Patterns of Proliferation and Differentiation of Hematopoietic Stem Cells After Compartment Depletion

By Paul A. Chervenick and Dane R. Boggs

The time of onset and rate of self-replication in the irradiation depleted pluripotential hematopoietic stem cell compartment of mice was measured by a split-dose irradiation method. Mice were irradiated to reduce the compartment size and at daily intervals thereafter were again irradiated; 10 days after the second irradiation the number of endogenous spleen colonies was determined. As irradiation interval was lengthened, colonies increased in an exponential fashion with no apparent lag interval after the first irradiation. The doubling time of the colony-forming cell compartment was calculated as 16 hr if severely reduced in size by 300 R or more but was slower if less irradiation was given. The time of resumption of erythropoiesis following irradiation was measured after 100–900 R by determining uptake of radioactive iron into marrow, spleen, and circulating red cells each day after irradiation. With 200 R or less, erythropoiesis was not abolished. With higher doses there was an interval of no apparent erythropoiesis which increased by 1.6 days for each increment of 100 R. These results are compatible with a pluripotential stem cell compartment, which (1) begins self-replication almost immediately following compartment size reduction, and (2) will not differentiate if reduced in size below approximately 10% of normal.

Hematopoietic stem cells are those cells that are capable of self-replication as well as differentiation. The pluripotential stem cell compartment of the mouse, capable of differentiating into erythrocytic, granulocytic, and megakaryocytic tissue, can be studied by the spleen colony technique. Irradiation of mice results in depletion of the colony-forming unit (CFU) cell compartment as well as depletion of mature cell compartments. Irradiation survival is dependent upon regeneration of mature cell compartments.

Previously reported studies of the kinetics of repopulation of the CFU compartment have suggested that there is a lag of 1 to 3 or more days before the depleted CFU cell compartment begins self-replication. Such studies have been done by lethally irradiating mice, transplanting hematopoietic tissue, and

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at given times thereafter using the primary recipient's spleen or marrow cells as a transplant for a secondary, lethally irradiated recipient. The number of spleen colonies observed in the secondary recipient has been considered representative of the rate of self-replication of CFU cells in the primary recipient. However, as knowledge of the CFU system has accumulated, a number of observations raise questions as to how such transplant studies are to be interpreted: namely, the recovery curves for spleen and marrow may differ in the same animal;\(^6\)\(^9\) the fraction of transplanted CFU cells that lodge in the spleen can be influenced by perturbing the donor;\(^10\) CFU cells continue to circulate for some time after transplantation;\(^11\) the number of CFU cells in a femur is different if measured by transplantation as compared to measurement by migration after shielding the femur;\(^12\) the change in the number of femoral CFU after transplantation fails to correlate with changes in L.D.\(^{10}\);\(^13\) and, finally, the number of transplantable CFU cells in a given organ is not necessarily representative of the total number of CFU in the animal as measured by a variety of techniques.\(^2\)

DeGowin and Johnson\(^14,15\) studied the radiosensitivity of the erythroid compartment by a split-dose technique with the femur shielded during the first irradiation. They suggested that repopulation of the stem cell compartment might begin almost immediately after irradiation and furthermore that this might precede differentiation from the compartment. Weisman and co-workers\(^16\) studied self-replication rate in femur, tibia, and tail at various times after 200 R whole-body irradiation by then shielding that area of marrow while irradiating the rest of the animals. Within 2 days after 200 R there was evidence for regrowth of the femur CFU compartment, but there was no change for more than 4 days in the tibia and an even longer delay in the tail. The authors suggested these differences might reflect different rates of cell migration from the different areas.

We have reexamined the rate of self-replication of CFU cells and the time of onset of regrowth of differentiated erythroid cells in irradiated mice in an endogenous system, avoiding transplantation or shielding. Self-replication was measured by a split-dose irradiation technique in which the CFU compartment is reduced by the first exposure, is allowed to regrow for varying periods, and is again reduced by a second irradiation to a degree that endogenous colonies are in a countable range 10 days later. Limited data on colony regrowth employing this technique were reported by Till and McCulloch\(^17\) and by Chaffey and Hellman.\(^18\) Onset of differentiation was measured by determining changes in uptake of radioactive iron into marrow, spleen, and blood at varying intervals following a single irradiation.

