CFU-GM Content of Bone Marrow Graft Correlates With Time to Hematologic Reconstitution Following Autologous Bone Marrow Transplantation With 4-Hydroperoxycyclophosphamide-Purged Bone Marrow


Autologous bone marrow transplants (BMTs) can repopulate the hematologic system of patients treated with marrow-ablative chemotherapy and/or radiotherapy. However, treatment of the bone marrow graft to eliminate residual tumor cells prior to reinfusion can delay the return of peripheral blood elements, presumably from damage to or loss of hematopoietic stem cells responsible for hematologic recovery. To develop a model predictive of hematologic recovery, we studied the progenitor cell contents of 4-hydroperoxycyclophosphamide (100 µg/mL)-purged bone marrow grafts of 40 consecutive patients undergoing autologous BMT at this center. Granulocyte-macrophage colonies (CFU-GM) were grown from all grafts after treatment with this chemotherapeutic agent, but erythroid (BFU-E) and mixed (CFU-GEMM) colonies were grown from only 44% and 33% of the grafts respectively. The recovery of CFU-GM after purging ranged from 0.07% to 23%. The logarithm of CFU-GM content of the treated grafts was linearly correlated with the time to recovery of peripheral blood leukocytes (r = −0.80), neutrophils (r = −0.79), reticulocytes (r = −0.60), and platelets (r = −0.68). The CFU-GM content of purged autologous bone marrow grafts may reflect the hematopoietic stem cell content of the grafts and thus predict the rate of hematologic recovery in patients undergoing autologous BMT.

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METHODS

Patient selection and transplant procedures. Hematopoietic progenitor cell assays were performed on the bone marrow grafts of 40 consecutive patients undergoing autologous BMT with 4-HC-purged bone marrows at this center. Eighteen of the patients were transplanted for the diagnosis of acute nonlymphoblastic leukemia in second remission and 16 for the diagnosis of refractory Hodgkin’s disease (9) or non-Hodgkin’s lymphoma (7). Five patients with pediatric solid tumors and one patient with acute lymphoblastic leukemia in first remission were also harvested. The age range for these patients was 2 to 53 years with a median of 29 years. Twenty-eight of the patients were male. The details of the induction regimens used have been previously reported.46 Patients with acute nonlymphocytic leukemia, Hodgkin’s or non-Hodgkin’s lymphoma with bulky disease, and pediatric solid tumors (neuroblastoma, melanoblastoma, and rhabdomyosarcoma) received busulfan (4 mg/kg daily × 4) and cyclophosphamide (50 mg/kg daily × 4) for induction. The remaining patients received an induction regimen consisting of cyclophosphamide (as noted above) and total body irradiation (TBI; to 1200 rad). All patients received appropriate supportive care, including prophylactic platelet transfusions for platelet counts less than 20,000/µL. Approval for the therapeutic

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application of autologous BMT at this center was obtained from the Joint Committee on Clinical Investigation of the Johns Hopkins Hospital and the Johns Hopkins University, and informed consent was obtained for all patients prior to bone marrow harvesting.

**Marrow processing.** The bone marrow grafts were collected, treated with 4-HC, cryopreserved, and thawed as previously described. Briefly, all bone marrows were incubated with 100 \( \mu g/mL \) of 4-HC at a nucleated cell concentration of 2 \( \times 10^7 \) cells/mL for 30 minutes at 37\(^\circ\)C. The red cell contents of the incubation mixtures were not adjusted (the range of packed red cell volumes was 5% to 23% for this series). The cells were then resuspended for cryopreservation with 10% dimethyl sulfoxide (DMSO) in the liquid phase of nitrogen. Subsequently the cells were rapidly thawed in a 37\(^\circ\)C water bath and immediately infused without further manipulation.

