

## Hematopoietic Stem Cells: Effect of Preirradiation, Bleeding, and Erythropoietin on Thrombopoietic Differentiation

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A method of measuring differentiation of stem cells towards platelets is described using syngeneic bone marrow injected into lethally irradiated mice. Fourteen days after such injection, the platelet counts are found to be proportional to the number of bone marrow cells injected and can be used as a measure of platelet progenitors. Perturbation of the milieu in which the transplanted marrow is placed by host preirradiation, bleeding, or erythropoietin administration leads to enhanced thrombopoiesis. It has been

shown previously that similar perturbation favors erythropoiesis at the expense of granulopoiesis. The data from these and other experiments appear to be consistent, with a model of the stem cell compartment as a continuum with proliferative activity increasing as commitment is restricted. These functions vary inversely with the capacity for self-renewal. The various stem cell assays measure different ranges of stem cells, but overlap within this continuum.

**W**HEN SYNGENEIC BONE MARROW is injected into lethally irradiated mice and sufficient time is allowed for proliferation and differentiation of progenitor cells, the progeny of such cells can be assayed. It has been demonstrated previously that these progeny are proportional to the number of cells injected for both the erythrocytic and granulocytic series.<sup>1,2</sup> In those systems, sufficient time must be allowed for committed cells to proliferate, mature, enter the circulation, and be lost, so that it is the progeny of more primitive cells that is being assayed. When the recipient animal is preirradiated 6–7 days prior to transplantation, made anemic by bleeding, or given erythropoietin, the injected progenitor cells differentiate toward erythropoiesis at the expense of granulopoiesis.<sup>3–5</sup> These results are interpreted as consistent with the notion of a common pluripotent stem cell pool on which are made competing proliferative demands. There is, of course, other evidence for such a pool of pluripotent hematopoietic stem cells. Whang et al. have demonstrated that the Philadelphia chromosome described in chronic myelogenous leukemia could be identified in erythrocytes, granulocytes, and megakaryocytes, suggesting that these cells descend from a single stem cell.<sup>6</sup> Later, Wu et al. studied the progeny precursor cells containing radiation-induced unique chromosomal markers that could be identified in hematopoietic spleen colonies produced after marrow transplantation into lethally irradiated mice.<sup>7</sup> Cell suspensions from individual colonies have been found to contain multiple types of recognizable differenti-

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ated cell forms, thus proving that single multipotent cells exist that can give rise to erythrocytes, granulocytes, or platelets.

In order to understand pluripotent stem cell differentiation toward thrombopoiesis, a platelet repopulation assay similar to that for erythropoiesis and granulopoiesis is required. This study describes such an assay, and it investigates the effects of perturbation of the milieu by preirradiation, bleeding, or erythropoietin treatment of the recipient animal. In addition, the platelet repopulating ability after perturbation of the donor by preirradiation is described.

### MATERIALS AND METHODS

Animals used in all experiments were male C3H/HEJ mice, 12-14-wk old. Whole animal irradiation was performed using a  $^{137}\text{Cs}$  irradiator through simultaneously opposing fields (less than 4% inhomogeneity). Animals were placed in cages of eight to ten animals each and supplied with food and water ad libitum. All results are indicated as the means with their standard errors. Bone marrow cell suspensions were prepared by flushing the medullary cavity of the tibia and femur with cold sterile Tyrode's solution. Cell suspensions were kept on ice until used, and were injected intravenously into the lateral tail vein not longer than  $1\frac{1}{2}$  hr after removal. Sufficient volume of solution was used so that the injected bolus varied between 0.05 and 1 ml. Cell counts were made using a hemocytometer and counting at least 200 cells. Platelet counts were performed by phase microscopy by previously described standard methods.<sup>8</sup> The normal platelet count in this particular strain of mice was found to be  $(980,000 \pm 40,000)/\text{cu mm}$  based on counts in 30 animals.

