Effect of Erythropoietin on Proliferation of Erythroid Stem Cells in the Absence of Transplantable Colony-forming Units

By Kurt R. Reissmann and Sommart Samorapoompichit

A single administration of Myleran eradicated colony-forming stem cells from marrow or spleen of mice for a period of two weeks or longer. Erythropoiesis was restored during that time, and the exclusion of cell influx from the colony-forming progenitors into the intermediary erythroid stem cell compartment permitted a study of the effects of erythropoietin on the kinetics of the latter. The results indicate that erythropoietin, in addition to its role in transforming immediate precursors into proerythroblasts, stimulated the proliferation of erythroid stem cells. The significance of this in regard to stem cell kinetics and rate regulation of erythropoiesis is discussed.

Erythroblasts represent a non-self-sustaining population, and continuous erythropoiesis requires influx of cells from less differentiated precursor compartments. The latter, referred to as stem cells, can not be identified morphologically, but are circumscribed by two basic observations: (1) Injection of marrow cells into lethally irradiated mice produces in their spleens colonies containing large numbers of erythroid, myeloid, or megakaryoid cells. Sufficient evidence indicates that each colony is derived from a single primitive stem cell. (2) Erythroblasts, absent in marrow of polycythemic mice, appear within 18 hours after injection of erythropoietin. This latter process was at first thought to represent the actual differentiation of a primitive pluripotential stem cell into a highly specialized erythroblast. Subsequent observations, however, have suggested more complex cytokinetics and led to the theory of two consecutive stem cell compartments, one capable of forming hematopoietic colonies, and the second committed to erythroid development. The origin, boundaries, cell kinetics and regulation of this intermediary erythroid stem cell compartment are little...
understood, and its existence has been questioned by those who believe that the immediate precursor of the proerythroblast is a primitive stem cell occupying a particular phase of its cell cycle during which it can be induced into erythroid differentiation.6,7

Erythropoietin-responsive cells, the immediate precursors of proerythroblasts, can be estimated by a test introduced by Gurney et al.8 It is based upon the observation that the rate of erythropoiesis elicited by a standard test dose of erythropoietin in a polycythemic mouse is proportional to the number of erythropoietin-responsive cells present at the time of injection. By employing this test, radiation or cytotoxic drugs were found to reduce these cells,8,9 whereas erythropoietin, given one or several days before the test dose, increased the response, presumably by increasing the number of responsive stem cells.10-12 The latter observation is of interest in regard to the mode of action of erythropoietin because it suggests the possibility that the level of erythropoietin not only exerts a rate regulation by transforming a proportional number of stem cells into proerythroblasts, but also by regulating the number of responsive stem cells. An enhancing effect of erythropoietin on the restoration of erythropoiesis was also demonstrated after its suppression by vincristine,5 5-fluorouracil, 14 or actinomycin D,15 and we have shown16 that the effect is not due to storage of erythropoietin in the regenerating cells or of information imparted by it. A recruitment of immediate precursors of proerythroblasts by erythropoietin thus appears likely, and the present study was undertaken to obtain further information on that effect and its underlying mechanism. According to the “one stem cell” theory, such an increase in erythropoietin-responsive cells could be brought about by inducing primitive stem cells to enter the responsive cell phase, or by lengthening this phase. In terms of the “two stem cell compartments” theory, a transformation of a greater number of colony-forming cells into erythroid stem cells would increase their number, or erythropoietin could stimulate the proliferation of erythroid stem cells. In this study, an experimental model is presented which permits further exploration of these possibilities. A single administration of busulfan (Myleran) to mice was found to result for a period of two weeks in the absence of transplantable stem cells (CFU) from their marrows or spleens. Erythropoiesis was wiped out for a few days, but it could be restored, and the effect of erythropoietin on the number of erythroid stem cells could thus be examined under conditions which eliminated their increase through influx of cells from primitive precursors.

