The Effect of Plethora on Growth and Differentiation of Normal Hemopoietic Colony-Forming Cells Transplanted in Mice of Genotype W/Wv

J. E. TILL, L. SIMINOVITCH and E. A. McCulloch
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Two functional assays are, at present, commonly used to investigate stem cells. First, Gurney and his collaborators, making use of findings which indicate that the hormone erythropoietin acts on relatively undifferentiated stem cells, devised an assay for such stem cells based on responsiveness to erythropoietin. Second, we have suggested that the cells that give rise to spleen colonies in heavily irradiated or genetically anemic recipient mice may be considered as stem cells, and that the spleen colony method provides a quantitative assay for such cells. Evidence has been summarized elsewhere that the stem cells which form spleen colonies are not identical with the cells which respond to erythropoietin. For example, it has been shown that exposure of mice to hypoxia, a procedure which might be expected to increase erythropoietin levels, had little effect on the colony-forming efficiency of cells from the marrow or spleen of these animals at a time when erythropoiesis was greatly increased.

Transfusion-induced plethora suppresses erythropoiesis, presumably by decreasing the production of erythropoietin. A number of workers have studied the formation of spleen colonies in irradiated plethoric mice, and in most cases, plethora has been found to cause a reduction in the number of spleen colonies and an increase in the proportion of colonies which are primarily granulocytic in composition. Such findings might be interpreted as indicating that a proportion of the colony-forming cells are indeed sensitive to erythropoietin, and so fail to proliferate in the plethoric animals. However, an alternative interpretation is also possible. Suppression of erythropoiesis might prevent differentiation of enough erythroblasts to form macroscopically visible colonies, and the predominantly granulocytic colonies which were observed might result from stimulation of the growth of multipotent colony-
forming cells by radiation-induced granulocytopenia in the irradiated plethoric hosts.

The difficulty in interpretation of the results obtained when irradiated plethoric mice are used as hosts for the formation of splenic colonies might be avoided by using genetically anemic mice of genotype $W^v/W^v$ as recipients. Such animals have a severe macrocytic anemia but their peripheral leukocyte and thrombocyte counts are nearly normal. The hemopoietic colony-forming cells of these genetically anemic mice are defective and unable to give rise to normal numbers of spleen colonies. However, the tissues of mice of genotype $W/W^v$ are competent and unirradiated mice of this genotype support colony formation when cells derived from coisogenic normal mice are injected into them. In the present paper, we report the results of a series of experiments in which the process of colony formation and the growth of colony-forming cells was studied following the injection of normal coisogenic bone marrow cells into plethoric mice of genotype $W/W^v$.

**Materials and Methods**

*Mice.* Genetically anemic mice and their normal coisogenic littermates were either obtained from the Jackson Laboratory, Bar Harbor, Maine, or were bred in the animal colony of the Ontario Cancer Institute. The breeding stock in the O.C.I. colony was obtained from the Jackson Laboratory and therefore mice from either source were used, as available. Mice of genotype $W/W^v$ were obtained by crossing mice from the inbred strain WB maintained heterozygous at the W locus (WB-W/+ and C57BL/6 maintained heterozygous at the W locus by repeated backcrossing (B6-W/+). This cross yields normal (WB x B6)F1 hybrid mice (WBB6F1) which served as donors of hemopoietic cells, and genetically anemic WBB6F1-$W^v/W^v$ mice which served as recipients of these cells. In addition, mice of genotype $W^v/W^v$ were obtained by crossing animals of genotype C3H-$W^v/+\) with animals of genotype C57BL/6-$W^v/+\). This cross yields normal C3B6F1-$+/+$ mice as donors and genetically anemic mice of genotype $W^v/W^v$ to serve as recipients. Similar results were obtained with $W/W^v$ and $W^v/W^v$ recipients and these will not be designated separately. Following injection with red blood cells and marrow cells the animals were housed 2 to 3 per cage and allowed food (Rockland Mouse Diet) and water freely.

