Altered Stem Cell (CFU-S) Function Following Infection of Hematopoietic Cells With a Virus Carrying V-src

By David Boettiger and T. Michael Dexter

Long-term murine bone marrow cultures were used to support the growth and development of hematopoietic cells. After hematopoiesis was established, the cultures were infected with a recombinant murine amphotropic virus carrying the avian sarcoma virus src gene and the CFU-S kinetics were examined. The CFU-S from the src-infected cultures displayed a reduced seeding efficiency in the standard spleen colony assay. The self-renewal capacity of these CFU-S was tested by their ability to reestablish hematopoiesis when serially transplanted on irradiated bone marrow cultures and by serial passage in spleens of irradiated mice. In both tests, cells from the src-infected cultures exhibited an enhanced ability to sustain a high level of self-renewal. The other property of stem cells which may be measured is the probability of self-renewal at each cell division which dictates the distribution between stem cells and differentiated type progeny. CFU-S from the src-infected cultures had higher average probabilities of self-renewal and therefore reduced differentiation. These differences suggest that expression of src had indirectly or directly altered the normal differentiation program of the stem cells.

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ONE OF THE prominent effects of at least some oncogenes is a reduction in the ability of the affected cell to maintain its normal differentiation program. This has been particularly well demonstrated for the src oncogene through the use of temperature-sensitive mutants along with specific cell markers available for particular differentiated cell types. These studies have demonstrated that the synthesis of muscle-specific proteins in myotubes, the synthesis of cartilage-specific extracellular matrix components by chondroblasts, the synthesis of extracellular matrix components by fibroblasts, and the production and maintenance of melanosomes by pigmented retina cells are suppressed by the expression of v-src in these cell types. Further experiments in the chondroblast and fibroblast systems have demonstrated that the differences in synthesis of these products is controlled primarily at the transcriptional level. Suppression of inhibition of expression of the mature cell phenotype is not an effect restricted to the src oncogene, but has also been demonstrated for the myb oncogene in macrophages and for the erb oncogenes in erythroid cells. In each of these cases, the affected cell is one which is already committed to particular lineage and which in most cases has initiated the synthesis of the definitive cell type-specific products. Few studies have examined the potential of oncogenes to affect properties of progenitor cell populations. An attempt has been made to infect chondroblast progenitors which are present in day 3 chick limb buds. These experiments demonstrated that the src oncogene did not prevent the differentiation of these cells into chondroblasts and may, in fact, have promoted the process of differentiation. This suggests that the effects of oncogenes in the progenitor cell compartments of differentiation may be different than their effects on the definitive cell types.

To investigate further the effect of oncogenes on progenitor cell compartments, we turned to the murine hematopoietic cell system. The chief advantages of this system are the availability of the long-term bone marrow (LTBM) culture for the maintenance of the hematopoietic stem cells and the availability of quantitative assays for both the stem cell and the committed progenitor cell stages of differentiation. The only effects on hematopoietic cells which may be ascribed to src are induction of hemangiomias and transformation of erythroid cells, but leukemias have not been reported. We have recently described the effects of a recombinant murine retrovirus carrying the avian v-src gene on murine LTBM cultures. There was an overall reduction in the production of nonadherent hematopoietic cells, reflecting a reduction in the granulocyte cell population and a dramatic increase in the proportion of blast cells. This was accompanied by a 25- to 50-fold increase in the level of GM-CFC (the granulocyte-macrophage progenitor cells) and CFU-S (the multipotential stem cells) on a per culture basis. These data suggested that the hematopoietic progenitor cells represented by these two compartments may be altered as a consequence of the infection. Cells from the src-infected LTBM cultures could be serially passaged using either the standard GM-CFC assay conditions or the in vivo spleen colony assay for more passages than the controls or than had been previously observed. This implied that the alteration in the progenitor cell population persisted in cells removed from the microenvironment of the src-infected cultures. Additional studies on the GM-CFC agar colonies have demonstrated that these colonies are actually initiated by multipotential cells rather than by cells committed to the GM-CFC lineage. The combined data imply that the primary lesion in the hematopoietic cells is intrinsic and appears to be primarily at the stem cell or multipotential cell level.