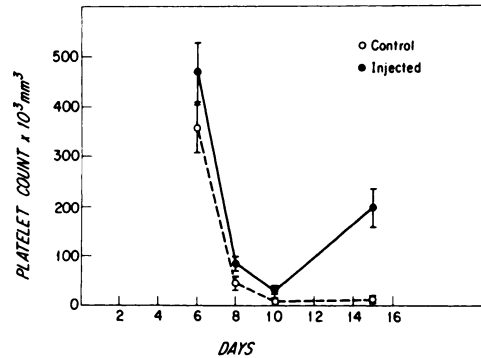
*Platelet repopulating ability of transplanted bone marrow in preirradiated animals.* Recipient animals were exposed to 150 rads whole body irradiation. Seven days later they received 950 rads whole body irradiation, followed by the injection of graded amounts of syngeneic bone marrow. Control animals were randomly selected litter mates not preirradiated, but who received 950 rads irradiation on the same day as the experimental groups and were injected with the same bone marrow suspension. The test animals and the control groups were sacrificed at the end of 14 days and platelet counts performed. Six or seven surviving animals were used for each dose of bone marrow in both the control and the preirradiated groups.

*Platelet repopulating ability of transplanted bone marrow in animals bled prior to irradiation and bone marrow transplantation.* Animals were divided into two groups: one group was bled of 0.6 ml from the orbital sinus 3 days and then again 1 day prior to irradiation. The mean hematocrit at the time of bone marrow transplantation in bled groups was  $25.0\%$  ( $\pm 1.2$ ), and in the nonbled group was  $49.0\%$  ( $\pm 1.1$ ). Both groups of animals then received 950 rads whole body irradiation. Each group was subdivided and graded doses of syngeneic bone marrow were injected. Platelet counts were performed from blood obtained at cardiac puncture 14 days later (six to ten mice per group).

*Platelet repopulating ability in recipients receiving erythropoietin.* Erythropoietin was prepared commercially from phenylhydrazine-treated sheep (Connaught Laboratories, Toronto). Mice were given 950 rads whole body irradiation and then divided into two groups. Both groups were given varying doses of syngeneic bone marrow cells. The group serving as a control received 0.5 ml of normal saline injected intraperitoneally on days 2-5 after lethal irradiation and bone marrow transplantation. The study group was injected with erythropoietin, 3-6 units, intraperitoneally in 0.5 ml of normal saline on days 2-5 after lethal irradiation. Platelet counts were performed in both groups (six to ten mice per group) on blood obtained from cardiac puncture 14 days after irradiation and bone marrow transplantation.

*Platelet repopulating ability of bone marrow from donors receiving 200 rads.* Mice were given 200 rads and then, at varying intervals from 0 to 20 days after irradiation, they were sacrificed, and graded amounts of their bone marrow was injected into syngeneic mice that had received 950 rads whole body irradiation. In each experiment, similarly prepared recipients were injected with marrow from unirradiated donors and used for comparison.

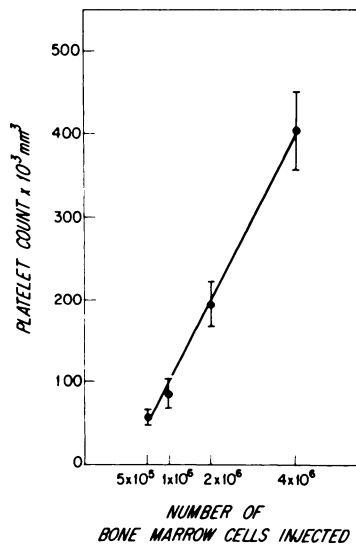
**Fig. 1.** Injected group (●) received 950 rads and  $2 \times 10^6$  bone marrow cells on day 0. The control group (○) received 950 rads and no bone marrow on day 0. Each point is the mean of at least six determinations with its standard error.



