Delayed Erythropoiesis in Irradiated Rats Grafted With Syngeneic Marrow: Effects of Cytotoxic Drugs and Iron-deficiency Anemia

By Pamela Rodday, Michael Bennett, and Joseph J. Vitale

Erythropoiesis in spleens of lethally irradiated Lewis rats grafted with 4–35 × 10⁶ syngeneic marrow cells was inhibited or delayed during the test period of 5 days; this was in marked contrast to observations in irradiated mice. The mechanism of this inhibition was the subject of this study. Pretreatment of recipients 9 days prior to irradiation with the cytotoxic drugs cyclophosphamide (CY), busulfan (BUS), or dimethylmyleran (DMM), or the induction of iron deficiency with anemia in recipients reversed this delayed erythropoiesis. However, neither iron-deficiency anemia nor pretreatment with BUS or DMM affected the ability of irradiated recipients to reject 20 to 50 × 10⁶ allogeneic marrow cells. The administration of commercial preparations of erythropoietin to hosts stimulated erythropoiesis moderately. However, proliferation of syngeneic marrow cells was not enhanced when infused into lethally irradiated Spontaneous Hypertensive (SH) inbred-strain rats which have high levels of endogenous erythropoietin. Finally, plasma from irradiated rats treated with phenylhydrazine to produce severe anemia was rich in erythropoietin but failed to stimulate erythropoiesis in the cell transfer system. Two hypotheses are considered: (1) Irradiation inhibits the secretion of a factor (not erythropoietin) responsible for initiating early stages in differentiation of transplanted stem cells; iron-deficiency anemia and cytotoxic drugs stimulate the secretion of this factor. (2) Normal rats secrete a factor which suppresses erythropoiesis; iron-deficiency anemia and cytotoxic drugs inhibit the production or function of this factor. Cellular rather than humoral factors may be involved.

When marrow stem cells are infused into lethally irradiated histocompatible recipients, they repopulate the hemopoietic tissues after seeding into reticular organs. The early stages of differentiation are controlled by properties of specific “hemopoietic inductive microenvironments” which determine the particular pathway for differentiation. The expansion of the erythropoietic foci or colonies is known to be stimulated by the hormones erythropoietin and testosterone, and inhibition of erythropoiesis can be accomplished by hypertransfusion of red blood cells. Plasma from polycythemic animals appears to have factors which inhibit erythropoiesis, although their exact physical and chemical nature is not known.

As donor stem cell proliferation proceeds, discrete hemopoietic colonies appear on the surface of the host spleen. In the mouse, these colonies appear between 7 and 9 days after marrow grafting, and both erythropoiesis and granulopoiesis occur. In marked contrast, the development of these colonies in the rat...
appears to be delayed, and almost all of the colonies are erythropoietic. The present study deals with the phenomenon of delayed erythropoiesis in rats and its abrogation by cytotoxic drugs and iron deficiency with anemia.

MATERIALS AND METHODS

Animals

Rats. Inbred Lewis (LEW) and Spontaneous Hypertensive (SH) rats were purchased from Charles River Breeding Laboratories, Wilmington, Mass. Fischer (F344) and Wistar Furth (WF) rats were purchased from ARS Sprague-Dawley, Madison, Wis. (Lewis × Buffalo)F1 hybrid [(LEW × BUF)F1] rats were obtained from Microbiological Associates, Walkersville, Md. All rats were 6-8-week-old males and were maintained on Purina Lab Chow and water ad libitum unless otherwise indicated. The major histocompatibility genotypes of these rat strains are: LEW, Ag-B'; F344, Ag-B1; WF, Ag-B2; (LEW × BUF)F1, Ag-B' /Ag-B6.

Mice. C57BL/6J and (C57BL/6 × A)F1 hybrid mice were purchased from the Jackson Laboratory, Bar Harbor, Me. WB and noninbred Swiss mice were from our colony. Adult mice of either sex were used. C57BL/6J mice are H-2b, (C57BL/6 × A)F1 are H-2b/H-25 and WB are H-2b at the major histocompatibility locus of the species.