MATERIALS AND METHODS

LTBM cultures were prepared from young adult B6D2F1 mice and were infected after five to eight weeks with the recombinant murine amphotropic virus using a Moloney leukemia virus helper virus. Cells were removed from the nonadherent pool at the time of feeding and were assayed for CFU-S by inoculation into...
B6D2F1 recipients which had been irradiated with 13.5 Gy\textsuperscript{37}
Cs-rays (dose-rate 0.85 Gy/hr). Mice were killed at 11 days, the
spleens removed, fixed in Bouin's solution, and the number of spleen
colonies counted. All assays included two to three different doses of
inoculated cells, and eight to ten mice were used per group; a
background group was included with each experiment. The SE was
calculated as given in Tables 1 through 5 or as error bars on Figs 1
through 3 as appropriate. For neuraminidase treatments, the cells
were incubated for 30 minutes at 37 °C with 0.2 U/mL of Clostridium
perfringens neuraminidase (Sigma) prior to inoculation.

To calculate the seeding efficiency (ie, the fraction of potential
CFU-S which seed in the spleen and produce a spleen colony),
lethally irradiated mice were inoculated intravenously (IV) with 10\textsuperscript{4}
to 10\textsuperscript{6} cells from src-infected cultures or from control cultures. Some
animals inoculated with low cell doses were kept for counting of
spleen colonies at 11 days. Some animals inoculated with higher cell
doses were killed at 24 hours; their spleens were removed, and single
cell suspensions were prepared. Aliquots of these single cell suspensions
were inoculated into secondary lethally irradiated recipients, and
spleen cell colonies were counted after 10 to 11 days. The f
number was calculated by the formula:\textsuperscript{11} Number of CFU-S colonies in
secondary recipients/number of CFU-S colonies in primary recipients, corrected for the differences in initial inoculum size.

RESULTS

CFU-S from Src-infected LTBM cultures have an altered spleen seeding efficiency. The seeding efficiency of CFU-S obtained from the src-infected cultures was measured at
different times after infection for two separate experiments. The
experimental procedure compares the spleen colony formation for cells that had to seed the spleen once to those
that have to seed the spleen twice. Table 1 shows a seeding efficiency (f number) for CFU-S obtained from control
cultures of 0.11 and 0.15 which is in close agreement with previous data obtained for either CFU-S from LTBM cul-
tures or CFU-S from fresh BM (references 17 and 18, and
Dexter, unpublished observations). In contrast, the seeding efficiency for the CFU-S from the infected cultures was
0.018 to 0.024, or about fivefold to sixfold less than the
the controls. The seeding efficiency of CFU-S in the spleen
appears to depend on cell surface properties.\textsuperscript{10} The difference
in the seeding efficiency implies an intrinsic difference
between the CFU-S obtained from the src-infected cultures and
CFU-S from normal LTBM cultures.

The hypothesis that seeding efficiency is determined by
properties of the cell surface is supported by the observation
that treatment of the CFSC (the cells which produce
CFU-S) with neuraminidase (which removes the sialic acid
from cell surface glycoproteins and glycolipid) results in a
decline in the seeding efficiency.\textsuperscript{19} To determine whether
neuraminidase treatment would have a similar effect on the
CFSC from the src-infected cultures, nonadherent cells from control and src-infected LTBM cultures were treated
with neuraminidase, and the efficiency of spleen colony
formation was measured. The results in Table 1 show the
relative decrease in colony formation by neuraminidase-
treated cells in comparison to untreated cells from the
LTBM cultures. Neuraminidase treatment reduced the
colony-forming efficiency of the normal CFU-S by approxi-
mately sixfold, which confirms the previous results.\textsuperscript{18,19} The
CFU-S from the src-infected cultures showed a reduction of
about 3.5-fold following neuraminidase treatment. Although this
result is compatible with differences in seeding efficiency
being determined by cell surface properties, it does not exclude the participation of additional factors.