**Cell culture.** Aliquots of bone marrow cells were obtained from the bone marrow graft buffy coats before and after the 4-HC treatment (prior to the addition of cryoprotectants). Light-density mononuclear cells (sp gr \( \leq 1.077 \ g/mL \)) from both the treated and untreated specimens were obtained by Ficoll-Hypaque (FH) separation. The cells were cultured in 1.32% methylcellulose containing alpha-medium (Gibco, Grand Island, NY), 5% phytobemegglutinin (PHA)-stimulated leukocyte-conditioned medium, 1% bovine serum albumin (Sigma Chemical Co. St Louis), 30% fetal bovine serum (FBS, Hyclone, Logan, UT), 10\(^-4\) mol/L 2-mercaptoethanol, and 10\(^-5\) mol/L 2-mercaptoethanol, and 1 U/mL erythropoietin (Amgen, Thousand Oaks, CA). Untreated cells were plated at a seeding density of 5 \( \times 10^6 \) cells per 1 mL plate (LX; Miles Laboratories, Naperville, IL). Treated cells were plated at multiple seeding densities from 5 \( \times 10^6 \) to 1 \( \times 10^7 \) cells/mL. All samples were cultured in quadruplicate and maintained at 37\(^\circ\)C in a humidified 5% CO\(_2\) in air atmosphere. CFU-GM-, BFU-E-, and CFU-GEMM-derived colonies were scored on an inverted microscope after 14 days of culture. The same bone marrow samples were also cultured for CFU-C as previously described. Briefly, 1 \( \times 10^5 \) untreated, and 5 \( \times 10^5 \) and 1 \( \times 10^6 \) treated light-density cells were cultured in 1 mL of McCoy’s medium (Gibco) containing 0.3% agar (Difco Laboratories, Napersville, IL). Untreated cells were plated at a seeding density of 5 \( \times 10^9 \) cells per 1 mL plate (LX; Miles Laboratories, Naperville, IL). Treated cells were plated at multiple seeding densities from 5 \( \times 10^9 \) to 1 \( \times 10^10 \) cells/mL. All samples were cultured in quadruplicate and maintained at 37\(^\circ\)C in a humidified 5% CO\(_2\) in air atmosphere. Colony cultures were maintained at 37\(^\circ\)C in a humidified 5% CO\(_2\) in air atmosphere, and colonies containing \( > 50 \) cells were enumerated on an inverted microscope after ten days.

**Determination of bone marrow graft-progenitor cell content.** The hematopoietic progenitor contents of the marrow grafts before and after incubation with 4-HC were determined by multiplying the number of colonies scored per number of cells seeded into culture by the number of nucleated cells (per kg recipient weight) within the grafts. Because the cloning efficiency of the treated cells fell to 80% of predicted at a seeding density of 10\(^7\) cells/mL, all calculations of graft progenitor cell content were based, when possible, on the lowest seeding density giving rise to at least five colonies per plate (usually 5 \( \times 10^5 \) cells/mL seeding density). We assumed that all progenitor cells were contained within the light-density fraction after density-gradient separation and multiplied the above product by the proportion of cells recovered within this fraction. CFU/\( kg \) = CFU/plat + cells/plat x cells/kg x gradient recovery.

**Analysis of hematologic recovery.** Daily peripheral blood leukocyte counts were obtained following the reinfusion of the thawed bone marrows (day 0). When the WBC was less than 1,000/\( \mu L \), the WBC and granulocyte count were determined with flow cytometry (Spectrum III, Ortho Diagnostic Systems Inc, Westwood, MA). Above a WBC of 1,000/\( \mu L \), the WBC was determined on an automated cell counter (ELT 800, Ortho Diagnostic Systems Inc), and the granulocyte fraction was determined visually with Wright-stained peripheral blood smears. Platelet counts were determined daily using the automated cell counter. Reticulocyte counts were measured twice weekly with the flow cytometer using acridine orange-stained specimens. The day of recovery is the first day after the reinfusion of the marrow on which the particular peripheral blood count (sustained leukocytes \( \geq 1,000/\mu L \), granulocytes \( \geq 500/\mu L \), reticulocytes \( \geq 2\% \)) is achieved. For analysis of platelet recovery we chose the time to the last platelet transfusion, which corresponded to a self-sustained peripheral blood platelet count of greater than 20,000/\( \mu L \) for most patients.