Cell Transfer Procedures

Prospective recipient inbred-strain and F1 hybrid animals were placed in a Gamma Cell 40 137Cs small animal apparatus and exposed to 800-900 rads of total-body irradiation a few hours prior to transplantation. Syngeneic or incompatible bone marrow cells were flushed from the long bones of donors with a 2-cc syringe and 23-gauge needle filled with Eagle's minimal essential medium (MEM). The cells were suspended by repeated aspiration of the marrow plugs into the syringe, and the resulting cell suspension was filtered through 200-mesh/in stainless steel wire mesh to remove particulate matter. Nucleated cells were counted in a hemocytometer and were then adjusted to the desired concentration with MEM. Inocula of donor marrow cells were infused into a lateral tail vein of lethally irradiated recipients. Radiation control animals received no cells.

Direct Splenic Injection of Cells Into Host Spleens

LEW rats were anesthetized with ether and a left lateral abdominal incision was made. The spleens were exposed and an inoculum of 5 × 10^6 marrow cells in 0.1 ml saline was injected directly into the spleen substance using a 27-gauge needle. Spleens of control animals were injected with 0.1 ml MEM.

Graft Evaluation

Graft cell proliferation was assessed 3-6 days after cell transfer by measuring the splenic incorporation of a specific DNA precursor, 5-iodo-2'-deoxyuridin-125I (IUdR), as described. Each recipient was injected intraperitoneally with 10^-7 moles of 5-fluoro-2'-deoxyuridine to inhibit the endogenous formation of thymidine precursors, which compete with IUdR for incorporation into DNA. After 60 min, each rat received 0.5 μCi of IUdR intravenously and was sacrificed 2 hr later. The 125I not incorporated into DNA of spleen cells was eluted by soaking the spleens in 70% ethanol for 3 days prior to counting in a gamma scintillation counter. The uptake of 125I by spleens was expressed as the percentage of IUdR injected. In addition, histological sections were prepared to confirm marrow graft proliferation. Under these conditions, especially in rats, erythropoiesis comprised more than 90% of the proliferating cells.

Pretreatment of Recipients With Immunosuppressive Agents

Busulfan (BUS, Burroughs Wellcome) was dissolved in dimethylsulfoxide, the proportion in the final solution being reduced to 10% with peanut oil. The drug was administered to prospective recipients intragastrically 9 days prior to marrow grafting. Rats received 30 mg/kg and mice received 50 mg/kg body weight sublethal doses used to suppress hemopoiesis.

Cyclophosphamide (CY, Mead Johnson) was administered intraperitoneally to rats either 1 or
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9 days prior to marrow grafting. Each rat received 200 mg/kg body weight, a dose which suppresses marrow allograft reactivity.\textsuperscript{12}

Dimethylmelaran (DMM, Lot No. CA-124, Drug Development Branch of the National Cancer Institute) was dissolved in propylene glycol, diluted 1:10 with saline, and injected intraperitoneally. Each rat received 5 mg/kg body weight, a sublethal dose which suppresses hemopoiesis,\textsuperscript{13} 9 days prior to marrow grafting.

Silica particles (5\textmu m average diameter) were injected intravenously (250 mg/kg body weight) 1 day prior to irradiation and marrow grafting. This dose of silica suppresses marrow allograft and xenograft reactivity.\textsuperscript{14,15}

Finally, rats were exposed to 500 rads 10 days prior to lethal (800 rads) irradiation and cell transfer. This procedure weakens marrow allograft reactivity in mice.\textsuperscript{16}

