Effect of Bleeding on Hematopoiesis Following Irradiation and Marrow Transplantation

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In previous studies, bleeding after irradiation did not affect the rate of regeneration of endogenous spleen colony-forming cells, but induced an early (4-6 days after irradiation) appearance of erythrocytic colonies which differentiated and disappeared by days 7-8. This “abortive” wave was associated with a similarly abortive wave of splenic $^{59}$Fe uptake. The present experiments were done to determine whether or not an abortive wave of erythropoiesis could be induced in the transplanted, exogenous stem cell system. Lethally irradiated mice were given normal bone marrow cells and one-half of the group were bled of about one-third their blood volume within 4 hr of irradiation. Groups were killed on days 3-10 after irradiation. Seventeen to twenty hours prior to killing, $^{59}$Fe was injected. Hematocrits, spleen weights, colony numbers, and per cent $^{59}$Fe uptake were determined. Hematocrits of bled mice averaged about 70% of those of cell-injected controls. Spleen weights, colony counts, and per cent $^{59}$Fe uptake per spleen began to increase about 1 day earlier in bled mice (days 4-5 as compared to days 5-6), and rates of increase were the same as those of controls. However, no abortive wave of erythropoiesis was detected. A large cell dose resulted in earlier increases in all parameters than a small dose. Thus, bleeding after injection of cells produced results similar to those obtained by increasing the cell dose. The inability of bleeding to induce an early abortive wave of erythropoiesis in transplanted as compared to endogenous colony-forming systems may reflect differences in the cell cycling characteristics of these systems.

IN PREVIOUS STUDIES the rate of regeneration of endogenous colony-forming cells (E-CFU), as assayed by subjecting mice to two doses of radiation separated by various intervals and counting spleen colonies 10 days after the last exposure, was found to be unchanged by differentiative stimuli such as bleeding after the first exposure. As long as the radiation dose was high enough to reduce the E-CFU compartment below about 10% of its normal size, increasing the radiation dose did not affect the rate of regeneration, but did delay the time of onset of differentiation as measured by $^{59}$Fe uptake per spleen. These studies and those of others suggest that after severe depletion of the E-CFU compartment, self-replication begins immediately and continues without differentiation until the compartment reaches a minimal level of about 10% normal, and thereafter both differentiation and regeneration occur. There is an interval immediately after irradiation, however, when appropriate stimuli can cause a brief wave of differentiation. When mice were bled shortly after a single radiation and studied at daily intervals, a large early increase in colony counts ap-
peared and peaked on the fifth day. A similar increase in iron uptake appeared and peaked on the sixth day. These then declined to a minimum on the seventh and eighth days, and then a second increase occurred at the same time as in the controls. Similar abortive waves of erythropoiesis were seen by others when mice were stimulated by bleeding or injections of erythropoietin after busulfan treatment.4

The purpose of the present studies was to determine whether or not exogenous CFU could be induced to produce a similar early wave of erythropoiesis in response to bleeding shortly after their transplantation into lethally irradiated recipients.

MATERIALS AND METHODS

Mice were (C57 Bl/6J × DBA/2)F1 bred in this laboratory from stock purchased from Jackson Laboratories. Groups of female mice were balanced as to age (12-16 wk) and weight (21-24 g) and given food and HCl water (pH 2.4) ad libitum. The radiation source was 137Cs, and mice were exposed in a plastic chamber at a dose rate of 50-60 rads/min (Fricke ferrous sulfate dosimetry). Recipient mice were exposed to 900 rads and given a suspension of normal bone marrow cells intravenously within 2 hr. Three to four hours after cell injection, one-half the mice were bled by puncturing the retro-orbital sinus and removing approximately 15 drops (0.4 ml) of blood. Groups of mice were killed on days 3-10, 17-20 hr after the intraperitoneal injection of 0.1 μCi of 59FeCl3 solution in citrate. Hematocrit, body weight, and spleen weight were determined, and spleens were placed in Bouin’s fixative. Per cent iron uptake was determined from scintillation counts of spleens and aliquots of injected 59Fe.

