Kinetics of erythroid precursors in mice infected with the anemic or the polycythemic strain of Friend leukemia virus

(erythroid colony-forming unit/erythroid burst-forming unit)


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Communicated by Charlotte Friend, November 9, 1979

ABSTRACT The kinetics of both erythroid burst-forming and colony-forming units (BFU-E, CFU-E) and myelomonocytic precursors [myelomacrophage colony-forming unit (CFU-C)] have been evaluated in tibial marrow, peripheral blood, and spleen of DBA/2 mice at time intervals after inoculation of either the anemic (FLV-A) or the polycythemic (FLV-P) strain of Friend leukemia virus. Either one of the viruses induced, at 7-10 days after infection, a massive increase in the number of BFU-Es in peripheral blood, in parallel with their depletion in tibial marrow and increase in spleen. A comparable increase in the BFU-E number was observed in spleen of FLV-infected mice. These results indicate a marrow-spleen migration of BFU-Es. In spleen, the increase of the BFU-E number was associated with an increase in the CFU-E pool. In tibial marrow, a sequence of expansion/depletion waves occurred reciprocally at the level of BFU-E and CFU-E. The cycling of BFU-E (3H)thymidine in vitro suicide index) in marrow, blood, and spleen was enhanced, whereas that of CFU-E and CFU-C showed little or no modification. These kinetic data suggest that the main target cell of FLV may be the BFU-E or a closely related precursor. In plates without added erythropoietin (but containing it in fetal calf serum), expression of CFU-E from FLV-P-treated animals was maximal; that of CFU-E from FLV-A-injected mice was either virtually absent or only slight in marrow or spleen, respectively. BFU-E growth always was fully dependent upon erythropoietin addition. Control studies in FLV-infected mice infected with normal marrow and spleen of DBA/2 mice given FLV-P have been observed (12). The present studies focused on the kinetics of CFU-C (proliferative rate) of BFU-E, CFU-E, and myelomacrophage colony-forming units (CFU-C) in marrow, spleen, and peripheral blood of highly susceptible DBA/2 mice and of resistant BALB/c and C57BL/6 mice inoculated with either one of the N-tropic FLV strains.

MATERIALS AND METHODS

Viruses. N-tropic, cell-free homogenates of FLV-A and FLV-P were prepared as described (1) and passaged in our laboratory. Virus stock titers were: FLV-P, 10^4.6 LD_50/0.2 ml; FLV-A, 10^3.2 LD_50/0.2 ml. Both strains were devoid of lactate dehydrogenase virus.

Mice. DBA/2 mice of both sexes, 7-12 weeks old (from CNEN-Casaccia, and Charles River Italia, Calco, Italy), and BALB/c or C57BL/6 female mice of comparable age (Charles River Italia) were housed 10 per cage and fed with laboratory pellets and tap water ad libitum. The animals received FLV-P or FLV-A intraperitoneally (0.2 or 0.3 ml per mouse, respectively).

Number and In Vitro Killing Index of BFU-E, CFU-E, and CFU-C. The numbers of BFU-E, CFU-E, and CFU-C in tibial marrow, spleen, or peripheral blood were evaluated at time intervals after FLV infection. In some studies, mice were subjected to splenectomy or sham-operation under light ether anesthesia 6 hr prior to virus infection. All experimental points, except those after splenectomy, were repeated at least three times. Each group consisted of at least three mice. Two plates per group were always used. The assays of BFU-E, CFU-E, and CFU-C were performed by means of methylcellulose cultures, according to a slight modification (13) of a previously reported method (9). Peripheral blood BFU-Es were analyzed as described (10). Each plate contained either erythropoietin (3 in-

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ternational units) or lung-conditioned medium (0.1 ml). Preliminary experiments indicated that these amounts induced maximal growth of BFU-E, CFU-E, and CFU-C colonies under the various conditions evaluated here. Human urinary erythropoietin was purified as described (13). The lung-conditioned medium was prepared as before (13).