Induction of Iron-deficiency Anemia

Weanling LEW rats were injected intramuscularly with deferoxamine mesylate (DFM, Ciba), a potent iron-chelating agent,\textsuperscript{17} and were fed a powdered whole milk diet ad libitum (Pilgrim Dairy, Weymouth, Mass.). Each animal received 5 mg DFM/100 g body weight 31, 28, 24, and 21 days (Table 2, Exp. 1) or 23, 17, and 10 days (Table 2, Exp. 2) prior to marrow transplantation. To insure that DFM was not directly exerting an effect on the marrow grafts, another group received 1 mg of iron dextran (ID, Lakeside) intramuscularly 24 hr after each injection of DFM, and was maintained on the milk diet. Control rats were injected with saline and were maintained on the whole milk diet supplemented with 50 mg iron/kg diet as ferrous sulfate. A final control group received DFM and was maintained on an iron-fortified diet. (LEW × BUF)\textsubscript{F\textsubscript{1}} rats were administered DFM as above, 29 and 20 days prior to transplantation and maintained on a whole milk diet. Because these \textsubscript{F\textsubscript{1}} rats were resistant to the induction of iron deficiency by this method, 1 ml of peripheral blood was withdrawn from the orbital sinus of each animal 11 and 5 days before cell transfer. Hemoglobin and hematocrit determinations and peripheral blood smears were performed to evaluate the severity of iron-deficiency anemia.

Erythropoietic Stimulation

Step I and step III erythropoietin, prepared from the plasma of phenylhydrazine-treated sheep, was purchased from Connaught Laboratories, Toronto, Ont. Step I was injected subcutaneously (s.c.) on the day of and the day following irradiation and marrow cell transfer (2.8 U/100 g body weight). Step III was injected on the day of and on each of the first 4 days following irradiation and cell transfer (3.0 U/100 g body weight).

WF rats were exposed to 800 rads of \gamma-rays. On the same day, and for the following 4 days, the rats were injected s.c. with 0.3 ml of a neutralized 1\textpermille solution of phenylhydrazine. The rats were bled on the day following the last injection to obtain plasma rich in erythropoietin.\textsuperscript{18} The hematoctrit values of the plasma donors ranged between 9\textpermille and 16\textpermille. The plasma samples were pooled. Irradiated LEW rats infused with LEW marrow cells were injected s.c. with 0.5 ml of this plasma on the first 3 days following cell transfer.

In two experiments, inbred SH rats were tested, since these rats have high blood levels of endogenous erythropoietin from birth.\textsuperscript{19}

Statistics

The IUdR uptake values were expressed as mean and standard error of the mean for each group. Student’s t test was used to determine statistical differences in mean values between groups. The method of least squares was used to determine the regression analysis between inoculum size and IUdR uptake (per cent), and to determine doubling time (\textit{t}\textsubscript{1/2}) of IUdR uptake (per cent) between days 3 and 6 following marrow transplantation (Fig. 2).

RESULTS

Delayed Proliferation of Syngeneic Marrow Cells Grafted Into Lethally Irradiated Rats

When LEW marrow grafts of 3.5 or 35 × 10\textsuperscript{6} cells were infused into lethally irradiated LEW hosts, the cells failed to proliferate well in the first 5 days, as
Table 1. Effect of Various Host Pretreatments on the Growth of Syngeneic Marrow Cells in Lethally Irradiated Rats