*Transfusion Procedure.* Red blood cells for transfusion were obtained either from normal coisogenic mice or from heterozygous littermates ($W^v/+\), $W^v/+\) or $W^v/+\) Red cell donors were lightly anesthetized and bled from the carotid artery. The red cells were washed three times in the cold with phosphate buffered saline. Anemic mice to be transfused received 1 ml. of washed packed red cells intraperitoneally on each of 3 or 4 successive days. One ml. of packed red cells usually contained in excess of $1.4 \times 10^{10}$ red cells. Genetically anemic mice did not tolerate the procedure well, and in some experiments as many as 50 per cent of the transfused animals died during the course of the experiment. Some of the survivors were found to have failed to retain elevated red blood cell counts (see "Results"). However, less extensive transfusion procedures, while permitting a greater survival, did not yield adequate suppression of erythropoiesis.

*Colony Formation.* The spleen colony technic using recipients of genotype $W/W^v$ has been described previously. In brief, hemopoietic cells were obtained from the marrows of coisogenic normal littermates, or from the spleens of transfused or control animals which had received transplants of marrow cells from normal coisogenic donors (see below). An appropriate number of these cells was injected intravenously into the recipient animals, which had been given a sublethal dose of irradiation (150 rads) prior to injection of the cells. After 10 days, the spleens were removed, fixed in Bouin's solution, and the
macroscopic spleen colonies were counted. Results were expressed as colony-forming units (CFU) per 10^5 nucleated cells injected.

Assay for the Growth of Colony-Forming Units. The technic for obtaining growth curves for CFU in irradiated recipients was adapted for measuring growth curves of normal hemopoietic cells transplanted into uniradiated mice of genotype W/W^v or W^v/W^v. The colony-forming efficiency of a marrow cell suspension was first tested by injecting 8 × 10^4 nucleated cells into lightly irradiated genetically anemic mice. Then, 2 × 10^6 nucleated marrow cells from the same cell suspension were injected into uniradiated recipients of genotype W/W^v or W^v/W^v. These uniradiated recipients consisted of animals which had been transfused prior to injection of the marrow cells, along with their nontransfused controls. At various times thereafter, individual mice from these groups were killed and cell suspensions prepared from spleen and marrow. The spleen cell suspension was tested for colony-forming efficiency by injecting appropriate numbers of cells into groups of lightly irradiated anemic mice of the appropriate W genotype. Tests for iron-incorporating capacity and for peroxidase-positive cells were also done on the spleen and marrow cell suspensions. At the time of the sacrifice a peripheral red cell count and reticulocyte blood count were made on each recipient animal.

Incorporation of Radioactive Iron. Erythropoiesis in spleen and marrow cell suspensions was tested by measuring the capacity of such cells to incorporate radioactive Fe^59 into heme in vitro, as described elsewhere. In brief, the technic used was as follows: 1.2 × 10^7 nucleated marrow cells were incubated at 35 C. for 45 or 90 minutes in a medium consisting of CMRL 1066 and mouse serum labelled with Fe^59 (obtained as the chloride from Abbott Laboratories, specific activity 10–25 me/mg.). The amount of Fe^59 incorporated into heme per unit time was found to be linearly related to the number of cells present in the incubation mixture. Results were expressed as percentages of the activity added to the incubation tube which was incorporated into heme by 1.2 × 10^7 marrow cells in 45 minutes.

Peroxidase Staining Technic. The number of granulocytes present in the spleen cell suspensions was determined using the peroxidase-staining technic of Ryto
toma as modified by Fowler using cells deposited on Millipore filters.

Irradiation Procedures. Radiation was delivered to recipient mice of genotype W/W^v, using a Ca irradiator designed by Cunningham, Bruce, and Webb at a dose rate of approximately 115 rads per minute.

Red Cell Counts and Reticulocyte Counts. Red cell counts were made using a Coulter counter (Coulter Electronics, Hialeah, Fla.), and reticulocytes were counted directly on smears stained with brilliant cresyl violet.

