Some Properties of the Circulating Hemopoietic Stem Cells

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Some properties of hemopoietic stem cells (CFU) circulating in the peripheral blood of the normal mouse (PCFU) were studied. Those parameters were chosen that are well-known characteristics of bone marrow stem cell populations. Leukocytes separated from the peripheral blood were the source of PCFU. The radiosensitivity (D₀ value), growth curve, seeding efficiency (f number), and the turnover state (number of PCFU in S phase measured by ³H-thymidine killing technique in vitro) of PCFU were compared to the same parameters for bone marrow CFU. Several characteristic changes in these parameters were found for PCFU: (1) Lower radiosensitivity (D₀ = 140 R), (2) Higher seeding efficiency (f number 20.9%), (3) Higher sensitivity to ³H-thymidine in vitro (killing effect 32%). The doubling time was the same both for CFU and PCFU (21 hr), but the logarithmic growth of the PCFU population started by approximately 36 hr later than that of the marrow-derived CFU population. All these findings support the idea that PCFU represent a subpopulation of the heterogeneous bone marrow CFU population.

Hemopoietic stem cells (CFU) can be demonstrated in the peripheral blood of mice even under physiologic conditions. Although the CFU content of the blood amounts only to 10–30 CFU/ml in normal mice, because of the rapid clearance of the cells (disappearance half-time 6 min), a considerable proportion of the total CFU content still migrates through the circulation every day.

Circulating CFU undoubtedly play a role in the regeneration of the bone marrow after severe local damage. However, relatively little is known about the properties and physiologic importance of circulating CFU and about the control of their appearance in and disappearance from the circulation.

The present experiments were designed to investigate some properties of CFU circulating in the peripheral blood (PCFU). Properties such as radiosensitivity, doubling time, fraction of the population in S phase of the cell cycle, generally characteristic of any cell population, plus seeding efficiency were studied. These properties were chosen since they have been extensively studied and are well known for bone marrow CFU.

MATERIALS AND METHODS

Animals

Twelve-to-fourteen-wk-old BALB/c x CBA/F₁ hybrid male mice were used as donors.

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Determination of the Stem Cell Count

Bone marrow cells were obtained from the femur. The hemopoietic stem cell count (from the bone marrow and peripheral blood) was determined by spleen colony counting, according to Till and McCulloch. About 10–12 female recipients irradiated with 850-R 60Co γ-rays were used for each determination. The spleen colonies were counted on day 9 posttransplantation.

Separation of Leukocytes

The mice were bled from their axillary veins, under ether anesthesia, using sterile conditions. The blood from 30–60 mice was pooled for each experiment. The pooled, heparinized blood was mixed with 3 volumes of 3% gelatine solution in siliconized tubes and allowed to sediment under 4°C. After sedimentation of the erythrocytes, the supernatant was sucked off. The pellet was mixed again with 2 volumes of gelatine solution and the former procedure repeated. The combined supernatants were diluted by 3 volumes of saline and centrifuged at 1300 rpm at +1°C for 15 min. The pellet was washed once with ice-cold Hank's solution, then suspended by repeated careful aspirations through a hypodermic needle and injected immediately after cell counting. By this method, the leukocyte yield ranged from 50% to 80%. The erythrocyte contamination was of the order of 10⁷/ml. The PCFU content of the leukocyte suspension ranged from 5.5 to 7.5 PCFU/10⁶ cells. The PCFU content for fresh blood and for the leukocyte suspension was determined in pilot experiments. Since this ratio did not change significantly during cell preparation, it may be assumed that the procedure does not reduce the viability of PCFU.

Determination of the Radiosensitivity of CFU and PCFU

Radiosensitivity was determined by the survival rate of in vitro irradiated CFU and PCFU. The cells suspended in ice-cold Hank's solution in siliconized tubes were irradiated with various doses of 60Co γ-rays (dose rate 139 R/min) in a homogeneous field, at room temperature. The unirradiated (control) cell suspensions were kept at room temperature for time periods identical with those required for irradiation. The erythrocyte contamination in the leukocyte suspension was greater, by an order of magnitude, than the usual erythrocyte content of the bone marrow suspension. Therefore, to equalize the erythrocyte contamination of the suspensions, before irradiation an appropriate volume of 1000-R in vitro irradiated washed erythrocytes was added to the bone marrow suspension. The irradiated samples were diluted to the required concentrations and transplanted into 10–12 recipients immediately.