<table>
<thead>
<tr>
<th>Exp.</th>
<th>No. Cells Grafted (x 10^6)</th>
<th>Strain</th>
<th>Pretreatment (days)</th>
<th>Mean ± SE Splenic IUdR Uptake (%)</th>
<th>No. Rats</th>
<th>Student's t Test</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>LEW</td>
<td>None</td>
<td>0.006 ± 0.0008</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>LEW</td>
<td>None</td>
<td>0.01 ± 0.003</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>LEW</td>
<td>CY (1)</td>
<td>0.003 ± 0.0001</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>LEW</td>
<td>None</td>
<td>0.01 ± 0.002</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>LEW</td>
<td>CY (1)</td>
<td>0.004 ± 0.0007</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0*</td>
<td>LEW</td>
<td>None</td>
<td>0.01 ± 0.001</td>
<td>3</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>5*</td>
<td>LEW</td>
<td>None</td>
<td>0.01 ± 0.003</td>
<td>7</td>
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<tr>
<td></td>
<td>5 Swiss mice</td>
<td>Silica (1)</td>
<td></td>
<td>0.47 ± 0.02</td>
<td>4</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>LEW</td>
<td>None</td>
<td>0.01 ± 0.001</td>
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<tr>
<td></td>
<td>5</td>
<td>LEW</td>
<td>500 rads (10)</td>
<td>0.05 ± 0.01</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>LEW</td>
<td>Silica (1)</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>LEW</td>
<td>None</td>
<td>0.03 ± 0.001</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>LEW</td>
<td>BUS (9)</td>
<td>0.73 ± 0.19</td>
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<td>p &lt; 0.05</td>
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<tr>
<td></td>
<td>4</td>
<td>LEW</td>
<td>None</td>
<td>0.06 ± 0.02†</td>
<td>3</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>LEW</td>
<td>BUS (9)</td>
<td>0.91 ± 0.17†</td>
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<td></td>
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<tr>
<td>5</td>
<td>4</td>
<td>LEW</td>
<td>None</td>
<td>0.14 ± 0.03</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>LEW</td>
<td>CY (9)</td>
<td>0.43 ± 0.02</td>
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<td>p &lt; 0.005</td>
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<tr>
<td>6</td>
<td>10</td>
<td>LEW</td>
<td>None</td>
<td>0.11 ± 0.03</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>LEW</td>
<td>DMAM (9)</td>
<td>1.04 ± 0.19</td>
<td>6</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

*Intrasplenic injection.
†Assay performed on day 6, all other experiments assayed on day 5.

shown by the low IUdR uptake values (Table 1, Exp. 1). Histologic examination of the host spleens revealed only small foci of primitive cells, confirming the poor growth of the graft. The presence of these small foci suggested, but did not prove, that a normal seeding mechanism existed in the LEW host, and that failure of the graft to proliferate was due to some other mechanism(s). To substantiate this possibility, marrow cells were injected directly into the spleens of lethally irradiated LEW hosts. The cells failed to proliferate (Table 1, Exp. 2).

The viability of the LEW marrow cells was determined by infusing them intravenously into Swiss mice given 5 mg silica 24 hr prior to lethal irradiation and marrow grafting, a procedure which prevents rejection of the xenogeneic cells. The LEW marrow cells grew well in these mice (Table 1, Exp. 2), indicating that the failure of the syngeneic grafted cells to proliferate resided in the LEW hosts.

Effects of Immunosuppression

The slow growth of syngeneic cells in the rat resembles the resistance to marrow allografts in mice. CY suppresses marrow allograft reactivity in mice and rats, but pretreatment of rats with CY 1 day prior to irradiation and transplantation had no effect on proliferation of syngeneic rat marrow grafts (Table 1, Exp. 1). LEW rats exposed to 500 rads of irradiation 10 days before
Fig. 1. Spleens of LEW rats 5 days after lethal irradiation and infusion of $20 \times 10^6$ syngeneic marrow cells. (A) Host pretreated with DMM 9 days before irradiation and cell transfer. Intense erythropoietic activity is evident, which extends to the capsule. (B) No host pretreatment. There is minimal evidence of hemopoiesis. Hematoxylin and eosin stain. × 48.6.
lethal irradiation (800 rads) failed to support the growth of syngeneic marrow cells (Table 1, Exp. 3). Intravenous administration of silica also failed to stimulate proliferation of the syngeneic grafts (Table 1, Exp. 3).

In addition, the following immunosuppressive host pretreatments failed to enhance proliferation of syngeneic marrow grafts (data not shown): (1) administration of 30 mg of cortisol (Hydrocortone, Merck, Sharp and Dohme) intraperitoneally 2 days prior to marrow grafting; (2) thymectomy of newborn rats 3 wk before irradiation and cell transfer; (3) administration of horse anti-rat thymocyte serum (Microbiological Associates, 1 ml intraperitoneally) 1 day prior to irradiation and marrow transplantation; and (4) pretreatment with $^{90}$Sr (50 $\mu$Ci at 21 days of age and 100 $\mu$Ci at 42 days of age). Irradiation and marrow cell transfer were performed when the rats were 63 days of age. $^{90}$Sr abrogates marrow allograft reactivity in mice.21