RESULTS

Effect of Plethora on Spleen Colony Formation in Mice of Genotype W/W^v

Preliminary experiments were designed to test whether or not the suppression of erythropoiesis in uniradiated mice of genotype W/W^v would affect the number of colonies observed in the spleens of such animals following the transplantation of marrow cells from coisogenic normal mice. Groups of five animals were rendered plethoric by the injection of 1 ml. of packed washed red cells on four successive days. Three days after the last intraperitoneal injection of red cells these animals and control anemic mice were injected with 8 × 10^4 normal nucleated marrow cells. After 10 or 14 days, the recipient animals were killed. Peripheral red cell counts and reticulocyte counts were done on each animal, and marrow from each group was pooled and its capacity to incorporate radioactive iron into heme was tested. The spleen of each animal was fixed in Bouin's solution and examined for spleen colonies. The results are presented in Table 1. From the table it is apparent
Table 1.—Effect of Transfusion-Induced Suppression of Erythropoiesis on the Formation of Spleen Colonies

<table>
<thead>
<tr>
<th>Item</th>
<th>Transfused</th>
<th></th>
<th>Not Transfused</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals tested</td>
<td>Exp. 1</td>
<td>Exp. 2</td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td>Mean red cells/ml. ( \times 10^{-9} )</td>
<td>9.23</td>
<td>9.67</td>
<td>5.89</td>
<td>5.60</td>
</tr>
<tr>
<td>Mean reticulocytes (%)</td>
<td>0.2</td>
<td>—</td>
<td>3.6</td>
<td>—</td>
</tr>
<tr>
<td>Fe(^{59}) uptake in marrow cells (%)</td>
<td>0.03</td>
<td>0.02</td>
<td>0.67</td>
<td>0.48</td>
</tr>
<tr>
<td>Cells/2 femora ( \times 10^{-7} )</td>
<td>5.2</td>
<td>3.0</td>
<td>3.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Cells/spleen ( \times 10^{-8} )</td>
<td>—</td>
<td>3.6</td>
<td>—</td>
<td>2.9</td>
</tr>
<tr>
<td>Granulocytes/spleen (%)</td>
<td>—</td>
<td>3.8</td>
<td>—</td>
<td>3.6</td>
</tr>
<tr>
<td>CFU/10(^5) marrow cells, day 10</td>
<td>—</td>
<td>0.0</td>
<td>24.6</td>
<td>7.2</td>
</tr>
<tr>
<td>day 14</td>
<td>0.0</td>
<td>0.3</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

that the transfused animals had elevated red cell counts and markedly reduced numbers of circulating reticulocytes, compared with mice which had not been transfused. In addition, the pool of marrow cells obtained from the transfused animals showed a very low capacity to incorporate radioiron into heme, in comparison with the nontransfused controls. These findings indicate that the transfusion procedure was successful in raising the red cell count of mice of genotype W/W\(^v\) and in suppressing erythropoiesis. The numbers of cells obtained from spleen and marrow, and the percentages of granulocytes present in the spleen, were not significantly changed by the suppression of erythropoiesis in these experiments. In contrast, it is apparent from the table that very few spleen colonies were seen in the transfused mice, and that those which were seen only became visible on the fourteenth day after injection of the marrow cells. Thus, suppression of erythropoiesis in mice of genotype W/W\(^v\) inhibits the development of detectable macroscopic spleen colonies by marrow cells from normal coisogenic donors.

Effect of Plethora on the Growth of Hemopoietic Cells in Mice of Genotype W/W\(^v\)

Macroscopic colonies might fail to develop in plethoric mice of genotype W/W\(^v\) following marrow cell transplantation, either because the transplanted colony-forming cells failed to grow in the recipient animals or because suppression of erythropoiesis prevented formation of erythroblasts in numbers sufficient to give rise to macroscopic colonies. To distinguish between these two alternatives, the growth of colony-forming cells in plethoric and anemic mice of genotype W/W\(^v\) was measured. In these experiments groups of mice received 1 ml. of packed washed red cells on three successive days. Three days later these animals and anemic controls received \(2 \times 10^6\) nucleated marrow cells derived from coisogenic normal mice. At intervals from day 7 to day 10 after injection of the marrow cells, individual mice were killed, samples of peripheral blood were taken for red cell and reticulocyte counts, and cell suspensions were made from spleen and bone marrow. The bone marrow and spleen cell suspensions were tested for their ability to incorporate
radioiron into heme; in addition, the percentages of peroxidase-positive cells in the spleen cell suspensions were determined. The colony-forming efficiencies of the cells in the spleen cell suspensions were measured by injecting suitable numbers of cells into groups of lightly irradiated mice of genotype W/Wv and counting the numbers of spleen colonies seen after 10 days.