Determination of the Growth Curves of CFU and PCFU

1 x 10⁵ bone marrow cells or 3 x 10⁶ leukocytes were injected into recipients irradiated with 850-R 60Co γ-rays. This injected bone marrow suspension contained 15.3–16.4 CFU, the leukocyte suspension 19.7–20.3 PCFU. On days 7, 9, and 11 posttransplantation, the spleen cells from these primary recipients were retransplanted into secondary recipients irradiated with 850 R. The total CFU (PCFU) content of the spleen was obtained by relating the spleen colony count measured on day 9 posttransplantation to the nucleated cell count of the spleen.

Determination of the Seeding Efficiency (f Number)

The seeding efficiency in the spleen of the injected colony-forming cells derived from bone marrow (CFC) or blood (PCFC) was determined using a slight modification of the method of Siminovitch et al. The CFU content of the original cell suspension was determined as described above. The primary recipients were irradiated with 1000 R, 24 hr before transplantation. Thus, on the one hand, at the time of injection, radiation-destroyed cells had disappeared from the spleen, and no embolism-induced death occurred among the secondary recipients, since it was not necessary to transplant a very large number of cells. On the other hand, this longer period between irradiation and injection of cells seemed to be more useful for avoiding the variation in seeding efficiency due to variations in splenic cell count. The primary recipients were transplanted with 3 x 10⁵ bone marrow cells or 1 x 10⁷ leukocytes and killed 2 hr after transplantation. A known amount of the cell suspension prepared from their spleens was transplanted into the secondary recipients (10–12 animals per group). Spleen colony count was determined as above.
Determining the Turnover State of CFU

The number of CFU in S phase was characterized by their in vitro sensitivity to $^3$H-thymidine, determined according to Becker et al. The cells were suspended in Fischer's medium (Gibco, Grand Island, N.Y.). The bone marrow suspensions were incubated in a concentration of $15-18 \times 10^6$/ml, the leukocyte suspension in $16-22 \times 10^6$/ml. After preincubation at $37^\circ$C for 15 min, 200 $\mu$Ci (0.2 ml) of prewarmed, isotonized $^3$H-thymidine (Amersham, England, spec. act. 21-26 Ci/mM) was added per 1 ml of the cell suspension studied. The same volume of saline was added to the control samples. After incubation at $37^\circ$C for 30 min, the samples were diluted to the required cell concentration by ice-cold Hanks' solution and transplanted into lethally irradiated recipients immediately. The difference between the CFU contents in control and $^3$H-TdR-incubated samples was expressed as the per cent of CFU in S phase at the time cells were removed from the animals.

RESULTS

Radiosensitivity of Bone Marrow CFU and PCFU, in Vitro

For the sake of comparison, the radiosensitivities of bone marrow CFU and PCFU were determined in all the experiments simultaneously. The radiosensitivity of PCFU was found to be appreciably lower ($D_0 = 140$ R) than that of bone marrow CFU ($D_0 = 100$ R) (Fig. 1).

Growth Curves for CFU and PCFU

About the same amounts of CFU and PCFU were transplanted into lethally irradiated recipients. The doubling time of CFU seeding in the spleen was found to be 21 hr for both groups between days 7 and 11 posttransplantation (Fig. 2).

However, a significant time shift was found for the starting of the logarithmic growth of stem cells derived from CFU or PCFU. The growth curve for PCFU was delayed by some 36 hr as compared to that of CFU.
Seeding Efficiency of Bone Marrow CFC and PCFC

According to the mean of three experiments, the f number proved to be 12.9% for bone marrow CFC. Since it has been known\textsuperscript{13,14} that seeding efficiency is dependent also on the time elapsed between irradiation and injection of cells, this relatively low seeding efficiency might be due to the longer postirradiation period. The seeding efficiency of PCFC was 20.9%. This difference is significant (\( p > 0.01 \)) (Table 1). Since seeding efficiency of both CFC and PCFC was measured under identical conditions, if the longer postirradiation period affected the seeding efficiency at all, it did affect both groups alike.

Fraction of Cells in S Phase of Cell Cycle of Bone Marrow CFU and PCFU

In normal steady state, less than 10% of bone marrow CFU are in the S phase of the cell cycle.\textsuperscript{12} The sensitivity to \(^3\text{H}\text{-thymidine of PCFU was examined in
Table 2. Ratio of Cells in S Phase in Bone Marrow CFU and PCFU Populations

