Effect of Hypertransfusion on Bone Marrow Regeneration in Sublethally Irradiated Mice. I. Enhanced Granulopoietic Recovery

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Hypertransfusion can enhance recovery from neutropenia in certain clinical and experimental situations. We have studied the pattern of myeloid recovery in mice hypertransfused after receiving 350 rads whole body irradiation. Both hypertransfused and control groups showed the degenerative phase, abortive rise, and regenerative phase that has been described following sublethal irradiation. The blood granulocyte counts in the hypertransfused group returned to normal more rapidly and were maintained at a significantly higher level during the regenerative phase. This difference is not the result of a shift in granulocytes from the marrow granulocyte reserve or marginal granulocyte pool to the circulating pool, but is associated with significantly enhanced bone marrow granulopoiesis. While the total bone marrow cellularity of the hypertransfused mice is less than that of the control mice, the hypertransfused group contains more CFU-GM and myeloid cells during the regenerative phase. The enhanced granulopoiesis is not due to increased colony-stimulating activity (CSA) levels in the hypertransfused mice, as the CSA levels were significantly lower in this group compared to the controls prior to and during the initial phase of granulopoietic recovery. This study suggests that hypertransfusion increases the rate of recovery of myelopoiesis by increasing the number of precursors available for myeloid differentiation from an earlier stem cell compartment.

RED BLOOD CELL (RBC) hypertransfusion has been shown to increase the rate of granulopoietic recovery in patients receiving induction chemotherapy for acute lymphocytic leukemia. While stem cell competition has been suggested as a possible explanation, the precise mechanism underlying this observation is not understood. Earlier studies had shown that granulopoiesis in polycythemic mice recovered more rapidly from radiation-induced bone marrow depression. However, in these studies, animals were rendered polycythemic before irradiation so that the experimental group began with reduced erythropoietic and expanded granulopoietic compartments. More recently, Firkin, Hays, and Cline studied mice given RBC hypertransfusions after bone marrow depletion and showed more rapid granulocytic recovery. However, from this study it is not apparent which phase of the bone marrow response to irradiation is affected by RBC hypertransfusion. In order to further define events in this experimental model and investigate underlying mechanisms, we have studied various aspects of bone marrow recovery in the postirradiation hypertransfused mouse. In this article we report the effect of hypertransfusion on myeloid recovery in the bone marrow, its relationship to colony-stimulating activity in the plasma, and its effect on distribution of mature granulocytes between the major functional granulocyte pools.

MATERIALS AND METHODS

C3H-He female mice weighing 18–26 g were obtained from Cumberland View Farms, Clinton, Tenn.; Harlan Industries, Indianapolis, Ind.; and Texas Inbred Mice, Houston, Texas, as available. Mice from only one supplier were used in each experiment. Platelet and white blood cell counts were performed before irradiation, and mice with only one supplier were used in each experiment. Platelet and white blood cell counts were performed before irradiation, and mice with abnormal values were not included. These initial counts were used as the basis for grouping mice so that the mean platelet and white blood cell counts and their standard deviations were approximately the same for all groups.

Irradiation

Mice were placed in plastic holders and received 350 rads whole body x-irradiation at 167 rad/min. The irradiation was delivered from a General Electric Maxitron 300 x-ray therapy unit operated at 300 KVP and 20 mA with 2-mm copper filtration using a 20 cm x 20 cm field size at 50 cm from the source. All red blood cells and plasma preparations used for transfusion received 1000 rad before administration.

Preparation of Red Blood Cells and Plasma for Transfusion

Blood was collected into acid citrate from untreated retired breeder female C3H-He mice via cardiac puncture under ether anesthesia. The red blood cells were separated by differential centrifugation. The platelet-rich plasma anduffy coat were removed and the platelets and white blood cells pelleted. The plasma was then used to resuspend the red blood cells and the separation procedure repeated twice. The resulting red blood cell suspensions had a packed cell volume (PCV) of 65%–73% and contained 28,000–135,000 platelets/cu mm and 200–4400 white blood cells/cu mm. The packed cells or plasma were given intraperitoneally 0.5–2 hr and 1 day following irradiation. The volume given was adjusted for body weight to achieve a PCV on day 2 of 65%–75%. This PCV range was chosen as one which would adequately inhibit...
erythropoiesis but not so high as to cause shortened platelet survival. For example, a 22-g mouse received 1.0 cc and then 0.9 cc of packed red blood cells. To control for (1) stimulatory factors that might be present in mouse blood, (2) possible endotoxin contamination of blood during processing, and (3) effect of intraperitoneal injection and blood volume expansion, control mice received an equal volume of plasma from the same blood used to prepare the packed red blood cells. The PCVs of all experimental mice were checked on day 2, and hypertransfused mice with PCVs outside the range 65%–75% were excluded.