The killing index of BFU-E, CFU-E, and CFU-C was evaluated according to a slight modification (14) of the in vitro [3H]thymidine method (9). Control studies (9, 14) excluded reutilization of [3H]thymidine in the culture dish.

The plates were incubated in a humidified atmosphere of 7.5% CO₂ in air at 37°C. CFU-C colonies containing a minimum of 50 cells were scored on days 7–8. CFU-E and BFU-E colonies, containing a minimum of either 8 or 200 cells, were scored at 36–45 hr or 8–12 days, respectively. The identification was performed in situ on the basis of morphological criteria. The validity of these criteria has been demonstrated by control studies involving benzidine staining of colonies smeared on glass slides (13).

RESULTS

Clonal Analysis of Erythroid Precursors in Marrow, Spleen, and Peripheral Blood. Infection with either FLV-P or FLV-A induced a dramatic increase in the BFU-E number in peripheral blood, which was characterized by an initial increase (days 5–10) and a subsequent plateau (FLV-A) or a further increase (FLV-P) (days 10–20) (Fig. 1). The increase caused by FLV-P was larger than that with FLV-A (470-fold and 75-fold, respectively, over control values). These findings suggest that a massive migration of erythroid precursors occurs. Therefore, the sizes of the splenic and marrow BFU-E pools were measured to determine whether the flow of this migration was from marrow to spleen. The increase in the number of BFU-E in the blood on days 5–10 paralleled an increase in the spleen and showed an inverse relationship with the BFU-E pool in marrow (Figs. 2 and 3). The postulate of marrow-to-spleen BFU-E migration is further strengthened by results of experiments in splenectomized mice. In spleen-ablated DBA/2 mice infected with FLV-P or FLV-A on day 0, there was a striking increase in the BFU-E number in blood (FLV-P, 861/ml; FLV-A, 672) on day 12 compared with that in noninfected animals (sham-operated, 9; splenectomized, 42). This increase was slightly larger than in the infected, sham-operated controls (FLV-P, 713; FLV-A, 599).

The number of BFU-Es in the blood continued to increase on days 10–20 after FLV-P infection and paralleled the levels of BFU-E in the spleen in spite of BFU-E/CFU-E differentiation (Figs. 1 and 3B). On the other hand, in FLV-A-infected mice, the plateau phase of BFU-E in the blood on days 10–20 was associated with a decrease in the number of BFU-E in the spleen, apparently due to differentiation of BFU-E to CFU-E (Figs. 1 and 2B). In this respect, it is noteworthy that the amplification of the splenic CFU-E pool was larger in FLV-A-than in FLV-P-infected mice (Figs. 2B and 3B).

In regard to marrow kinetics, the size of both the BFU-E and the CFU-E pool showed an oscillatory pattern after FLV-A

Fig. 1. Number of BFU-E in peripheral blood of DBA/2 mice at various times after injection of FLV-P (A) or FLV-A (B) inoculation. Each point represents the mean ± SEM of at least three separate experiments, except that only one experiment was performed on day 7. —, Erythropoietin added at 3 international units per plate; ---, no erythropoietin added. **, P < 0.001 for comparison with day 0 group.

Fig. 2. Total number of BFU-E, CFU-E, and CFU-C in tibial marrow (A) and spleen (B) of DBA/2 mice at various times after injection of FLV-A. Each point represents the mean ± SEM of at least three separate experiments. ---, Erythropoietin added at 3 international units per plate; ---, no erythropoietin added. For comparison with day 0 group: *, P < 0.05; **, P < 0.001.
infection (Fig. 2A). An early increase in the BFU-E number on
days 1 and 2.5 was followed by a progressive decrease with a
nadir on day 10, a second wave of amplification on day 15, and
a final decrease on day 20. The kinetic pattern of CFU-E was
the reverse of that of BFU-E, with peak values on days 10 and
20. It is of interest that the kinetics of the CFU-C pool in both
marrow and spleen were similar to the kinetics observed for the
BFU-E compartment, although the early amplification at the
level of the tibial BFU-E pool was not observed for the CFU-C
population (Fig. 2A and B).