For preparation of bone marrow cells for injection, marrow cells of one humerus were washed quantitatively6 into a known quantity of ice-cold Hanks’ medium, diluted to give a certain part of a humerus counted with a Coulter Model B electronic particle counter, and injected intravenously in 0.5-ml volumes.

Statistical evaluations include calculations of mean ± SE for all groups. When experiments were repeated, weighted means were calculated, and estimates of standard errors were calculated to include both between and within experiment sources of error.7 Statistical evaluation was done by paired t tests for experimental and control groups of the same experiment.

Times of onset of increases in spleen weight or per cent Fe uptake were estimated as described previously.2 A “no-erythropoiesis” level was established by studying mice given 900 rads and no marrow. They have a small but significant uptake of iron which reaches a minimum on day 2 after irradiation, and thereafter neither iron uptake nor spleen weight increases until death. In mice given marrow, iron uptake initially declines to, or near, the no-erythropoiesis baseline. However, at some point thereafter it begins to rise. A least squares fit for the period of increasing values was done, and the resultant line was extrapolated to the no-erythropoiesis level. The intercept of these two lines was considered the time of onset. When ratios were calculated, standard errors were estimated, taking into account variation of both numerator and denominator.8

RESULTS

In five repeated experiments, hematocrits were determined on days 3-10 after mice were exposed to 900 rads and injected with 1/75 of the cells of a humerus (mean of 1.2 × 103 cells) from a normal donor (Fig. 1). In two additional experiments, 1/40 humerus and 1/160 humerus or no cells were injected. Bleeding within 4 hr after injection of 1/75 humerus reduced the hematocrit at the time of killing to about 70% of controls. The lines shown in Fig. 1 represent the expected rate of decrease from normal red cell death when there was no replacement by red cell production, assuming a mean red cell life span of 41.5 days.9 Both control and bled groups seem to follow this line for 6 days, with some
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Fig. 1. Hematocrit in bled and nonbled lethally irradiated, marrow-injected mice at various times after treatment. Per cent hematocrit was determined on blood collected from the retro-orbital sinus just before killing mice on days 3-10 after exposure to 900 rads (X) and injection of 1/75 (e), 1/40 (m), or 1/60 (a) of the cells of a humerus of a normal donor, or 900 rads and 1/75th humerus plus bleeding of 15 drops within 4 hr of cell injection (c). Lines represent the rate of decrease in hematocrit expected if red blood cells that die are not replaced. Points represent mean ± 1 SE for groups of 5-10 mice in repeated experiments.

Evidence of renewed erythropoiesis thereafter (values fell above the line). There was no apparent difference between the rate of change in hematocrit in bled and control groups or between controls receiving different quantities of cells.

The effect of bleeding on changes in iron uptake per spleen, spleen weight, and colonies per spleen in five experiments is shown in Fig. 2. These mice were exposed to a lethal dose of radiation, injected intravenously with 1/75 of the cells from the humerus of a normal donor, and either bled or not bled. The per