**Statistical evaluation.** The relation of the logarithm of the progenitor cell content of the bone marrow graft to the time of hematologic recovery of the recipients was evaluated by linear regression analysis and calculation of Pearson’s correlation coefficient. The significance of the correlation parameters was tested by Student’s \( t \) test.

**RESULTS**

The hematopoietic culture data for the various progenitor cells before and after incubation with 4-HC are shown in Table 1. The mean recovery of each progenitor cell type was similar (approximately 5% to 2% for CFU-C). However, CFU-GM-derived colonies were obtained from all bone marrows following the ex vivo incubation. BFU-E- and CFU-GEMM-derived colonies were grown from all specimens before treatment but from only 44% and 33% respectively of the specimens after treatment. CFU-C-derived colonies were cultured from only 17 (42%) of the treated bone marrow grafts.

The clinical protocols prescribed a minimum number of nucleated cells to be harvested and cryopreserved for the patients undergoing autologous BMT; therefore most patients had similar numbers of nucleated cells and CFU-GM within the bone marrow grafts prior to the purging steps. The mean CFU-GM content of the bone marrow grafts before the 4-HC incubation was 1.4 \( \pm 0.7 \times 10^5 \) (\( \pm SD \)) per kg body weight (range, 0.5 \( \times 10^5 \) to 4.0 \( \times 10^5 \)). The mean CFU-GM content after the 4-HC incubation was 7.6 \( \pm 13.3 \times 10^5 \) (\( \pm SD \)) per kg with a range from 5.0 \( \times 10^5 \) to 5.7 \( \times 10^6 \) per kg. The recovery of CFU-GM within the graft after incubation with 4-HC ranged from 0.07% to 23%.

No relation was evident between the nucleated cell, CFU-C, BFU-E, or CFU-GEMM content of the treated bone marrow grafts and time to recovery of peripheral blood cells. The logarithm of the CFU-GM content showed a linear correlation with time to leukocytes \( \geq 1,000/\mu L \), granulocytes \( \geq 500/\mu L \), reticulocytes \( \geq 2\% \), and time to last platelet transfusion (Figs I A to D). The logarithm of CFU-GM content also predicted the day of recovery when analyzed for subgroups of patients. This included the time to a WBC \( \geq 1,000/\mu L \) for both the acute nonlymphocytic leukemia (ANLL) (\( r = -0.79, n = 15 \)) and all lymphoma patients (Hodgkin’s and non-Hodgkin’s lymphomas, \( r = -0.74, n = 11 \)), and time to 500 granulocytes/\( \mu L \) (\( r = -0.78 \) and \( r = -0.63 \) respectively). Sufficient evaluable patient courses are not available for similar comparative analysis of time to recovery of platelets and reticulocytes. However, these data suggest that the relationship of CFU-GM to pluripotent stem cell contents of the bone marrow grafts after purging is similar for these patient groups and that the patient diagnoses do not affect the subsequent transplant course when the grafts are purged ex vivo with 4-HC. Only four patients with ly-
IN VITRO ASSAY PREDICTS HEMATOLOGIC RECOVERY

**DISCUSSION**

Ex vivo manipulation of bone marrow grafts can affect the ability of these grafts to reconstitute the hematopoietic system of the recipients. In the phase I trial of purging autologous bone marrows with 4-HC reported from this center, the time to hematopoietic recovery was related to the concentration of 4-HC to which the bone marrow grafts were exposed. Therefore an assay that demonstrates a strong correlation with the ability of the bone marrow graft to repopulate the hematopoietic system of the recipient is valuable both in terms of patient management as well as in the development or modification of purging techniques.