The individual spleen colonies produced following the
inoculation of cells from the src-infected LTBM cultures were
morphologically normal and displayed the same distri-
bution of erythroid, granulocyte, and mixed colonies as do
spleen colonies derived from cells taken from normal LTBM
cultures.

Serial transplantation of CFU-S from Src-infected cul-
tures. The serial transplantation of CFU-S from normal
BM in vivo in the spleens of lethally irradiated recipients
leads to a progressive decline in the number of recoverable
CFU-S per input CFU-S until there are insufficient CFU-S
in the inoculum to continue the transplantation.\textsuperscript{30} To
optimize the number of transplant generations, transplantations
are separated by about four weeks. In contrast to the results
obtained with normal BM CFU-S and those obtained with
CFU-S from normal LTBM cultures, CFU-S from the
src-infected cultures could be passaged more frequently and
did not show the usual decline in CFU-S self-renewal.\textsuperscript{14}

To examine the self-renewal potential of the CFU-S from
the src-infected cultures, nonadherent cells were serially
passaged in spleens of irradiated recipients at 9- to 20-day
intervals. The data for this experiment are given in Table 2.

Table 2. Serial Transplantation of CFU-S From SRC-Infected Cultures

<table>
<thead>
<tr>
<th>Transplant Generation</th>
<th>Transplant Interval (d)</th>
<th>CFU-S Transplanted</th>
<th>CFU-S Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>1.90</td>
<td>13.0 ± 1.2</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>0.13</td>
<td>15.9 ± 6.7</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>0.20</td>
<td>21.8 ± 4.0</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>0.44</td>
<td>04.7 ± 3.3</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>0.40</td>
<td>05.3 ± 3.2</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>0.53</td>
<td>05.0 ± 1.6</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>0.50</td>
<td>00.6 ± 1.1</td>
</tr>
</tbody>
</table>

Cells from src-infected long-term bone marrow cultures were inocu-
lated IV into syngeneic, irradiated recipients in the standard CFU-S assay.
After the indicated transplant interval, mice were sacrificed, spleen
colonies were counted, and spleen cell suspensions were made from a
pool of two to three mice and diluted and inoculated into the next
generation of irradiated recipients. The CFU-S transplanted per recipient
mouse based on the spleen cell counts and the mean CFU-S recovered
per mouse are given. The recovered values give the SEM.
Using this data in combination with the seeding efficiency data given in Table 1 and the fact that no increase in CFU-S can be detected during the first three days after inoculation, the growth curve for CFU-S shown in Fig 1 was calculated. The lack of increase in CFU-S during the first three days after inoculation has been demonstrated in previous experiments on normal BM CFU-S \(^2\) and was confirmed for CFU-S from the src-infected cultures (data not shown). The seeding efficiency was only determined experimentally for the initial point and not at each serial passage. Figure 1 shows that a relatively constant doubling time for these CFU-S of 23 hours was maintained throughout the 60 days of the experiment. There was no indication of a slowing of the growth curve at the higher passages and hence no suggestion that the passing could not have been continued for more passages. The ability of the CFC-S from the src-infected cultures to be serially passaged in this fashion implies that there is an intrinsic change in these CFU-S which allows them to maintain their capacity for self-renewal under conditions inimical to the maintenance of normal stem cells.

Because the injected cells were taken from cultures producing the src virus, it was possible that it was the virus rather than the cells which was responsible for the results in Fig 1. In control experiments, either the src virus or cells from src-infected cultures, which were irradiated with 15 Gy, were inoculated: no spleen colonies were produced. Because the irradiated cells would still continue to produce virus, the possibility that the spleen colonies produced were due to virus spread and endogenous spleen colony stimulation can be ruled out. It is formally possible that some transformed cells were inoculated as part of the nonadherent cell population taken from the infected LTBM cultures, and that these participated in the rescue of endogenous CFU-S. Although this may explain the initial transplantation, it does not appear to explain the serial passages or the production of secondary colonies from individual spleen colonies (Table 3).