## RESULTS

Platelet counts were studied in animals that had received 950 rads whole body irradiation and graded amounts of syngeneic bone marrow cells varying from 0 to  $1 \times 10^7$  cells/ml (Fig. 1). In all groups of animals, the nadir of the platelet count was at day 10 after irradiation. In animals that received no bone marrow injection, there was a slight rise in the platelet count after day 10, with 8 of 12 animals dying by day 15. Animals receiving syngeneic bone marrow developed a rapid rise in the platelet count after day 10. These animals did not die, and when followed for as long as 4 wk after transplantation demonstrated a return of hematocrit, white count, and platelet count to normal or nearly normal levels.

Perturbation of the recipient animal could be performed only if an assay system could be developed in which the platelet count on a particular day after irradiation and transplantation was proportional to the number of bone marrow cells injected. Figure 2 demonstrates that when between  $5 \times 10^5$  and  $4 \times 10^6$  cells were injected, there was a linear relationship between the number



**Fig. 2.** Relationship between bone marrow cells injected and the platelet count 14 days after 950 rads and bone marrow injection. Each point is the mean of at least six determinations with its standard error.

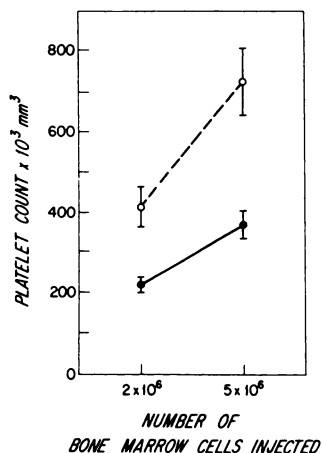


Fig. 3. The preirradiated group ( $\circ$ ) received 150 rads 1 wk prior to 950 rads and graded doses of bone marrow. The control group ( $\bullet$ ) received only 950 rads and graded doses of bone marrow. Platelet counts were performed 14 days after irradiation and injection. The platelet counts in the preirradiated group were significantly higher ( $p < 0.001$ ) at both doses of bone marrow injected. Each point is the mean of at least six determinations with its standard error.

of cells injected and the platelet count when assayed on day 14. Thus, this procedure could be used to assess stem cell progenitors in the injected marrow as regards their ability to restore thrombopoiesis.

Having developed this assay for platelet progenitor cells, we then sought to vary the recipient milieu in fashions similar to those previously described for erythropoiesis and granulopoiesis. Animals that had received 150 rads preirradiation 1 wk prior to 950 rads and syngeneic bone marrow injection had significantly higher platelet counts with both cell numbers injected when compared to non-preirradiation control (Fig. 3). This observation was consistent with preirradiation favoring thrombopoiesis.

In a subsequent series of experiments, the host milieu was varied by transplanting syngeneic marrow into recipients that were made anemic by bleeding 3 and 1 days before irradiation and transplantation. Again, thrombopoiesis was favored (Fig. 4). The effects of erythropoietin administration to the recipient were studied. The differences in platelet counts were less marked in the animals receiving intraperitoneal erythropoietin than in the bled recipients. However, in repeated experiments and with multiple cell numbers injected, ani-

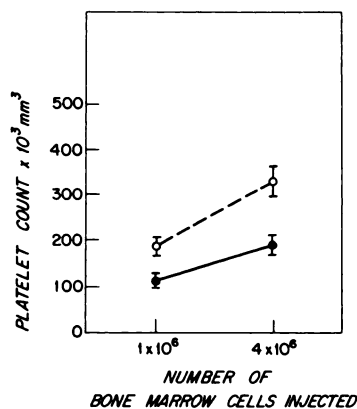


Fig. 4. Animals were bled at 3 days and again 1 day before 950 rads and graded doses of bone marrow ( $\circ$ ). The control group received only 950 rads and graded doses of bone marrow ( $\bullet$ ). Platelet counts were performed 14 days later and were significantly higher ( $p = .05 - 0.001$ ) in the bled group. Each point is the mean of at least six determinations with its standard error.

