Kinetics of Cell Proliferation of Murine Bone Marrow Cells Cultured in Diffusion Chambers: Effect of Hypoxia, Bleeding, Erythropoietin Injections, Polycythemia, and Irradiation of the Host

By Arne Boyum, Arland L. Carsten, Ole Didrik Laerum, and Eugene P. Cronkite

Mouse bone marrow cells were cultured in diffusion chambers implanted in the abdominal cavity of host mice that were subjected to different kinds of treatment. Preirradiation of the chamber host enhanced the cell growth in the chambers as follows: the number of cells capable of proliferation and differentiation in the chambers (diffusion chamber progenitor cells, DCPC) as determined by a limiting dilution method increased significantly. Similarly, the spleen colony-forming units (CFU's) in the chambers proliferated more rapidly in irradiated than in normal hosts. The total number of cells in the granulocytic series harvested after culture periods of 3-7 days was also significantly increased. The number of cells in the granulocytic series harvested per DCPC in irradiated animals was twice normal. These findings indicate that the progenitor cells probably proceed through multiplicative mitoses before differentiation in the irradiated animals. A higher cloning efficiency or a shortened generation time of stem cells are other possibilities. The increased number of differentiating granulocytes may simply be a consequence of the events taking place in the stem cell compartment, but a stimulating effect of the irradiation environment on proliferating cells in the granulocytic series cannot be excluded. Preirradiation also stimulated the growth of macrophages but less consistently. When normal mice carrying chambers with normal marrow were subjected to intermittent hypoxia during the culture period, the number of DCPC's and of cells in the granulocytic series and macrophages were reduced approximately to the same extent. Hypoxic treatment of donor animals for 3-5 days resulted in a similar reduction in number of DCPC's and CFU's at both intervals. These findings may suggest competition for a common pool of stem cells for erythrocytes, granulocytes, and macrophages. However, a toxic effect cannot be excluded. Erythropoiesis, which is usually absent, was significantly increased when hypoxia and irradiation of the chamber hosts were combined. Bleeding and erythropoietin injections also enhanced erythropoiesis to some extent.

From the Medical Research Center, Brookhaven National Laboratory, Upton, N.Y.

Received September 28, 1971; revised February 7, 1972; accepted March 31, 1972.

Supported by the United States Atomic Energy Commission and the Norwegian Defense Research Establishment.

Arne Boyum, M.D.: Scientist, Division of Toxicology, Norwegian Defense Research Establishment, Kjeller, Norway; Research Collaborator, Medical Research Center, Brookhaven National Laboratory, Upton, N.Y. Arland L. Carsten, Ph.D.: Scientist, Medical Research Center, Brookhaven National Laboratory, Upton, N.Y. Ole Didrik Laerum, M.D.: Scientist, Division of Toxicology, Norwegian Defense Research Establishment, Kjeller, Norway. Eugene P. Cronkite, M.D.: Chairman, Medical Research Center, Brookhaven National Laboratory, Upton, N.Y.
The culturing of bone marrow in diffusion chambers implanted in the mouse peritoneum is a convenient method for study of the kinetics of proliferation of cells in the granulocytic series and macrophages. Since the cells grow in a closed system, one is not concerned with migration or interchange of cells from elsewhere in the body. In this system, the proliferation of spleen colony-forming units (CFU's), granulocytic stem cells and their differentiated progeny, can be measured. Erythropoiesis is not generally observed in the diffusion chamber cultures. However, some Fe uptake has been induced by exposing the host animals to hypoxia or by suspending the inoculated cells in erythropoietin-rich serum. Benestad and Breivik in similar experiments observed a small increase in number of nucleated erythroid cells.

Transplantation of bone marrow into otherwise fatally irradiated mice results in reconstitution of all hematopoietic lines. However, erythropoiesis commences earlier than granulopoiesis. Stimulation of erythropoiesis by exogenous erythropoietin injections results in diminished granulopoiesis, suggesting competition for a common pool of stem cells. When short-term cultures of bone marrow cells are treated with serum from mice irradiated 10 days previously, an increased uptake of H-thymidine has been observed. In addition, serum obtained 5 days after irradiation of the donor enhanced the growth of granulocytic colonies in soft agar. When bone marrow is grown in diffusion chambers implanted into irradiated hosts, granulocyte production was increased.