Serial transfer of CFU-S in vitro. The self-renewal capacity of the CFU-S from the src-infected long-term bone marrow cultures was examined in vitro by serial passage of the non-adherent cells on 4- to 10-week-old normal long-term bone marrow cultures which had been irradiated with 15 to 20 Gy prior to transfer. When normal cultures were given this dose of radiation, endogenous hematopoiesis was completely ablated, but the intact stromal layer could be used as a feeder to support the growth of the inoculated hematopoietic cells. Table 4 shows the results obtained from six serial transfers which were performed at approximately biweekly intervals. The numbers in the boxes indicate the number of CFU-S which were inoculated per flask to initiate each successive transfer. For passages 3 through 6, these numbers were calculated by interpolation and were not measured directly at the time of transfer. The other numbers in Table 4 are the measured number of CFU-S per flask in the nonadherent cell population at different assay times. The cultures were fed at the times given in column 1 using the standard procedure for LTBM cultures (removal of half of the medium and half of the nonadherent cell population). At each transfer, there was both maintenance and growth of the CFU-S population in the culture. The initial burst in CFU-S production, particularly prominent in the first transfer, may have been the result of altered growth factor conditions, since these are known to control CFU-S multiplication in these cultures. The relative decrease in CFU-S proliferation at later times probably resulted from the degeneration of the irradiated adherent monolayer. This degeneration occurred primarily as a result of the initial irradiation of the stromal cells. Although this resulted in some impairment of the ability of the new adherent layers to support the CFCS, it is worth noting that the passed CFCS proliferated following each successive passage. Furthermore, the irradiated stromal layers continued to support the proliferation of CFCS after the parental cultures had become nonhematopoietic. The ability to maintain sufficient CFCS self-renewal to permit reestablishment of hematopoiesis through multiple serial passages distinguished the CFCS in the src-infected cultures from CFCS in control cultures. The CFCS in the nonadherent cell population of the control LTBM cultures rapidly lose their self-renewal potential when serially trans-

![Fig 1. Cumulative growth curve for CFC-S from src-infected cultures. The growth curve was calculated from a successive retransplantation of CFU-S from cultures infected with the src-containing recombinant virus 24 weeks before the initial transplant. The CFU-S values were corrected for the seeding efficiency, and it was assumed that there was no growth for the first three days after inoculation. The 66.1 cell doublings calculated correspond to an increase of 7.9 x 10^2-fold.](image)

<table>
<thead>
<tr>
<th>Colony</th>
<th>Colonies Analyzed</th>
<th>Mean CFU-S per Colony ± SD</th>
<th>CFU-S*</th>
<th>P†</th>
<th>Tc‡ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal LTBM cultures§</td>
<td>106</td>
<td>10.54 ± 23.30</td>
<td>81</td>
<td>0.582</td>
<td>6.9</td>
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<td>Normal bone marrow§</td>
<td>362</td>
<td>28.0 ± 37.24</td>
<td>215</td>
<td>0.683</td>
<td>8.6</td>
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<tr>
<td>Src-infected LTBM cultures</td>
<td>28</td>
<td>06.11 ± 7.96</td>
<td>290</td>
<td>0.700</td>
<td>9.3</td>
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<tr>
<td>Src-infected fifth in vivo TP</td>
<td>15</td>
<td>07.60 ± 8.75</td>
<td>ND</td>
<td>0.728</td>
<td>10.2</td>
</tr>
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</table>

*CFU-S are spleen colony-forming cells calculated from the CFU-S and the f number (Table 1). Values are mean CFU-S per spleen colony at 11 days.
†P values were determined using the formula of Vogel et al.\(^3\) and Schofield et al.\(^4\) where m = mean no. of CFU-S per colony; and v = SD/m; v² = (2 - 2p)/((2p - 1) + 1/m or p = (2 m + m² - 1)/(2 m² + 2 m - 2).
‡Tc is the cell cycle time calculated from the formula of Schofield et al.\(^5\) where c (no. of cell cycles) = In m/In (2) [p - 1] and Tc = 192/c for the 11-day spleen colonies.
§Data from Schofield and Dexter.\(^2\)
‡These cells were derived from the fifth in vivo passage as spleen colonies of cells derived from src-infected cultures.
Table 4. In Vitro Passage of CFU-S From src-Infected Cultures

<table>
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<tr>
<th>Days*</th>
<th>P 1</th>
<th>P 2</th>
<th>P 3</th>
<th>P 4</th>
<th>P 5</th>
<th>P 6</th>
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<td>20</td>
<td>1,760</td>
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<td>26</td>
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<td>504</td>
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<tr>
<td>110</td>
<td>150</td>
<td>19</td>
<td>44</td>
<td>198</td>
<td></td>
<td>38</td>
</tr>
</tbody>
</table>

*Time of feeding and removal of one-half of the cells.