Fig. 2. Effect of bleeding on hematopoiesis in lethally irradiated, marrow-injected mice. Changes in per cent 59Fe uptake per spleen (A), spleen colony count (B), and spleen weight (C) 3-10 days after irradiation and cell injection. Points represent weighted mean values ± 1 SE calculated to take into account both between and within experimental errors for one experiment on day 3, three on day 5, five on days 5-8, and two on days 9 and 10. Each group in each experiment represents 5-10 mice, and weighted means represent 14-47 mice. Half of the groups received 900 rads followed within 2 hr by the intravenous injection of 1/75 of the cells of a normal humerus (mean cell number injected was 1.2 × 10^7) and are represented by open circles (c). The rest of the groups received that treatment, plus 15 drops of blood were removed from their retro-orbital sinus 3-4 hr after cell injection (e). Colony counts for bled animals are not shown on days 8-10 as many of the spleens could not be included because of coalesced colonies.
cent $^{59}$Fe uptake for both bled and control groups remained at the baseline (no-erythropoiesis) level for several days, and the mean time of onset of increased uptake was earlier in bled groups (4.9 ± 0.24 days) as compared to controls (6.0 ± 0.29) (Fig. 2A). The mean difference in times of onset for the five experiments was 1.1 ± 0.32 days ($p < 0.02$). The counts in bled mice remained above those of controls through day 8. Colonies first became visible on the fourth day after transplantation (Fig. 2B). The number of colonies increased through day 6 and tended to level off between days 7 and 10. The average time of onset of increase from the 0.13 colony level of 900-rad controls was 2.66 ± 0.34 days for bled as compared to 3.86 ± 0.25 days for controls, and the average difference in times of onset for the five experiments was 1.19 ± 0.21 days ($p < 0.005$). Spleen weights in bled mice were significantly greater than their paired controls on days 6–10 (Fig. 2C). With the baseline value of 21.0 ± 0.45 mg, the mean times of onset of weight increases were 6.3 ± 0.19 days for controls and 5.6 ± 0.22 days for bled ($p < 0.05$). The average difference in times of onset of increase averaged 0.69 ± 0.18 days for the five experiments ($p < 0.02$). In another experiment (not shown) mice were bled 24 hr after transplantation and compared to groups bled at 3 hr or not bled. Bleeding at 24 hr hastened onset of iron uptake and spleen weight to the same extent as did bleeding at 3 hr, but colonies were not apparent any earlier than in nonbled controls.

Since onset of proliferation is known to be delayed for 24–48 hr after transplantation, it seemed possible that bleeding soon after transplantation caused an early onset of stem cell proliferation which then resulted in the described effects. Ara-C is known to kill cells in DNA synthesis. We therefore tried to
prevent any early onset of erythropoiesis by injection of ara-C after bleeding. Ten milligrams of ara-C were given to irradiated recipients 3, 12, or 24 hr after injection of 1/75 of the cells of one humerus (1.7 × 10^5 nucleated cells). Mice were bled 3 hr after cell injection. Groups of mice were killed 4–8 days after irradiation; spleen colonies, spleen, weight, and per cent ^59^Fe uptake per spleen were measured (Fig. 3A, B, D). Colony counts of bled mice given no ara-C increased through day 6 and then leveled off (Fig. 3A). Injection of 10 mg of ara-C was increasingly effective at reducing the number of colonies as the interval between cell and drug injection increased. Colony counts of ara-C-injected groups continued to increase through day 8, while those of nontreated controls leveled off or even decreased somewhat between days 7 and 8, resulting in a smaller difference between groups on these later days than on earlier days (Fig. 3C).

The time of onset of erythropoiesis was delayed by ara-C injection as judged by ^59^Fe uptake in the spleen (Fig. 3D). Spleen weight increases were also delayed by ara-C injection. The differences were about 1 day for the groups given ara-C at 24 hr.

Colony counts after ara-C expressed as per cent of non-ara-C-treated control counts for bled and nonbled recipients are shown in Table 1. The effects of ara-C were similar in mice which were or were not bled and, in fact, there is a suggestion that its effect may have been less, rather than greater, in bled as compared to nonbled mice.

To see whether or not bleeding had the same effect on patterns of erythropoiesis as increasing the cell dose, two experiments were done in which growth of cells of 1/160 humerus was compared to growth of four times that number from the same donor. Weighted mean values for per cent iron uptake per spleen, spleen weight, and visible spleen colonies are shown in Fig. 4 (A, B, and C). Onset of increases began roughly 1 day earlier in the groups given the larger cell dose. Onset of erythropoiesis, as judged by spleen ^59^Fe, began at 5.9 ± 0.07 days in mice given 1/40 of a humerus and at 6.6 ± 0.36 days in mice given 1/160 of a humerus. Once iron began to increase, the rate of increase was similar in the two groups, with the rate of increase declining between days 9 and 10.