**Table 1. Platelet Count  $\times 10^3$  on Day 14 After Saline or Erythropoietin Administration**

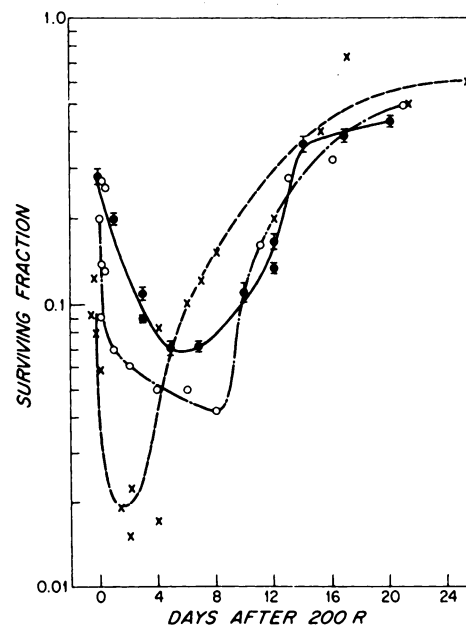
Cells Injected $\times 10^6$	Saline Group	Erythropoietin Group
1.0	61.0 $\pm$ 2.0	140.0 $\pm$ 10.0
1.0	168.0 $\pm$ 41.0	207.0 $\pm$ 28.0
4.0	340.0 $\pm$ 50.0	410.0 $\pm$ 60.0
4.0	410.0 $\pm$ 33.0	500.0 $\pm$ 99.0
5.0	402.0 $\pm$ 76.0	651.0 $\pm$ 109.0

mals receiving erythropoietin had higher platelet counts than the uninjected controls (Table 1).

In the final experiment, donor mice received 200 rads and, at intervals varying from 0 to 20 days after the irradiation, graded amounts of their bone marrow were injected into syngeneic mice that had received 950 rads. Fourteen days later, platelet counts were determined. The data were compared to platelet counts 14 days following the injection of marrow from control donors into similar recipients. From these results, a survival fraction of platelet repopulating ability could be determined in a fashion similar to that for the granulocytic repopulating ability.<sup>2</sup> Fractions of cells surviving 200 rads, as measured by the platelet repopulating ability at various times after the donor irradiation, are demonstrated in Fig. 5. Fractions of cells surviving 200 rads as measured by the erythrocytic and granulocytic repopulating abilities in our laboratory are also presented for comparison.<sup>9</sup> The platelet repopulating ability appears to follow that of the granulocytic, rather than the erythrocytic, repopulating ability.

#### DISCUSSION

The use of irradiated recipients injected with syngeneic bone marrow allowed an assay for platelet precursors. After 10 days, there was a rapid rise in the

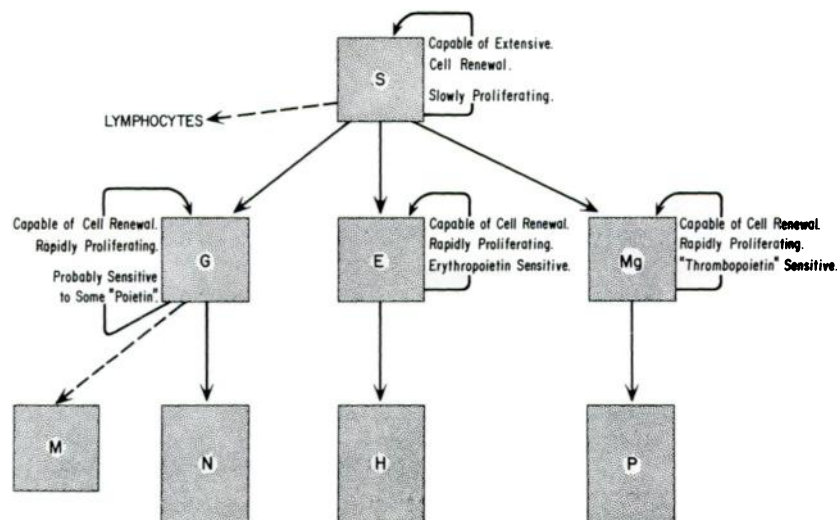


**Fig. 5. Fractions of cells surviving 200 rads as measured by the platelet repopulating ability (●) at various times after irradiation. Each point is the mean of at least six determinations with its standard error. Erythrocytic (×) and granulocytic (○) repopulating abilities are also graphed.**