**Materials and Methods**

Female MF1 (Manor Farm) mice (20 to 25 Gm.) received by stomach tube 60 mg. Myleran per kilogram of body weight. The drug was dissolved in a small quantity of acetone and then diluted with corn oil. Erythropoietin * was injected subcutaneously. For measurement of colony-forming units (CFU), femoral marrow was collected from one to 22 days

* Supplied by the Erythropoietin Committee of the National Heart Institute. The erythropoietin was procured by the Department of Physiology, University of the Northeast, Corrientes, Argentina, and processed by the Hematology Research Laboratories, Children’s Hospital of Los Angeles.
Table 1.—Colony-forming Units in Marrow of Mice After 60 mg./Kg. Myleran (Explantation Method)

<table>
<thead>
<tr>
<th>Days After Myleran</th>
<th>Cells per Femur x 10^6</th>
<th>Fraction of Femur Injected</th>
<th>No. of spleen colonies per group</th>
<th>Spleen colonies per femur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>21.6±2.4</td>
<td>1/100</td>
<td>22</td>
<td>278</td>
</tr>
<tr>
<td>1</td>
<td>14.4±3.3</td>
<td>1/6</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>2.6±1.2</td>
<td>1/20</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2.0±0.8</td>
<td>1/4</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>2.5±0.9</td>
<td>1/4</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>3.8±4.1</td>
<td>1/4</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>4.4±2.4</td>
<td>1/10</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>5.4±2.8</td>
<td>1/5</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>7.7±3.1</td>
<td>1/10</td>
<td>31</td>
<td>13</td>
</tr>
<tr>
<td>10</td>
<td>6.8±3.4</td>
<td>1/10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>18</td>
<td>6.8±3.4</td>
<td>1/3</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>22</td>
<td>12.3±4.1</td>
<td>1/5</td>
<td>26</td>
<td>255</td>
</tr>
<tr>
<td>No marrow</td>
<td>0</td>
<td>22</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

after Myleran administration. The marrow was flushed out of the bone with Hank's solution as completely as possible, and a cell suspension of the pooled marrow of at least ten mice was made by drawing it back and forth through a 22 gauge needle. Nucleated cells were counted in a Coulter Counter using Cetrimide as an erythrocyte-lysing agent. One-half ml. of each cell suspension, representing a suitable fraction of one femoral marrow, was injected in the tail vein of each of 50 lethally irradiated mice (950 rad total body radiation, 60Co source). Ten days later the recipients were killed and the spleens fixed in Bouin's solution. Visible colonies on the surface of the spleen were counted and their number expressed as per one donor femur. In three experiments, spleen cells were obtained from mice given Myleran two, four, or eight days before, and a volume of cells equivalent to one-tenth of the spleen was injected into each of the radiated recipients. Endocolonization experiments were carried out by irradiating mice at from 1 to 14 days after Myleran administration. The mice were anesthetized with Nembutal and, in each experiment, a group of 40 mice was taped on a board in such a manner that the left lower leg of each was shielded by 2 inches of lead during irradiation (950 rad, 60Co source). Their spleens were removed 11 days after radiation for colony counts.

Erythropoietin-sensitive stem cells were assayed in mice made polycythemic by three weeks exposure at 390 to 340 mm. Hg barometric pressure. Two days after their removal from the decompression chamber, the mice received Myleran and erythropoietin as described. They were then divided into groups of eight mice, and serial measurements of their erythropoietic response were made by injecting a test dose of 1 unit of erythropoietin per mouse in each group. Each mouse received 0.5 µCi 55Fe 48 hours later, and 55Fe incorporation was ascertained on heart blood collected 24 hours thereafter. Blood volume was calculated as 7 per cent of body weight. Data from mice with hematocrits below 58 per cent at time of counting were discarded. In the data presentation, the 55Fe incorporation reported on days 10/11 means injection of 55Fe on day 10 after Myleran and its counting 24 hours later. Estimates of marrow cell composition were made from the total nucleated femur count and differential counts on 2000 cells.

**RESULTS**

**Colony-forming Units**

The absence of transplantable CFU in the bone marrow of mice which had received Myleran from 1 to 10 days prior to marrow collection is seen in
Table 2.—Spleen Colonies in Myleran-treated Mice*

<table>
<thead>
<tr>
<th>Radiated days after Myleran</th>
<th>Spleen colonies 11 days after radiation</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>57</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>0.2</td>
<td>65</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>17</td>
</tr>
<tr>
<td>10</td>
<td>3.3</td>
<td>12</td>
</tr>
<tr>
<td>12</td>
<td>8.7</td>
<td>24</td>
</tr>
<tr>
<td>14</td>
<td>13.2</td>
<td>11</td>
</tr>
<tr>
<td>No Myleran</td>
<td>&gt;50</td>
<td>12</td>
</tr>
</tbody>
</table>

* Endocolonization: 950 R, left lower leg shielded.