Effects of BUS, DMM, or CY

Pretreatment of LEW rats with BUS, DMM, or CY 9 days prior to irradiation and transplantation markedly enhanced the proliferation of syngeneic marrow cells (Table 1, Exps. 4–6). Histologic examination revealed erythropoiesis expanding the red pulp and extending to the capsule in spleens of drug-pretreated rats (Fig. 1A). The spleens of control animals showed minimal evidence of hemopoiesis (Fig. 1B) consistent with the low IUdR uptake values. Bone marrow uptake of IUdR was also determined in several experiments and only negligible values were obtained, even when uptake was high in spleens.

To determine whether or not quantitative experiments with marrow cell grafts could be performed in irradiated rats which did not restrict growth of syngeneic stem cells, LEW rats were treated with DMM 9 days prior to irradiation and infusion of 5–40 $\times$ 10$^6$ syngeneic marrow cells. There was a linear
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Fig. 3. Growth curve of $20 \times 10^6$ transplanted syngeneic LEW bone marrow cells in irradiated hosts pretreated with 5 mg/kg DMM 9 days earlier. The IUdR uptake (%) values in spleens of irradiated hosts not pretreated with DMM were low (0.01%–0.07%), and the values did not increase with time after cell transfer (data not shown).

relationship between inoculum size and IUdR uptake (per cent) in spleens 5 days after cell transfer (Fig. 2). In a second experiment, LEW hosts pretreated with DMM were grafted with $20 \times 10^6$ syngeneic marrow cells. Splenic IUdR uptake (per cent) was determined on days 3, 4, 5, and 6. Growth of grafted cells was exponential between days 3 and 6 following marrow transplantation, and the mean doubling time ($t_2$) was 14.5 hr (Fig. 3). These results were similar to those obtained in mice not pretreated with cytotoxic drugs.7 IUdR uptake values in rats only irradiated and infused with $2 \times 10^7$ marrow cells were 0.01%–0.07% between days 3 and 6; the mean values did not increase with time.

Effect of Iron-deficiency Anemia

Iron deficiency is known to stimulate differentiation and proliferation of hemopoietic stem cells. Mice with hereditary iron-deficiency anemia have greatly increased erythropoietic stem cell activity in the marrow and spleen.22 In a double crossover experiment, both prospective LEW donors and hosts were made iron deficient. At the time of transplantation, mean red cell indices in the iron deficient and control groups, respectively, were: hemoglobin 2.8 and 10.9 g/100 ml, hematocrit 18.6% and 39%, and mean corpuscular hemoglobin concentration (MCHC), 5% and 28%. Donor iron deficiency had no effect on graft proliferation, but iron deficiency in the host markedly enhanced proliferation of syngeneic marrow cells in irradiated LEW hosts (Table 2, Exp. 1).

DFM did not exert an independent effect on the marrow grafts. One control group of LEW rats received no DFM and was maintained on an iron-fortified diet. Two other groups of control animals did receive DFM, but were administered iron either in the form of iron dextran injections or as ferrous sulfate in the diet. In this experiment, hematocrits were 19, 41, 40, and 40 in experimental and the three control groups, respectively. Peripheral blood smears of
only the iron-deficient group demonstrated evidence of iron-deficiency anemia. The iron-deficient rats, but none of the controls, allowed extensive proliferation of syngeneic marrow cells (Table 2, Exp. 2). Histologic examination confirmed the extensive erythropoiesis in the spleens of the anemic, but not of the control, hosts.

**Inability of BUS, DMM, or Acute Iron Deficiency to Prevent Rejection of Incompatible Marrow Grafts**

C57BL mice were pretreated with BUS 9 days prior to lethal irradiation and transfer of incompatible WB marrow cells. (C57BL × A) F₁ mice were similarly pretreated and infused with incompatible parental strain C57BL marrow cells. In both cases, pretreatment with BUS had no effect on rejection of the incompatible grafts (Table 3, Exps. 1 and 2). However, the donor cells grew well when infused into untreated lethally irradiated syngeneic mice.