platelet count, which continued toward normal. This repopulation appeared to be due, at least in part, to the earliest progenitor cells injected, since there was no abortive rise that might be expected if more mature cells had been transplanted and exhibited only limited division. A fourteen-day point was chosen for the assay, since it was in the phase of rapid platelet repopulation, allowed sufficient time for proliferation, and permitted an assay whose sensitivity was similar to that for granulopoiesis and erythropoiesis.

Preirradiation, bleeding of recipient animals, and erythropoietin administration to the recipient animals all resulted in enhanced platelet production from transplanted bone marrow. One must be cautious in interpreting the erythropoietin results, since this erythropoietin was a crude preparation and may have contained thrombopoietic substances distinct from erythropoietin.

Bleeding experiments, as a means of enhancing endogenous erythropoietin, have been performed so as to avoid possible misinterpretation arising from the use of crude exogenous erythropoietin. Previous experiments in this laboratory have demonstrated that these same stimuli of the recipient animal favored differentiation of stem cells towards erythropoiesis at the expense of granulopoiesis.<sup>4</sup> This favoring of specific differentiation pathways has been explained by hypothesizing the existence of a limited number of common hematopoietic stem cells on which competing proliferative demands are made by erythropoiesis, granulopoiesis, and thrombopoiesis.<sup>1-5</sup> Such data, as well as the previously mentioned experiment of Wu et al., argue for a common stem cell whose differentiation pathways can be altered by the milieu in which proliferation occurs. Figure 6 represents a traditional view of the various stem cell compartments. Since erythropoiesis and thrombopoiesis are affected similarly by the described milieu changes, it is possible that these cell lines share a common early cell distal to the multipotent cell and that there is not a thrombo-



**Fig. 6.** Traditional model of the early stages of hematopoiesis: S, stem cell; E, committed early erythropoietic cell; G, committed early granulocytic cell; Mg, committed early thrombopoietic cell; N, granulocyte; M, monocyte; P, platelet; H, erythrocyte.

poietic substance distinct from erythropoietin. Studies by De Gabriele and Penington,<sup>10</sup> Shreiner and Levin,<sup>11</sup> and Odell,<sup>12</sup> however, suggest that thrombopoietin and erythropoietin are distinct. The present experiments demonstrate that following manipulation of the donor by preirradiation, the platelet repopulating ability has recovery kinetics similar to the granulocytic repopulating ability. However, when the recipient is preirradiated, bled, or treated with erythropoietin, both thrombopoiesis and erythropoiesis are favored at the expense of granulopoiesis. Thus, the platelet repopulating ability appears under differing circumstances to be different from either the granulocytic or erythrocytic repopulating ability, consistent with platelets having separate control mechanisms.

Recent experiments by Constable, Blackett, and Millar challenged this traditional rigid compartmentalization of stem cell differentiation.<sup>13-15</sup> Donor bone marrow was treated with a variety of agents including dimethylmyleran, cyclophosphamide, methotrexate, actinomycin D, and tritiated thymidine. Granulocytic repopulating cells proliferated more slowly than the erythroid counterpart, suggesting to them that these techniques measured progenitor cells, which were already committed to single lines of differentiation as opposed to measuring the multipotent stem cell. Such data, however, cannot explain the experiment of Hellman et al. supporting competition between erythropoiesis and granulopoiesis for a common stem cell pool.<sup>2-5</sup>

