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Increase in Circulating Stem Cells Following Chemotherapy in Man

By Carol M. Richman, Roy S. Weiner, and Ronald A. Yankee

The number of circulating granulocytic stem cells (CFU-C) was determined by the in vitro methylcellulose technique in cancer patients receiving intermittent chemotherapy. In 17 patients studied prior to therapy, the median CFU-C concentration per 2×10^5 mononuclear cells plated was six, compared to a posttreatment median of 23 in 21 patients ($p < 0.001$). Large numbers of stem cells were obtained by leukopheresis and cryopreserved with a 99.5% median CFU-C recovery. Cyclical changes in the concentration of stem cells with maximum values of 20 times baseline were demonstrated in a patient studied at weekly intervals during multiple courses of treatment. It was estimated that, at peak CFU-C concentrations, a quantity of stem cells equivalent to that present in a bulk bone marrow harvest could be obtained from the peripheral blood by a 17-liter pheresis. These results suggest that it may be practical to obtain an adequate number of stem cells from the peripheral blood to study autologous stem cell infusion as a means of averting myelosuppression in patients receiving intensive chemotherapy.

IN ANIMALS, pluripotential stem cells capable of repopulating the hematopoietic system circulate in the peripheral blood.[1,2] Autologous stem cells obtained by leukopheresis have been used successfully to rescue animals from otherwise lethal marrow aplasia induced by radiation or chemotherapeutic agents.[3-7] In humans, the evidence that pluripotential stem cells circulate is derived from cases where engraftment occurs following transfusion of blood products to immunosuppressed patients.[8,9] As yet, no direct assay for the pluripotential stem cell is available in man.[10-12]

There is abundant evidence that human peripheral blood contains committed granulocytic stem cells which form colonies in semisolid medium (CFU-C). The concentration of these cells in the peripheral blood is 1%-10% of that found in bone marrow.[13-15] Because of the small number of stem cells in the circulation, utilization of autologous peripheral blood to alter myelosuppression in patients receiving intensive chemotherapy is currently impractical.

It has been noted that the bone marrow CFU-C concentration increases above baseline values in patients recovering from chemotherapy.[16-18] We are not aware of any published observations showing a similar increase in peripheral blood CFU-C in man.

In this report we demonstrate serial changes in peripheral blood CFU-C in patients with solid tumors receiving intermittent chemotherapy.[19] The increase in circulating CFU-C observed may be of sufficient magnitude to consider

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From the Blood Research Laboratories, Sidney Farber Cancer Center, and Harvard Medical School, Boston, Mass.

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Address for reprint requests: C. Richman, M.D., Sidney Farber Cancer Center, 35 Binney St., Boston, Mass. 02115.

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leukopheresis as a practical means of procuring autologous stem cells for early hematologic reconstitution following high dose chemotherapy.

MATERIALS AND METHODS

Assays for committed stem cells (CFU-C) were performed on peripheral blood mononuclear cell concentrates using a modification of the culture method described by Worton and Iscove.[20,21] Methylcellulose was prepared by adding 10.4 g of 4000 cps premium Methocel powder (Dow, Midland, Mich.) to 200 ml of hot, autoclaved deionized distilled water. The mixture was allowed to boil with continuous stirring and then cooled to room temperature. An equal volume of double strength alpha medium (Flow Laboratories, Inc., Rockville, Md.) was then added and the resulting 2.2% (w/w) solution stirred continuously for 48 hr at 4°C.

Conditioned medium containing colony-stimulating factor (CSF) was prepared using human peripheral blood leukocytes obtained by sedimenting whole blood.[21,22] These cells were suspended in 0.5% agar at a concentration of 1×10^6 cells/ml. A top layer containing 80% alpha medium and 20% fetal calf serum (Flow Labs., Inc.) was applied to the agar layer and the flask incubated with 10% CO_2 in a 37°C moist atmosphere. After 7 days, the top layer was decanted and centrifuged at 12,000 *g* for 20 min to remove cellular debris and agar particles. The supernatant solution was then filtered through a 0.2-μ Millipore filter (Bedford, Mass.) and stored at 4°C. Fetal calf serum used in the preparation of conditioned medium and in all culture procedures was derived from a single lot selected after a screening procedure to determine optimum colony growth.

