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Assessing permanent damage to primitive hematopoietic stem cells after chemotherapy using the competitive repopulation assay

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Abstract. The competitive repopulation assay was used to document the effects of six chemotherapeutic agents on primitive hematopoietic stem cells. The assay measures the relative abilities of donor cells to produce circulating erythrocytes and lymphocytes in lethally irradiated congeneic mice over a period of 6 months. Long-lasting marrow reconstitutive deficits in cells of donor origin occurred after exposure to 5-fluorouracil (5FU), bis-chloronitrosourea (BCNU), cyclophosphamide (CTX), vincristine (VCR), and actinomycin D (ACT) but not after exposure to cytosine arabinoside (ARA). Repopulating abilities were reduced after as little as a single dose of CTX or BCNU. A second dose of BCNU caused even more severe effects. A single dose of 5FU had no effect on repopulating abilities despite a temporary 10-fold reduction in marrow cell number, but multiple doses reduced the marrow stem-cell replicative ability to less than half of the normal control levels. These effects were not reliably predicted or detected by colony-forming assays or by reductions in marrow cell number. Thus, long-lasting proliferative defects in the primitive hematopoietic stem-cell (PHSC) population can result from the use of chemotherapeutic agents. Such findings may have clinical implications, especially in individuals receiving repeated or prolonged administration of these agents or in instances of marrow transplantation.

Introduction

The presence and extent of long-term chemotherapy-induced damage to pluripotent hematopoietic progenitor cells is difficult to define conclusively. Although there are anecdotal clinical reports of residual cytopenias that have been noted after intensive drug administration [15, 24, 26], studies of long-term hematologic dysfunction as a result of chemotherapy remain rare [1, 2, 5, 7, 18, 20].

The effects of drugs on hematopoietic stem-cell function have been studied most often using in vitro assays [23]. The colony-forming unit-spleen (CFU-S) assay [29] is the most widely used in vivo assessment of marrow reconstitutive function in mouse model systems. However, it measures only the pluripotent myeloid precursor cells that are active in the first 2 weeks after transplantation, not the cells responsible for reconstitution over large fractions of an individual's life span [10, 11, 28].

It is vital to target for study those cells that are responsible for the long-term repopulation of bone marrow after drug-related or other marrow depletion, i.e., primitive hematopoietic stem cells (PHSC), and in which drug-induced damage would have the most serious consequences to long-term health. The competitive repopulation assay [10] enables sensitive and accurate assessment of the long-term functional capacities of PHSC by measuring the relative abilities of mixtures of cells from mice of differing genotypes to repopulate stem-cell-depleted animals. Marrow cells from drug-treated mice and controls are mixed with genetically distinguishable competitor marrow, and the mixtures are injected into irradiated recipients. After 90 and 180 days, the percentages of donor and competitor erythrocytes and lymphocytes are measured in those recipients. Diminished percentages from drug-treated donors quantitatively define the drug-related damage to PHSC.

The results of competitive repopulation assays performed after exposure of stem cells to several chemotherapeutic agents are reported. Because the assay directly measures the replicative potential of cells responsible for long-term repopulation of both myeloid and lymphoid lineages, it should be valuable for preclinical testing of chemotherapeutic agents.

Materials and methods

Mice. Genetically defined inbred, or congeneic, mice were used in all experiments. They have been bred and housed under specific-pathogen-

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Table 1. Effects of chemotherapy on long-term repopulation

Drug	Dose (mg/kg) per injection [mean cumulative dose (mg)]	Schedule	% Donor type (mean ± SE)		
			L	E	
Control	None	NA	49 ± 3	46 ± 3	28
ARA	160 [8]	qd × 2	52 ± 4	48 ± 3	10
ARA	160 [32]	qd × 2, q2w × 4 (8 doses)	45 ± 4	42 ± 3	24
4FU	150 [4]	q2w × 4	23 ± 5*	19 ± 4*	21
BCNU	40 [1]	× 1	32 ± 4*	29 ± 3*	11
BCNU	40 [2**]	× 2	10 ± 3*	6 ± 2*	11
CTX	200 [5]	1	33 ± 6*	29 ± 6*	8
CTX	200 20]	q2w	27 ± 3*	31 ± 6*	13
CTX	200 [40]	qd × 2, q2w × 4	35 ± 6*	19 ± 2*	13
VCR	0.1 [0.4]	q2w × 4 (8 doses)	37 ± 3*	35 ± 3*	14
ACT	0.2 [0.8]	qd × 2, q2w × 4	35 ± 3*	33 ± 3*	14