Numbers in boxes indicate the number of CFU-S initially added per culture of irradiated bone marrow feeder cells (for passages 3 through 6, the starting values were calculated by interpolation). The other values indicate the number of CFU-S per culture taken at the various sampling times. On day 95 after initiation of the serial transfer, the original src-infected cultures contained 5 CFU-S per culture and none by day 139.

CFU-S: Self-renewal vs Differentiation. The growth of CFSC populations is characterized by the production of progeny which retain the properties of the parental CF-S (self-renewal) as well as of progeny cells with a more limited potential which produce the functional differentiated cells. Thus, the actual increases in the stem cell numbers may be described by two parameters: (a) the average cell cycle time, and (b) the P value, or probability that a daughter cell following division of a stem cell is also a stem cell, i.e., the probability of self-renewal. In normal homeostasis, it is likely that both P value and cell cycle time vary in response to the demands on the system. Unfortunately, neither of these parameters can be satisfactorily measured independently, and available methods depend upon secondary assumptions.

The value of P was determined using the assumptions of the stochastic model. The model assumes that at each CFSC cell division each daughter has the same probability (P) of being a CFSC. This probability can be determined from the analysis of the mean and coefficient of variation SD/mean CFU-S per colony) for the CFU-S content of individual spleen colonies. Individual spleen colonies initiated by CFSC harvested from src-infected cultures and from the fifth in vivo passage of CFSC from src-infected cultures were excised, and each colony was divided and injected into three lethally irradiated recipients to determine the number of CFU-S in each colony. The cells were taken from the long-term cultures at the normal weekly feeding, at which time the normal CFU-S are quiescent. This cell cycle stage difference as well as the time in culture may account for the lower P value for CFU-S from control cultures when compared with CFU-S from fresh bone marrow (Table 3). Cells taken directly from the src-infected cultures or from the fifth serial in vivo passage of cells from these cultures exhibited a P value somewhat higher than that of CFU-S from the uninfected LTBM cultures and slightly above the value obtained for fresh BM. Although CFU-S from normal BM may achieve P values similar to that observed for the CFU-S from the src-infected cultures, these higher P values were not maintained in the LTBM cultures in the absence of src.

Previous experiments have demonstrated that increase in CFU-S begins at approximately day 3 in the typical spleen colony assay. Hence, it was assumed that eight days' growth of CFU-S occurred in the production of the 11-day spleen colonies. From the P value, the measured mean increase in the CFU-S population, and the time of eight days, the average cell cycle time for each population was calculated (Table 3).

Proliferation of CFU-S during spleen colony formation. When CFU-S are inoculated into irradiated recipients, there is an initial lag phase followed by a phase of logarithmic CFU-S growth (usually between four and 12 days after inoculation but dependent on the size of the inoculum). CFU-S from different embryonic and adult tissue sources had characteristic doubling times which ranged from 16 to 25 hours. This parameter appears to measure an intrinsic growth property of the CFU-S, which would include both the cell cycle time and the P value. The rate of CFU-S doubling during the logarithmic growth phase was determined for CFU-S taken from the src-infected cultures as an alternative measure of CFU-S growth and differentiation.