**Materials and Methods**

Mice were (C57 BL female × DBA male) F\(_1\), bred in our laboratory from breeding stock purchased from Jackson Laboratories. Both male and female mice ranging in age from 7 to 14 wk were employed. However, in any given study all mice were of the same sex and within 2 wk of the same age.

Irradiation source was a Norelco NG 300-kV machine with 1 mm copper filtration. Mice were irradiated in a lucite chamber and positioned 35 cm from the source. As measured by a Victoreen-R-Meter, dose rate was 150 R/min. The system used to shield all of the
mouse except the spleen from irradiation has been described. Briefly, the mouse is encased within a thick lead shield. The spleen is exteriorized through a flank incision with the blood supply intact. The spleen is placed on top of a 3-mm-thick lead plate composing the top of the shield and kept moist with saline during irradiation. By this means, the spleen can be heavily irradiated while only a small amount of whole-body irradiation is delivered.

Iron uptake into marrow, spleen or blood was determined by injecting 0.1 μCi of radiactive iron (59Fe) intraperitoneally. The mice were killed 24 hr afterward, and the spleen and the femur or humerus stripped of all muscle, and were counted directly in a well-type gamma detector. Blood was obtained by orbital sinus puncture, the hematocrit was determined, and a sample was then weighed and counted.

Slopes of all lines were determined by least-squares fit. Slopes of increasing colonies, and the iron and spleen weight after a split dose of irradiation were clearly best described by a first-order equation. However, the iron uptake curves following single irradiation were often neither clearly exponential or arithmetic in shape. Least-squares fit in both types of slope was tested and the confidence of the slope was greater for an exponential than an arithmetic fit. Therefore, the slope was expressed from a first-order equation.

RESULTS

Splenic Hematopoiesis Following Two Whole-Body Irradiation Exposures

One hundred ten mice, weighing 23–27 g, were exposed to 395 R. At daily intervals thereafter, ten mice from the group were again exposed to 395 R. Ten days after the second exposure, the mice were killed.

![Graph showing the increase in number of colonies and spleen iron uptake as the interval between two whole-body irradiation exposures was increased. Mice were irradiated with 395 R and at intervals ranging from 1 hr to 10 days were again irradiated with the same exposure. Ten days after the second exposure, mice were killed, and the number of colonies in the spleen and uptake of 59Fe by the spleen were measured. Each point represents the mean of 8-10 mice and the brackets surrounding the point indicate the standard error of the mean.](image-url)
As exposures were spaced further apart, there was a rapid increase in splenic hematopoiesis (Fig. 1). The largest increment in number of colonies and $^{59}$Fe uptake per spleen was observed between day 0 and day 1. The doubling time for colonies was 18 hr between day 1 and day 5 and for $^{59}$Fe was 26 hr between day 1 and 7. Thereafter, the colonies were too numerous to count and the increase in $^{59}$Fe uptake slowed. Spleen weight also increased (mean of 21 mg/

![Fig. 2.—Increase in number of colonies and spleen iron uptake after split irradiation exposures in which spleen was selectively irradiated during second exposure. Mice were exposed to 395 R whole-body irradiation; at intervals ranging from 1 hr to 4 days later spleen was exteriorized and irradiated with 750 R followed immediately by 200 R whole-body irradiation. Each point is mean of 10–14 mice; brackets surrounding point show SEM.