LEW and F344 strain rats were administered DMM 9 days prior to irradiation and transplantation of Ag-B-incompatible WF marrow cells. The WF cells failed to grow in these incompatible hosts even at large cell inocula, but LEW cells did proliferate in the DMM-pretreated LEW hosts (Table 3, Exp. 3).

Finally, (LEW × BUF) F₁ hybrid rats were made iron deficient and given syngeneic or incompatible WF marrow cells. Peripheral blood smears and hematocrits confirmed the iron deficiency. Incompatible marrow grafts were rejected, whereas syngeneic cells grew well in the iron-deficient hosts (Table 3, Exp. 4). Thus, neither pretreatment with DMM nor induction of acute iron deficiency abrogated the resistance to incompatible marrow grafts in rats, and BUS did not abrogate resistance to incompatible marrow grafts in mice.

**Effect of Erythropoietic Stimulation**

Syngeneic marrow cells were injected into lethally irradiated SH inbred strain rats which have high blood levels of erythropoietin from birth. The grafts failed to proliferate (Table 4, Exps. 1 and 2).

Step I and step III erythropoietin preparations moderately stimulated the growth of syngeneic marrow cells (Table 4, Exps. 3 and 4).
Table 3. Inability of BUS, DMM, or Acute Iron-deficiency Anemia to Prevent Rejection of Incompatible Marrow Grafts

<table>
<thead>
<tr>
<th>Donor</th>
<th>No. Cells Grafted (x 10^6)</th>
<th>Host</th>
<th>Mean ± SE Splenic 125 I Udr Uptake (%)</th>
<th>No. Rats</th>
<th>Student's t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp.</td>
<td>Strain</td>
<td>Strain Pretreatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>—</td>
<td>C57Bl None</td>
<td>0.02 ± 0.005</td>
<td>3</td>
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</tr>
<tr>
<td></td>
<td>WB 2</td>
<td>C57Bl None</td>
<td>0.05 ± 0.01</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WB 2</td>
<td>C57Bl BUS</td>
<td>0.02 ± 0.002</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WB 2</td>
<td>WB None</td>
<td>0.61 ± 0.04</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>(C57Bl x A)F1 None</td>
<td>0.03</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C57Bl 2</td>
<td>(C57Bl x A)F1 None</td>
<td>0.02 ± 0.001</td>
<td>5</td>
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<tr>
<td></td>
<td>C57Bl 2</td>
<td>(C57Bl x A)F1 BUS</td>
<td>0.02 ± 0.001</td>
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<td>LEW 20</td>
<td>LEW DMM</td>
<td>0.23 ± 0.08</td>
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<td>p &lt; 0.025</td>
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<td>WF 20</td>
<td>LEW DMM</td>
<td>0.01 ± 0.001</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>F344 None</td>
<td>0.01 ± 0.001</td>
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<td>WF 20</td>
<td>F344 None</td>
<td>0.01 ± 0.001</td>
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</tr>
<tr>
<td></td>
<td>WF 20</td>
<td>F344 DMM</td>
<td>0.01 ± 0.002</td>
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<td></td>
<td>WF 50</td>
<td>F344 DMM</td>
<td>0.02 ± 0.002</td>
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<tr>
<td>4</td>
<td>(LEW x BUF)F1 30</td>
<td>(LEW x BUF)F1 DFM, Fe Diet</td>
<td>1.36 ± 0.35</td>
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<td>p &lt; 0.02</td>
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<tr>
<td></td>
<td>WF 30</td>
<td>(LEW x BUF)F1 DFM, Fe Diet</td>
<td>0.05 ± 0.02</td>
<td>5</td>
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</tr>
<tr>
<td></td>
<td>—</td>
<td>(LEW x BUF)F1 DFM, Fe Diet</td>
<td>0.07</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*SH rats with high levels of endogenous erythropoietin.
†Epo, erythropoietin.
§X, φH plasma obtained from irradiated (800 rads) or DMM treated (7.5 mg/kg) rats injected with phenylhydrazine for 4 days.
§Assay performed on day 4, all other experiments assayed on day 5.
Irradiated rats injected with phenylhydrazine to induce anemia have high circulating levels of erythropoietin. However, plasma from these animals failed to stimulate the growth of 20 x 10⁶ syngeneic marrow cells when injected into lethally irradiated hosts (Table 4, Exp. 5). In contrast, plasma from rats injected with a lethal dose of DMM (7.5 mg/kg) and subsequently injected with phenylhydrazine did stimulate the growth of syngeneic marrow cells (Table 4, Exp. 6), although the mean values were not significantly different.