Peripheral blood for the CFU-C assay was drawn in plastic syringes containing 10–20 U/ml of preservative-free heparin (Weddel, London, England). Mononuclear cell concentrates were prepared by the Ficoll-Hypaque density gradient technique as follows: peripheral blood was diluted with two volumes of alpha medium containing 5 U/ml of heparin. Then 35 ml were layered over 15 ml of 9% Ficoll–34% sodium diatrozoate (S.G. 1.078) and the tube centrifuged at 400 *g* for 40 min at 22°C.[23,24] The mononuclear fraction was washed three times in alpha medium containing 5% fetal calf serum with 5 U/ml of heparin. The cells were resuspended in alpha medium and adjusted to appropriate cell concentration. Heparinized bone marrow specimens were sedimented at room temperature for 45 min, and the cell-rich plasma was collected and washed three times in alpha medium.

A semicontinuous flow cell separator (Haemonetics Corp., Natick, Mass.) was used in selected individuals to obtain large quantities of peripheral blood cells for cryopreservation studies. By this technique, a 30-ml buffy coat fraction was obtained which consisted of 90% of the mononuclear cells present in each 500 ml of blood processed. The mononuclear fraction was diluted with 4 volumes of medium and separated on Ficoll-Hypaque as described above to reduce the number of erythrocytes.

Triplicate cultures were plated in 35×10-mm Falcon petri dishes at a final cell concentration of 2×10^5 mononuclear cells/ml. Each plate contained 40% methylcellulose, 20% fetal calf serum, 20% cells in alpha medium (containing 100 U/ml penicillin, 100 μg/ml streptomycin), and 20% CSF. In previous studies, concentrations of CSF $\geq$ 10% resulted in maximal colony formation by normal human bone marrow. Therefore, 20% CSF was chosen to ensure excess stimulating factor. Plates were incubated at 37°C in 10% CO_2 with high humidity and counted at 14 days using an inverted microscope at $\times$ 50. Aggregates of greater than 20 cells were designated as colonies.[21] The morphology of each colony was noted according to whether the cells formed loose or compact aggregates. Over 95% of all colonies counted in both normals and in patients were compact. Results were expressed as mean CFU-C per 2×10^5 cells plated $\pm$ standard error of the mean (SEM).

Single compact colonies were extracted from the culture plates using a drawn-out Pasteur pipette. The cells were suspended in alpha medium with 50% fetal calf serum, processed by a cytocentrifuge (Shandon-Elliot, Sewickley, Pa.) and stained with Wright–Giemsa. Cytologically these colony cells could be recognized primarily as differentiating neutrophils, including mature polymorphonuclear leukocytes.

Healthy volunteer platelet donors served as normal controls, and six of these individuals were tested on multiple occasions (Table 1).

Patients with solid tumors were studied on multiple occasions following intermittent chemo-

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Table 1. Peripheral Blood CFU-C Values in Normal Subjects

Normal Donors	Age (yr)	Sex	Peripheral Blood CFU-C*
1	22	F	2 ± 1
2	55	M	2 ± 1
3	47	M	2 ± 0
4	23	M	4 ± 1
5	22	M	6 ± 1
			8 ± 1 (7)
6	29	F	8 ± 1
			7 ± 0 (8)
			3 ± 1 (14)
7	28	M	9 ± 2
			10 ± 3 (23)
			13 ± 2 (36)
8	51	F	10 ± 2
9	48	M	10 ± 3
10	38	M	13 ± 1
11	29	F	13 ± 1
			12 ± 0 (1)
12	22	F	21 ± 3
13	26	M	29 ± 2
			49 ± 6 (5)
			25 ± 5 (7)
14	25	F	48 ± 4
			19 ± 1 (1)
			19 ± 5 (3)
			34 ± 0 (17)
Median			9.5