ARA, Cytosine arabinoside; 5FU, 5-fluorouracil; BCNU, bis-chloronitrosourea; CTX, cyclophosphamide; VCR, vincristine; ACT, actinomycin D; T, lymphocytes; E, erythrocytes; NA, not applicable. All drugs were reconstituted in water and were given by intravenous bolus. Controls received 1-alpha-MEM alone. Marrow mixtures containing 1×10^6 control or drug-exposed B6 donor and 1×10^6 untreated B6-*Hbb^d Gpi-1^a* marrow cells were infused into lethally irradiated B6 recipients. Lymphocytes and erythrocytes sampled after 90 and 180 d gave the same results, so only the 180 d data shown. When identical recipients were given only 4×10^5 B6-*Hbb^d Gpi-1^a* marrow cells to estimate amounts of B6-type cells due to recovery of recipient cells, the percentages of B6 host-type lymphocytes and erythrocytes were very low, 2.2% ± 2.2% (12) and 4.0% ± 5.0% (12) respectively, giving data as mean values ± SE (number of means of groups averaging 5.3 mice each), pooling data at 90, 180, and 350 days. Repopulating units (RU) ≈% (/100-%; *1* RU = repopulating ability of 1×10^5 untreated B6 marrow cells, as detailed elsewhere [10]

* $P < 0.05$ lower than control

free conditions at The Jackson Laboratory, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Donor cells were obtained from 8- to 12-week-old C57BL/6J (B6) mice, and competitor cells were acquired from the B6 congeneic line B6-*Hbb^d/Hbb^d*, *Gpi-1^a* (B6-*Hbb^d*, *Gpi-1^a*). B6-*Hbb^d-Gpi-1^a* congeneic mice were prepared from the B6.C-H-1b/By (*Hbb^d/Hbb^d*) and B6.CAST-Gpi-1^a/Ei (*Gpi-1^a/Gpi-1^a*) congeneic lines.

B6 and competitor B6-*Hbb^d Gpi-1^a* mice have electrophoretically distinguishable hemoglobin (Hb) patterns since they bear different alleles at the *Hbb* locus. B6 mice are homozygous for the *Hbb^s* locus specifying $\alpha_2\beta_2$s (single) Hb, whereas competitor mice are homozygous for the *Hbb^d* locus, having $\alpha_2\beta_2$ dmaj and $\alpha_2\beta_2$ dmin (diffuse) Hb. These mice also differ at the glucosephosphate isomerase (GPI-1) isoenzyme locus. B6 animals produce *Gpi-1^b* isoenzyme; B6-*Hbb^d Gpi-1^a* mice produce *Gpi-1^a*. B6-*Hbb^d Gpi-1^a* and B6 animals also differ at the H-1 locus. B6 mice are *H-1^a*, whereas B6-*Hbb^d Gpi-1^a* mice are *H-1^b*, but this difference has not affected marrow transplantations involving these congeneic strains of mice [11]. In the ensuing experiments in which the competitive repopulation assay was employed, competitor cells were obtained from B6-*Hbb^d Gpi-1^a* mice, whereas donor cells were acquired from B6 mice. Recipient groups comprised B6 animals that had been lethally irradiated (1050 R) using a cesium 137 irradiator (Shepherd Mark I).

In vitro assays. Mice used in these experiments were killed by cervical dislocation. Femurs and tibias were excised and flushed with 1-alpha-MEM (Gibco, Grand Island, N. Y.) to obtain a single-cell suspension of bone marrow. For the CFU-GM assay, 0.6 ml cells in a final concentration of 2×10^5 cells/ml were plated in 3% methylcellulose (DOW, Midland, Mich.) containing 30% heat-inactivated fetal calf serum (HIFCS; Hyclone, Logan, Utah), 1% 200 m*M* 1-glutamine (Gibco), and 20% colony-stimulating activity (CSA). CSA was prepared by suspending the peritoneal linings of B6 mice in RPMI-1640 (Gibco) supplemented with 10% HIFCS and a standard penicillin-streptomycin mixture and then incubating them at 37° C in a humidified atmosphere for 72 h. The supernatant was removed and stored at –4° C. Cultures were incubated at 37° C in 5% CO_2 in air for 7 days and the plates were scored for colonies.