Table 1. Marrow taken 14 days after Myleran produced a few colonies, and the latter rose to 49 per one femoral marrow obtained 22 days after Myleran administration. In three experiments, spleen cells were obtained on days 2, 4, and 8, respectively, after Myleran and injected in amounts equivalent to one-tenth of one spleen per recipient. No colonies were found in the latter. In order to exclude possible artifacts induced by the extracorporeal handling of cells, measurements of CFU in the marrow of Myleran-treated mice were also made by the endocolonization method (shielding of one lower leg during radiation). In control mice without Myleran, a rapid colonization took place and already, seven days after irradiation, their spleen colonies were too numerous and confluent to be counted. As seen in Table 2, no significant endocolonization was found in mice irradiated from 1 to 10 days after Myleran administration.

Marrow Composition After Myleran

The total number of nucleated cells in femoral marrow declined from $21.6 \times 10^6$ before to $2.3 \times 10^6$ on day four after Myleran (Table 1). This rapid decline was mainly due to the nearly complete disappearance of erythroblasts and to a marked reduction in nondividing myeloid cells (Fig. 1). Myelocytes showed a slow but progressive decrease, and a severe peripheral neutropenia was present after the second week. Erythroblasts, practically absent within 72 hours after Myleran, reappeared around day 8, and a significant peripheral reticulocytosis was noted on day 12. The appearance of erythroblasts was accompanied and preceded by a large but variable number of small lymphoid-like cells with very small or no visible cytoplasm.

$^{59}$Fe Incorporation

Figure 2 shows the results of serial $^{59}$Fe incorporation measurements in groups of polycythemic and normal mice after Myleran. The hemoglobin concentration in the latter remained in the normal range until the end of the second week. There was no detectable $^{59}$Fe incorporation from days 4 to 7 after Myleran. A sharp rise was noted on days 8/9, and the $^{59}$Fe reached a peak of more than 50 per cent of normal on days 12/13 after Myleran.
Fig. 1.—Marrow composition and peripheral blood counts in mice after 60 mg. Myleran per kilogram.

Thereafter, the $^{59}$Fe dropped and showed a second rise on days 20/21. Measurements later than day 16, however, are not entirely reliable because of a bleeding tendency in the mice resulting from severe thrombocytopenia. The recovery of erythropoiesis was markedly accelerated and accentuated by large doses of erythropoietin. Groups of polycythemic mice, given 6 units erythropoietin daily after Myleran, showed the first reappearance of proerythroblasts in their marrows on day 4. On subsequent days, the marrow became distinctively erythroblastic and the 24-hour $^{59}$Fe incorporation rose to 25 per cent on days 8/9 after Myleran. Injections of 1 unit of erythropoietin daily after Myleran resulted in an erythropoiesis very similar to that seen in nonpolycythemic mice. Polycythemic mice which received no daily erythropoietin injection after Myleran responded to the standard test dose of 1 unit given 48 hours before $^{59}$Fe with very minor increases in iron incorporation. The latter rose from 0.2 per cent on days 5/6 to 1.5 per cent on days 12/13, and very few proerythroblasts appeared in their marrows or spleens. All polycythemic mice required red cell transfusions after day 12 in order to maintain the polycythemia.

Table 3 shows experiments in which the erythropoietin administration was
limited to three days. In column 1, each group of mice received 8 units erythropoietin daily on days 1, 2 and 3 after Myleran, and each group was given an additional dose of 1 unit 48 hours before the $^{59}$Fe injection. No erythroblasts were seen in the marrow of these mice until day 5 after Myleran, i.e., 96 hours after injection of a relatively large dose of erythropoietin, and their 24-hour $^{59}$Fe incorporation rose sharply after day 7. In the experiments reported in column 2, groups of mice received 8 units erythropoietin on days 1, 2 and 3, but no further erythropoietin before $^{59}$Fe injection. These animals showed a few early erythroblasts in their marrows on day five but the subsequent erythroblastosis seen in the groups of column 1 was absent, and their iron incorporation was much lower than that in the former. In column 3, groups of mice received 8 units of erythropoietin on days 3, 4 and 5 after Myleran and 1 unit, 48 hours before $^{59}$Fe injection. Fewer erythroblasts appeared in their marrow and the maximal iron incorporation reached only one-third of that in column 1.
Table 3.—Per Cent $^{59}$Fe Incorporation in Polycythemic Mice Given 60 mg./Kg. Myleran on Day 0