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Table 1.—Rate of Increase (Doubling Time) of Splenic Hematopoiesis with Different Irradiation Exposures Spaced at Various Intervals*

<table>
<thead>
<tr>
<th>First Exposure (R)</th>
<th>Second Exposure (R)</th>
<th>Daily Intervals Studied</th>
<th>Doubling Time (hours)</th>
<th>Spleen Weight</th>
<th>$^{59}$Fe Uptake</th>
<th>Spleen Colonies</th>
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<td>100</td>
<td>650</td>
<td>0–5</td>
<td>†</td>
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<td>31</td>
<td>26</td>
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<td>150</td>
<td>600</td>
<td>0–5</td>
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<td>36</td>
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<tr>
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<td>600</td>
<td>0–7</td>
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<tr>
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Mean of last 13 studies: 31 34 21

* Groups of 8–10 mice were studied 10 days after the second irradiation. Slopes of increase were calculated by least-square fit, excluding day 0 values from consideration.
† Slope of the line was not statistically significant by t test.
‡ Colonies became confluent too soon in the study to construct a line.
Fig. 3.—Mean doubling time for colonies after split-dose irradiation. These curves were constructed by taking the mean change in number of colonies between each day (6 to 10 values per point in upper curve and 2 to 3 per point in lower curve) so that actual colony number cannot be noted. The brackets surrounding each point indicate the standard error for the main change of various groups on that day.

spleen in a day 0 group, mean of 112 g/spleen in a day 10 group) as did hematocrit (mean of 35.5% in a day 0 group, 40.1% in a day 10 group).

To determine whether splenic hematopoiesis was primarily the result of cells in the spleen surviving the second irradiation or whether cells could migrate to the spleen following the second irradiation, the following experiment was made.

Fifty male mice, weighing 24–27 g, were exposed to 395 R whole-body irradiation. At intervals thereafter, the spleen was exteriorized without interrupting the blood supply and exposed to 750 R with the remainder of the mouse shielded. The spleen was then replaced, and within 5 min of the splenic irradiation the mouse was exposed to 200 R whole-body irradiation. Therefore, the spleen received 950 R, a dose that will kill more than 99.9% of hematopoietic cells. Any hematopoiesis subsequently observed in the spleen should be the result of cells migrating to the spleen following the second irradiation.

As the interval between irradiations was increased, splenic hematopoiesis increased (Fig. 2). Therefore, we concluded that the observed colonies following a second whole-body irradiation (Fig. 1) could be derived from cells migrating to the spleen.

A series of groups of mice were then exposed to different doses of irradiation, and the rate of increase in splenic hematopoiesis with an increasing time interval between irradiation exposures was studied (Table 1). The doubling time for number of spleen colonies was longer for the three groups that received 200 R or less in the first irradiation exposure than in any of the 10 groups that
Fig. 4.—Mean doubling time for spleen iron and spleen weight in groups of mice in which the initial dose in the split irradiation was 300 R or more. The curves were constructed as noted for Fig. 3 and each point represents the mean change for 10 to 13 groups.

received 300 R or more. However, there was no significant correlation \( R = +0.12 \) between initial irradiation dose and doubling time if mice receiving less than 300 R were excluded. There was a similar lack of correlation between initial irradiation dose and the doubling time of iron uptake or spleen weight. Therefore, it appeared that the kinetics of hematopoietic recovery after irradiation of 300 R or more were different from recovery after 200 R or less. There was no significant difference in doubling time between mice of different age (ranging from 7 to 14 wk in various groups in Table 1) or between male and female mice. There was no correlation between doubling time and total irradiation dose. The mean doubling time for colonies was significantly \( p \leq 0.01 \) faster than the doubling time of iron intake and spleen weight, but the latter two did not differ significantly.

The statistical confidence of the slope for doubling time differed widely in various studies due to differences in the number of groups per experiment as well as other factors. Therefore, an alternative method of deriving a mean doubling time was also used. For each group, in each study the mean change from one day to another was calculated. The mean curve for colonies (Fig. 3) derived in this fashion indicated a doubling time of 16 hr for groups receiving an initial exposure of 300 R or more and of 31 hr for those receiving 200 R or less. For groups receiving 300 R or more in the first exposure, the mean doubling time for spleen iron uptake was 29 hr and for spleen weight was
Mean hematocrit values also increased significantly from 34% to 38% as the interval between irradiation increased from 0 to 6 days.