Hemorrhage, hypoxia, polycythemia, irradiation, and immunosuppressive chemicals are known to influence growth and differentiation of hematopoietic cells. The present study was designed to evaluate the influence of some of these factors on the growth and differentiation of bone marrow within diffusion chambers. Chambers containing normal bone marrow were implanted in mice that had been treated in different ways. The mice in which the diffusion chambers were implanted were subjected to (1) irradiation and/or hypoxia, (2) bleeding, (3) transfusion polycythemia, and (4) erythropoietin injection. The effect of the preceding treatments on stem cells and their differentiation in the chambers was studied. In addition, the influence of hypoxia on bone marrow CFU's and diffusion chamber progenitor cells (DCPC) of donor animals was also studied.

Materials and Methods

Eight to 10-wk-old female mice of the Hale-Stoner-Brookhaven (HSB) strain and (C3H × DBA/2) F1 hybrids were used as donors. (Different strains were used because some experiments were performed in Norway and some at Brookhaven.) HSB marrow was placed in HSB recipients, whereas (C3H × DBA/2) F1 marrow was placed in female NMRI/BOM mice. Bone marrow cells were obtained by grinding the femurs from each of four donor mice in Medium 199. The cell concentrations were determined by counting in a hemocytometer. Irradiated, recipient mice received whole-body 250 kVp x-ray exposures at a dose rate of approximately 120 rads/min to HSB animals or 40 rads/min to C3H × DBA/2. Hypoxia was accomplished by keeping animals in a decompression chamber at 0.44 atmospheres for 16 hr a day. Mice were bled from the orbital sinus 2 days (0.65 ml/animal) and 1 day (0.5 ml/animal) before implantation, as well as 1 day after implantation (0.5 ml/animal). For determinations involving polycythemic mice,
animals were injected intravenously with 0.5 ml of erythrocytes in saline (75% by volume) 1 and 2 days before chamber implantation.

In erythropoietin studies, each diffusion chamber contained 1.6 U of erythropoietin at implantation. Human erythropoietin was provided by the Hematology Research Labs., Children's Hospital in Los Angeles. The erythropoietin activity determined in the exhyepoxic polycythemic mouse by the $^{59}$Fe-incorporation method was 58.8 U/mg dry weight. The Millipore filters were saturated by incubating the chambers for 30 min at room temperature in Medium 199, containing 2 U of erythropoietin/ml. The host animals were injected every 8 hr with erythropoietin dissolved in 0.5% albumin and saline. Each animal received a total of 40 U during the 5 treatment days. Control animals were injected with the 0.5% albumin and saline solution. The diffusion chamber technique used in these studies has been previously described in detail.2

The concentration of DCPC's was determined using the principle of limiting dilution.2-3 If myeloblasts and promyelocytes were found in a chamber smear after a culture period of 7 days, it was assumed that this chamber initially contained one or more DCPC's. If a chamber did not contain any DCPC's at implantation, no granulocytes or only mature granulocytes were found after 7 days. These chambers were scored as "empty."

The stem cell directed down the granulopoietic pathway is believed to divide once at the myeloblast and promyelocyte level and perhaps twice at the myelocyte level, for an amplification of 16 in man.15 The amplification in mouse is not known. In the chambers containing only one or a few DCPC's, there must be multiplication of these cells to a sufficient number to produce enough myeloblasts and promyelocytes in the chamber so that they can be observed on the seventh day. Thus, when few DCPC's are placed in a chamber it is mandatory that there be multiplication of the DCPC before differentiation ensues.

When each chamber contains an equal volume (100 μl) from the same cell suspension, the distribution of stem cells in the chambers can be calculated from the Poisson probability law. If the average number ($\lambda$) of stem cells in the chambers is small, then some chambers will not contain any stem cells. The probability, P(o) of getting no stem cells in a chamber is given by the equation:

$$P(o) = e^{-\lambda}, \text{ where } P(o) = \frac{\text{number of empty chambers}}{\text{total number of chambers}}$$

1.

$$\lambda = -\ln P(o).$$

2.

For instance, if 37% of the chambers are empty, then $\lambda = -\ln 0.37$, and the average stem cell number is one. Therefore, if the fraction of empty chambers can be determined, the average DCPC's per chamber can be calculated from equation 2. The DCPC population is assumed to be composed of both multipotent (=CFU) and unipotent stem cells,8 but this has not been proven as yet.