*Mean number of colonies in triplicate plates ± SEM per 2×10^5 Ficoll–Hypaque separated mononuclear cells. Numbers in parentheses indicate weeks from initial determination. Only initial determinations were used in calculation of the median.

therapy. The majority of patients were previously untreated females with stage III ovarian carcinoma participating in an autologous bone marrow transplantation protocol[25] in which marrow was harvested and cryopreserved prior to therapy with adriamycin (ADR) and cyclophosphamide (CY). The diagnoses, CFU-C values, and mononuclear cell counts before and after treatment are presented in Table 2. In the first 14 patients, peripheral blood CFU-C concentrations were determined prior to and at 21 days following successive courses of ADR/CY up to a total of five courses (mean = three courses). Only the maximum postchemotherapy value observed for any individual patient is shown. The initial dose of ADR/CY was 45/500 mg/sq m. In subsequent courses, patients received 70/750 mg/sq m. Ten patients (Nos. 1-5, 7, 8, 10, 11, 14) received at least one course at each dose level during the study period.

Aliquots of Ficoll-Hypaque separated peripheral blood specimens were frozen in 2-ml plastic vials (Costar, Cooke Laboratories) using 10% dimethylsulfoxide (DMSO) as a cryopreservative.[26,27] The freezing rate was controlled at −1°C per minute to −40°C. The cells were then rapidly cooled to −100°C and placed in the vapor phase of liquid nitrogen (−150°C). Vials were thawed rapidly in a 40°C water bath and diluted ten-fold over 10 min at 0°C with medium containing 25% fetal calf serum and 20 U/ml heparin. Cells were washed three times in alpha medium containing 5% fetal calf serum and plated as above. These samples were used to determine the effect of cryopreservation on peripheral blood stem cells. Recovery of CFU-C was calculated by comparing the total number of colonies per vial prior to freezing with the number after thawing. The total CFU-C before freezing was obtained by multiplying CFU-C per 2×10^5 cells by the total number of cells in each vial. Similarly, after thawing, the total CFU-C per vial was obtained by multiplying the total number of cells recovered from the vial by the colonies per 2×10^5 cells plated (Table 3).

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Table 2. Peripheral Blood CFU-C and Mononuclear Cell Concentrations in Patients Before and After Chemotherapy

Patient	Age (yr)	Diagnosis and Stage	Peripheral Blood CFU-C*		Peripheral Blood Mononuclear Cells† (per cu mm × 10^3)	
			Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
1	38‡	Terato ca	5 ± 0	101 ± 12	1.82	1.52
2	51	Ovarian sarc	1 ± 1	68 ± 19	2.98	2.47
3	41	Ovarian III	13 ± 2§	63 ± 6	1.36	1.50
4	48	Ovarian IV (tumor in bone marrow)	16 ± 4	39 ± 9 (day 14)	2.18	1.42
5	28	Ovarian III	1 ± 0	37 ± 6	1.30	2.85
6	24‡	Hemangiopericytoma	4 ± 1	34 ± 1	2.05	1.68
7	50	Ovarian III (granuloma in bone marrow)	13 ± 2	31 ± 6	2.00	2.32
8	44	Ovarian III	23 ± 2	19 ± 6	2.70	1.94
9	25	Mesothelioma	27 ± 2	16 ± 2 (day 18)	1.17	1.36
10	56	Ovarian III	0 ± 0	16 ± 1 (day 18)	1.78	2.86
11	46¶	Ovarian III	1 ± 1	12 ± 2	1.73	0.61
12	40¶	Breast III	5 ± 1	8 ± 2	1.96	2.14
13	29¶	Breast III	6 ± 2	6 ± 1	3.72	2.84
14	41	Ovarian III	9 ± 4	4 ± 1	2.20	2.04
15	50	Ovarian III	3 ± 2	—		
16	50	Ovarian III	6 ± 0	—		
17	49¶	Breast IV	13 ± 1	—		
18	36¶	Breast IV	—	157 ± 26		
19	29¶	Synovial sarc	—	89 ± 10		
20	28	Ewing's sarc	—	52 ± 8		
21	55	Ovarian IV	—	23 ± 1		
22	55‡	Sarcoma	—	9 ± 2		
23	62	Ovarian IV	—	7 ± 2		
24	53	Ovarian III	—	6 ± 1		
Median			6	23	1.98	1.99