After infection with FLV-P, the BFU-E pool in tibia showed
a less-well-defined pattern than after FLV-A (Fig. 3A). However,
the size of the CFU-E pool was characterized by a se-
quence of depletion/amplification waves which resemble those
observed after FLV-A infection (Figs. 2A and 3A). The kinetics
of the CFU-C pool apparently resemble those of the BFU-E
population (Fig. 3A and B).

Cycling of Hemopoietic Precursors in Marrow, Spleen,
and Peripheral Blood. The number of BFU-E in DNA syn-
thesis in marrow, blood, and spleen, as evaluated on the basis
of the percentage in vitro killing by [3H]thymidine, was
markedly enhanced after FLV-A or FLV-P infection (Table
1). The results of two representative experiments at 10 and 15
days after infection are shown in Table 1. These results confirm
that the killing index for these precursors in peripheral blood
is virtually null (15). In contrast, the number of CFU-E and
CFU-C in DNA synthesis was only slightly modified or not
modified at all by infection with either strain of FLV.
Furthermore, the cycling activity of CFU-E appeared to be similar
when these precursors were plated in cultures with and without
added erythropoietin (except that tibial CFU-E from day 15
FLV-A-infected mice required erythropoietin addition for
significant in vitro growth).

Growth of Erythroid Colonies Without Addition of
Erythropoietin. In all studies, the growth of BFU-E was totally
dependent upon addition of erythropoietin (Figs. 1–3). In
contrast, the growth of CFU-E colonies from FLV-P-infected mice
was independent of the addition of erythropoietin starting
from days 5–7 after virus inoculation (Fig. 3A and B). On
the other hand, the splenic CFU-E pool from FLV-A-infected mice
was only to a minor extent independent of the addition of
erthropoietin—i.e., the ratio between Ep+ and Ep− colonies
was always >3:1—whereas tibial CFU-E were totally eryth-
ropoietin dependent (Fig. 2A and B).

Control Studies. The temporal correlation observed between
virus infection and fluctuations of BFU-E, CFU-E, and CFU-C
pool sizes was further investigated to verify the existence of a
cause/effect relationship. The pools of spleen BFU-E and
CFU-E from DBA/2 mice evaluated at days 10–20 after
injection of either heat-inactivated virus (FLV-P or FLV-A held
at 56°C for 1 hr) or spleen homogenate from normal DBA/2
mice did not show any significant change (data not shown).
In addition, the results of a dose-response experiment indicated
that, on day 10 after FLV-P infection, the magnitude of BFU-E,
CFU-E, and CFU-C amplification in the spleen was clearly
dependent upon virus concentrations (Fig. 4).

The specificity of the effect was demonstrated by experiments
performed in mouse strains partially (BALB/c, Fc-1+/−,
Fc-2+/−) or fully (C57BL/6, Fc-2+/−) restrictive for replication
of N-tropic FLV strains. The BFU-E, CFU-E, and CFU-C pool
sizes in BALB/c mice given undiluted FLV-A showed no sig-
nificant modification at the tibial level (Fig. 5A). An increase
in the CFU-E number was observed in the spleen, but to a
smaller extent than in DBA/2 mice (Fig. 5B). This is in line with
the fact that the Fc-1 gene restricts FLV replication or trans-
formation only quantitatively—i.e., by 2–3 orders of magni-
tude. In C57BL/6 mice, the increase in the splenic CFU-E
number upon injection of the more potent FLV-P virus was
decreased to approximately 1/25th of that in DBA/2 mice (Fig.
6). The small difference in CFU-E number between FLV-
infected and control C57BL/6 mice is probably due to non-
specific mechanism(s) contingent upon inoculation of hetero-
logous antigenic material such as DBA/2-derived FLV-P
preparations. This is further confirmed by the observation of
similarly small increases of splenic CFU-Es in C57BL/6 mice
given spleen homogenates from normal DBA/2 mice (data not
shown).