Finney's method of calculating the weighted mean and its standard error was used.16

RESULTS

Effect of Irradiation of Host on DCPC and CFU Proliferation in Implanted Chambers

Chambers containing normal, murine bone marrow were implanted into either unirradiated controls or mice receiving 800 R 1 day preimplantation. DCPC's are determined on the basis of the number of empty chambers found. Within the limit of accuracy imposed by sampling of the marrow suspension, the same number of stem cells were introduced into the chambers. However, the results were influenced by implantation of the chambers into the irradiated host. The number of DCPC's in 85 chambers implanted in irradiated hosts was $62 \pm 9/10^5$ cells, and $46 \pm 6/10^5$ cells in 101 chambers implanted in
KINETICS OF MARROW CELL PROLIFERATION

Fig. 1. Effect of irradiation of host on number of cells in granulocytic series and CFU's in chambers. Normal bone marrow cells (C3H × DBA/2) from same cell suspension were cultured in diffusion chambers implanted into normal and irradiated (800 R) hosts. On days 1–4, cells from six chambers in each group were harvested, counted, and pooled. These cells were injected into irradiated (950 R) mice, and spleens were removed after 7 days. Figure shows CFU's/10^5 cells cultured. Twelve to 13 spleens for each experimental point.

normal mice (p < 0.05). In other words, fewer empty chambers were found when they were grown in the irradiated host. Apparently, there is an increased probability for self-renewal, or the irradiated environment is more conducive to initial survival and proliferation of DCPC's (cloning efficiency).

CFU proliferation in the chambers was measured daily for 4 days (Fig. 1). After 1 day in culture, the number of CFU's decreased to about 40% of the initial value. Thereafter, CFU's proliferated more rapidly in the irradiated than in the normal host. The difference between the two was highly significant on the third day in culture (p < 0.001). The number of cells in the granulocytic series fell to a low on day 1. After day 2, the number in the chamber increased more rapidly in the irradiated than in the normal host. The continuing increase of cells in the granulocytic series as the CFU's leveled off between days 3 and 4 suggests that the rate of differentiation of CFU's into differentiated cell lines is approximately equal to the rate of proliferation.

Effect of Hypoxia on DCPC and CFU Concentration

Marrow was harvested from mice that had been hypoxic for 3 or 5 days, and the progenitor cell concentration was determined. The number of DCPC's per femur was reduced to about 40%, and the number of CFU's was reduced to about 55% of normal values (Table 1). Whether the DCPC and CFU may be the same cell is not known.

The effect of hypoxia was further studied by comparing growth of normal
Table 1. Effect of Hypoxia on Diffusion Chamber Progenitor Cells (DCPC's) in Bone Marrow

<table>
<thead>
<tr>
<th>Duration of Hypoxia (days)</th>
<th>No. of DCPC's/10^5 BM* Cells</th>
<th>DCPC and CFU's/10^5 BM* Cells of Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCPC</td>
<td>3</td>
<td>34.1±8.8, 40.2±12.7</td>
</tr>
<tr>
<td>CFU</td>
<td>23</td>
<td>22.2±1.7, 55.1±3.4</td>
</tr>
<tr>
<td>DCPC</td>
<td>5</td>
<td>33.2±8.6, 38.0±11.2</td>
</tr>
<tr>
<td>CFU</td>
<td>12</td>
<td>19.5±1.6, 48.5±3.0</td>
</tr>
</tbody>
</table>

Marrow was harvested from male HSB mice exposed to hypoxia, 0.44 atmospheres, 16 hr/day for 3–5 days, and the concentrations of DCPC's and CFU's (spleen colony-forming units) were determined. Seven control series for DCPC determination comprised 110 chambers. Normal controls for CFU determinations were taken at weekly intervals and comprised several hundred spleens.
* BM, bone marrow.

Marrow in mice at normal oxygen tension and in mice exposed to hypoxia 2 1/2 days before implantation and throughout the subsequent culture period. Hypoxia reduced the concentration of DCPC’s to 30% of normal values (Table 2).