Erythropoietin Determinations of Various Rat Plasma Samples

Pooled plasma from LEW rats which were treated in various ways were sent to Dr. Eugene Goldwasser, who kindly performed erythropoietin assays using cultures of rat bone marrow cells. Normal rat plasma has values of 20-90 milliunits (mU) per ml. Plasma from iron-deficient rats used in these experiments had 100 mU/ml. The plasma erythropoietin value from rats treated with 5 mg/kg DMM 9 days earlier was 0 mU/ml, from rats treated with 7.5 mg/kg DMM followed by four phenylhydrazine injections (Exp. 6 of Table 4) was 1200 mU/ml and from irradiated (800 rads) rats injected with phenylhydrazine (Exp. 6 of Table 4) was 600 mU/ml. These data indicated that erythropoietin levels did not correlate with the rapid rate of growth of transplanted marrow cells.

Histologic Observations

Spleens of rats pretreated with irradiation, cytotoxic drugs, or rendered iron deficient were examined histologically, using standard techniques. These rats were not lethally irradiated and grafted with marrow cells. The red pulp of the normal adult rat had many less myelopoietic cells, e.g., normoblasts or megakaryocytes, than did the red pulp of normal mice. The spleens of iron-deficient rats showed extensive erythropoiesis in the red pulp, and the spleens were enlarged. The spleens of rats treated with 5 mg/kg DMM 9 days earlier had no myelopoiesis in the hypocellular red pulp; the white pulp was essentially normal, including the marginal zone of macrophages. Few follicles were discerned in the samples examined. The spleens of rats exposed to 500 rads 10 days earlier were hypocellular, with only few lymphocytes and macrophages in the white pulp and only few erythroid cells in the red pulp.

DISCUSSION

The failure of marrow stem cells to undergo rapid erythropoiesis in spleens of irradiated syngeneic hosts (Table 1) could be due to a lack of stimulation or to an active inhibition of erythropoiesis. The erythropoietic hemopoietic inducive microenvironment (HIM) may not induce differentiation of stem cells as rapidly in rat spleens as it does in mouse spleens after irradiation. In this study, lack of erythropoietin did not appear to be the major cause of the delayed erythropoiesis observed. The administration of step I and step III preparations of erythropoietin moderately enhanced erythropoiesis (Table 4). While erythropoietin levels are increased in iron-deficiency anemia, syngeneic marrow grafts grew poorly in SH rats, which have high levels (i.e., 150–270 mU/ml) of endogenous erythropoietin. Moreover, the plasma from rats irradiated before injection of phenylhydrazine did not stimulate erythropoiesis, whereas the plasma from rats pretreated with DMM before injection of phenylhydrazine did stim-
ulate it (Table 4). Such plasma is rich in erythropoietin, and several investigators have shown that heavily irradiated rats are capable of secreting erythropoietin.

Rapid proliferation of syngeneic marrow cells has been observed in rats if immunosuppressive drugs are used in place of lethal irradiation. Sublethal doses of the three cytotoxic drugs BUS, DMM, and CY temporarily suppress stem cell proliferation. Since recovery from this stem cell suppression is maximal within 9 days after drug administration, it seemed likely that whatever stimulus exists at this time would also permit grafted syngeneic stem cells to proliferate in lethally irradiated rats. Indeed, the prior administration of these drugs to irradiated rats resulted in the rapid proliferation of the grafted erythropoietic stem cells (Table 1, Fig. 2).