The effect of the transfusion procedure on the peripheral red cell and reticulocyte counts, and on the capacity of spleen and marrow cells to incorporate radioiron into heme, is shown in Figure 1. Results obtained from transfused animals are shown as closed symbols, while results obtained from control anemic animals are shown as open symbols. It is evident from this figure that individual mice were not uniformly affected by transfusion. In some animals which received transfusion of red cells, plethora was successfully achieved, in that low peripheral reticulocyte counts and low levels of incorporation of radioiron into heme in spleen or marrow cells were observed 10 to 13 days after the last transfusion of red cells. In other transfused animals, plethora was not achieved, in that suppression of erythropoiesis was not maintained throughout the entire duration of the experiment. On the basis of the results shown in Figure 1, erythropoiesis in individual animals was con-
Fig. 2.—Growth of CFU in the spleens of unirradiated recipient animals of genotypes W/W\textsuperscript{v} which had been given \(2 \times 10^6\) marrow cells derived from normal littermates. \(\blacktriangle\) = recipients successfully plethorized; \(\bigtriangleup\) = recipients not successfully plethorized; \(\bigcirc\) = nontransfused controls. Data shown are the means of results obtained for 3 to 8 individual mice per point. The horizontal dotted line indicates the initial number of CFU per spleen, obtained by multiplying the mean number of CFU per \(2 \times 10^6\) marrow cells by 0.17, the fraction of injected marrow CFU which lodge in the spleen.\textsuperscript{x}

sidered to have been suppressed successfully only if their peripheral red counts were \(7.5 \times 10^6\) cells per ml. or greater, at the time of test.

The results of the assays of spleen cells for their colony-forming ability are shown in Figure 2 as a function of the time when the animals were killed. Time zero in this case was taken as the time the marrow cells were injected, 3 days after the last transfusion of erythrocytes. The animals were tested individually for successful suppression of erythropoiesis (Fig. 1), and on the basis of these tests they were separated into 3 groups. These groups consisted of animals in which erythropoiesis was successfully suppressed (closed triangles in Fig. 2), animals in which transfusion was unsuccessful in suppressing erythropoiesis (open triangles), and animals which were not transfused (open circles). The values shown in Figure 2 are means of the results obtained for the individual animals. The solid curve shown in Figure 2 represents the results obtained previously\textsuperscript{1a} for the growth of normal marrow CFU in the spleens of irradiated normal C3B6F1 recipient mice. Also shown
in the figure is the mean initial number of CFU present in the spleen (dashed horizontal line), calculated from the measured colony-forming efficiency of the original marrow cell suspensions by assuming that 17 per cent of the CFU initially injected lodged in the spleens of the recipient animals. An experiment, carried out in the same way as those described previously, confirmed that this value is also applicable to unirradiated, genetically anemic recipient mice, as well as to the irradiated normal recipients used in the previous experiments.

It is evident from the data shown in Figure 2 that although the growth of injected normal CFU in the spleens of unirradiated W/W<sup>+</sup> recipients was delayed in comparison with the growth of CFU in normal, heavily irradiated animals, growth occurred in the W/W<sup>+</sup> recipients whether or not erythropoiesis had been suppressed. Indeed, the number of CFU per spleen obtained from animals in which erythropoiesis had been successfully suppressed did not differ significantly (p > 0.1) from the number of CFU per spleen obtained from control animals or from animals in which transfusion did not result in suppression of erythropoiesis. Thus, the growth of normal CFU in animals of genotype W/W<sup>+</sup> appears to be controlled independently from the control of erythropoiesis.