The plasma clot method was used for production of erythroid colonies as previously described [28], with the following modifications: cells were adjusted to a concentration of 1×10^7 cells/ml, and 0.1 ml of this suspension was mixed with 30% F12 nutrient mixture (Gibco), 5% erythropoietin (Connaught, Willowdale, Ontario) at 10 units/ml in NCTC-109 (Gibco), and 1% citrated bovine plasma for a final total volume of 1.0 ml. This mixture was then scored at 2 and 7 days, respectively. Fixation, staining, and counting of colonies were performed according to standard procedure [28], with CFU-E and BFU-E being scored at 2 and 7 days after plating, respectively.

Drug administration in vivo. Some of the drugs employed in these experiments have been reported to have either stimulatory or inhibitory effects on normal hematopoietic stem cells, sometimes with an accompanying clinical impact [1, 2, 5, 18, 20, 24]. 5-Fluorouracil, a common chemotherapeutic agent, has been reported to spare primitive hematopoietic stem cells [3, 17, 30]. The effects of single doses of this agent were compared with its effects when it is given at 2-week intervals for 8 weeks.

All drugs used in this study were reconstituted in water. In all, 13 groups of mice comprising 4–6 animals each were then injected by intravenous bolus with one of the drugs. Each dose was documented during preliminary experiments to cause a reduction of 50% in nucleated bone marrow counts or a decrease of 10%–40% in survival of CFU-S. Each drug experiment was performed at least in duplicate. Details regarding the doses and schedules of administration are given in Table 1. Mice in control groups received an intravenous bolus of 0.5–1 ml 1-alpha-MEM in lieu of chemotherapeutic agents.

CFU-S assay. B6 males aged 10 weeks were killed 24 h after injection of the respective chemotherapeutic agents. Their femurs and tibias were excised and the bone marrow (BM) was flushed with 1-alpha-MEM. After the cells had been washed, they were counted and numbers were adjusted to a concentration of 1×10^5 cells/ml, which was injected via the tail vein into B6 males that had received 900 R Cs-137 irradiation 24 h prior to BM injection. These animals were killed 9 days later and their spleens were scored for macroscopic colonies using standard methods [29].

Competitive repopulation assay. Mice receiving cytosine arabinoside (ARA), 5-fluorouracil (5FU), cyclophosphamide (CTX), and bis-chloronitrosourea (BCNU) were killed 4 weeks after the last drug dose had been given. Mice that had been injected with actinomycin D (ACT) or vincristine (VCR) were killed 2 weeks after they had received their last drug dose. For all drugs, the 2- or 4-week interval allowed numbers of nucleated BM cells to return to normal in all cases. Femoral and tibial

marrow cells were suspended in 1-alpha-MEM. Cellular concentrations were adjusted to mix 1×10^6 cells of the B6 donor type from each drug group with equal numbers of competitor cells obtained from unmanipulated B6-*Hbb^d Gpi-1^a* mice. These cell mixtures were then infused into B6 recipients that had received 1050 R Cs-137 irradiation 18–24 h previously. Marrow reconstitution was complete at 90 days postinfusion [10, 12, 22]. Blood was obtained from the animals by retroorbital puncture at 90 and 180 days, and lymphocyte GPI-1 isoenzyme and Hb electrophoretic determinations were made.

In other experiments, B6 mice received 150 mg/kg 5FU in a single i.v. injection. Untreated syngeneic mice served as controls. At 1, 5, 8, and 15 days after 5FU administration, the mice were killed and their femurs were then flushed with a constant volume of medium. Nucleated BM cell numbers were recorded in each respective group, and fractions of the femoral cells of 20%, 40%, 60%, and 80% of the total were mixed with corresponding volumes of B6-*Hbb^d Gpi-1^a* competitor cells, e.g., 80%, 60%, 40%, and 20%, respectively. These mixtures were then injected into B6 mice that had received 1000 R at 18–24 h prior to the transplantation procedure. Blood samples were taken for Hb electrophoresis at 83 and 180 days [12].