Collection of Blood and Bone Marrow Samples

This study was designed so that specimens would be collected and analyzed separately for each mouse. Mice were warmed under an examination lamp to cause vasodilatation. Blood was obtained by puncturing a tail vein using a 27-gauge needle. Blood for white cell counts was collected in a 10-mU Unopette pipet and diluted in a Unopette reservoir containing 0.99 ml of 1% ammonium oxalate. White blood cell and platelet counts were determined using phase contrast microscopy, and differential counts were determined on methanol-fixed, Wright-stained peripheral blood films. Blood for PCV determinations was collected in oxalated 32-mm capillary tubes (Drummond Scientific Co., Broomall, Pa.).

After blood collection, mice were killed by cervical dislocation. Bone marrow was flushed from both femora of each mouse with alpha medium with 5% fetal calf serum and pooled for each mouse separately. The cells were washed once in 10 ml of alpha with 5% fetal calf serum and resuspended. The total nucleated cell count of each marrow suspension was determined by phase contrast microscopy, and viability determined by erythrosin dye exclusion. As the erythropoiesis but not be so high as to cause shortened platelet duration, but replacing the mouse L-cell-conditioned medium with 0.1 ml of test plasma and adjusting the final concentrations with alpha medium.

The committed granulocyte-macrophage colony-forming cells (CFU-GM) were assayed in methyl cellulose by a modification of the method of Worton, McCulloch, and Till. Nucleated bone marrow cells (2 x 10^3) were plated (35 x 10 mm culture dishes, Lux Scientific Corp., Newbury Park, Calif.) in 0.8% methyl cellulose with alpha medium, 20% fetal calf serum, and 20% conditioned medium from cultures of mouse L cells. The L-cell-conditioned medium was harvested on the seventh day from mouse L929 cells (Microbiological Associates, Bethesda, Md.) grown to confluence in Eagle's Minimum Essential Medium with Earle's balanced salt solution, 10% fetal calf serum, and 1% l-glutamine, filtered through a 0.45-μm Millipore filter and stored at −20°C. To provide maximum stimulation of CFU-GM sensitive to this stimulus, a concentration of conditioned media was chosen that was on the plateau portion of the curve generated from a dose-response study. Plasma was assayed for colony-stimulating activity (CSA) using this procedure, but replacing the mouse L-cell-conditioned medium with 0.1 ml of test plasma and adjusting the final concentrations with alpha medium.

The marginalizing granulocyte pool (MGP) was estimated by measuring granulocyte counts before and 15 min after a tail vein injection of 25 μg epinephrine (Park Davis & Co., Detroit, Mich.).

The marrow granulocyte reserve (MGR) was estimated by measuring granulocyte counts before and 2 hr after a tail vein injection of 1 ng of Escherichia coli endotoxin (Bureau of Biologics, Bethesda, Md.). The epinephrine response was measured in the same mice during the afternoon. Different groups of mice were used for the studies on day 3 and day 11 after irradiation. Statistical analysis was performed using a two-tailed Student's t test, nonpaired or paired as appropriate.

RESULTS

The PCV of irradiated mice injected with plasma (XRT-PL) fell from a pretreatment level of 45% to 41% on day 3 and then gradually rose to baseline by day 21 (Fig. IA). The PCV of irradiated mice hypertransfused with RBCs (XRT-RBC) were increased to 73%, decreased gradually until day 10, and then declined more rapidly, reaching baseline by day 21. The blood granulocyte counts for both groups reached a nadir on day 8, increased steadily until day 11, subsequently declined, and then recovered (Fig. IB). Granulocyte counts of the XRT-RBC groups were significantly greater on day 6 (p < 0.05), at the day-8 nadir (p < 0.02), the day-11 peak (p < 0.005), and day-17 nadir (p < 0.02).