Other investigators have reported that the granulocyte-macrophage progenitor cell (CFU-GM) content of the autologous bone marrow graft relates either in a logarithmic fashion or in a step fashion to the time to return of peripheral blood leukocytes but not platelets or reticulocytes after autologous BMT. These studies involved bone marrow grafts that were not exposed ex vivo to purging agents that could be toxic to normal committed and pluripotent progenitor cells or hematopoietic regulatory cells. In allogeneic transplantation a correlation between the CFU-GM content of the graft and hematologic reconstitution has been observed and has not been reported. Common features of those studies that do not find a correlation are both a limited range in the number of progenitor cells infused and a usual dose of CFU-GM greater than 10^7/kg of recipient weight. Because our data support a model of exponential growth of the infused bone marrow, the effects of minor (even twofold) variations in the number of CFU-GM infused may not be discernible in clinical studies. Thus patient-to-patient differences in cell loss during freezing or in the assumed FH recovery upon which our calculations are based may have little effect upon the overall correlation of progenitor cell content with hematologic reconstitution. The discrepancy

![Graphs showing correlation between log CFU-GM per kg and days to specific hematologic recovery.](image-url)
between our current data and the lack of correlation of 
CFU-C (CFU-GM) with hematologic reconstitution previously 
reported from this center results from the different 
progenitor cell assays used. The culture conditions described 
in this report have a lower threshold for detection of progeni-
tor cells following the 4-HC incubation (Table 1), and 
cultures of the grafts in this series using the previously 
reported assay techniques again demonstrated almost com-
plete abrogation of clonal growth at this concentration of 
4-HC. The two assay techniques differ greatly, and we 
cannot attribute the better growth in our current assay to any 
particular reagent(s). Because a threshold of detection also 
exists for the current assay, failure to grow only implies that 
the period of aplasia may be prolonged.

Many factors can affect the recovery of CFU-GM for 
bone marrow grafts manipulated ex vivo including incuba-
tion with 4-HC (as shown in this study) or other purging 
agents. Presumably the decreases in measurable (by in vitro 
culture) CFU-GM reflect a similar effect upon the 
primitive hematopoietic stem cell responsible for reconsti-
tution following bone marrow transplantation. Other purging 
regimens, especially those that use monoclonal antibodies 
(MoAbs), which theoretically have the potential of distin-
guishing between committed and pluripotent progenitor 
cells, may dissociate the close relationship of CFU-GM to 
the hematopoietic stem cell implied by our results. The 
development of pluripotent stem cell assays may sim-
plify the investigation of this aspect of BMT.

Although it is conceivable that the granulocyte colonies we 
observed arose from pluripotent stem cells, we would in 
this circumstance have expected to identify other colony types 
(erythroid, mixed granulo-erythroid) in the culture dishes as 
well. The stronger correlation of graft CFU-GM content to 
platelet and reticulocyte recovery may reflect the lineage commitment of this progenitor cell. How-
ever, other factors may have contributed to the relatively 
weaker correlation of the CFU-GM content with platelet and 
reticulocyte recovery. We routinely transfused platelets pro-
phylactically for platelet counts below 20,000/μL, and for 
medical complications such as severe liver disease, CMV 
infection, and immune destruction may affect platelet 
 survival or production. Similar considerations may affect 
the recovery of red cell production in these patients. In this series 
three patients died while still requiring platelet support after 
platelet recovery was predicted, but all had developed comp-
lications of transplantation prior to expected platelet recov-
ery. Two patients who relapsed with acute myelogenous 
leukemia had prolonged platelet requirements without other 

These data confirm that the in vitro quantitation of 
CFU-GM can correlate with engraftment following autolo-
gous BMT with manipulated bone marrow grafts. Although 
other purging regimens may not demonstrate the same 
relationship, attention should be given to in vitro models of 
engraftment. The development of assays for more primitive 
pluripotent hematopoietic stem cells may simplify this task.

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