Electrophoretic assays. Details of the Hb electrophoretic techniques have been reported elsewhere [10, 11, 14]. Hbb^s (s, single) forms a rapidly migrating band on standard cellulose acetate gels, whereas Hbb^d (d, diffuse) has two bands, Hbb-b1^d and Hbb-b2^d, which are respectively intermediate and slow in migration. The glucosephosphate isomerase electrophoretic assay has also been described previously [13, 14]. The *Gpi-1^b* allele at the glucosephosphate isomerase (*Gpi-1*) locus is identified by a band that moves more rapidly from positive to negative than does that formed by the *Gpi-1^a* allele. Lymphocytes used for the latter assay are separated from other blood cells by a density gradient after a suspension of peripheral blood cells in saline/citrate solution has been layered over Ficoll [14]. This mixture is centrifuged for 1 h at 1600 rpm. Lymphocytes are visualized as a band atop the Ficoll, whereas most erythrocytes pellet at the bottom of the tube. Erythrocytes contaminating the lymphocyte preparation are lysed using distilled water.

Results

Determination of drug doses

We used the doses of 5FU that had been used by other investigators and had increased marrow stem-cell concentrations [3, 17, 30]. For ARA, BCNU, and CTX, we first examined the acute toxicity to the CFU-S compartment and the effects on marrow cellularity (Table 2). Animals were killed 24 h after the administration of the respective drug and their marrow was used in the CFU-S assay as described below. Levels of CFU-S survival were about 10%–40% at 24 h after drug administration, a method of dose selection previously used by Botnick et al. [1, 2]. The marrow cell numbers of treated mice were compared with those of untreated or control animals in a search for drug doses that would temporarily reduce cellularity at least 50% without producing excessive morbidity or mortality among the groups of test animals.

As increasing doses of drugs did not necessarily yield proportionate declines in CFU-S values (Table 2), drug doses meeting either one or both of the criteria mentioned above were selected for the study. An intermediate dose of BCNU (40 mg/kg) was selected. In the cases of CTX and ARA, the final doses were chosen because they reduced marrow cellularity, although CFU-S concentrations were not reduced significantly. Finally, doses of ACT and VCR were not tested but were designed to approximate the doses used in humans during clinical trials.

Table 2. Effects of chemotherapy on CFU-S

Drug	Dose (mg/kg)	CFU-S (10^5 cells)	Control
ARA	30.0	18 ± 2.3	18
	50.0	16 ± 0.5	18
	80.0	17 ± 1	18
	160.0	17 ± 0	13
BCNU	3.6	18 ± 0.9	18
	9.6	21 ± 0.7	18
	30.0	9 ± 0.6*	18
	50.0	5 ± 1.8*	13
CTX	18.0	18 ± 1.7	18
	200.0	11 ± 3.6	13
	300–600	3 ± 1.5*	13

Numbers of macroscopic spleen colonies (CFU-S) are given per 1×10^5 marrow cells. Control values for CFU-S from untreated mice averaged $13 \pm 1.4 \times 10^5$ in one study and $18 \pm 1.5 \times 10^5$ in the other, as listed above. In each case, macroscopic colony numbers were averaged from 4–10 spleens. Asterisks denote statistically significant differences in CFU-S numbers between groups of mice that received chemotherapy and control groups in which mice received no treatment, with P values ranging from <0.01 to <0.05

Table 3. Effects of chemotherapy on colony-forming cells in vitro

Drug	Cumulative dose (mg)	Colony number (mean ± SE)	
		CFU-GM	CFU-E
ARA ($n = 6$)	32	223 ± 67	34 ± 9
5FU ($n = 6$)	3–4	149 ± 46	39 ± 17
BCNU ($n = 6$)	1	227 ± 70	49 ± 18
CTX ($n = 3$)	20	63 ± 26	26 ± 15
Control ($n = 6$)	–	173 ± 40	35 ± 12

CFU-GM were scored as colonies of 50 cells or more after 7 days of incubation; CFU-E were scored after 2 days of incubation. The marrow was collected 10–12 weeks after the chemotherapy had been given

In vitro colony assays

Results of the in vitro assays (CFU-GM and CFU-E) are displayed in Table 3. These assays were performed 10–12 weeks after the initial drug administration. The number of myeloid colonies formed after exposure of BM cells to the various drugs was compared with that of control cultures, i.e., those in which untreated marrow cells were used. After exposure to CTX, marrow contained reduced concentrations of CFU-GM but not CFU-E; other drugs had no effect on either colony type.