Experiments by Reincke et al. also indirectly challenged this traditional view.<sup>16</sup> They administered <sup>55</sup>Fe to donor mice and demonstrated that erythropoiesis was severely depressed after marrow transfer. In addition, spleen colony-forming units were decreased, and the ability of the transplanted marrow to prevent irradiation death was much reduced. They suggested that these phenomena might be explained by a shift of the pluripotent stem cell toward erythropoiesis at the expense of other cell lines, theoretically supporting the traditional concept of differentiation. They also suggested that the decreased content of the colony-forming units might be due to migration of the CFU from marrow to the spleen. An alternative explanation could be that some cells able to form spleen colonies were sufficiently differentiated to incorporate sufficient <sup>55</sup>Fe to cause their death.

As a possible synthesis of these results and the experiments here reported, we suggest that the strictly compartmentalized model (Fig. 6) should be modi-

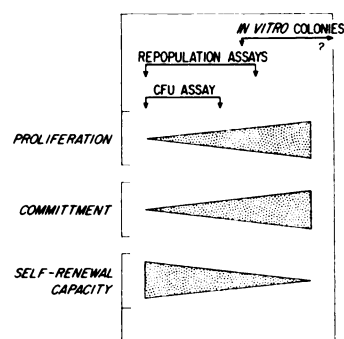


Fig. 7. Proposed stem cell model with regions assayed by CFU, repopulation, and in vitro colony techniques.

fied to consider the pluripotent stem cell and early committed stem cell as a continuum, with self-renewal a characteristic of both. Proliferative activity probably increases with restricted commitment. The most primitive cell might be envisioned as the so-called "immortal stem cell" of Cairns.<sup>17</sup> Such a cell would be quiescent at most times, and would be called into proliferation only when the stem cell compartment is reduced by toxic agents. A diagram of such a model is shown in Fig. 7, with a proposal for what the various stem cell assays measure. If the repopulation assays measure both the most primitive and the committed stem cell (granulocyte, erythrocyte, and platelet), then our data on competition and those of Blackett and co-workers<sup>13-16</sup> can be reconciled. Competition for the pluripotent stem cell could occur, while observations of proliferative differences of the repopulating abilities could be explained by the proliferative differences in the committed erythropoietic and granulopoietic stem cells. Even the cells which form spleen colonies are likely to be heterogeneous, with some cells which are able to form such colonies being committed to erythropoiesis and able to incorporate iron. Such <sup>55</sup>Fe incorporation by the committed colony-forming unit (CFU) would explain the Reincke et al. experiments. With <sup>55</sup>Fe, even a small amount of incorporation may cause cell death, and, perhaps, this is a characteristic of late committed CFUs. Schofield and Lajtha have performed experiments which would seem to indicate that the spleen colony-forming cell (CFU-S) is, in fact, heterogeneous; there are classes of colonies with entirely different self-renewal potential.<sup>18</sup> Similarly, Micklem et al.<sup>19</sup> studied blood and bone marrow stem cells under identical conditions and demonstrated marked differences in self-renewal characteristics and in the size of the descendant population, supporting the concept of the heterogeneity of stem cells.

In summary, the experiments here described demonstrate a repopulation assay for stem cell differentiation to thrombopoiesis. Alteration of the recipient milieu by host preirradiation, bleeding, or erythropoietin administration, all cause enhanced differentiation toward thrombopoiesis. The platelet repopulating ability following 200 rad to the donor resembles the granulocytic repopulating ability and suggests that thrombopoiesis is distinct from erythropoiesis. These data, as well as those from the other repopulating assays and spleen colony assays, can be explained with the stem cell pool as a continuum from the most primitive cells having the most capacity for cell renewal and the least proliferative activity, to proliferatively active stem cells committed to one particular formed blood element; these latter can have some self-renewal capacity. The various assays measure portions of this continuum to differing extents.

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