MASTER

QUANTIFICATION OF ERYTHROID AND GRANULOCYTIC PRECURSOR CELLS
IN PLATELETPHERESIS RESIDUES

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ABSTRACT

Mononuclear cell fractions of human blood and plateletpheresis residues were compared for their content of hemopoietic precursor cells. Erythroid burst-forming units (BFU-E) averaged 560 ± 130 per ml of blood and granulocyte-monocyte colony forming units (CFU-C) averaged 240 ± 90 per ml blood. Estimates based on a blood volume of 7% of body weight indicate that the total blood pools of BFU-E and CFU-C are about 3.5×10^6 and 1.5×10^6 cells respectively.

Plateletpheresis residues prepared after a mean of 3.4 liters of blood had been processed contained $1.3 \pm 0.22 \times 10^6$ BFU-E and $0.50 \pm 0.13 \times 10^6$ CFU-C. These values represent a 68% and 63% recovery respectively of BFU-E and CFU-C from the processed 3.4 liters of blood.

Sequential studies were performed over 3 days following one plateletpheresis in 4 donors. CFU-C and BFU-E approximately doubled between 48 and 72 hours after a plateletpheresis. During this time there was no significant alteration in the percent of null, T or B lymphocytes in blood. Thus, plateletpheresis appears to lead to a mobilization of precursor cells, which results in a transient increase in their concentration in blood. Therefore, pheresis 48 to 72 hours after an initial short-term procedure could harvest much larger numbers of precursor cells. Moreover, such techniques would put blood precursor cell content of plateletpheresis residues within reach of the precursor cell content in the volume of human marrow used for transplantation.

INTRODUCTION

The presence in the human circulation of cells with hemopoietic capabilities was suggested by the observation that blood leukocytes from patients with chronic myelogenous leukemia were capable of producing allogeneic marrow engraftment in myelosuppressed patients (1). Early bone marrow recovery was observed after myelosuppressive chemotherapy when blood leukocytes from normal donors were transfused into allogeneic and syngeneic recipients (2).

The availability of semisolid cell culture techniques led to the identification and quantitation of granulocyte-monocyte precursor cells (3-5) and, more recently, of erythropoietic precursors (6-8) in human blood. The stemness of circulating precursor cells in healthy human donors is uncertain. Granulocytic, erythroid and megakaryocytic proliferation has been reported following implantation of immunologically null, human blood lymphocyte-like cells into diffusion chambers (9). Also, cells that produce colonies containing both granulocytic and erythroid cells have been described in human blood and marrow (10). These studies suggest that cells closer to a multipotent hemopoietic precursor cell may be present in blood. Moreover, reconstitution of marrow has been achieved in patients with accelerated chronic myelogenous leukemia by transfusion of stored autologous blood cells (12,13).

Our laboratory has previously reported that the residues of plateletpheresis preparations are a convenient and rich source of blood mononuclear cells (11). In the present studies we have examined the recovery of circulating erythroid and granulocytic precursor cells in plateletpheresis residues. In addition, we have measured blood precursor cell concentration following plateletpheresis. We have found that plateletpheresis residues

are a rich source of erythroid and granulocyte progenitors for study. Further, such material from normal human blood may also prove to be a source of hemopoietic cells for transplantation.

MATERIALS AND METHODS

Collection of Mononuclear Cells from Blood and Plateletpheresis Residues

Normal donors from the Rochester American Red Cross Blood Center were used throughout the study. Fifteen to twenty mls of heparinized blood were obtained prior to initiation of plateletpheresis with a Haemonetic Model 30 cell separator (14). In three donors additional blood samples were obtained at the termination of the procedure and at daily intervals over three days for sequential studies of blood precursor cell growth. Blood mononuclear cells were prepared following centrifugation over an Isopaque-Ficoll step gradient (density = 1.077) as described by Boyum (15).

Plateletpheresis residues were diluted to 250 ml with medium 199 containing Earle's salts (TC-199) and heparin, 5 units/ml. This suspension was centrifuged at 200 g for fifteen minutes and the platelet rich supernate was discarded. The cell pellet was resuspended in 270 mls of TC 199 supplemented with 5 percent heat-inactivated fetal bovine serum. A twice-concentrated lymphocyte separating reagent containing iron filings in balanced salt solution was added and the suspension was incubated for 30 minutes at 37°C (16). The mononuclear cells depleted of erythrocytes and iron adherent cells were obtained following centrifugation over an Isopaque-Ficoll step gradient. The cells were washed twice and resuspended in TC 199 with 20% heat-inactivated fetal bovine

serum at a concentration of 5×10^6 cells/ml. Mononuclear cells were used the same day of preparation for quantitation of erythroid and granulocytic precursor cells.

The studies of the relationship of mononuclear cell number to BFU-E and CFU-C growth were performed on mononuclear cells following overnight incubation in tissue culture flasks (150 cm²) under 7 percent CO₂ and at 37°C. We have previously established that cell counts, viability and cell responsiveness do not change under these conditions.

Cell Counts and T and B Lymphocyte Determination

Mononuclear cells were counted using a Coulter Model ZBI particle counter. The percent of lymphocytes and monocytes were determined by integration of the curves of the cell volume distribution (16) at an amplification 2 and baseline threshold of 6. We have confirmed that this method of monocyte-lymphocyte differential counting is as accurate as morphologic, histochemical or phagocytic assays.

T lymphocytes were identified as those cells which formed rosettes with sheep red cells after an overnight incubation at 4°C (17).

B lymphocytes were identified as those cells forming rosettes when 0.1 ml of a suspension of 2×10^6 lymphocytes/ml with 0.1 ml of a 1:10 dilution of Immunobeads (Biorad) in Dulbecco's phosphate buffer without Ca⁺⁺ or Mg⁺⁺. A total of two hundred cells were counted in the hemocytometer (18).

Viscous Culture of Erythroid and Granulocytic-Monocytic Colonies

Three to 5×10^5 mononuclear cells were plated according to the method of Gregory and Eaves (19) into a tissue culture dish containing 0.8% methylcellulose in alpha medium, 30% fetal bovine serum, 10% deionized bovine serum albumin and 0.1 mM monothioglycerol. Erythropoietin (Step III of Connaught) was used at 2 units/ml. Colonies with cells containing hemoglobin were counted after 12-14 days.

The same assay condition described for erythroid cells were used to study granulocyte-monocyte colonies. The source of colony stimulating activity was human GCT cell line-conditioned medium added in a concentration of 0.1 ml/ml of culture unless specified otherwise (21). Colonies of greater than fifty cells were counted after 12-14 days of incubation. The incubations were conducted at 37°C under high humidity ($\approx 100\%$) and 5 percent CO₂.

The concentration of colonies was derived by dividing the total colonies per plate by the total number of mononuclear cells cultured. The number of colonies per ml blood or per ml of plateletpheresis residue was obtained by multiplying the number of colonies per mononuclear cell by the blood or plateletpheresis residue mononuclear cell count per ml.

RESULTS

Determining Conditions for Cultures: The Effects of Cell, Erythropoietin, and Conditioned Medium Concentrations on Erythroid and Granulocytic Colony Growth

Mononuclear cells obtained by plateletpheresis were used

to determine the concentration of erythropoietin or colony stimulating activity required for erythroid or granulocytic precursor cell growth. The number of BFU-E colonies was dependent on the amount of erythropoietin added per ml of culture. The requirement for erythropoietin is a function of the proportion of monocytes. Under the conditions of our studies 2 units/ml of erythropoietin produced maximal growth of BFU-E. Therefore, studies of BFU-E growth were performed at two units per ml. The growth of BFU-E at an increasing density of mononuclear cells cultured was approximately linear (Fig. 1A). Importantly a proportionality of erythroid growth was present between 2.5 and 5.0×10^5 mononuclear cells per ml, 60 versus 133 colonies.

The number of CFU-C was dependent on the concentration of GCT conditioned medium. There was some colony growth in the absence of conditioned medium, presumably because of endogenous colony stimulating activity production by monocytes present. Maximal stimulation of CFU-C growth occurred at a concentration of 5% conditioned medium. Ten percent cell line-conditioned medium was used for quantification of CFU-C in blood and in plateletpheresis residues. This resulted in maximal stimulation. The CFU-C growth also was near to linear when plotted against mononuclear cell concentration, and a near proportional increase was present between 2.5 and 5.0×10^5 cells per ml (24 to 43 colonies) (Figure 1B).

Mononuclear Cell Recovery from Plateletpheresis Residues

The mononuclear cell yield by semicontinuous flow centrifugation in seven normal donors is shown in Table 1. For an average 3.4 liters of

blood processed, the mean mononuclear cell number collected was 5.0×10^9 , which represents a 67% yield of the total number of mononuclear cells processed throughout the procedure. The recovery following ficoll-hypaque density separation and iron treatment was 38% of the total mononuclear cells pheresed.

Colony Appearance and Morphology

BFU-E were tightly clustered and increased in size and hemoglobin content with increasing dose of erythropoietin.

CFU-C grown from blood and plateletpheresis residues tended to reach a very large size by 14 days of incubation. Wright-Giemsa staining of individual colonies revealed that 45% of the granulocyte-monocyte colonies had an increased affinity for eosin. Microscopic appearance was similar to colonies in blood described recently by Nissen-Druey and Speck (22).

Precursor Cell Content in Blood and Plateletpheresis Residues

BFU-E were present in an average of 560 per ml of blood and the range of their content was from 220 to 1320 per ml in seven healthy donors. The total blood pool of BFC-E was 3.5×10^6 with a range from 1 to 8.7×10^6 per ml. Table II shows the yield of BFC-E in the plateletpheresis residues. Plateletpheresis residues contained a mean of 30,000 BFC-E per ml and a total of $1.3 \pm 0.22 \times 10^6$ BFC-E was collected by a 2 hour pheresis. This represented a yield of 68% of the BFC-E processed.

CFU-C in the blood ranged between 70 to 740 per ml with an average of 240 per ml. Table II shows the yield of CFU-C obtained by plateletpheresis. The total blood CFU-C pool was 1.5×10^6 , with a range from

0.45 to 4.8×10^6 cells. CFU-C per ml of plateletpheresis residue averaged 12,000 and a total of 0.5×10^6 CFU-C was collected after a 2 hour plateletpheresis procedure.

There was no correlation between the absolute lymphocyte count of the donor and the total CFU-C collected, although variations in the lymphocyte counts were small and, thus, such an analysis was difficult. However, either BFU-E or CFU-C colonies per 10^5 mononuclear cells in the blood of a donor was strongly correlated with its concentration in pheresis residues (Figure 2), indicating that no significant loss or enrichment of CFU-C and BFU-E per number of mononuclear cells occurred by plateletpheresis.

Precursor Cell Content of Blood After Plateletpheresis

We studied three donors sequentially following one pheresis procedure to determine if there was a change in the concentration of blood CFU-C or BFU-E. Table III shows the quantitative changes in precursor cells per ml of blood. The absolute lymphocyte count increased about 35% from a baseline value of $2.0 \pm 0.58 \times 10^6$ ml, to $2.7 \pm 0.63 \times 10^6$ /ml blood at 72 hours. There was no appreciable change, however, in the percent of null, T or B lymphocytes.

There was no depletion of either BFU-E or CFU-C in blood immediately following the pheresis; rather, both precursor cell types showed a 1.5 to a two-fold increase from prepheresis blood concentrations by 48 to 72 hours after the procedure in each of the four donors studied. Baseline values for blood precursor cell concentrations had not been restored by 96 hr in two patients.

DISCUSSION

The application of semicontinuous or continuous flow centrifugation techniques to harvest granulocytic progenitor cells has been reported in man (23-28) and in dogs (29). Our results show an efficiency of collection of about 65% of processed colony-forming cells, and confirm the data of Weiner (24) showing a strong correlation between CFU-C in the blood and those obtained by semicontinuous flow centrifugation.

Exercise, ACTH injection (30) as well as endotoxin (31) and recovery from chemotherapy (23) all have been reported to raise circulating CFU-C. Our study shows that immediately following a limited pheresis procedure BFU-E and CFU-C levels nearly doubled between 48 and 72 hours. Korbling et al. found an increase in CFU-C when the same donor was subjected to up to three procedures per week, with increments of 37-232% in collected CFU-C's over that obtained by one procedure (32). Our long-term postpheresis observations suggest that this increase in blood precursor cells may not require multiple pheresis procedures but may be a delayed (48-72 hour) reaction to a single pheresis.

In dogs dextran sulfate injections coupled with leukopheresis has been found to be effective in stem cell mobilization (29). This was associated with a striking extramedullary hemopoiesis localized to the spleen (33). Weetman (34) observed increased numbers of CFU-C in the blood of children with hereditary spherocytosis with a moderately severe degree of hemolysis. Elevation in circulating erythroid precursors was also noted by Ogawa in patients with hemoglobinopathies (8). Thus, precursor cell mobilization occurs under conditions of hemopoietic stress.

Although data is available in a canine model regarding bone marrow repopulation following autologous or allogeneic leukocyte transfusions (29), corresponding quantitative data are not yet available in man. Extrapolating from their data in a canine model, Korbiling et al. (32) calculated that a minimum reconstitution dose equalled 0.4×10^4 CFU-C/kg body weight, and for a seventy kg recipient this value is within reach of a single pheresis procedure.

Correlation of transplantation outcome with CFU-C or BFU-E assays in man have not been reported widely. In reports of partial marrow repopulation following combined radiation and chemotherapy in solid tumor patients (35,36), the CFU-Cs transfused ranged between 0.5×10^4 /kg to 6.9×10^4 /kg. Spitzer (35) et al. found that CFU-C in transfused autologous marrow correlated only with neutrophil recovery and not with platelet or lymphocyte recovery. Techniques for cryopreservation of both erythroid and granulocytic progenitor cells (BFU-E and CFU-C) are available (37,38) and single donor progenitor cells could be stored so as to accumulate sufficient number for transplantation. Moreover, cryopreserved blood precursor cells could be used for autotransplantation in patients undergoing intensive cytotoxic or radiation therapy. Recently, a study of patients with malignant lymphoma resistant to conventional therapy (39) has demonstrated that cryopreserved autologous marrow infusions shortened the chemotherapy induced leukopenia by half, as compared to control patients not receiving this form of treatment. Abrams and colleagues (36) have reported a partially successful reconstitution in a tumor patient given buffy coat cells from his identical twin. A

dose of 0.83×10^4 CFU-C/kg was administered in that case as compared to their experience with doses of autologous marrows that provided 6.9×10^4 CFU-C/kg.

The total blood CFU-C in our donors (1.5×10^6) could provide 3×10^4 CFU/kg to a 50 kg recipient, moreover, during the postpheresis overshoot the total blood CFU-C pool was doubled. Thus pheresis especially if precursor cell mobilization is enhanced by an antecedent short pheresis or by pharmacologic means, could provide numbers of precursor cells that may exceed the threshold for transplantation.

Further studies that quantify erythroid and granulocytic progenitors when blood cells are used as a transplantation product will be important. They may establish the parameters required for successful transplantation with blood cell and may be the ultimate proof of the stemness of circulating cells.

Moreover, there is no information to our knowledge on the effect of random-donor or related-donor hemopoietic precursor cells given inadvertently to patients receiving platelet or leukocyte transfusions. Inadvertent chimerism is probably rare because of the low dose of precursor cells administered and the immunologic barriers present in HLA-incompatible transfusions. The possibility, for example, that remission in aplastic anemia patients given high-dose cyclophosphamide and component transfusion is related to precursor cell infusion should be considered.

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LEGENDS

Figure 1 (A). Relationship of circulating erythropoietic precursors (BFU-E) to mononuclear cell number cultured. Mean $\pm$ SEM in five normal donors. Erythropoietin dose was 4 units/ml. (B). Relationship of CFU-C colony number to mononuclear cell number cultured. GCT conditioned medium at 0.1ml/ml:mean and range in two normal donors, each point done in triplicate.

Figure 2. The association of blood BFU-E (closed circles) and CFU-C (open circles) with plateletpheresis BFU-E and CFU-C.

TABLE 1. MONONUCLEAR CELL RECOVERY AFTER PLATELETPHERESIS

Donor	Total white count (# x 10 ⁶ /ml)	Mononuclear cells ¹ (%)	Blood volume processed (L)	Mononuclear cells processed (# x 10 ⁹)	Mononuclear cells in plateletpheresis packs (# x 10 ⁹)	Mononuclear cells recovered after iron adherence and isopaque-ficoll separation (# x 10 ⁹)
1	4.5	25	3.7	4.2	3.5	1.7
2	3.9	35	3.7	5.0	3.6	2.0
3	6.3	45	3.4	9.6	6.9	4.0
4	6.7	41	3.7	10	6.9	4.8
5	5.3	66	3.1	11	4.5	1.7
6	4.4	37	3.1	5.0	4.0	3.0
7	5.9	35	3.4	7.0	5.4	3.1
Mean	5.3	41	3.4	7.4	5.0	2.9
SE	0.39	4.8	0.11	1.0	0.55	0.45

¹ Mononuclear cells include blood lymphocytes and monocytes. The monocyte % in the blood of donors ranged from 2 to 6%.

TABLE 11. ERYTHROID (BFU-E) AND GRANULOCYTIC (CFU-C) PRECURSOR CELLS IN
BLOOD AND PLATELETPHERESIS RESIDUES

Donor	Weight (kg)	Estimated blood volume (l)	Blood precursor cell concentration		Total blood precursor pool		Volume blood processed (l)	Total precursor cells processed (A)		Plateletpheresis pack volume (ml)	Plateletpheresis precursor concentration		Total precursor cells collected (B)		Precursor cell recovery (B/A)	
			BFU-E (No/ml)	CFU-C	BFU-E (No x 10 ⁶)	CFU-C		BFU-E (No x 10 ⁶)	CFU-C		BFU-E (No x 10 ³ /ml)	CFU-C	BFU-E (No x 10 ⁶ /pack)	CFU-C	BFU-E	CFU-C
1	87	6.1	480	270	2.9	1.6	3.7	1.8	1.0	45	41	20	1.8	0.88	1.0	0.88
2	84	6.2	310	77	1.9	0.50	3.7	1.2	0.28	27	27	5.2	0.73	0.14	0.60	0.50
3	93	6.5	450	70	2.9	0.45	3.4	1.5	0.24	50	12	2.8	0.59	0.14	0.40	0.58
4	68	4.8	220	100	1.0	0.48	3.7	0.80	0.37	64	19	5.0	1.2	0.32	1.5	0.86
5	95	6.6	1300	740	8.7	4.9	3.1	4.0	0.23	42	34	23	1.4	0.96	0.35	0.41
6	81	5.7	530	210	3.0	1.2	3.1	1.7	0.65	40	27	11	1.1	0.44	0.65	0.67
7	86	6.0	640	200	3.8	1.2	3.4	2.2	0.68	48	48	15	2.3	0.72	1.0	1.0
Mean	86	6.0	560	240	3.5	1.5	3.4	1.9	0.79	45	30	12	1.3	0.50	0.68	0.63
SE	3.40	0.23	130	90	0.94	0.60	0.10	0.40	0.27	4	4.9	3.2	0.20	0.13		

(A) = precursor cells/ml blood x volume blood processed in ml

(B) = precursor cells/ml plateletpheresis pack x volume of pack in ml

TABLE III. SEQUENTIAL STUDIES OF FOUR DONORS UNDERGOING PLATELETPHERESIS

	Prepheresis	Postpheresis 0 hrs	24 hrs	48 hrs	72 hrs	96 hrs
Blood lymphocyte count x 10 ⁶ /ml (mean $\pm$ S.D.)	2.1 $\pm$ 0.50	2.7 $\pm$ 0.15	2.9 $\pm$ 0.95	2.3 $\pm$ 0.50	2.7 $\pm$ 0.46	2.8 $\pm$ 0.80
% T-lymphocytes	59 $\pm$ 6.9	66 $\pm$ 0.57	64 $\pm$ 4.9	64 $\pm$ 1.5	65 $\pm$ 4.3	60*
% B-Lymphocytes	8.8 $\pm$ 4.1	8.7 $\pm$ 3.5	11 $\pm$ 1.7	12 $\pm$ 1.3	11 $\pm$ 1.7	10*
CFU-C/ml (mean $\pm$ se)	130 $\pm$ 72	130 $\pm$ 42	220 $\pm$ 41	250 $\pm$ 100	190 $\pm$ 40	210 $\pm$ 36
BFU-E/ml (mean $\pm$ se)	440 $\pm$ 110	420 $\pm$ 130	510 $\pm$ 210	590 $\pm$ 78	670 $\pm$ 130	680 $\pm$ 260

*Performed at 96 hrs in 2 donors, only.