Nonadherent cells were taken from src-infected cultures at different times after infection and inoculated IV into irradiated recipients. At times between five and 12 days after inoculation, pairs of mice were sacrificed, and the CFU-S content of their spleens was determined. The data were plotted to determine the growth rate of the CFU-S during the initial logarithmic growth phase (Fig 2). The three separate growth curves obtained from the src-infected cultures show very different growth rates, with doubling times ranging from 15.7 to 79 hours. It was possible that the different input levels of CFU-S in the different experiments were responsible for these differences, although previous experiments had demonstrated that CFU-S doubling times were independent of inoculum size for inoculations up to 10^6 BM cells. However, due to the high CFU-S input of the Src 2 (7 week) time point (see Table 5), it was necessary to extend the study of the normal CFU-S. When 5 x 10^6 cells containing 950 CFU-S were inoculated, the doubling time was similar to that of the previous studies, although the plateau level was achieved earlier. This confirmed that it was the intrinsic differences in the cells which was responsible for the differences in the growth rate.

The nonadherent cells used in these determinations were derived from cultures at different times after injection. Src 2 (seven weeks) was taken at the peak of the rise in CFU-S in the cultures following a 50- to 100-fold rise in a period of less than two weeks. After this peak, there was a slight decline in the total CFU-S per culture and the establishment of a new equilibrium between the CFU-S and the more differentiated...
cells. Thus, the sample was taken at the transition between rapid increase in CFU-S and stabilization. This may explain the long CFU-S doubling time. Src 2 (15 weeks) was taken during the plateau phase, during which the level of CFU-S was stable and 50- to 100-fold greater than that of the control cultures. The doubling time for these CFU-S was similar to that observed for fresh BM. Src 1 (32 weeks) was taken late in the infection during the early part of the decline in CFU-S due to the transformation of the adherent stromal layer. These cultures ceased active hematopoiesis within three to four weeks following this sample. The shortening of the doubling time could be a response to the early phase of this hematopoietic decline.

The doubling time calculated from these growth curves is the product of two components, the cell cycle time and the $P$ value. Using the assumptions above and cell cycle times similar to those calculated in the previous section, corresponding $P$ values may be calculated (Table 5). These $P$ values display a very wide variation, as expected from the variation in the doubling times. The variation contrasts sharply with results from normal BM, and suggests that there is a looser control of these parameters in the CFU-S from the src-infected cultures.

### Table 5: Calculated Data From In Vivo Growth Curves

<table>
<thead>
<tr>
<th>Input CFU-S per Mouse</th>
<th>Doubling Time of CFU-S* (h)</th>
<th>$P$ Value for Times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tc, 8 hr Tc, 10 hr</td>
</tr>
<tr>
<td>Src 2 at 7 wk.†</td>
<td>1170.0</td>
<td>79.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.55 0.56</td>
</tr>
<tr>
<td>Src 2 at 15 wk.</td>
<td>67.0</td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.67 0.71</td>
</tr>
<tr>
<td>Src 1 at 32 wk.</td>
<td>37.5</td>
<td>15.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.75 0.82</td>
</tr>
</tbody>
</table>

*Doubling time for CFU-S was calculated from the slope of the growth curve in Fig 2.
†Time is given in weeks after infection of the culture with the virus containing src.

**Long-term regulation of CFU-S in vivo.** The hematopoietic cell balance was altered following the infection of LTBM cultures with a virus carrying the src oncogene. Part of this change may be explained by the altered properties of the CFU-S derived from these cultures. The capacity for serial cloning of multipotential cells from these infected cultures either in vitro or in vivo and the altered regulation of $P$ values and cell proliferation kinetics suggest that there may be a modification in the response of these CFU-S to normal regulatory factors or environment. Because the altered response results in the overproduction of the CFU-S relative to the differentiated cell types, it might be anticipated that in vivo inoculation of these cells could lead to leukemia. However, neither leukemia nor production of leukemic cells which could be amplified in LTBM cultures has been observed in normal, sublethally or lethally irradiated, syngeneic hosts. This failure to induce leukemia suggests the presence of in vivo regulatory factors which are capable of compensating for the defect demonstrated in vitro.

To investigate the long-term effects of the inoculation of CFU-S from src-infected cultures in vivo, the growth of CFU-S in the spleens of irradiated recipient mice reconstituted with these CFU-S was followed for a period of 60 days. A smaller inoculum of CFU-S from src-infected LTBM cultures in comparison to control cultures was required to reconstitute the lethally irradiated recipients. Figure 3 shows that the growth curves for both inoculae produced nearly identical growth curves, except that the plateau CFU-S level established in the spleen was ~50% higher for CFU-S from the src-infected cultures. The ability of cells from the src-infected cultures to establish a steady-state level of CFU-S in the recipient animals implies that the growth is regulated in
in the long-term, which may explain the failure of these cells to induce a leukemia.

