Collection of Pluripotential Hematopoietic Stem Cells by Cytapheresis

By Larry C. Lasky, Robert C. Ash, John H. Kersey, Esmail D. Zanjani, and Jeffrey McCullough

Successful complete hematopoietic reconstitution (CHR) using nonleukemic peripheral stem cells (PSC) after marrow ablation has been reported in animals but not man. Previous studies of cytapheresis products from humans, as a prelude to use for CHR, have documented the presence of committed myeloid (CFU-GM) and erythroid (BFU-E) precursors. We have examined mononuclear cell (MNC) products collected on the Fenwal C53000 Blood Cell Separator for these plus the more primitive mixed (granulo-, erythro-, mono-, and megakaryocytic) cell colony-forming units (CFU-GEMM) and for various lymphocytic subpopulations (LSP). One to two-hour products contained 36 ± 7 CFU-GEMM/10^6 MNC (mean ± SE, n = 8) or 490 ± 131/ml product. This compared favorably with blood (23 ± 4/10^6 MNC or 46 ± 8/ml, n = 14) and bone marrow (146 ± 58/10^6 MNC, n = 12). Collection efficiency for E-rosette-positive cells approximated that for total lymphocytes and was variable for other LSP. Recovery of CFU-GEMM after freezing in 10% dimethylsulfoxide at a controlled rate and storage in liquid N₂ was 54% ± 8% (n = 8). Cytapheresis collection of large numbers of pluripotential hematopoietic precursors and demonstration of adequate recovery of these after cryopreservation, both previously unreported, are significant steps toward eventual CHR using nonleukemic PSC.

COMPLETE HEMATOPOIETIC reconstitution following marrow ablation with radiation and/or chemotherapy may be possible in humans using stem cells collected from normal peripheral blood. Committed stem cells, including granulocyte-monocyte colony-forming units (CFU-GM) and/or erythocyte burst-forming units (BFU-E) have been assayed in cytapheresis products collected with several different pheresis devices. Nonleukemic hematopoietic stem cells collected from peripheral blood have rescued animals from marrow ablation following radiation and chemotherapy. Successful hematopoietic reconstitution has yet to be achieved in humans.

Several recent developments in quantitative assessment of hematopoietic progenitor cells, storage techniques, and pheresis technology that may affect the goal of human hematopoietic reconstruction using peripheral stem cells have come about. (1) Messner and coworkers have described an in vitro culture for a pluripotential stem cell measured by growth in single colonies of granulocytic, erythocytic, mononuclear, and megakaryocytic cell types (CFU-GEMM). The CFU-GEMM possesses at least some features analogous to the pluripotential murine splenic colony-forming unit (CFU-S). (2) Controlled rate freezing in 10% dimethylsulfoxide (DMSO) and storage in liquid nitrogen has been shown to adequately preserve hematopoietic stem cells from bone marrow, as measured functionally by reconstitution with stored marrow and by CFU-GM in stored marrow and peripheral blood concentrate. (3) A computer controlled blood cell separator, the Fenwal CS3000, has been introduced and evaluated for collection of platelets and granulocytes from normal donors. The device eliminates the need for a rotating seal between the centrifuge and the donor/patient, potentially allowing a completely "closed" collection system that vastly minimizes the possibility of introduction of foreign material into the product and/or donor/patient. The computer control and adjustable but reproducible separation and collection chambers make this machine ideal for collection of a particular cell type consistently from donor-to-donor, with minimal operator-to-operator variation.

We report the use of the Fenwal CS3000 Blood Cell Separator for collection of mononuclear cells (MNC) from normal subjects. We also report the hematopoietic stem cells content, including CFU-GM, BFU-E, and the more primitive CFU-GEMM, as well as various lymphocytic subpopulations for these MNC. Recovery after controlled rate freezing in 10% DMSO and storage in liquid nitrogen for the various stem cell types is also reported.

MATERIALS AND METHODS

Cell Collection

Healthy male volunteers that met the American Association of Blood Banks criteria for whole blood donation were used. Informed consent was obtained. Both the consent form and the procedures were approved by our institutional human studies committee. Donors were not treated with steroids.

The two-armed technique, as is required by the CS3000, was used in all subjects. Sixteen-gauge Terumo needles were inserted in
anticubital veins, one in each arm. The needles were connected to the pheresis set, already primed automatically by the CS3000, as they were inserted. The microprocessor was then instructed to perform standard leukocytic collections, with certain modifications, notably nonstandard centrifuge speeds. Standard granulocyte separation (D36) and collection (A35) chambers were used. Whole blood flow rate was 50 ml/min. Acid citrate dextrose NIH solution A (ACD) was mixed with whole blood in a ratio of 1:10 to 1:12. Hydroxyethyl starch was not used, although this substance is routinely used to collect granulocytes. Two 1-hr collections, separated by collection of specimens from the donor and product, or single 2-hr collections were performed. At the end of each procedure, the contents of the pheresis set, other than the collection bag, were reinfused into the donor, again under microprocessor control.