PHSC function

A single dose of 5FU had little or no effect on erythroid repopulating abilities tested 1, 5, and 8 days later (Table 4). The repopulating capacity of 5FU-exposed B6 marrow cells, obtained 15 days after 5FU dosing and at 40%–80%

Table 4. Effects of single 5FU doses on long-term erythropoietic repopulation

Days posttreatment	% Donor erythrocytes for % donor femur contents			
	20	40	60	80
Control	21 ± 2	37 ± 1	53 ± 2	72 ± 2
1	16 ± 2	32 ± 2	52 ± 2	71 ± 3
5	17 ± 2	34 ± 2	53 ± 1	82 ± 3
8	25 ± 1*	51 ± 2*	71 ± 1*	81 ± 2
15	13 ± 2	21 ± 2*	43 ± 2*	60 ± 3*

B6 nucleated bone marrow cells in fractions of 20%, 40%, 60%, and 80% were mixed with matching volumes or fractions of the congenic B6-*Hbb*^{d} *Gpi-1*^{a} bone marrow cells and this mixture was injected into lethally irradiated B6 mice. Erythrocytes were measured at 83 days after reconstitution; ensuing measurements made at 180 days did not significantly differ [22]. Bone marrow from normal syngeneic animals that had not been given 5FU served as controls. The asterisks indicate statistically significant differences between values (P <0.05 Student-Newman-Keuls test) relative to controls. Data are expressed as mean values ± SE, n = 4 in each case

of the total donor : competitor cell mixture, was inferior to that of control cells. At 8 days after drug dosing, marrow cells exposed to 5FU and contained in marrow mixtures with 20%–60% 5FU-exposed B6 cells had a slightly better repopulating ability than did controls. However, these effects probably resulted from random variation (Table 4).

The effects of all the other drugs on PHSC long-term repopulating abilities were tested in the competitive repopulation assay 2–4 weeks after drug administration (Table 1). Mice were killed 2–4 weeks after the last drug dose had been given. This interval allowed numbers of nucleated BM cells to return to normal levels in all cases.

Lymphocyte repopulation was measured as the amounts of donor GPI isoenzyme type in separated blood lymphocytes, and erythrocyte repopulation was measured as the amounts of donor Hb. These were extremely closely correlated (r = 0.963), suggesting that lymphocytes and erythrocytes had descended from the same precursor, the PHSC [12, 14]. Control mixtures of BM cells containing equal numbers of unmanipulated B6 donor and B6-*Hbb*^{s}*Gpi-1*^{a} competitor cells produced the expected results: approximately equal amounts of each cell population's respective Hb and lymphocyte GPI-isoenzyme type (Table 1). Stem cells (PHSC) from groups of mice that had received ARA at a cumulative dose of 8 or 32 mg failed to exhibit any adverse effects of drug exposure. Levels of donor-type lymphocytes and erythrocytes were comparable with those produced by control-cell populations (Table 1). All other drugs damaged PHSC. Marrow from donors exposed to 5FU repeatedly over a period of 8 weeks repopulated less than half as well as did control marrow in both the erythrocyte and the lymphocyte compartments.

Discussion

Intensification of chemotherapeutic regimens has often meant increased long-term survival of cancer patients. However, there may be acute and chronic multisystem disorders signaling long-lasting chemotherapeutic effects in these survivors. The long-term viability of PHSC after exposure to intensive chemotherapeutic regimens is a vital issue, since myeloid and lymphoid cell functions must be preserved over the long term. In autologous transplantation, "rescue" of the patient involves the infusion of autologously derived, often cryopreserved bone marrow cells that have frequently been exposed to extended or high-dose chemotherapy through prior treatment courses or ex vivo purging [9, 21, 25, 27], such that the vitality of the transplanted PHSC is a pressing concern.