### Table 1. Comparison of Effects of Cytosine Arabinoside (10 mg) Given After Irradiation, Cell Injection, and Bleeding or Not Bleeding

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Time of Ara-C Injection</th>
<th>Colonies as % of Control* ± 1 SE†</th>
<th>Days Recipients Killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bled 1</td>
<td>24 hr (10)</td>
<td>19 ± 8.8* (20) 39 ± 7.3 (20) 56 ± 8.6 (14) 65 ± 13.1</td>
<td></td>
</tr>
<tr>
<td>Nonbled 1</td>
<td>24 hr (10)</td>
<td>11 ± 8.1* (20) 21 ± 8.1 (19) 31 ± 4.8 (16) 71 ± 11.9</td>
<td></td>
</tr>
<tr>
<td>Bled 2</td>
<td>12 hr (19)</td>
<td>53 ± 9.9* (18) 68 ± 10.4 (18) 67 ± 6.1 (20) 86 ± 7.9</td>
<td></td>
</tr>
<tr>
<td>Nonbled 2</td>
<td>12 hr (19)</td>
<td>15 ± 8.4* (19) 32 ± 8.5 (17) 43 ± 5.8 (18) 74 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>Bled 3</td>
<td>3 hr (18)</td>
<td>74 ± 16.6* (16) 86 ± 10.2</td>
<td></td>
</tr>
<tr>
<td>Nonbled 3</td>
<td>3 hr (15)</td>
<td>128 ± 79.1* (16) 72 ± 11.5</td>
<td></td>
</tr>
</tbody>
</table>

*Control received 900 rods and 1/75 humerus, was bled or not bled for appropriate groups, but received no ara-C. The results in the nonbled groups have been published previously. 
†SEs were calculated to include both variation of numerator and denominator. 
‡Numbers in parentheses are total numbers in control and experimental groups.
Fig. 4. Effect of changing cell dose on hematopoiesis in lethally irradiated recipients. Changes in per cent $^{59}$Fe uptake per spleen (A), spleen weight (B), and spleen colony count (C) 3–10 days after irradiation and cell injection are shown for two different cell doses. Points represent weighted mean values ± 1 SE calculated to take into account both between and within experimental errors for two repeated experiments. Weighted means represent 14–20 mice. The groups received either 900 rads plus cells of 1/40th humerus (e) (mean cell number injected was $1.6 \times 10^8$), or 900 rads plus cells of 1/160th humerus (o) (mean cell number, $0.4 \times 10^8$), or 900 rads alone (line at the bottom of curves A and B). Mean values 10 days after 900 rads to controls were $21 \pm 1$ mg spleen weight, $0.49 \pm 0.058$ per cent Fe uptake per spleen, and $0.28 \pm 0.12$ colonies per spleen. Colony counts are not shown on days 9–10 in the group receiving the high cell dose because coalescing colonies prevented accurate enumeration.

Spleen weights increased in both groups through day 10, and colony counts leveled off after day 6 for the large cell dose and tended to slow after day 5 but continued to increase through day 9 in the mice receiving the smaller cell dose of nucleated cells. Thus, increasing the cell dose resulted in earlier onset of erythropoiesis, as did bleeding, but, unlike bleeding, increasing the cell dose caused an increase in 7–10-day colony count.

DISCUSSION

The effect of bleeding upon patterns of erythropoiesis from colony-forming cells of exogenous source (CFU) is quite different from that from endogenous colony-forming cells (E-CFU). Bleeding$^1$ or erythropoietin injection$^2$ induces a very early but abortive wave of colonies and erythropoiesis from E-CFU. Similar abortive waves follow erythropoietic stimulation after stem cell depletion by busulfan injection.$^4$ Following the decline of the abortive wave, a second wave of colonies and erythropoiesis was observed, and the time of onset of that wave was not consistently different after bleeding. Conversely, the present results provide no evidence for an abortive wave from CFU induced by bleeding, but the eventual time of onset of colonies and erythropoiesis was hastened.