The cellularity of the bone marrows of both groups showed the three phases—degenerative phase, abortive rise, and regenerative phase—classically de-
scribed after sublethal irradiation (Fig. 2A). The bone marrow of the XRT-RBC group was significantly less cellular than that of the XRT-PL mice on days 6 ($p < 0.025$), 10 ($p < 0.01$), and 11 ($p < 0.025$). The nucleated bone marrow cell viability of the XRT-RBC group was consistently higher than that of the XRT-PL group (Fig. 3). The viability of cells from the XRT-PL group was markedly lower on day 10, which was a consistent finding in three other experiments not included here. The marrow plugs of XRT-PL group were difficult to suspend on day 10 and required more prolonged mechanical manipulation with a Pasteur pipet, which may have damaged cells and decreased viability. No problems with marrow suspension were encountered at any of the other sampling times.

Marrow erythroid cells in the XRT-PL group were reduced to 21% of normal on day 3, recovered to 54% of normal by day 6, followed by a sharp rise to normal between days 8 and 10 (Fig. 2C). The XRT-RBC group showed the expected inhibition of erythropoiesis with erythroid cells being less than 4% of normal at day 3 and less than 1% of normal from day 6 to day 11, after which recovery began to occur. The bone marrow lymphocytes of both groups were depressed to approximately 15% of normal on day 3 and remained low through day 10 (Fig. 2D). Between days 10 and 11, a rapid rise in bone marrow lymphocytes occurred with levels reaching 158% and 198% of control for the XRT-PL and XRT-RBC groups, respectively. The bone marrow lymphocyte counts for both groups then returned to control levels.

Myeloid cells (Fig. 2B) were present in greater numbers in the bone marrow of the hypertransfused mice on day 11 ($p < 0.005$), day 13 ($p < 0.01$), and day 17 ($p < 0.1$). The post-mitotic bone marrow myeloid pool varied in proportion to the total myeloid cell count and was 85% and 91% on day 11 and 82% and 80% on day 13 for the XRT-PL and XRT-RBC groups, respectively.

The CFU-GM in the bone marrow of both groups were reduced to approximately 17% of control at day 3 (Fig. 4). The CFU-GM of both the XRT-RBC and
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Fig. 3. Effect of hypertransfusion after 350 rad x-irradiation on viability of mouse nucleated bone marrow cells. Solid circles, irradiated hypertransfused mice (4–19 mice/point); open circles, irradiated plasma controls (4–19 mice/point); solid square, no-treatment controls (35 mice). Vertical lines represent SEM.

XRT-PL groups showed a similar pattern from day 3 to day 8. By day 11, there were significantly more CFU-GM in the XRT-RBC group ($p < 0.01$) with the largest mean difference in CFU-GM between the two groups occurring on day 13 ($p < 0.02$). Thereafter the difference between the two groups diminished.

In order to determine if alteration in the distribution of granulocytes between the circulating compartment, marginating granulocyte pool (MGP), and marrow granulocyte reserve (MGR) could explain, in part, the differences in blood granulocyte counts, mice were given epinephrine and endotoxin on days 3 and 11. The results are shown in Fig. 5. Normal mice showed the expected response to stimulation with epinephrine; however, neither XRT-PL nor XRT-RBC mice on day 3 or day 11 showed significant release of granulocytes in response to epinephrine, suggesting a very small or absent MGP. Normal, XRT-PL, and XRT-RBC mice all showed significant release of granulocytes from the MGR with probabilities of $<0.005$, $<0.05$, and $<0.005$ on day 3 and $<0.01$, $<0.005$, and $<0.005$ on day 11, respectively, for the various groups. On day 11, the final granulocyte counts reached by the XRT-RBC group were greater than those reached by the XRT-PL mice in response to endotoxin ($p < 0.1$), and the final count for both groups represented an approximate doubling of the pre-endotoxin count.

The evidence for increased granulopoiesis in the hypertransfused mice, the fact that increased granulopoiesis in some circumstances, such as following lithium or endotoxin administration, is mediated by increased production of CSA and the possibility that hypertransfusion might enhance CSA production led us to study plasma CSA levels in the test mice (Fig. 6). Plasma CSA levels in the XRT-RBC mice were significantly lower on days 8 and 11 ($p < 0.01$). These experiments indicate that the enhancing effect of hypertransfusion on granulopoiesis is not mediated through higher CSA levels.