**Effect of the Transfusion Procedure on Granulopoiesis**

Hemopoietic colony-forming cells appear to be capable of giving rise to granulocytic as well as erythrocytic descendants. It was of interest to see whether or not the suppression of erythropoiesis by plethora would affect granulopoiesis. In the same experiments which yielded the results given in Figures 1 and 2, the histochemical test for peroxidase was used as an assay for granulocytic cells in the spleens of individual transfused and anemic mice of genotype W/W<sup>+</sup> which had been injected with marrow cells from normal donors. The results of these tests are presented in Figure 3. In the figure, granulopoiesis in individual animals, as measured by the total number of peroxidase-positive cells in their spleens, is compared with erythropoiesis in the same animals, as measured by percentage iron incorporation into heme in marrow cells. Data from animals in which suppression of erythropoiesis was successfully achieved by transfusion of erythrocytes are shown along with data from nontransfused controls and from animals in which suppression of erythropoiesis was not successfully achieved. As shown in Figure 1, animals in which erythropoiesis had been effectively suppressed by transfusion all yielded cells which incorporated very little radioiron, in comparison with cells from control animals and animals in which transfusion had been ineffective, which showed a high level of iron incorporation. However, there was no correlation between successful suppression of erythropoiesis and numbers of granulocytes per spleen. Although animals which had been transfused had a significantly higher total granulocyte count per spleen than control animals (p < 0.02), this increase in granulocytes was observed whether or not the transfusion procedure had been effective in suppressing
Fig. 3.—Number of granulocytes per spleen as a function of Fe$^{59}$ incorporation into the heme of marrow cells obtained from unirradiated recipient mice of genotype W/W$^v$ which received $2 \times 10^6$ normal marrow cells 7 to 10 days prior to test. ▲ = recipients successfully plethorized; △ = recipients not successfully plethorized; ○ = nontransfused controls. The stippled area indicates the zone occupied by 85 per cent of the points.

erythropoiesis. The granulocyte counts in the two groups of animals which received transfusions did not differ significantly ($p > 0.5$).21

The design used in the experiments reported above does not permit one to distinguish between host erythropoiesis and granulopoiesis, and that derived from the transplanted cells. However, cells with colony-forming capacity found in the recipient animals may be considered to be of graft origin since endogenous cells of mice of genotype W/W$^v$ are ineffective in the process of colony formation.7 One would expect, therefore, that if granulopoiesis observed in the recipient mice of genotype W/W$^v$ were largely derived from the transplanted cells, then the number of peroxidase-positive cells observed should be correlated with numbers of CFU; however, if granulopoiesis were largely derived from the host, no such correlation should be observed. Since in the experiments described above, determinations were made of both the number of peroxidase-positive cells per spleen in individual animals (Fig. 3) and the number of CFU per spleen in these same animals (averaged to obtain the results of Fig. 2), it was possible to test for such a correlation. The results are given in Figure 4. Data are shown for animals in which erythropoiesis was successfully suppressed, along with data for nontransfused controls and
Fig. 4.—Number of CFU per spleen versus number of granulocytes per spleen in individual unirradiated recipient animals of genotype W/W<sup>+</sup> which received 2 × 10<sup>6</sup> normal marrow cells 7–10 days prior to test. ▲ = recipients successfully plethorized; △ = recipients unsuccessfully plethorized; ○ = nontransfused controls. The stippled area indicates the zone occupied by 85 per cent of the points.

for animals in which suppression of erythropoiesis was unsuccessful. It is evident from the figure that a correlation exists between the granulocyte count per spleen and the number of CFU per spleen in individual recipients. A test of association between the two measurements showed a highly significant association (p < 0.001) between granulocytes and CFU, although neither granulopoiesis nor colony-forming ability was correlated with erythropoiesis.

**DISCUSSION**

Results presented in this paper show that suppression of erythropoiesis by transfusion will prevent the appearance of macroscopic colonies in the spleens of mice of genotype W/W<sup>+</sup> following the transplantation of marrow cells from normal coisogenic donors. However, this suppression of colony formation appears to result from a failure to develop the large numbers of erythropoietic cells required for the recognition of macroscopic colonies and not from any direct effect on the growth of colony-forming cells.