By measuring the relative production of two competing and genotypically distinctive PHSC populations, the competitive repopulation assay precisely defines the long-term repopulating functions of primitive stem cells that have been exposed to various drugs relative to fresh marrow competitors. With this assay, the proliferative abilities of PHSC can be compared over long periods with quantitative detection of even subtle replicative defects [10–14].

Our results confirm the previously noted deleterious effects of alkylating agents [1, 2], indicating that as little as a single dose of either BCNU or CTX is sufficient to cause a significant and, at times, drastic decline in the ability of exposed B6 cells to repopulate normally in competition with untreated B6-*Hbb*^{d} *Gpi-1*^{a} cells. This is demonstrated by drops of 80% or greater in both erythrocyte and lymphocyte production after exposure of cells to two doses of BCNU. In all instances, damage to myeloid and erythroid production is well correlated; this suggests that damage occurs to their common precursor – a primitive stem-cell target. This suggestion is supported because defective replicative performance did not improve over the entire study period of between 90 and 180 days (Table 1).

Modest but significant replicative damage (a decline of almost one-third) was seen after the use of VCR or ACT. It is conceivable that this toxicity could be lessened by extending the period from drug administration to sacrifice to 4 weeks. Further studies will have to be performed to examine this possibility.

The administration of ARA, at least at the doses used in these experiments, resulted in no long-term marrow defect. Cells exposed to this agent consistently performed as well as fresh marrow in these tests (Table 1). Possibly, ARA acts only on cells that are synthesizing DNA rapidly [4], a condition not met by PHSC in this experiment.

5FU appears to be a drug that in a single dose eliminates most proliferating cells but spares noncycling PHSC [3, 17, 30]. Our use of a single dose of 5FU (150 mg/kg) fits this pattern, as cells obtained at 1, 5, and 8 days after 5FU administration repopulated at least as well as did fresh marrow, suggesting that PHSC are not permanently injured (Table 4). These results have been confirmed, and the slight deleterious effects observed at 15 days probably resulted from physical changes in the regenerating marrow that made it more vulnerable to damage when removed [22]. The exact nature of the damage cannot be exactly ascertained at this time, although there is some evidence that the mere handling of bone marrow PHSC may perhaps be enough cause for injury to PHSC to occur, with ensuing drops in repopulating ability [8].

To study the effects of 5FU on regenerating PHSC and also since multiple doses of drugs are generally used in clinical medicine, we gave 5FU repeatedly over 8 weeks. In these experiments, 5FU exposure led to severe PHSC functional impairments, exposed cells having less than half of the normal replicative potential (Table 1). Thus, when given in multiple doses, 5FU does not spare undifferentiated or uncommitted cell populations, unlike the results reported for hydroxyurea [19].

Importantly, damage to PHSC resulting from one dose of BCNU and multiple doses of 5FU was observed despite normal concentrations of colony-forming cells in vitro (Table 3). Apparently, damage to PHSC may escape detection by such indicators. Qualitative effects of drugs have previously been shown to be similar in mouse and man [6]. Therefore, our studies suggest that PHSC damage can be severe and permanent after intensive or prolonged chemotherapy. Cells from individuals who have received extensive chemotherapeutic treatment may later be characterized by "proliferative exhaustion," especially if they are used to repopulate the ablated hematopoietic microenvironment of autologous bone-marrow transplant patients. Drug-induced stromal injury [16] or forced differentiation of PHSC at the expense of self-renewal may contribute to such exhaustion.

The competitive repopulation experimental design eliminates stromal effects, since donor and competitor cells are in the same recipient exposed to the same stroma. Therefore, the defective repopulation of drug-exposed cells (Table 1) demonstrates permanent PHSC defects that are independent of stromal injury. Instead, the severe myelopoietic and erythropoietic defects seen in the present study suggest that PHSC injury or loss is a probable cause of the dysfunction. Further examination of the effects of drugs on PHSC is warranted, and competitive repopulation should be a useful tool in such studies.

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