Based on observations in the E-CFU system, the following hypothesis concerning control of stem cell differentiation was formulated.$^1$ Very shortly following stem cell depletion by irradiation, a strong differentiative stimulus (bleeding) can induce stem cell differentiation leading to the abortive wave.
However, if this stimulus is delayed, no differentiation can be induced until the stem cell compartment proliferates to replenish approximately 10% of its original size. An alternative hypothesis to explain the abortive wave is that the colonies in that wave are from committed, erythropoietin-sensitive stem cells rather than from pluripotent stem cells. If this last hypothesis is true, as has been suggested, the lack of an abortive wave in the CFU system and in the E-CFU system when the stimulus is given 24 or 48 hr after irradiation must be explained. Possibly the stimulus causes extra divisions of committed stem cells sufficient to produce a colony, but if the stimulus does not appear soon after irradiation, differentiation of available cells renders them unable to respond by extra divisions at a later time. In this case the committed stem cells cannot be transplantable.

The observations can be explained within the context of the first hypothesis. The lack of an abortive wave in the CFU system may simply be due to the failure of cycling cells to be present shortly after irradiation and transplantation. The failure to produce an abortive wave of erythropoiesis in the E-CFU system by bleeding 24 hr or later after irradiation can also be explained by taking into account the cycling characteristics of these cells. Previous studies using split radiation exposures and cycle active drugs have produced evidence that E-CFU are in cycle and proliferate immediately after irradiation. Conversely, studies utilizing cycle active drugs or retransplantation from the recipient suggest that about 1 day elapses after transplantation before onset of proliferation of CFU. This is true even when the donor of the cells was irradiated, suggesting that stem cells left in situ are in cycle, but when they are transplanted proliferation is delayed.

It has been suggested that cells are susceptible to erythropoietin only during certain stages of the cell cycle, and Bedard and Goldwasser have evidence that this stage is G2. It is possible that by the time CFU come into cycle after transplantation, the hypothetical recognition signal for compartment damage is in effect, and differentiation does not occur until the stem cell compartment has regrown to a critical threshold size, then it is too late for an abortive rise despite increased erythropoietin.

Earlier onset of erythropoiesis in bled, transplanted mice could not be explained by bleeding inducing an earlier onset of cycling in the CFU compartment. Cytosine arabinoside, given within 24 hr of transplantation, had no more effect on CFU from bled mice than from nonbled. Therefore, the earlier onset of colony formation and erythropoiesis must reflect faster cell growth in colonies. An increased rate of migration of CFU to the spleen might occur in response to bleeding and lead to visible colonies at an earlier time, but unless CFU migrated to existing colonies the end result should be more colonies. Erythroid differentiation might have taken precedence over myeloid as a means of accelerating appearance of erythroid colonies, but unpublished studies from our laboratory have failed to show any diminution in neutrophil recovery rate in spleen or marrow of mice irradiated, transplanted, and bled. Accelerated growth rate of colonies would cause them to become visible at an earlier time, but not be more numerous at a later time, and such appeared to be the case in our experiments.
Increasing the cell dose fourfold resulted in an earlier onset of erythropoiesis similar to that caused by bleeding, but unlike bleeding, the number of late colonies was also increased. Thus, increasing the cell dose caused effects in the CFU system more like the effects seen in the E-CFU system when the irradiation dose was reduced; i.e., time of onset of erythropoiesis appears inversely proportional to stem cell compartment size.

In summary, substantial differences are observed in the effect of postirradiation bleeding on colony growth and erythropoiesis in the CFU and E-CFU systems. However, these differences can be explained by known differences in cycling characteristics between the two systems and within the context of the model of control of differentiation in stem cell compartments of subnormal size which we and others have proposed.

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