<table>
<thead>
<tr>
<th>Day $^{59}$Fe Injected/ Counted</th>
<th>(1) 8 units Erythropoietin, days 1, 2, 3 + 1 unit Erythropoietin†</th>
<th>(2) 8 units Erythropoietin, days 1, 2, 3 + 1 unit Erythropoietin†</th>
<th>(3) 8 units Erythropoietin, days 3, 4, 5 + 1 unit Erythropoietin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/4</td>
<td>1.4±0.3</td>
<td>0.3±0.2</td>
<td></td>
</tr>
<tr>
<td>4/5</td>
<td>1.4±0.2</td>
<td>2.8±1.0</td>
<td></td>
</tr>
<tr>
<td>5/6</td>
<td>3.3±0.3</td>
<td>3.0±0.6</td>
<td>1.1±0.6</td>
</tr>
<tr>
<td>7/8</td>
<td>10.5±2.9</td>
<td>2.9±0.7</td>
<td>3.6±1.3</td>
</tr>
<tr>
<td>8/9</td>
<td>16.1±2.7</td>
<td>1.1±0.6</td>
<td></td>
</tr>
<tr>
<td>9/10</td>
<td>16.5±4.5</td>
<td></td>
<td>5.9±2.3</td>
</tr>
<tr>
<td>10/11</td>
<td>14.6±4.6</td>
<td>2.6±1.4</td>
<td>4.2±2.1</td>
</tr>
<tr>
<td>11/12</td>
<td>9.3±1.4</td>
<td>2.9±1.9</td>
<td>5.9±3.1</td>
</tr>
<tr>
<td>12/13</td>
<td></td>
<td></td>
<td>5.8±2.8</td>
</tr>
</tbody>
</table>

* Mean and SEM, eight mice per group.
† 1 unit erythropoietin given 48 hours before $^{59}$Fe.

**Transplantation of Erythroid Stem Cells**

Several attempts were made at transplanting the erythroid precursors which gave rise to the described waves of erythropoiesis. Marrow from Myleran-treated animals, given 8 units erythropoietin daily, was collected from four to 10 days after the drug and was injected into lethally radiated recipients which received 1 unit of erythropoietin daily to insure normal erythropoietin levels. $^{59}$Fe incorporation was measured in recipients at from five to 11 days after cell injection and their spleens and marrows examined for presence of erythroblasts. No erythropoiesis was detected and the results are therefore not presented in detail.

**Discussion**

The alkylating agent Myleran is thought to prolong the intermitotic interval possibly by forming cross-links between DNA strands. The absence of demonstrable colony-forming units (CFU) in marrow or spleen for nearly two weeks after its administration indicates that the drug either killed or sterilized most or all of these cells, and thus prevented their forming hemopoietic spleen colonies, which requires cell multiplication and differentiation. Thus, the erythropoietic recovery in Myleran-treated mice toward the end of the first week could not have resulted from a differentiation and subsequent multiplication of CFU into erythropoietin-sensitive cells. On the other end of erythroid development, there were no erythroblasts present, and the erythroid recovery could only have originated in the intermediary compartment, namely, erythroid stem cells. The reason for their faster regeneration than that of CFU is still under investigation. Aside from possible differences in drug uptake, sensitivity or disposal, the erythroid stem cells may be endowed with inherently shorter generation times, and the few erythroid stem cells which escaped the killing or sterilizing action of the drug were thus capable of building up demonstrable population levels at an earlier date than the slower multiplying CFU. The principal finding, however, is the marked acceleration of erythroid stem cell regeneration by erythropoietin and
the discernible dose-response relation. Since the experimental parameters
excluded other possibilities, the conclusion is inescapable that erythropoietin
stimulated proliferation of the erythroid stem cells which ultimately fur-
nished the cells that were transformed into erythroblasts. The effect was
observed on regenerating marrow, but the recruitment of erythropoietin-sensi-
tive stem cells by erythropoietin under conditions not involving regenera-
tion\textsuperscript{11,19–21} suggests the general applicability of the present observations. The
stimulatory effect of erythropoietin on the rate of division of erythroid stem
cells is complimentary to its well documented role in transforming erythro-
poietin-sensitive stem cells into proerythroblasts. This dual action is empha-
sized by the data in columns 1 and 2 of Table 3. Injection of erythropoietin
on days 1, 2 and 3 after Myleran obviously sufficed to restore a sizable popu-
lation of erythropoietin-sensitive stem cells, and the small standard dose
injected on day 5 induced a considerable wave of erythropoiesis. Without
this late dose of erythropoietin, the restored erythroid stem cells did not
progress into the erythroblast stage and practically no iron incorporation
was seen. The presence of erythropoietin was thus required over a period of
five or more days after Myleran, suggesting a continued action of the hor-
mone on several generations of erythroid stem cells, including the latest,
which is transformed into proerythroblasts. It remains to be seen whether
this multifocal action is exerted by one hormone or whether erythropoietin
contains several biologically active moieties, as it is suggested by the recently
reported differences in the activities of various erythropoietin preparations
when measured in vivo versus different in vitro assays.\textsuperscript{22}