The maximum posttherapy value is shown when multiple determinations were available. Specimens were assayed on day 21 following a course of chemotherapy unless indicated by parentheses. All patients received adriamycin (ADR) and cyclophosphamide (CY) every 21 days with the exception of patient No. 19, who received methotrexate in addition to ADR and CY, and patient No. 18, who received methotrexate and dimethyl triazeno imidazole carboxamide (DTIC).

*Mean number of colonies in triplicate plates ± SEM per 2 × 10^5 Ficoll–Hypaque separated mononuclear cells.

†Product of whole blood white count and per cent lymphocytes plus monocytes.

‡Male patients.

§Pre- and posttreatment (Rx) values from cryopreserved specimens.

¶Prior chemo- or radiotherapy.

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Table 3. Cryopreservation of Peripheral Blood Cells and CFU-C

Specimen	Weeks Stored	Total Cells $\times 10^6$ Fresh	Total Cells $\times 10^6$ Thawed	Per Cent Recovery of Cells*	Total CFU-C $\times 10^2$ Fresh	Total CFU-C $\times 10^2$ Thawed	Per Cent Recovery of CFU-C†
1	0	13.5	8.8	65	0	0	—
2	1	30.0	13.0	43	117	117	100
3	1	30.0	15.5	52	106	142	134
4	2	35.0	15.5	44	114	81	71
5	3	10.5	12.6	120	2	2	100
6	3	50.0	31.0	62	100	98	98
7	4	22.5	9.2	41	42	92	219
8	4	30.0	34.0	113	106	105	99
9	5	21.0	8.0	38	21	10	48
10	6	16.5	8.4	51	2	1	50
11	7	18.5	8.4	45	3	7	233
12	7	22.0	21.0	95	41	48	117
13	8	16.0	11.0	69	81	68	84
Median				52			99.5

*Per cent recovery of cells, calculated for each sample by using the total number of cells per vial before freezing and the total number of cells after thawing.

†Per cent recovery of CFU-C, calculated for each sample by using the total CFU-C content of vials before freezing and after thawing.

RESULTS

In 14 normal donors, the median peripheral blood CFU-C concentration was 9.5 (range 2–48) per 2×10^5 mononuclear cells plated (Table 1). The median CFU-C concentration in patients studied prior to chemotherapy was 6 (range 0–27) (Table 2). There was no statistical difference between the patients' pretreatment values and the values for normal donors ($p > 0.3$ Mann–Whitney U Test). Following chemotherapy, the median CFU-C concentration for the entire group of patients was 23 (range 4–157). This value was significantly higher than pretreatment values ($p < 0.001$ Mann-Whitney U Test).

Fourteen patients were studied both before and after treatment, and in nine there was an increase in circulating CFU-C following chemotherapy (Nos. 1–7, 10, 11, Table 2). The increase in CFU-C occurred in eight patients after the first course and was present after each subsequent course of therapy. In this group of 14 patients, the posttherapy CFU-C concentrations were significantly higher than those prior to therapy ($p < 0.01$, Wilcoxon Paired Signed-Ranks). There was no significant difference between the number of circulating mononuclear cells (lymphocytes plus monocytes) before chemotherapy as compared with the number after chemotherapy (Table 2, $p > 0.3$).