DISCUSSION

A comprehensive evaluation of BFU-E, CFU-E, and CFU-C
kinetics in marrow, spleen, and peripheral blood of mice
infected with FLV-P or FLV-A has been carried out. Both viruses
induce impressive fluctuations of the pool size of these pre-

Table 1. In vitro [3H]thymidine-induced killing index (%) of BFU-E, CFU-E, and CFU-C from marrow, spleen, and peripheral blood of control or FLV-A- or FLV-P-infected mice on day 10 or 15 after infection

<table>
<thead>
<tr>
<th>Time, days</th>
<th>Control</th>
<th>Marrow</th>
<th>Spleen</th>
<th>Blood</th>
</tr>
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<tbody>
<tr>
<td>BFU-E</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>54</td>
<td>54</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>33</td>
<td>59</td>
<td>61</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>75</td>
<td>86</td>
</tr>
<tr>
<td>CFU-E*</td>
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<td></td>
<td>81(−)</td>
<td>71(−)</td>
<td>82(−)</td>
<td>81(−)</td>
</tr>
<tr>
<td>15</td>
<td>70</td>
<td>80(+)</td>
<td>84(+)</td>
<td>74</td>
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<tr>
<td></td>
<td>80(−)</td>
<td>80(−)</td>
<td>78(−)</td>
<td>79(−)</td>
</tr>
<tr>
<td>CFU-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>33</td>
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<td>10</td>
<td>13</td>
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</table>

ND, not done.
* With (+) or without (−) erythropoietin added to the culture dish.
cursors. The cause/effect relationship between virus infection and these kinetic phenomena was established. The effect was dependent upon virus dosage and was not observed in DBA/2 mice given heat-inactivated FLV or in genetically restricted animals treated with live, undiluted FLV preparations.

The kinetic markers of FLV-induced erythroleukemia are represented by (i) a markedly enhanced proliferative rate of BFU-E in marrow, blood, and spleen, as evaluated on the basis of their in vitro $[^3]$H-thymidine suicide index and (ii) massive migration of BFU-E from marrow to spleen via the blood. These phenomena suggest that the BFU-E may be the main target cell of FLV. These in vitro observations are compatible with results of recent in vitro studies (16) showing growth of erythroid bursts in cultures of murine marrow cells infected with FLV-P. However, the remote possibility exists that the main FLV target cell is distal to the BFU-E: both enhanced cycling and migration of BFU-E would thereby constitute a "secondary" phenomenon. This hypothesis is unlikely in view of the marked and selective enhancement of BFU-E proliferation and the dramatic increase of their pools in blood and spleen. It must be pointed out, however, that migration of both early hemopoietic precursors and stem cells is not restricted to leukemic diseases but has also been reported in stress erythropoiesis (11) and after injection of zymosan (17). On the other hand, in view of the fact that the BFU-E is closely related to the CFU-S (18), the possibility should be considered in future studies that the latter also represents a target cell for FLV. Finally, it is emphasized that FLV action on BFU-E may be direct or mediated via enhanced production of burst-enhancing factor(s) (18).

On the basis of these observations, it is tempting to postulate that the basic kinetic mechanism underlying FLV-induced erythroleukemia is represented by the following sequence of events. (i) The main target cell of FLV is the BFU-E, which is induced to proliferate and migrate from marrow to spleen via peripheral blood. (ii) The affected BFU-Es give rise, in the splenic microenvironment, to an explosive expansion of the CFU-E pool, which is larger in FLV-A- than in FLV-P-infected mice; this phenomenon may be accounted for by a partial blockade of CFU-E terminal differentiation after FLV-A infection, which results in both CFU-E accumulation and profound anemia. (iii) In the bone marrow of FLV-A-infected

![Figure 4](image1.png)

**FIG. 4.** Total number of BFU-E, CFU-E, and CFU-C in the spleens of DBA/2 mice given various dilutions of FLV-A and sacrificed 10 days later. C, control; +Ep, 3 international units of erythropoietin added per plate; −Ep, no erythropoietin added.