It seems well established that erythropoiesis is suppressed in plethoric animals because production of the hormone erythropoietin is inhibited under these conditions. It has been suggested previously that although the progeny of hemopoietic colony-forming cells may respond to the action of erythropoietin, the colony forming cells themselves are not sensitive to
the hormone. The observations presented in this paper provide further support for this view, since the absence of a stimulus for erythropoiesis did not inhibit the proliferation of colony-forming cells (Fig. 2). Moreover, the observation that suppression of erythropoiesis prevented the development of macroscopic colonies (Table 1) is consistent with the view\textsuperscript{9,10} that cells responsive to erythropoietin ("erythropoietin-sensitive cells") are included among the progeny of colony-forming cells.

It should be pointed out that no correlation was observed in our results between the suppression of erythropoiesis and granulopoietic activity (Fig. 3). This lack of correlation was not due to a complete suppression of granulopoiesis in unirradiated W/W\textsuperscript{v} mice, since an increase in granulocytes was observed in the spleens of W/W\textsuperscript{v} animals given transplants of genetically normal marrow (Fig. 4). It is possible that the growth of transplanted colony-forming cells in W/W\textsuperscript{v} hosts (Fig. 2) was related to a continuing stimulus for granulopoiesis even in the absence of a stimulus for erythropoiesis. This finding is consistent with the view\textsuperscript{19} that the control of granulopoiesis is independent of the control of erythropoiesis.

Previous results\textsuperscript{10} had shown that a stimulation of erythropoiesis by exposure of animals to hypoxia did not produce any significant increase in the numbers of colony-forming cells in spleen or marrow. The observations presented in this paper show that the proliferation of colony-forming cells is not inhibited in the absence of a stimulus for erythropoiesis. Taken together, these findings provide strong support for the view\textsuperscript{9,10} that colony-forming cells differ from erythropoietin-sensitive cells.

**Summary**

Suppression of erythropoiesis by transfusion of animals of genotype W/W\textsuperscript{v} was found to prevent the development of macroscopic spleen colonies following injection of normal coisogenic marrow cells. This inhibition of colony-formation was not due to a failure of colony-forming cells to proliferate in the absence of erythropoietic stimulation, since the growth rate of normal colony-forming cells in plethoric animals did not differ significantly from that seen in anemic hosts. It is likely that, in plethoric hosts, insufficient differentiated erythroblasts were produced to permit the development of macroscopically visible spleen colonies. Evidence was obtained that granulocytic differentiation proceeded during the growth of the transplanted colony-forming cells, and that this mode of differentiation was not affected by the suppression of erythropoiesis. These results indicate that both granulocytic differentiation and the process of self-renewal by which colony-forming cells increase in numbers are controlled independently of the control of erythropoiesis. These experiments provide additional support for the view that colony-forming cells differ from erythropoietin-sensitive cells.

**Summario in Interlingua**

Le suppression, per transfusiones, del erythropoiese in animales del genotypo W/W\textsuperscript{v} se ha provate capace a prevenir le disveloppamento de macroscopic colonias splenic post le injection de normal cellulas medullari coisogene. Iste
inhibition del formation de colonias non eseva un effecto del non-proliferation de cellulas a formation de colonias in le absentia de stimulation erythropoietic, viste que le intensitate crescential de normal cellulas a formation de colonias in animales plethoric non differe significativamente ab illo incontrote in hospites anemic. Il es probable que, in hospites plethoric, insufficiente erythroblastos differentiate eseva producite pro render possibile le disveloppamento de macroscopicamente visible colonias splenic. Esseva obtenite evidentia in supporto del conclusion que le differentiation granulocytic procedeva durante le crescentia del transplantate cellulas a formation de colonias e que iste modo de differentiation non eseva afficite per le suppression del erythropoiese. Iste resultatos indica que tanto le differentiation granulocytic como etiam le processo del auto-renovamento per le qual le cellulas a formation de colonias augmenta lor numeros es regulate de maniera independente del regulation del erythropoiese. Le experimentos hic reportate supporta additionalmente le conception que cellulas a formation de colonias differe ab cellulas sensihile pro erythropoietina.

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EFFECT OF PLETHORA ON COLONY-FORMING CELLS