DISCUSSION

The ability of red cell hypertransfusion to potentiate granulopoietic recovery when given postirradiation has been investigated in sublethally irradiated mice. This study has confirmed and extended the original observations of Firkin et al. with this system to show that hypertransfusion causes the circulating granulocytes to decrease less initially, show a larger abortive rise, and decline less after the abortive rise. Endotoxin and epinephrine administration did not reveal differences in the distribution of granulocytes among the circulating compartment, marginating granulocyte pool, and marrow granulocyte reserve that could account for the higher granulocyte levels in the hypertransfused mice.

The largest difference in blood granulocytes
between the hypertransfused and control groups occurred at the peak of the abortive rise. This is interesting because of the explanations that have been given for the abortive nature of early hematopoietic recovery. Originally, the abortive rise was attributed to survival of damaged pluripotential stem cells that could differentiate but had limited proliferative capacity. More recently, abortive recovery has been explained as the differentiation of surviving cells committed to specific hematopoietic cell lines that lack or have limited self-renewal capabilities. This explanation is supported by the observation of Chen and Schooley that CFU-GM of mice exhibited an abortive recovery phase after 200 R exposure but that CFU-S did not. If damaged pluripotential stem cells are responsible, the larger granulocyte abortive rise in the hypertransfused mice may be explained by decreased competition for stem cells as discussed below. However, if committed stem cells are responsible, the enhancing effect of hypertransfusion on the abortive rise may be a different type of interaction, such as that of potentiation by red cells of CFU-GM formation described by Bradley et al.

Hypertransfusion did not alter the overall pattern of changes in marrow cellularity after sublethal irradiation of a degenerative phase, an abortive rise, and finally recovery. Hypertransfused mice showed the same initial decrease in marrow cellularity as irradiated controls, however, marrow cellularity during the abortive recovery phase was lower in the hypertransfused group due to the almost complete absence of erythropoiesis. All hematopoietic cell lines for which there was demand showed pronounced expansion between days 8 and 11 in both XRT-PL and XRT-RBC mice, suggesting that the pluripotential hematopoietic stem cells must recover to some finite level before differentiation can occur, as proposed by earlier investigators.

Marrow myeloid cells and CFU-GM were reduced to the same level in both irradiated groups during the degenerative phase, indicating that hypertransfusion did not alter the damage to the myeloid compartment. However, myeloid cells decreased less after the abortive recovery phase, and the abortive phase of CFU-GM recovery was eliminated by hypertransfusion. Thus, increased granulopoiesis during the second postirradiation week would seem to be responsible for most of the increase in granulocyte counts in the hypertransfused mice. However, the lower CSA levels and same time of onset of recovery indicate that the more rapid myeloid regeneration is not the result of greater CSA production or earlier precursor recovery in the hypertransfused mice.

The more rapid recovery of the nonerythroid compartments in the XRT-RBC mice mainly resulted from the burst of activity between days 8 and 11, suggesting that the primary effect of hypertransfusion was to alter the direction of differentiation. One way that has been proposed for this to occur is that reduced demand for erythropoiesis in XRT-RBC mice leaves more stem cells available for differentiation into nonerythroid cell lines. Whether this effect is mediated by competition of humoral regulators (erythropoietin and GM-CSA) for the same precursor cells has been the subject of recent studies that have yielded varying results that appear to depend on the cell concentration used in the in vitro assays. A note of caution that should be mentioned with this simple compartmentalized interpretation comes from the studies of Hellman et al. who observed that the fraction of marrow cells surviving 200 rad that would repopulate the granulocytic and erythrocytic compartments differed. This may only reflect differences in the proportions or radiosensitivity of committed cells for these two cell lines but these findings should be considered in interpretations of data such as those presented here.

Viability of nucleated marrow cells as estimated by dye exclusion was consistently higher in the XRT-RBC mice. This difference in viability was not due to differing times between marrow collection and viabil-
ity determination, since at sacrifice mice were killed alternately from each experimental group. The higher viability in the hypertransfused mice may be explained by (1) a mechanical effect of the greater concentration of RBCs in the marrow, (2) an alteration in the intercellular marrow matrix, or (3) an absence of erythroid cells that may be more fragile. The observation that on day 10 the marrows of the XRT-PL mice were more difficult to suspend than those of the XRT-RBC group suggests that, at least on this day, there was a difference in the composition of the marrow matrix.

In summary, hypertransfusion produces more rapid myeloid recovery in mice after irradiation. Thus, hypertransfusion might be expected to enhance myeloid recovery in clinical or experimental situations where there is transient marrow depression. There is evidence that it is effective in one such clinical situation—acute lymphocytic leukemia during induction therapy.1,2

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