![Figure 5](image2.png)

**FIG. 5.** Total number of BFU-E, CFU-E, and CFU-C in tibial marrow (A) and spleen (B) of BALB/c mice at various times after injection of FLV-A. The data are from a representative experiment. —, Erythropoietin added at 3 international units per plate; ---, no erythropoietin added.
mice, the fluctuations of BFU-E and CFU-E pool sizes are apparently interconnected in that amplification of the former population is accompanied by depletion of the latter one and vice versa. This correlation may perhaps reflect a cyclic sequence of "stop and go" kinetic events, initiated by BFU-E proliferation leading to amplification of this pool and followed by differentiation of these precursors toward the CFU-E compartment. These phenomena occur in parallel to sustained BFU-E marrow-to-spleen migration, which possibly masks a similar "stop and go" mechanism at the spleen level. The kinetics of marrow CFU-E after FLV-P infection apparently resemble those after FLV-A treatment.

However, several differences have been observed in response to the virus strain used. The amplification of the marrow BFU-E pool in FLV-A-infected mice represented the earliest kinetic signal of FLV infection, whereas in FLV-P-infected mice the BFU-E pattern was less well defined. In addition, the ability of CFU-E colonies to grow in the absence of added erythropoietin also differed between mice infected with FLV-A and those given FLV-P. In the former, expression of CFU-E was virtually not independent of erythropoietin addition in tibia and only slightly independent in spleen, whereas it was always totally independent in the latter. Erythropoietin-independent CFU-Es from FLV-P-infected mice have been reported (12). These differences suggest that this independence of CFU-E is largely mediated by the spleen focus-forming component of FLV-P. This defective virus may, therefore, simply play the role of mimicking erythropoietin. Accordingly, it may facilitate the erythropoietin expression of the leukemogenic transformation induced by the FLV complex. This hypothesis is supported by results obtained in studies of in vitro transformation by FLV-A (19) or FLV-P (20). In cells infected with FLV-A only, those cultures to which erythropoietin was added developed malignantly transformed erythroid colonies. On the other hand, transformation was accomplished in cells infected with FLV-P without the addition of erythropoietin.

The erythropoietin-dependent growth of BFU-E from FLV-infected mice is in apparent contrast with results obtained with the partial or total independence of CFU-E—i.e., the progeny of BFU-E. However, this phenomenon may be explained in terms of a progression toward malignancy in the course of in vivo BFU-E-to-CFU-E maturation in FLV-infected mice. These data are compatible with the contention that BFU-E differentiation is modulated also by erythropoietin (13, 14, 21) and not only by the burst-enhancing factor(s) (22).

Kinetics of CFU-C pool size are fairly similar to those of the BFU-E population. However, the likelihood of CFU-C as target cells of FLV is dampened by lack of enhanced cycling of these precursors after FLV infection. The above similarity, already observed after erythroid perturbations (23), may reflect the strict interrelationship existing between these precursors. An additional explanation is that granulopoiesis and monocytopoiesis are enhanced by intense antigenic stimulation (24), as induced here by FLV infection.

We express our gratitude to Dr. N. N. Iscove (Basel, Switzerland) for his cooperation in preparing the "purified" human urinary erythropoietin. We also express our appreciation to Mr. P. Ciaglia for his excellent technical help. This work was supported in part by grants from CNR, Rome (Progetti Finalizzati "Virus", 78.385.84 to G.B.R. and "Controllo della cresita neoplastica", 78.02558.96 to C.P.), the North Atlantic Treaty Organization (1152), EURATOM Bruxelles (159-76-B101), and Volkswagen Foundation, Hannover.