**Onset of Differentiation After Irradiation**

Groups of mice were irradiated with exposures ranging from 100 to 900 R. At daily intervals thereafter, $^{59}$Fe was injected and 24-hr uptake into blood, spleen, and marrow was determined. After 900 R, iron uptake reached a minimum value by 2 days after irradiation and no significant increase in uptake into spleen, marrow, or blood was observed until the mice died at 9-13 days. Therefore, iron uptake after 900 R was considered representative of a “no-erythropoiesis” level. With each experiment a 900-R irradiated group was studied 4 days after irradiation as a no-erythropoiesis control and an unirradiated control group was also included. An example of the results of iron uptake into spleen and marrow in such an experiment is shown in Fig. 5.

After 400 or 600 R, iron uptake by the spleen declined to a level indistinguishable from the 900-R group within 1 day of irradiation and iron uptake by the marrow declined to this level within 2 days. Spleen iron uptake did not again rise significantly ($p \geq 0.5$) above the “no-erythropoiesis” level until 5 days after 400 R ($p \leq 0.001$) and 8 days after 600 R ($p \leq 0.05$). Marrow iron uptake did not rise above the “no-erythropoiesis” level ($p \geq 0.5$) until 6 days after 600 R and 4 days after 400 R. With 200 R, iron uptake into marrow never declined to the no-erythropoiesis level and was at this level in the spleen only on day 2. In this experiment iron uptake into marrow began to increase at an earlier time than in the spleen. In 16 separate studies in which the time of iron uptake increase was measured in both spleen and marrow, marrow increased before spleen in 8, spleen before marrow in 3 and there was an identical time of increase in 5. This tendency is not statistically significant ($p \leq 0.2$). This pattern of decrease to a low level at iron uptake followed by a sudden increase was observed for spleen, marrow, and blood in all groups studied (uptake into spleen studied in 17 groups, marrow in 16, and blood in 4). In all groups that received 300 R or more, iron uptake decreased initially to a level not significantly different from the 900-R control. Once an increase began, iron uptake rapidly rose until it exceeded that of unirradiated mice. A significant degree of fairly regularly occurring undulations were frequently noted after iron uptake returned to normal levels (Fig. 5).

Data from all groups are summarized in Fig. 6. For each group at each irradiation exposure level, the time of onset of increasing iron uptake was determined in the following fashion. A least-squares fit for the period of increasing iron uptake was done and the resultant line was extrapolated to the no-erythropoiesis level (900-R exposed mice). The intercept of these two lines was considered the time of onset of erythropoiesis. With 200 R or less the extrapolation point was less than 0 in certain studies. Day of onset of erythropoiesis proved directly proportional to irradiation exposures (Fig. 6). From these data, it appeared that splenic erythropoiesis was transiently interrupted by more than 200 R and marrow erythropoiesis by more than 215 R (Fig. 6). Iron uptake into blood increased with approximately a 2-day lag behind the increase in spleen and marrow (Fig. 6).
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The rate of increase of erythropoiesis, as judged by the slope of the line describing the increase in $^{55}$Fe uptake into spleen and marrow did not differ in any consistent fashion between different irradiation exposures.

Discussion

These data suggest a working model for the kinetics of self-replication and differentiation of the irradiation-depleted CFU cell compartment (Fig. 7).

Self-replication is considered to begin almost immediately after the CFU cell compartment size is reduced. This is evidenced by the absence of a lag in onset of increasing spleen colony number as split irradiation doses were spaced farther apart. The increase in colonies observed between day 0 and day 1 was larger than on subsequent days. This may reflect, at least in part, the phenomenon of irradiation repair. If cells are exposed to sublethal irradiation, a degree of repair of damage occurs which is maximal within 5 hr or so following irradiation. Following maximal repair at about 5 hr, there is some decrease in “repair,” with a secondary rise beginning before the end of the first day. The secondary rise may represent proliferation. Because of this re-
Fig. 6.—Relation between time of resumption of erythropoiesis following irradiation and irradiation dose. Each point in the figure represents a group of at least 50 mice in which iron uptake was determined daily following irradiation. Resumption of erythropoiesis was considered to be the time when the line describing increasing iron uptake intercepted the 900 R (no erythropoiesis) level (see Fig. 5).

pair effect, the exact time of onset of proliferation cannot be determined from our data.