Growth of Differentiating Bone Marrow Cells Cultured in Irradiated and/or Hypoxic Mice

Chambers containing 20,000 normal bone marrow cells were implanted into normal, irradiated, and hypoxic hosts. Some of the latter were irradiated 1 day before implantation. The exposure to hypoxia started 2 1/2 days before implantation and lasted throughout the culture period. Chambers were removed daily for 7 days to determine cell numbers. The data from several experiments (173 chambers) were pooled, and the results are shown in Figs. 2–5. The growth of granulocytic cells is shown in Fig. 2. In all cases, cells in the granulocytic series decreased after implantation. A significant increase started after 3 days in the hypoxic group and after 2 days in the other groups. Initial growth in all instances was exponential. After the fifth day the growth rate appeared to taper off, except in the irradiated hypoxic hosts. The largest number of cells

Table 2. Effect of Hypoxia on DCPC's

<table>
<thead>
<tr>
<th>No. of Chambers</th>
<th>No. of DCPC's/10^5 BM* Cells</th>
<th>DCPC Concentration In % of Normal (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia</td>
<td>32</td>
<td>32.7±7.6, 30.8±10.5</td>
</tr>
<tr>
<td>Normal controls</td>
<td>31</td>
<td>106.0±26.4</td>
</tr>
</tbody>
</table>

Normal marrow cells were cultured in normal mice and in mice exposed to hypoxia beginning 2 1/2 days before implantation and continuing throughout culture period (7 days).
* BM, bone marrow.
Fig. 2. Growth of cells in granulocytic series in diffusion chambers. Chambers containing 20,000 normal bone marrow cells were implanted into normal hosts (female HSB mice), into hosts irradiated (750 rads) 1 day before implantation, into hosts exposed to hypoxia (0.44 atmosphere) from 2 1/2 days before implantation and throughout culture period, and into irradiated hypoxic hosts. Four hundred cells were counted in each smear.

in the granulocytic series was produced in the chambers grown in the irradiated hosts (p < 0.01 compared with normal hosts). From day 3 to 7 the number of cells in the granulocytic series in chambers grown in hypoxic mice was increased, but to a lesser degree than in the other treatment groups (p < 0.001).

The per cent proliferating granulocyte precursors (myeloblast through myelocyte) is shown in Fig. 3. After 1 day, the chambers from normal animals contained a higher percentage of proliferating cells than chambers from irradiated animals (p < 0.05). However, each group contained only three to
Fig. 4. Growth of macrophages in diffusion chambers implanted into same chambers and hosts as described in legend of Fig. 2.

four chambers on day 1, and the smears were difficult to examine due to the low cell numbers; in a subsequent experiment with larger cell inocula, no difference was found between the two groups after 1 day. From day 2, the percent proliferating granulocyte precursors increased and reached a plateau at days 3–4, followed by a gradual decrease from day 5 to values between 40% to 60% on day 7. Cells grown in the normal and irradiated host had a larger fraction of proliferating granulocytes on day 3 than cells grown in hypoxic hosts ($p < 0.05$). This situation was reversed at the end of the culture period when chambers from hypoxic hosts contained relatively more proliferating granulocyte precursors than chambers from normal and irradiated hosts ($p < 0.01$), suggesting that hypoxia delays granulocyte proliferation. There was no significant difference between normal and irradiated animals from day 2 to 6, but chambers from irradiated animals contained a higher percentage of proliferative granulocytic cells on day 7 ($p < 0.01$). The latter finding was confirmed in experiments with another strain of mice (C3H × DBA).

To see if a relatively short, initial exposure to irradiation environment would influence the cell yield after 5 days, chambers were transferred from irradiated hosts to normal hosts during the culture period (Table 3). The reimplantation of chambers from normal primary hosts to a secondary normal host did not significantly influence growth of cells in the chamber. One day in irradiated host and 4 days in the normal host significantly increased the yield of granulocytic cells compared to growth in normal host only ($p < 0.02$). This may suggest a stimulation of DCPC proliferation within 24 hr after implantation. The yield of granulocytic cells increased further with duration (2 or 5 days in the irradiated host, $p < 0.001$).

Macrophage growth is shown in Fig. 4 and Table 3. There were so few macrophages in the initial cell implant that they could not be quantified. In all treatment groups detectable macrophage growth occurred during the first day, being the least in the irradiated hypoxic host. The growth rate in the normal and in the irradiated hosts was comparable throughout the culture period, the
KINETICS OF MARROW CELL PROLIFERATION

Table 3. Effect on Cell Yields of Primary Culturing in Normal or Irradiated Hosts, Followed by Transfer of Diffusion Chambers to Normal Hosts