A dose of 500 rads of γ-rays suppresses stem cell proliferation to a similar extent as does the administration of these drugs. However, in rats exposed to 500 rads of γ-irradiation 10 days prior to lethal irradiation and cell transfer, growth of marrow cells is poor (Table 1, Exp. 3). Thus it is conceivable that irradiation has a qualitatively different effect upon hemopoiesis. In irradiated (560 rads) rats, erythropoiesis occurs early if irradiation follows, but not if irradiation precedes phenylhydrazine-induced anemia. Perhaps drug treatment but not irradiation leads to the production of an erythropoietic factor other than erythropoietin. Thus, anemia in most instances may be associated with the production of at least two factors or hormones, and both may be present in most currently available preparations of erythropoietin. An observation supporting this concept of two different factors has been reported. Both unirradiated and irradiated rabbits were injected with phenylhydrazine to induce anemia. The plasma from both groups of rabbits stimulated erythropoiesis in rats, but the response was delayed by several days in recipients of plasma from the irradiated rabbits.

Iron-deficiency anemia increases marrow and spleen erythropoietic activity at the level of the stem cell in mice, and we observed an increase in splenic erythropoiesis histologically in rat donors (Table 2) which were iron deficient. One possibility is that iron-deficiency anemia may increase the inductive capacity of the erythropoietic inductive microenvironment so as to allow greater proliferation of the grafted marrow cells. In addition to anemia, other possibilities exist. Iron deficiency with or without the associated anemia has been shown to influence the metabolism of other nutrients such as folic acid and fatty acids, and is associated with altered morphology, not only of the erythron but of the liver, gastric mucosa, and granulocytic cells. Metabolic and/or functional changes in these nutrients and systems could be expected to alter the metabolism of other nutrients or hormones which might affect stem cell differentiation and cell proliferation. It would be of interest to determine the effect of pyridoxine deficiency on delayed repopulation. The microcytic, hypochromic anemia of pyridoxine deficiency is associated with hyperferremia, in contrast to the hypoferrremia of iron-deficiency anemia. Thus, we cannot be certain whether iron deficiency and/or the anemia is specific for the observed effects, or whether induced secondary metabolic or functional changes play the major role in the abrogation of the delayed erythropoiesis in irradiated rats grafted with marrow cells.
The delayed repopulation of rat spleens by syngeneic marrow cells resembled the lack of repopulation of mouse spleens by allogeneic or incompatible parental marrow cells. However, pretreatment of rats with agents capable of suppressing marrow allograft reactivity, e.g., CY 1 day before irradiation, had no effect on grafts of syngeneic marrow cells (Table 1). BUS, DMM, and iron deficiency were able to accelerate growth of syngeneic rat stem cells, but were unable to weaken the resistance to marrow allografts in rats or mice (Table 3). The immune system does not appear to be involved in this delayed erythropoietic phenomenon, since a variety of immunosuppressive agents, e.g., cortisol or antithymocyte serum, had no effect on graft proliferation. It is conceivable, however, that irradiated rats exert an active inhibitory influence on erythropoiesis by elaborating a humoral factor, or by a cellular mechanism. Iron deficiency or drug treatment would suppress its secretion or function.

Why erythropoiesis occurs rapidly in mice but not in rats following marrow grafting is presently unknown. In preliminary experiments, we have observed that hemopoiesis in irradiated dogs grafted with autologous marrow cells was also delayed for several days. Species differences in susceptibility to ionizing irradiation have been noted. For example, the LD50/30 dose of x-rays was 600 rads for mice and 300 rads for sheep. Moreover, hematologic recovery from a two-thirds LD50/30 dose (400 rads to mice, 200 rads to sheep) was much more rapid in the smaller animals. Hemopoiesis may be delayed in man grafted with autologous marrow cells.

The elucidation of the mechanism by which erythropoiesis is delayed in irradiated rats and how this delay is prevented by cytotoxic drugs or iron-deficiency anemia may give new insights into the physiologic control of hemopoiesis, and may provide a more rational approach to the use of bone marrow transplants in man.

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