The doubling time of the CFU cell compartment, as judged by the slope of

Fig. 7.—A model of stem cell replication and differentiation following irradiation.
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increasing colonies after split-dose irradiation, was significantly faster when the first irradiation exposure was 300 R or more (16 hr) than after 200 R or less (31 hr). This appeared to be a threshold phenomenon, since increasing the dose above 300 R did not further increase the doubling time. Till and McCulloch suggested that "patterns of regeneration might be different" following 150 R as compared with 400 R, and differences in recovery pattern between 250 R and 500 R were observed in the studies of Chaffey and Hellman.22 We considered the possibility that after 300 R a period of self-replication might precede any differentiation. Differentiating activity within a self-replicating compartment will reduce the doubling time; for example, if half of the cells are differentiating and half are replicating, the compartment is of stable size.

This hypothesis was explored by studying the time of resumption of erythropoiesis after different irradiation exposures. Uptake of $^{59}$Fe into marrow and spleen was used as a measure of erythropoiesis within these organs. The assumption that this measure reflected erythropoiesis appears reasonable since $^{59}$Fe uptake into circulating red cells paralleled the increase in $^{59}$Fe uptake in spleen and marrow. Erythropoiesis was considered to be absent if iron uptake declined to the same level observed in mice given 900 R. With 300 R or more, all groups declined to that level within 2 days after irradiation. With 200 R or less, this degree of decline was not regularly observed. These data suggested that erythropoiesis was transiently interrupted by irradiation levels about 200 R and that the duration of interruption was extended by an average of 1.6 days for each 100-R increment (Fig. 6). Thus, irradiation threshold for interrupting erythropoiesis was at approximately the same level (200 to 300 R) as the level at which the slope of the doubling time for colonies changed. Given the assumption that erythropoiesis was truly interrupted, these results support the hypothesis concerning self-replication without differentiation when the CFU cell compartment is reduced below a certain size.

Iron uptake presumably parallels hemoglobin synthesis and thus would not measure erythropoiesis until differentiation beyond the erythropoietin-sensitive cell compartment had occurred. Thus, an increase in the size of this cell compartment must precede the onset of erythropoiesis as measured in our studies. The similarity of the slope of increase in iron uptake after high or low doses of irradiation suggests that this cell compartment was of similar size when erythropoiesis began after each irradiation dose. From our data we cannot distinguish between a gradual or an abrupt maturation of cells from the CFU cell to the erythropoietin-sensitive cell compartment. We cannot rule out the possibility that a very low level of erythropoiesis, undetectable by iron uptake, persisted in all groups throughout the postirradiation period.

These results of curves of iron uptake are compatible with the reduced or absent response to erythropoietin in the first few days after irradiation or irradiation and transplantation. On the basis of a period of unresponsiveness to erythropoietin, DeGowin suggested that regeneration of the CFU system might precede differentiation.

The critical compartment size for beginning differentiation is placed at 10% in our working model (Fig. 7) since this is the level of CFU cell killing cal-
culated in these mice from the determined $D_\alpha$ of 78 R and an assumed extrapolation number of 1.5.\textsuperscript{19}

The 16-hr doubling time for colonies is equivalent to the generation time, given the assumption that there was no cell death and that all cells were in cycle as well as the assumption that differentiation was not occurring. Till and McCulloch\textsuperscript{22} calculated a 32-hr doubling time in a study of regrowth following split-dose irradiation, but the number of points between 1 and 6 days was limited and consisted of pooled data from mice of different age and sex.

Chaffey and Hellman\textsuperscript{18} studied the number of endogenous colonies after split-dose irradiation, with the split varying from 0 to 4 days. With 500 R as an initial dose they observed a steady increase in colonies as the second irradiation (250 R) was spaced from 0 to 4 days from the first. However, the slope of increase that they observed is less than in our studies. When the first dose was 250 R and the second 500 R, a transient increase was observed, maximal with an interval of approximately 6 hr, but then a decrease occurred and colonies were not again increased until the split was extended to 4 days. We have no ready explanation for the differences between our results and theirs, but are in complete agreement with respect to a difference in the recovery patterns following a low as compared to a high irradiation dose.