<table>
<thead>
<tr>
<th>No. of Chambers</th>
<th>Irradiated Hosts (days)</th>
<th>Normal Hosts (days)</th>
<th>Average Cell Number/Chamber (× 10^6 ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Granulocytic Cells</td>
<td>Macrophages</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>5</td>
<td>282±39 40±6</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>1+4</td>
<td>332±38 44±6</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>4</td>
<td>501±49 61±8</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>3</td>
<td>659±51 102±5</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>0</td>
<td>794±119 78±10</td>
</tr>
</tbody>
</table>

Pooled data from two sets of experiments. 10^5 normal bone marrow cells from male (C3H × DBA) F1 hybrid mice were cultured for 5 days. In two series cells were cultured for 5 days in normal hosts only, in two others for 1 day in normal hosts, followed by transfer to other normal hosts. In other series, cells were cultured either 1 day (two series) or 2 days (one series) in hosts irradiated with 800 R 1 day before implantation, and chambers were transferred to normal hosts for the rest of the period. In two series, cells were cultured for 5 days in irradiated hosts.

rate diminishing after the fifth day. On the seventh day the difference was significant (p < 0.05). Growth in the irradiated hypoxic hosts was significantly less than in the irradiated or normal animals throughout the entire period (p < 0.05) to (p < 0.001). Growth in hypoxic hosts was comparable to normal and irradiated hosts on days 1 and 2. Between days 2 and 4 a marked reduction in numbers occurred. From day 4 to 7 approximate exponential growth resumed. Compared to irradiation plus hypoxia, the number of cells in hypoxia alone was significantly less on days 4–6 (p < 0.05).

Macrophage growth was not influenced by reimplantation from a normal to another normal host (Table 3). When chambers were grown for 1 or 2 days in an irradiated host and then implanted into normal hosts, the growth was enhanced over that when grown only in the normal host, being greater when conditioned by 2 days in the irradiated host (p < 0.01).

Doubling Times for Growth of Differentiated Cells

The doubling time was estimated from the slopes during the period most closely approximating exponential growth (days 3–5). The estimated values are tabulated in Table 4.

Table 4. Doubling Times (Hr) From Day 3 to 5. Chamber Cultures in Normal Hosts and in Those Subjected to Hypoxia, Irradiation, or Hypoxia Plus Irradiation

<table>
<thead>
<tr>
<th></th>
<th>Normal Control</th>
<th>Hypoxia</th>
<th>Irradiation</th>
<th>Hypoxia + Irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytic series</td>
<td>(14.5–20.4)</td>
<td>14.3</td>
<td>11.8</td>
<td>12.4</td>
</tr>
<tr>
<td>Macrophages</td>
<td>14.8</td>
<td>21.5</td>
<td>13.1</td>
<td>14.7</td>
</tr>
<tr>
<td>Granulocytic series + macrophages</td>
<td>(12.1–18.9)</td>
<td>14.4–42.8</td>
<td>(11.1–12.5)</td>
<td>(11.8–19.5)</td>
</tr>
<tr>
<td></td>
<td>15.7</td>
<td>16.8</td>
<td>11.7</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>(13.5–20.3)</td>
<td>(12.8–24.7)</td>
<td>(11.1–12.5)</td>
<td>(10.7–16.4)</td>
</tr>
</tbody>
</table>

For details, see text to Fig. 1. Table gives average values and ranges corresponding to ± 1 SE. Female HSB mice.
The doubling time of cells in the granulocytic series was significantly decreased when the cells were grown in irradiated hosts, 11.6 hr compared to 16.9 hr in normal hosts \((p < 0.02)\). The doubling time was shorter when cells were grown in hypoxic and in hypoxic-irradiated hosts, but the difference is not significant. Macrophage growth was not changed by irradiation. The doubling time for macrophages was prolonged by hypoxia, but the standard error was large due to variable and low cell numbers so that the prolongation was not statistically significant.

**Growth of Bone Marrow Cells During Erythropoietic Stimulation or Inhibition**

Increased erythropoiesis \((p < 0.001)\) was seen from day 5 to 7 when hypoxia and irradiation of chamber hosts were combined (Fig. 5), but only 4\%-7\%