These growth curves were not corrected for the decreased seeding efficiency of the CFU-S from the src-infected cultures (Table 1). However, when the seeding efficiencies of CFU-S taken from the spleens of these reconstituted animals was measured two months after reconstitution, no difference was found between animals reconstituted with CFU-S from control cultures and CFU-S from src-infected cultures. This suggests that the altered CFU-S phenotype induced following infection of the LTBM cultures with the src virus reverted to a more normal phenotype during the long-term reconstitution.

DISCUSSION

Infection of the LTBM cultures with the murine virus construct containing the src oncogene derived from Rous sarcoma virus resulted in an altered balance of the normal cell types present in the cultures. There was an increase in the proportion, as well as in the absolute level, of cells which produced normal spleen colonies in irradiated recipients (CFU-S) and in cells which produced colonies in agar using WEHI-3 cell-conditioned medium as a source of colony-stimulating factor. Additional analysis of the founder cells for these agar colonies has revealed that they are not the same as the cells from normal BM which produce colonies using these assay conditions, in that agar colonies derived from the src-infected cultures could be serially recloned and were capable of producing CFC-Mix colonies. These data suggest that the CFU-S or multipotential stem cells present in the src-infected cultures were altered as a consequence of the infection. In previous experiments using the various murine retroviruses, no similar alterations at the stem cell level have been observed, suggesting that in some way the expression of the src oncogene is responsible for these alterations. The data presented here provide additional evidence that src induces an alteration in stem cell properties.

The possibility that the cell surface properties of the CFU-S from the src-infected cultures were altered using the seeding efficiency assay. It was expected that the efficiency with which CFU-S would seed the spleen would be related to properties of the cell surface. This supposition was supported by the reduction of seeding efficiency observed following the removal of sialic acid from the cell surface glycoproteins and/or glycolipids with neuraminidase. It was found that the seeding efficiency for the CFU-S from the src-infected cultures was about sixfold lower than that for the CFU-S from the control cultures. Treatment with neuraminidase reduced the seeding efficiency for control CFU-S by an additional fivefold and for CFU-S from the src-infected cultures by threefold, implying that the CFU-S from the src-infected cultures may have an altered cell surface. Indeed, alterations in cell surface glycoproteins and glycolipids have been a regular finding in cells transformed by viruses containing src. The reduced seeding efficiency for the CFU-S from src-infected cultures also means that the previously published measurements of CFU-S from these cultures were underestimates. Use of the measured seeding efficiencies to calculate the actual number of CFU-S from the previous CFU-S measurements reveals that the increase in CFU-S in the src-infected cultures was 500-fold. At late culture times, this would mean that about 30% of the total nonadherent cells were CFU-S, whereas only 15% were morphologically mature granulocytes. Thus, the normal hierarchy of cell type distribution appears to be reversed, with a predominance of the more primitive types.