During the exponential phase of growth after transplantation, doubling time estimates have been 20 hours or more.\textsuperscript{3,10} Since a doubling time of 12 hr or less is required, if colonies contain a million or more cells by 10 days,\textsuperscript{25} our estimate of 16 hr may still be a bit long.

In considering the increase in colonies after split-dose irradiation to be a measurement of growth of the CFU cell compartment, we have assumed that the number of endogenous spleen colonies reflects the total size of the mouse's CFU cell compartment. There are a number of reasons for considering this a valid assumption. It was demonstrated that colonies on the spleen 10 days after the second irradiation can be the result of migration of cells to the spleen rather than from cells in the spleen surviving the second irradiation (Fig. 2). The hematocrit increased as exposures were spaced further apart, suggesting that the increasing number of colonies reflected increasingly effective output of mature cells. In previously reported studies we found an increase in endogenous colonies to be the only type of spleen colony technique that would measure the increase in CFU cells in mice injected with foreign plasma.\textsuperscript{2} Endotoxin injection and bleeding afford irradiation protection,\textsuperscript{26,27} so in these instances the increase in endogenous colonies probably reflects an expanded stem cell pool. Thus, there is reason to believe that the number of endogenous colonies does mirror the total size of the CFU cell compartment.

However, we cannot rule out the possibility that the increasing colonies with increasing intervals between irradiation are due to such factors as migration of an increasingly larger percentage of total CFU cells to the spleen. Another possibility is that radiation sensitivity of the CFU compartment changes significantly as irradiation doses are spaced farther apart. This appears unlikely, since appreciable changes were not found in the studies of Till and McCulloch.\textsuperscript{22}

Our data suggesting that self-replication of the CFU cell compartment be-
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Proliferation almost immediately after irradiation is in agreement with that of De Gowin but in disagreement with previously reported studies of replication of transplanted CFU cells. In the latter studies there was an apparent lag of 1 to 4 days before self-replication began after transplantation. Kretchmar demonstrated that the proportion of transplanted CFU cells in marrow, spleen, blood, and lung changes during the first 3 days after transplantation and suggested that the growth rate of CFU might be “grossly underestimated” during the first 3 days after transplantation. CFU cells proliferate after foreign plasma injection, but such proliferation cannot be demonstrated by transplanting marrow or spleen. Therefore, the apparent lag in self-replication after transplantation may reflect shifting CFU cells rather than an absence of replication.

The hypothesis that self-replication has precedence over differentiation in a markedly reduced stem cell compartment represents a protective mechanism for the animal. A reduced stem cell compartment capable of extensive differentiation without self-replication, as suggested by others, is a potentially suicidal compartment.

Should this hypothesis prove correct, a phase of cell growth refractory to differentiating stimuli could be explained easily by either of the following mechanisms. If self-replicating and differentiating stimuli are in competition for surviving stem cells, the level of self-replicating stimuli might be high enough after heavy irradiation to effectively block differentiating stimuli. In theory, a cell might be responsive to differentiating stimuli only during the G1 phase of the generative cycle. This phase may be foreshortened to the point of virtual absence in rapidly growing cell populations.

Till et al. analyzed the distribution of transplantable CFU cells in individual colonies and found the distribution was more compatible with random, rather than precisely controlled, differentiation versus replication of individual CFU cells. The mathematics of this model have been extensively analyzed by Vogel et al. Our two models are, in theory, compatible. As they noted, “It appears possible that in our studies, the progeny of a single cell display that random feature of hematopoietic function, while a study of large populations of cells would reveal the orderly behavior of the whole system.” Our studies were designed to measure the latter aspect. Assuming the overall probability for differentiation or self-replication is dependent upon the relative concentration of specific regulating substance, then the model we propose can allow “random” behavior of an individual CFU cell.

REFERENCES

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