<table>
<thead>
<tr>
<th>Incubated Without 3H-thymidine*</th>
<th>Incubated With 3H-thymidine*</th>
<th>CFU in S Phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonies per 6 x 10⁶ Bone Marrow Cells or 2 x 10⁶ Leukocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU</td>
<td>14.33 ± 1.42</td>
<td>14.00 ± 2.42</td>
</tr>
<tr>
<td></td>
<td>12.70 ± 2.89</td>
<td>12.30 ± 2.71</td>
</tr>
<tr>
<td></td>
<td>12.20 ± 1.02</td>
<td>11.95 ± 1.64</td>
</tr>
<tr>
<td>PCFU</td>
<td>8.76 ± 1.02</td>
<td>5.58 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>10.50 ± 2.59</td>
<td>7.50 ± 1.38</td>
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<tr>
<td></td>
<td>9.11 ± 2.06</td>
<td>5.80 ± 1.57</td>
</tr>
<tr>
<td></td>
<td>11.12 ± 2.14</td>
<td>8.33 ± 1.41</td>
</tr>
</tbody>
</table>

*Mean ± SD.
†Significant at 0.01 level.
§Significant at 0.02 level.

vitro and compared to the 3H-thymidine sensitivity of bone marrow CFU from the same normal animals. As found, in normal steady state, about 25.0−36.3%, of PCFU could be sterilized by 3H-thymidine, while in the bone marrow CFU population from the same mouse, the number of CFU in S phase did not exceed the usual control level (2.3%) (Table 2).

DISCUSSION

In the experiments reported, certain properties of PCFU (radiosensitivity, seeding efficiency, fraction of cells in S phase) were compared with the same parameters for bone marrow CFU. For bone marrow CFU, these parameters have been reported previously. As a control, determinations were repeated in the present studies. This permitted us to compare the results obtained for PCFU with the parameters obtained for bone marrow CFU under our own laboratory conditions.

Three properties of PCFU were found to differ from those of bone marrow CFU (Table 3).

The doubling time measured in the logarithmic growth phase was identical both for CFU and PCFU: 21 hr. However, the doubling of transplanted PCFU started about 36 hr later than the doubling of bone marrow CFU. Since the CFU and PCFU contents of the graft were almost identical, the shift in time could not be explained by different graft sizes. The lag period of PCFU before the starting of doubling seems to be longer than that of bone marrow CFU. This phenomenon seems to contradict our finding that more cells of the PCFU
population are in S phase than of the CFU population. However, the same divergence has been found for splenic CFU, as well.\textsuperscript{16,17}

It is generally known that the various properties of CFU derived from various hemopoietic sites show certain differences. Although CFU both from the fetal liver and from the spleen are pluripotent and capable of differentiation (i.e., have the same properties as hemopoietic stem cells) their radiosensitivity, doubling time, \( f \) number, repopulation capacity, and differentiation ratio are characteristically different from the respective parameters of bone marrow CFU.\textsuperscript{18-22}

These differences might be interpreted as the result of each hemopoietic organ having its own type of CFU developing under the influence of microenvironments characteristic of each organ. This assumption, however, fails to explain why PCFU that, according to present knowledge, mainly develop in and migrate out of the bone marrow\textsuperscript{4,23,24} should differ from bone marrow CFU.

Recent investigations suggest that bone marrow CFU itself might be a heterogeneous population.\textsuperscript{25,26} True, CFU fractions of various densities might be isolated by cell fractionation methods, fractions with different self-renewal capacities, and turnover states.\textsuperscript{27-29}

The differences observed between bone marrow CFU and PCFU might be interpreted in two ways: (1) by assuming that the migration from the heterogeneous bone marrow CFU population into the peripheral blood does not occur at random but selectively, so that a certain part of the population is released into the periphery or (2) by assuming that PCFU have two sites of origin, being a mixed population of bone marrow CFU and splenic CFU.

This latter assumption is contradicted by the observation that, on adequate stimulus, CFU mobilization into the circulation might be induced in splenectomized animals just as well as in normal ones.\textsuperscript{8,30} Doubling times identical for bone marrow CFU and PCFU, however, appreciably differing for splenic CFU,\textsuperscript{17} are a further objection.

On the one hand, PCFU have the properties of the pluripotent stem cell (spleen colony-forming capacity and differentiation);\textsuperscript{31} on the other hand, as confirmed by these experiments, they differ from the pluripotent stem cell in many properties. The higher turnover rate measured as against bone marrow CFU was considered to be one of the most characteristic of these differences. Since it is known that the turnover rate of the committed stem cells is appreciably higher than that of the pluripotent CFU,\textsuperscript{12-14} it might be supposed that PCFU represent some subpopulation of the assumedly heterogeneous bone marrow CFU population. As to the degree of differentiation, this hypothetical subpopulation seems to be somewhat closer to the committed populations.

\textbf{ACKNOWLEDGMENT}

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