Quantitation of circulating stem cells at weekly intervals revealed a cyclical change in CFU-C content following consecutive courses of chemotherapy (Fig. 1). There were no CFU-C detectable in the peripheral blood at 7 days following chemotherapy. By day 15 the CFU-C content was 5 times the baseline value, at a time when the granulocyte count was at the nadir. Following each course of chemotherapy, the maximum peripheral blood CFU-C content occurred on day 21, with values up to 20 times baseline. Despite marked

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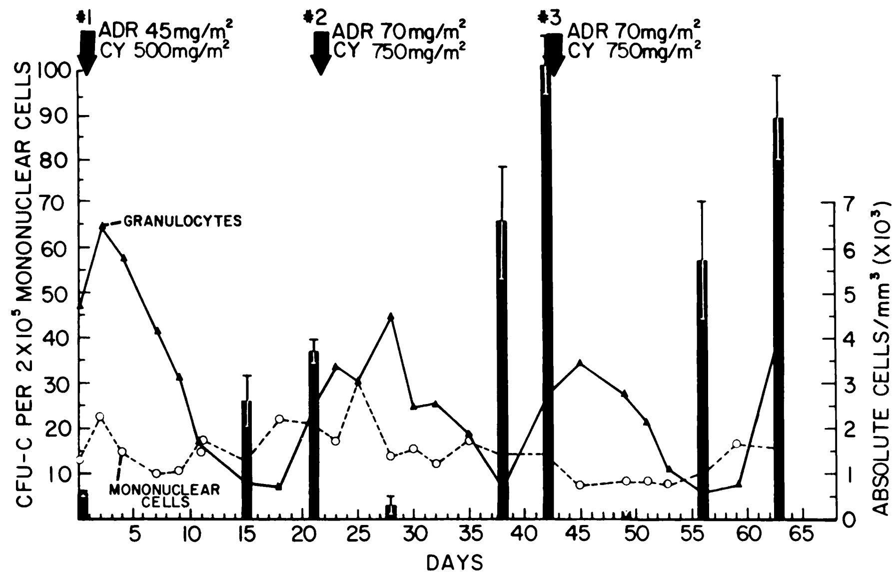

Fig. 1. Serial absolute granulocyte counts (▲——▲), mononuclear cell (lymphocytes + monocytes) counts (○------○), and mean peripheral blood CFU-C values (▬) following three successive courses of adriamycin (ADR) and cyclophosphamide (CY) for patient No. 1. The dose of drug and day of administration are shown by the arrows. Vertical bars represent SEM.

fluctuation in the concentration of peripheral blood leukocytes during each 21 day period, the total mononuclear cell concentration showed little variation.

Peripheral blood CFU-C activity was maintained with cryopreservation for up to 2 mo as shown in Table 3. The median total cell recovery and the median CFU-C recovery were 52% and 99.5%, respectively.

DISCUSSION

This study indicates that myelosuppressive chemotherapy with cyclophosphamide and adriamycin profoundly affects committed granulocytic stem cells in the peripheral blood. The observed increase in CFU-C concentration represents an increase in the total number of circulating stem cells, and is not an artifact produced by changes in the differential counts. The posttherapy CFU-C concentration per 2×10^5 mononuclear cells is significantly increased without a significant change in the total number of circulating mononuclear cells. These findings are consistent with the transient increase in bone marrow CFU-C concentration previously noted during recovery from cyclophosphamide administration in man[16] and the increase in bone marrow and peripheral blood CFU-C observed following chemotherapy in animals.[28,29]

A similar rebound in the more primitive hematopoietic stem cell (CFU-S) has been demonstrated in both peripheral blood and bone marrow following chemotherapy in mice.[30-32] Although no direct assay is available for quantitating pluripotential stem cells in man, one can speculate that a parallel increase in the primitive and in the committed stem cells might occur after cyclophosphamide/adriamycin therapy. Murine studies showing parallel changes in bone marrow CFU-S and CFU-C after vinblastine[31] and cyclophosphamide[32]