**Table 5. Effect of Erythropoietin Injections and Bleeding on Cell Growth in Diffusion Chambers**

<table>
<thead>
<tr>
<th></th>
<th>Nucleated Erythroid Cells (( \times 10^6 \pm SE))</th>
<th>All Granulocytic Cells (( \times 10^6 \pm SE))</th>
<th>Macrophages (( \times 10^6 \pm SE))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythropoietin-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injected mice</td>
<td>6</td>
<td>21.0(\pm)4.1</td>
<td>237(\pm)34</td>
</tr>
<tr>
<td>Albumin-injected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>controls</td>
<td>7</td>
<td>7.5(\pm)4.7</td>
<td>218(\pm)68</td>
</tr>
<tr>
<td>Bled animals</td>
<td>12</td>
<td>7.4(\pm)1.5</td>
<td>173(\pm)13</td>
</tr>
<tr>
<td>Normal controls</td>
<td>6</td>
<td>1.1(\pm)0.5</td>
<td>234(\pm)17</td>
</tr>
</tbody>
</table>

Chambers containing 50,000 normal bone marrow cells and 1.6 U erythropoietin were implanted. In one group, mice were injected three times daily with erythropoietin, 40 U total. Controls were injected with albumin three times daily. In another group, mice were bled from orbital sinus 2 days (0.65 ml/animal) and 1 day (0.5 ml/animal) before implantation, and 1 day after implantation (0.5 ml/animals). Table gives average number of cells \((\pm \text{SE})\) per chamber after a culture period of 5 days. Female HSB mice.
Table 6. Effect of Polycythemia and Irradiation of Chamber Host on Cell Growth in Diffusion Chambers

<table>
<thead>
<tr>
<th>No. of Chambers</th>
<th>Nucleated Erythroid Cells (× 10³ ± SE)</th>
<th>All Granulocytic Cells (× 10³ ± SE)</th>
<th>Macrophages (× 10³ ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycythemia</td>
<td>7</td>
<td>162±25</td>
<td>72±10</td>
</tr>
<tr>
<td>Polycythemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ irradiation</td>
<td>6</td>
<td>0</td>
<td>295±38</td>
</tr>
<tr>
<td>Irradiation</td>
<td>8</td>
<td>1.4±0.8</td>
<td>265±50</td>
</tr>
</tbody>
</table>

Twenty-thousand normal bone marrow cells were implanted into polycythemic mice, polycythemic irradiated mice, and irradiated mice. Table gives average number of cells per chamber (± SE) after a culture period of 6 days. Female HSB mice.

of total cell numbers were nucleated erythroid cells. Irradiation alone also slightly enhanced erythropoiesis in the chambers on day 7 (p < 0.05).

Erythropoietin increased the number of nucleated erythroid cells (p < 0.05) after a culture period of 5 days (Table 5), without any significant effect on cells in the granulocytic series or macrophages. Bleeding of chamber hosts also stimulated erythropoiesis (p < 0.02).

In some experiments, irradiation was combined with polycythemia to see if depression of erythropoiesis would further enhance the growth of granulocytes. Polycythemia alone (Table 6) gave cell yields similar to normal animals in other experiments. Irradiation increased the number of cells in the granulocytic series (p < 0.02) in polycythemic hosts. The number of macrophages was not significantly affected. There was no difference in cell yields from irradiated or irradiated and polycythemic host mice.

DISCUSSION

The relationship between spleen colony-forming units (CFU's) and diffusion chamber progenitor cells (DCPC's) has been discussed previously.4,8 CFU's may comprise multipotent, as well as committed granuloid and erythroid stem cells. DCPC's may be multipotent stem cells and committed granuloid stem cells. In the following discussion CFU's and DCPC's will be considered as stem cells for granulocytes.

In a closed culture system, where one can observe changes in the number of CFU's and differentiated cell lines, there are several possibilities for the stem cells to take. If the stem cell production rate and differentiation into the multiplicative compartment of granulopoiesis remain constant, the production of nondondividing granulocytes is determined by the number of multiplicative mitoses between the stem cell and the termination of mitosis. The production of nondondividing granulocytic cells will be directly proportionate to the input of stem cells into the granulopoietic pathway when generation time and number of mitoses are constant. Increased numbers of differentiated progeny can be obtained by an increased production of stem cells followed by the usual amplification from mitoses in the differentiated compartment, or with constant stem cell number, by additional mitoses before cell cycling ceases.15

Vogel et al.17 have suggested that a slight increase of the self-renewal prob-
ability of stem cells (CFU’s) during the initial phase of growth will significantly increase the number of spleen colonies, and thereby the number of differentiating cells. The irradiation environment enhanced the growth of all members of the granulocytic series compared with the growth in normal animals. However, while the number of DCPC’s detected increased by a factor of only 1.35, the yield of granulocytic cells (Table 3) increased by a factor of 2.8 in the irradiated animals. Hence, the number of cells in the granulocytic series produced per DCPC was twice normal. Whereas the increase in DCPC’s could result from increased cloning efficiency, the combined effect on DCPC’s and differentiating cells may best be explained by an increased self-renewal rate of DCPC’s prior to differentiation. The results are in agreement with other reports showing that DCPC’s proliferating rapidly during regeneration after vinblastine treatment yielded twice as many cells in the granulocytic series as did DCPC’s in normal marrow.