Because little is known about specific molecular markers for stem cells or CFU-S, the experiments focused on the functional properties which serve to define stem cells. Stem cells have the dual properties of self-renewal and multipotentiality which allows for the maintenance of the stem cell population and the ability to produce differentiated cell progeny (ie, the functional cells for the several myeloid lineages). It is clear from the serial reconstituting the CFU-S, the ability of the CFU-S to reestablish hematopoiesis when transferred onto an irradiated BM stromal layer, and the previously published data in the in vitro reconstituting agar, that the CFU-S in the src-infected cultures retain the ability to differentiate into the various myeloid lineages and produce functional progeny. The property of self-renewal is described by the P value, which represents the probability that each daughter cell following cell division will retain the properties and potentialities of the parental cell. For the CFU-S, this means the capacity to found a spleen colony. When the P value drops below 0.5, the cell population loses its stem cell character. In the assays of stem cells, the ability to reconstitute does not depend only on the maintenance of a P value > 0.5—the P value must also be sufficiently >0.5 to overcome any inherent inefficiency in the assay. Furthermore, the CFU-S assay is performed in a potentially lethal irradiated animal, which implies conditions of extreme hematopoietic stress. Previous experiments have demonstrated that CFU-S derived from normal BM or from LTBM cultures are not able to sustain a sufficient P value to avoid extinction in serial passage. In contrast, CFU-S derived from the src-infected LTBM cultures were able to sustain a high P value and to achieve continual logarithmic growth during serial transplantation at short intervals. The unusual ability to maintain high P values was also demonstrated more directly by the statistical analysis of CFU-S content of individual spleen cultures. These higher P values did not exceed those which have been reported for CFU-S from normal BM. This may suggest that the increased P value measured in the irradiated host reflected not an absolute change in the steady-state P value but a slower response time to changes in the microenvironment. This type of altered response suggests that the intrinsic change may be caused by an altered sensitivity to regulatory factors. These delayed response times could explain both the altered cell balance obtained in the LTBM cultures and the potential for serial passages and serial recloning.

The short-term CFU-S growth rate in the irradiated host showed remarkable differences, which appeared to depend on the phase of the culture from which the cells were taken, suggesting that the growth rate at these different times is a function of the inoculated cells. A similar conclusion was reached in measuring the growth rate for CFU-S taken from spleen as compared to BM. The variation was interpreted in
terms of a mixture of CFU-S with different inherent growth rates. The alterations in rate observed for the CFU-S from the src-infected cultures exhibited a greater oscillation than for the normal CFU-S and appeared to change in response to culture conditions. The growth rate was decreased in cells from cultures which had undergone a recent proliferation and which produced a relative increase in CFC-S of about 500-fold, and was highest in the late phase of cultures in which the total CFU-S was starting to decline due to failure of the stromal cell layer. These results suggest that the differences in CFU-S growth rate may reflect the responses of the whole CFU-S population to external factors. The 500-fold increase in CFC-S followed by an exaggerated drop in CFU-S growth rate suggests that a component of the altered regulation could be a delay in the response to overproduction and perhaps also underproduction of CFU-S.

The altered self-renewal and proliferation of CFU-S from the src-infected cultures, which could be demonstrated either in the in vitro assays or in irradiated hosts prior to stable reconstitution, appeared to be lost in the later phases of the reconstitution. The longer term growth curves demonstrated little difference in the CFU-S content of spleens of irradiated animals inoculated with cells from control or src-infected LTBM cultures by 10 to 14 days after inoculation, and reached similar plateau values. This stabilization is consistent with our previous failure to demonstrate that cells from the src-infected cultures were leukemic. Most of the animals display a mild anemia, as demonstrated by a reduction in their hematocrit. This apparent numerical stabilization of the CFU-S suggests the establishment of an intermediate stage in the development of leukemia and bears some analogies of the situation of human chronic myelogenous leukemia. In this disease, it is apparent that several alterations occur, often sequentially, and give rise to a succession of clinically recognizable states culminating in an acute blast crisis.

Although the demonstration of some intrinsic alteration in the CFU-S in the src-infected LTBM cultures would be most easily explained by the infection of these cells by the virus containing the src oncogene, this may not be the case. Factor-dependent cell lines derived from src-infected cultures remained multipotent, although they do not produce spleen colonies (Spooncer and Dexter, unpublished observations), and contained an integrated Moloney leukemia virus, which was used as a helper virus for the defective src virus; however, integrated src virus could not be detected (Stoker, Wyke, Spooncer, and Dexter, unpublished observations). Thus, the alterations in the CFU-S described here may be induced by a "hit and run" type of mechanism, which would also help explain the failure of these cells to induce leukemia. In this respect, it is worth noting that although the factor-dependent cell lines derived from the src-infected cultures carry the Moloney leukemia virus, this tissue culture-passaged virus is only weakly leukemogenic in adult animals with the C57B1/6 genetic background. This may help explain the absence of leukemia during the six-month observation period following the inoculation of these cells.

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