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support this possibility. The analogy may not, however, be true for other cytotoxic drugs, especially those with different sites of pharmacologic action.[33]

The mechanism for the appearance of increased numbers of circulating stem cells is unclear. It may represent an expansion in the total number of marrow stem cells during recovery from myelosuppression and a subsequent spillover of these cells into the peripheral blood, as postulated in the murine and canine models.[29,30] Injury to the supporting structure of the marrow by cytotoxic drugs may also contribute to the release of stem cells into circulation. In addition, chemotherapy may destroy or alter cells which ordinarily inhibit colony formation,[34] thereby allowing CFU-C to proliferate. In our studies, 5 of 14 patients failed to show an increase in circulating CFU-C (Nos. 8, 9, 12–14, Table 2). Prior irradiation may have contributed to the reduced response in two of these patients. It is also possible that the maximum increase in CFU-C was missed in several of the patients who were studied on only one occasion (usually day 21) after a course of therapy. The day of highest peripheral blood CFU-C varied in the small group of patients studied here. For example, in patient No. 4, the CFU-C content was highest on day 14 (39 colonies), compared with day 21 (18 colonies) following chemotherapy. Observations at more frequent intervals would be useful in establishing the significance of these findings.

The viability of cryopreserved human stem cells, as measured by the CFU-C assay, has been well documented.[26] In our limited study, there was considerable variation in range of CFU-C recovery (from 48% to 233%). Values greater than 100% recovery may be partially explained by the difficulty in quantitating differences when the baseline CFU-C values are low.

We propose that the observed increase in circulating CFU-C may facilitate procurement of a quantity of autologous stem cells sufficient to support patients receiving intensive chemotherapy. Cryopreserved peripheral blood stem cells have been used successfully in dogs to avert otherwise lethal marrow aplasia induced by high dose radiation or chemotherapy.[3-7] In order to estimate the feasibility of this approach in humans, we studied one patient undergoing leukopheresis at periodic intervals during chemotherapy. From these results, the pheresis time required to collect a bone marrow transplant equivalent dose of CFU-C may be calculated. For example, a $1\frac{1}{2}$-hr bone marrow harvest from this patient prior to therapy yielded 4.3×10^9 mononuclear cells which contained 231 CFU-C/10^5 cells. The total number of bone marrow CFU-C available for reinfusion was therefore 9.9×10^6. Prior to chemotherapy, the number of CFU-C obtained from the peripheral blood by pheresis was 3.2 per 2×10^5 mononuclear cells. Since a 1-liter pheresis took 40 min and yielded 1.4×10^9 mononuclear cells, we estimated that a bone marrow equivalent dose of CFU-C would necessitate clearance of 442 liters of blood requiring 296 hr. Following chemotherapy (day 21), 1.5×10^9 cells were obtained by a 1-liter pheresis and yielded 78 colonies per 2×10^5 mononuclear cells plated. A dose of stem cells equivalent to a bone marrow harvest could, at that time, be obtained from the peripheral blood by a 17-liter pheresis requiring 11 hr. It is premature to draw conclusions from these observations in a single patient. However, it should be emphasized that, in the majority of patients studied, there was a significant increase in circulating CFU-C after chemotherapy. Obtaining autologous stem cells by several successive days of pheresis would have a

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distinct advantage over a bone marrow harvest since the suggested amount of pheresis (i.e., 11 hr) is generally well tolerated and can be performed repeatedly on an outpatient basis without the risk of anesthesia or the discomfort of multiple bone marrow punctures. If this form of autologous support is effective in promoting early hematopoietic recovery following cytotoxic drug treatment, it may facilitate the study of high dose chemotherapy as a means of improving therapeutic response in patients with cancer.

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