The importance of the events taking place during the initial phase was clearly demonstrated in the present experiments, when 1 day of culturing in irradiated hosts, followed by transfer to normal hosts for 4 days, resulted in significantly increased cell yields (Table 3).

The CFU number decreased remarkably during the first 24 hr, indicating differentiation or death of the CFU’s. Thereafter, the number increased rapidly, more so when grown in the irradiated host, suggesting that the irradiated host environment is stimulating stem cell renewal (Fig. 1). The increased number could result from suppression of differentiation, a shortened generation time, or both.

It may appear that the stimulating effect of irradiation on DCPC’s (Table 3) precedes the effect on CFU’s (Fig. 1). It is possible that DCPC’s present at implantation are stimulated to self-replication within the first 24 hr, while proliferation of resting CFU’s in Go starts later. Furthermore, the initial depression of CFU’s might suggest that some of them become committed to granulopoiesis shortly after implantation.

After the second day there was a marked increase in the number of cells in the granulocytic series, being much greater in chambers from irradiated than from normal hosts. This finding may be explained on the basis of events taking place in the stem cell compartment. However, it cannot be excluded that the irradiation environment stimulates myeloblast and myelocytes, either by shortening their generation time or by prolonging the maturation process and thus, allowing more multiplicative mitoses in the differentiated proliferating compartment before cell cycling ceases.

The enhancement of cell growth in irradiated animals must depend on stimulation by a humoral agent, or lack of an inhibiting agent. A stimulatory effect of materials released from dead cells is one possibility. Morley et al. suggested that serum from irradiated animals contains a stimulating factor. They found increased numbers of granulocyte colonies on agar supplied with serum from irradiated mice, which indicates that this factor acts directly on stem cells by increasing their self-renewal tendency or cloning efficiency. The proliferation of granulocytes is influenced by humoral substances; granulo-
cytic chalone is said to prevent, and antichalone probably promotes, the entry into DNA synthesis. Chalone is asserted to be produced by mature granulocytes, and a reduction or disappearance of this factor in irradiated animals could explain the enhanced growth. An increased concentration of antichalone in irradiated animals would have a similar effect. Chalone and antichalone might also have a direct, but yet unproved, effect on stem cells.

Growth of macrophages in irradiated hosts produced equivocal results. In HSB mice there was almost no effect. In contrast, with (C3H × DBA) F1 bone marrow cells, macrophage proliferation was significantly enhanced by growing in irradiated hosts, suggesting a strain difference.

Effect of Hypoxia on DCPC's, CPU's, and Differentiating Cells

In mice exposed to hypoxia for 3–5 days, the number of CPU's per femur was reduced to approximately 50% of normal values (Table 1). Bruce and McCulloch\textsuperscript{21} and Kubanek et al.\textsuperscript{22} did not find this reduction of femoral CFU's, though the spleen CFU numbers were reduced to about the same extent as bone marrow CFU's in the present work. This may be due to strain differences, as elsewhere reported by Kubanek,\textsuperscript{23} or somehow depend on the strength of the hypoxic stimulation. Rickard et al.\textsuperscript{24} found a depression of agar colony-forming units during hypoxia consistent with our observations.

Hypoxia for 3–5 days reduced the number of DCPC's approximately to the same extent as CPU's (Table 1), indicating a close relationship between the two types of progenitor cells. It is premature to conclude that the two techniques measure the same type of hemopoietic stem cell. When chambers with normal marrow were grown in hypoxic mice throughout the culture period, the concentration of DCPC's was reduced to 30% of normal (Table 2). Hypoxia of the donor produces a similar decrease in DCPC's (Table 1). The formation of cells in the granulocytic series and macrophages was also reduced when animals carrying chambers with normal marrow were subjected to hypoxia. This reduction was similar to that observed for DCPC's. The growth rates of cells in the granulocytic series and macrophages were not significantly affected. This indicates that hypoxia primarily effects the DCPC's and not their differentiating progeny. The results suggest a common stem cell for macrophages and granulocytes (and erythrocytes). In other experiments, the sum of cells in the granulocytic and macrophage series remained quite constant, even though the actual number of each varied considerably (Table 5), also suggesting a common stem cell (Table 5).

