differentiation of pluripotential stem cells (CFUs) towards the leukemic cell line emptying the CFUCs compartment. More work remains to be done to test this hypothesis, particularly the study of the stem cell compartment in this leukemic model. This work seems to indicate that, contrary to L 1210 leukemia, hematopoiesis is perturbed at the in vitro stem cell level, although the number of granulocytic blood cells are often normal.

Experiments are now being conducted to study the pluripotential CFUs using techniques that eliminate the leukemic cells.

REFERENCES

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10. Metcalf D, Moore MAS, Warner NL:


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