The presented data also show the limitations of the erythropoietin-stimu-
lated proliferation of erythroid stem cells in regard to the maintenance of
a normally responding population. In the nonpolycythemic mice, the \(^{59}\)Fe
incorporation reached a peak of more than 50 per cent of normal on days
12/13 after Myleran, but the number of available erythropoietin-sensitive
stem cells declined thereafter as indicated by the lower incorporation of \(^{59}\)Fe
injected on days 14 through 18. Very similar results were obtained in poly-
cythemic mice given 1 unit of erythropoietin daily. An abortive erythro-
poietic recovery after Myleran in rats has also been described earlier by
Elson.\textsuperscript{21} We attribute this inability of maintaining high population levels of
erthroid stem cells to an imbalance between outflow (into erythroblast com-
partment) and proliferation rate. The latter remained subnormal either due
to a lingering effect of the drug or, more likely, due to the lack of an influx
of cells from the primitive precursor compartment. It was sufficient, however,
to build up a demonstrable population of erythropoietin-responding cells as
long as their outflow rate was small, but when larger numbers were trans-
formed into erythroblasts, the pool size decreased.

No significant number of erythropoietin-sensitive stem cells was regener-
ated within 14 days after Myleran in polycythemic mice given no erythropoietin.
This finding does not necessarily imply the necessity of erythropoietin
for the normal multiplication of erythroid stem cells. It merely indicates that
erythropoietin is required to restore a severely decimated population, and
the finding is not in contradiction to the perseverance of erythropoietin-
sensitive stem cells during prolonged periods of suppressed erythropoietin formation in polycythemic mice. In the latter, the influx from the primitive progenitor compartment might continue and this alone could maintain an erythroid stem cell population. It is not known whether the latter is of normal size in the polycythemic mouse, and the relative contribution of progenitor transformation in regard to the physiological maintenance of the erythroid stem cell pool thus remains undecided.

**Conclusions**

1. Erythroid stem cells exist as a distinct compartment comprising several successive generations of cells with probably relatively short cell cycles.
2. Erythropoietin effects the transformation of their latest generation (erythropoietin-sensitive cells) into proerythroblasts, but it also enhances the proliferation of earlier generations of erythroid stem cells. Both these processes provide the rate regulation of erythropoiesis, and, moreover, the same agent which causes a greater outflow of erythroid stem cells into the erythroblast compartment also stimulates their proliferation and thus provides at least a degree of stability of the erythroid stem cell population. (3) It remains to be decided whether the erythroid stem cells require erythropoietin for their normal proliferation or whether it acts as an accelerator when present in high concentrations. (4) It is likewise unclear whether erythroid stem cells are self-sustaining under physiological conditions or whether they require a continuous inflow from a more primitive compartment. After their decimation by cytotoxic drugs, influx appeared necessary for a permanent restoration of a normally responding cell population. Requirements and regulation of this transformation in erythroid stem cells are obscure. It may well entail a random process proceeding at a nearly fixed rate, whereas the rate regulation sets in at later stages as described above. (5) The concept of erythropoietin exerting its sole action during the differentiation of a primitive, pluripotent cell into a proerythroblast is untenable. At increased levels, erythropoietin stimulates proliferation of erythroid stem cells, possibly through several generations, and the derepression of genetic information, which it most likely induces, pertains to cell division as well as to differentiation.

**References**

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