Bruce and McCulloch\textsuperscript{21} suggest that the loss of splenic CFU's during hypoxia may result from an increased demand for differentiating cells. In contrast, injections of erythropoietin increased the number of CFU's in the spleen, and a toxic effect of hypoxia, unrelated to erythropoietin, has been considered (Kubanek et al.\textsuperscript{22} and Stohlman et al.\textsuperscript{29}). In the preceding experiments there was no effect on femoral CFU's. Fried et al.\textsuperscript{26} found a slight increase in femoral CFU's during hypoxia. Accordingly, the toxic effect in these experiments\textsuperscript{22,25} must be restricted to splenic CFU's. In the present work, hypoxia reduced the number of DCPC's but had no effect on the growth rate of cells in the granu-
locytic series. It is possible that hypoxia stimulates multipotent stem cells to differentiate along the erythroid line, and there is a reduced number of stem cells available for producing granulocytic cells and macrophages. The erythropoietin produced in response to hypoxic stimulation may not act directly on multipotent stem cells but on an intermediate pool of committed erythroid stem cells. A depletion of this cell pool may lead to subsequent differentiation and decrease of multipotent stem cells. The slight erythropoiesis may not appear compatible with competition for a common stem cell. However, the stimulus to differentiation may be so strong that the cells mature to erythrocytes after a few divisions; thus, each stem cell produces only a few differentiated cells. This is in agreement with the effect of preirradiation; a large cell number cannot be produced unless the DCPC’s multiply during the initial phase. Significantly enhanced erythropoiesis was found only by combining hypoxia and irradiation. Hypoxia alone produces high erythropoietin levels only during the first days. After 4–5 days, the levels are higher in animals where hypoxia and irradiation are combined. A strong hypoxic stimulation during the first days after implantation appears essential to deplete the stem cell pool by differentiation. Preirradiation of the chamber host has an opposite effect that may tend to enlarge the stem cell pool during the first part of the culture period, and these cells produce increased numbers of erythroid cells on days 5–7 in response to stimulation by erythropoietin.

Bleeding and erythropoietin injections enhanced erythropoiesis to some extent. There was no depressive effect on the proliferation of cells in the granulocytic series and macrophages. Apparently, the experimental setup is important. Hellman and Grate transplanted normal bone marrow into irradiated animals. Erythropoietin injections during the phase of regeneration resulted in diminished granulopoiesis, indicating competing proliferative demands on the stem cell pool.

The difficulty with which erythropoiesis is induced might suggest that the ecologic conditions within the chambers are not favorable for erythropoiesis. However, it may somehow depend on properties of the inoculated cells. When diffusion chambers containing autologous hemopoietic cells were implanted into severely bled rabbits, the erythroblasts comprised about 50% of total cell number.

Irradiation alone seems to induce maximal stimulation of granulopoiesis. If granulocytes and erythrocytes have a common stem cell, depression of erythropoiesis by polycythemia could be expected to result in further enhancement of granulocytic cell growth. No such effect was seen.

We conclude that preirradiation of chamber hosts enhances the growth of cells in the granulocytic series, most probably by increasing the self-renewal tendency of DCPC’s (their progenitor cells) during the early phase of the culture period. Hypoxia depresses granulopoiesis by depleting the pool of DCPC’s. This effect must either be toxic or must result from a strong stimulus to differentiation, thereby reducing the number of multipotent stem cells.

ACKNOWLEDGMENT

We wish to thank Lawrence Cook, Shirley Dunwoody, Wenche Heimli, and Randi Andreassen for excellent technical assistance.
REFERENCES


27. Curry, J. L., Trentin, J. J., and Wolf, N.: Hemopoietic colony studies. II. Erythro-


Kinetics of Cell Proliferation of Murine Bone Marrow Cells Cultured in Diffusion Chambers: Effect of Hypoxia, Bleeding, Erythropoietin Injections, Polycythemia, and Irradiation of the Host

Arne Boyum, Arland L. Carsten, Ole Didrik Laerum and Eugene P. Cronkite

Updated information and services can be found at:
http://www.bloodjournal.org/content/40/2/174.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml