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Enrichment of Committed Stem Cells (CFU-C) from Human Peripheral Blood (40556)

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Granulopoietic stem cells (CFU-C) can be identified by their capacity to produce colonies of maturing progeny in culture (1). Colonies of maturing granulocytes and macrophages can be grown from peripheral blood or bone marrow cells in semisolid agar (1, 2). Colony formation in this system is absolutely dependent on specific colony-stimulating factor(s) (CSF) (3).

A double-layer agar culture technique is widely used to study stem cell differentiation (2). CFU-C are placed in the overlayer with a source of CSF in the underlayer. Appropriate modification of the assay permits study of either CFU-C or sources of CSF. CFU-C are usually obtained from murine or human bone marrow aspirates (4).

Other investigators have found that the residues from single-donor plateletpheresis are a source of immunocompetent cells (5) and CFU-C (6). We have used these residues to prepare a convenient, reproducible supply of CFU-C-enriched fractions which may be cryopreserved without loss in colony-forming activity.

Materials and methods. Plateletpheresis residue. A Haemonetics Model 30 Cell Separator was used to obtain platelet-rich plasma from normal donors. Leukocytes and platelets were collected from each donor by centrifugation of 6 to 8 successive vol of approximately 500 ml of blood (a total of approximately 3500 ml) (7). After each 500 ml of blood had been centrifuged, the leukocytes and platelets were aspirated from the bowl and the plasma and red blood cells were returned to the donor. A total of approximately 320 ml of leukocyte- and platelet-rich plasma was collected and sedimented at 150*g* for 8 min at room temperature (5). The platelet-rich plasma supernate was removed for clinical use and the residue was used to prepare concentrates of CFU-C. Informed consent was obtained from each donor and the research was carried out according to the Declaration of Helsinki.

Lymphocyte-enriched suspension. The residue consisted of mononuclear cells suspended in 50 ml of platelet-rich plasma. Cells were processed using sterile techniques at room temperature unless otherwise noted. Ten milliliters of the suspension was mixed with 40 ml of calcium-and-magnesium-free Hank's buffered salt solution (HBSS) into each of five 50-ml, conical glass centrifuge tubes. The tubes were centrifuged at 150*g* for 15 min, the supernates were discarded, and each pellet was gently resuspended in 40 ml of HBSS (5). Ten milliliters of a solution of Ficoll–diatrizoate (lymphocyte separation medium or LSM, Litton Bionetics, Inc.) was underlayered in each tube, and the mixture was centrifuged at 400*g* for 30 min (8). The cells at the interface were collected, combined in a 50-ml conical centrifuge tube, and washed twice with 50 ml of HBSS. The cells were collected between washes by centrifugation at 150*g* for 15 min. The pellet was then resuspended in a total of 300 ml of RPMI 1640. For convenience, the suspension was transferred to three 100-ml bottles which were stoppered and stored overnight (16 hr).

Macrophages were then depleted by pouring the suspensions into five or six 150 × 15-mm plastic petri dishes and incubating for 1 hr at 37° in a humidified atmosphere of 5% CO_2–95% air (9). The nonadherent cells were decanted and distributed into 50-ml conical centrifuge tubes. The cells were collected by centrifugation at 150*g* for 15 min and resuspended in 20 ml of HBSS, yielding approximately 100 × 10^6 cells/ml of HBSS. The suspension was subjected to isopycnic centrifugation to obtain further enrichment of CFU-C or was cryopreserved.

Cryopreservation. Freezing. The cell sus-

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pension was adjusted to 10×10^6 cells/ml by adding RPMI 1640 containing 50% fetal bovine serum, chilled in melting ice, and further diluted to a concentration of 5×10^6 cells/ml by drop-wise addition of ice-cold 20% dimethyl sulfoxide in water. Sixty-milliliter aliquots were transferred to 300-ml Fenwal transfer packs and rapidly frozen at −70° in a Harris freezer.

Thawing. The transfer packs containing the frozen cells were immersed in a water bath at 37° and agitated at intervals until the last ice crystal had disappeared. The suspension was then diluted with an equal volume of RPMI 1640 containing 50% fetal bovine serum, transferred into 50-ml conical tubes, and centrifuged at 150*g* for 15 min. The supernate was discarded, and the pellet was washed three times by resuspension in 50 ml of HBSS and recentrifugation at 150*g* for 15 min. The final pellet was resuspended in 20 ml of HBSS. Cell viability was determined by eosin exclusion.

Isopycnic centrifugation. A suspension of 200×10^6 cells in 20 ml of HBSS was placed in a 50-ml conical glass tube. The cells were collected by centrifugation at 150*g* for min and the supernate was decanted. The cells were then suspended in the residual medium and mixed with 3 ml of a solution of 23% bovine serum albumin (BSA) in Shortman's balanced salt solution (density 1.071 g/cm^3) (10). This suspension was overlaid with 1 ml of a solution of 17% BSA in Shortman's balanced salt solution (density 1.053 g/cm^3) and centrifuged at 3600*g* for 10 min at 4°. The cells accumulated at the interface were collected and washed twice by resuspension in 50 ml of HBSS and recentrifugation at 150*g* for 15 min at room temperature. The final pellet was suspended in 20 ml of HBSS. Cell viability was determined by eosin exclusion.

In vitro CFU-C assay. The double-layer agar culture technique of Pike and Robinson was used (2). The underlayer in all of these studies contained peripheral blood leukocytes from the same normal donor. Peripheral blood for preparation of leukocytes was collected in a syringe rinsed with preservative-free heparin. The syringe was inverted and the red cells were sedimented by gravity. The leukocyte-rich plasma was removed and diluted to a concentration of 1×10^6 cells/ml with McCoy's 5A medium containing 15% fetal bovine serum and 0.5% agar. The overlayer contained various mononuclear cell fractions suspended at concentrations of 5 to 9×10^5 cells/ml in McCoy's 5A medium containing 15% fetal bovine serum and 0.3% agar. The cultures were incubated for 14 days at 37° in a humidified atmosphere of 5% CO_2–95% air. Aggregates of greater than 40 cells were counted as colonies. Cell morphology was determined from smears of aspirated colonies stained with Wright Giemsa.

Results. CFU-C. Unfractionated peripheral blood leukocytes obtained from 20 healthy volunteers formed 4 ± 4 (SD) colonies when 5×10^5 cells were cultured in the overlayer of the standard assay system (Table I). Cultures of 5×10^5 cells of the mononuclear cell fraction of the plateletpheresis residue prepared by centrifugation on LSM formed 10 ± 3 colonies, the macrophage-depleted mononuclear cell fractions yielded 13 ± 4 colonies, and the mononuclear cell fractions sedimented on BSA yielded 25 ± 4 colonies (Table I).

The data in Table II show that the yield of CFU-C was maintained by cryopreservation. The cells from Donor 3 were assayed at weekly intervals for 4 weeks and the number of colonies formed remained unchanged. The number of colonies increased linearly from 7 ± 2 to 51 ± 14 when the inoculum of cryopreserved cells sedimented on BSA was increased from 1 to 9×10^5 cells (Fig. 1).

Viability studies. The various freshly prepared cell fractions assayed for colony-forming activity maintained a cell viability of

TABLE I. COLONY-FORMING ACTIVITY AT VARIOUS STAGES OF CFU-C ENRICHMENT

Source	Colonies/5 × 10^5 cells plated	Range
Unfract. P.B.[a]	4 ± 4 (20)[b]	0–10
P.R.[c] + LSM[d]	10 ± 3 (5)	4–13
P.R. + LSM + Macrophage depletion	13 ± 4 (5)	6–18
P.R. + LSM + Macrophage depletion + BSA[e]	25 ± 4 (10)	19–41

[a] Peripheral blood.
[b] Mean ± SD number of subjects studied in parentheses.
[c] Plateletpheresis residue.
[d] Lymphocyte separation medium.
[e] Bovine serum albumin gradient.

TABLE II. FROZEN AND THAWED CFU-C CONCENTRATES[a]

Source	Day frozen prior to thawing	Colonies/5 × 10^5 cells plated
Donor 1		
	0	24 ± 3[b]
	2	19 ± 2
	3	17 ± 5
	21	29 ± 8
Donor 2		
	0	24 ± 3
	6	44 ± 6
	7	51 ± 14
Donor 3		
	0	25 ± 5
	7	28 ± 12
	14	30 ± 10
	21	26 ± 6
	28	38 ± 6

[a] Cells obtained from plateletpheresis residue were separated on LSM and macrophages removed by adherence. These mononuclear cells were frozen and thawed as described under methods. After thawing, the cells were subjected to isopycnic centrifugation on BSA prior to culture.

[b] Mean ± SD.

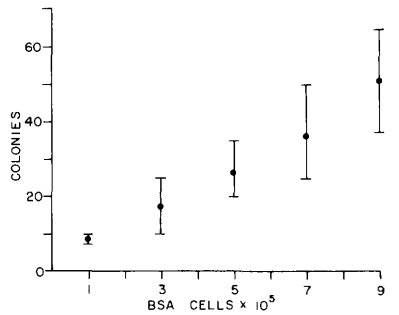

FIG. 1. The frozen and thawed cells obtained from Donor 3 were sedimented on BSA. This figure illustrates the relationship between the number of BSA cells plated and the number of colonies formed. The bars on the graph represent ±1 SD.

>99% as determined by eosin exclusion. After freezing and thawing (Donors 1, 2, and 3, Table II) the cell viability was 87 ± 7%. After isopycnic centrifugation of these cells in BSA, the cell viability was 96 ± 2%.

CFU-C morphology. All of the various freshly prepared and cryopreserved cell fractions formed predominantly macrophage colonies in culture.

Discussion. The usual source of CFU-C for study of normal hematopoietic cell differentiation is human bone marrow. However, both the availability of normal human bone marrow and the yield of CFU-C from marrow specimens are unpredictable. Murine bone marrow is a reliable source of CFU-C, but is not a satisfactory substitute for human cells in many studies. For example, some degree of species specificity is readily demonstrated in studies of colony-stimulating factor(s) (4).

CFU-C are present in the mononuclear cell fraction of normal peripheral blood in very small numbers (11), and therefore large volumes of blood must be processed in order to obtain a sufficient number of CFU-C for detailed studies. It has been reported by others that mononuclear cells including immunocompetent cells (5) and CFU-C (6) may be recovered from the residue of a single-donor plateletpheresis. In the present studies a greater than fourfold (mean, sixfold) enrichment of the CFU-C was achieved by centrifugation of the plateletpheresis residue on LSM, macrophage depletion, and density gradient centrifugation on a BSA gradient. The final preparations consistently yielded 25 ± 4 colonies when plated at a concentration of 5 × 10^5 cells per culture. A linear relationship was observed between the number of cells plated and the number of colonies produced in culture.

Comparable colony-forming activity was reported by another group of investigators (6) using human peripheral blood mononuclear cells purified by semicontinuous flow centrifugation. These mononuclear cell preparations were not separated as extensively as in our study, yet formed 5.3–41 colonies at a concentration of 2 × 10^5 cells in culture. The discrepancy between these results and our data may be explained by differences in the CFU-C assay (methylcellulose vs agar), feeder layer (human leukocyte-conditioned media vs human peripheral blood cells), and the criteria for determining a colony (greater than 20 cell aggregates versus greater than 40 cell aggregates).

The present study demonstrates the practicability of plateletpheresis residues as a continuous source of CFU-C. Approximately 3 × 10^8 cells are recovered from the plateletpheresis residue of a single-donor and are sufficient for 150 quadruplicate cultures of freshly prepared cells, or for aliquots of cells

to be cryopreserved for subsequent study. The increasing use of single-donor plateletpheresis as a source for platelet transfusion in clinical medicine permits the starting material to be easily obtained, and the cell fractionation can be conveniently accomplished in a few hours. Such preparations provide a consistent supply of human CFU-C, and have proven very useful in the study of human hematopoietic cell differentiation.

Summary. The residues obtained from single-donor plateletpheresis are a reproducible and convenient source of CFU-C as detected by a double-layer agar culture technique. Enrichment of CFU-C is achieved by sequential sedimentation on LSM, macrophage depletion by adherence to plastic, and isopycnic centrifugation on BSA gradients. The final preparation from a single-donor contains approximately 3×10^8 cells, sufficient for 150 quadruplicate cultures. Enrichment of CFU-C was about sixfold and the yield of CFU-C was 76%. Cells from the macrophage-depletion step were suspended in 10% dimethyl sulfoxide, frozen, and stored at −70°. After thawing at 37°, CFU-C were enriched by centrifugation on BSA. Frozen cells retained full CFU-C activity for at least 4 weeks.

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