**Cell Preparation and Marker Studies**

The method for purification of monoclonal cells (MNC) for analysis was similar both for putative stem cell preparations and for accompanying peripheral blood to be used for comparison purposes. Pairing each product with simultaneously drawn blood allowed calculation of the degree of concentration for various cell populations. The product or blood was collected in heparinized syringes and was diluted 1:2 in tissue culture media. Nine milliliters of suspension was layered on 3 ml of Ficoll-Hypaque at a specific gravity of 1.078. This was centrifuged at 400 g for 30 min. The opaque layer of lymphocytes at the interface was removed and concentrated with the supernatant. The concentration was determined by specific gravity measurement. The cell suspension was resuspended in 100% absorbed newborn calf serum to be used for comparison purposes. The antibodies used to accompany peripheral blood to be used for comparison purposes were directed against the following: sheep erythrocyte rosette, sheep erythrocyte rosetting, OKT4 (s), OKT8 (t), OKT3 (s), OKT1, OKT4, OKT8, and OKT12.

**Freezing and Storage**

Cells to be frozen, suspended in either autologous plasma or Hank's Balanced Salt Solution (Hanks) with 5% human albumin at concentration of less than 200 x 10^6/ml, were mixed with an equal volume of 20% DMSO in Hanks with 5% human albumin, and placed at zero degrees centigrade in 4-ml polypropylene tubes. Samples were then frozen in a Cryo-Med controlled rate freezer at 1°C/min, before and after phase change, down to -60°C, and at 3°C/min, down to -90°C. The samples were then placed rapidly into liquid nitrogen and stored until analysis.

Thawing was accomplished by placing the sample tubes in lukewarm water. As soon as the samples became liquid, they were diluted 1:10 with Hanks with 5% human albumin and 50 U heparin/ml. Samples were then washed twice in Hanks with albumin and cultured.

**Culture Techniques**

A modification of the CFU-GEMM (granulocyte, erythrocyte, megakaryocyte, monocyte precursor) assay of Messner et al. was performed on stem cell preparations from peripheral blood and pheresis concentrates. This modification, developed by Ash et al., is important in that it, unlike Messner's original technique, produces more colonies for bone marrow, by a factor of three, than for MNC for peripheral blood. The coefficients of variation, both within and between normal individuals, is also smaller (as is the extent of the "normal range"). Phytomagglutinin (PHA) leukocyte conditioned medium was made by culture of human peripheral blood leukocytes for 7 days in the presence of 1% (v/v) PHA and 10% fetal calf serum. Human erythropoietin was made from the urine of patients. Mononuclear cells were mixed with 30% fetal calf serum, 5 x 10^{-5} M 2-mercaptoethanol, 5% percent PHA-leukocyte conditioned medium, 1 U/ml erythropoietin, Iscove's modified Dulbecco's medium, and methylcellulose to a concentration of approximately 1% (w/v). The cultures were incubated at 37°C in a humidified 5% CO2 atmosphere. The cultures were examined after incubation for 15 days. Colonies containing visible hemoglobin were, on a random sampling basis, removed by micropipette and mounted on slides using cytocentrifugation, or examined in situ with an inverted microscope. Wright's and other stains (including nonspecific esterase and peroxidase) were applied in order to identify the nature of the colonies. CFU-GMs and BFU-Es were also quantitated on these plates.

**RESULTS**

The volume of the products ranged from 180 to 200 ml (Table 1). The total product white count averaged...
Using the 1000-rpm centrifuge speed, a mean of 392 ± 143 CFU-GEMM/ml (mean ± standard error of the mean) were collected in five 1–2 hr runs (Table 1). This compares favorably to several determinations made on peripheral blood, in which a mean of 46 ± 8 CFU-GEMM/ml (n = 14; range 8–100/ml) were found. Expressed per 10^6 MNC, the cellular concentrates contained 32 ± 10 CFU-GEMM (n = 5) and peripheral blood 23 ± 4 (n = 14). A comparable set of determinations on bone marrow from healthy male volunteers showed 146 ± 58 CFU-GEMM/10^6 MNC (n = 12). The CFU-GEMM colonies from the concentrate were qualitatively similar to those found in culture of bone marrow. A single determination performed on a 1-hr 800-rpm product held 45 CFU-GEMM/10^6 MNC or 358/ml. Two 1400-rpm 1-hr products held 22 and 65 CFU-GEMM/10^6 MNC or 425 and 1180/ml. Collection efficiency for CFU-GEMM ranged from 45% to 175% for 1000-rpm runs, was 78% for the 800-rpm run, and was 103% and 113% for the 1400-rpm runs. The efficiencies were invariably larger than the corresponding MNC collection efficiency for a given donor, implying a relative concentration of CFU-GEMM over MNCs.

CFU-GM and BFU-E averaged 366 and 1541/ml of concentrate, respectively, considering all speeds (5 at 1000, 1 at 800, and 2 at 1400 rpm). This translates to 27.5 and 139/10^6 MNC, respectively. Peripheral blood contained an average of 40 and 184/ml or 25 and 93/10^6 MNC, respectively (n = 14). The collection efficiency for BFU-E and CFU-GM both averaged 68%.

Recovery after controlled rate freezing in 10% DMSO and storage for 15–42 days in liquid nitrogen is summarized in Table 2. The mean CFU-GEMM recovery was 54%, while that for BFU-E was 63% and for CFU-GM 64%.

**DISCUSSION**

Complete hematopoietic reconstitution using normal human peripheral blood stem cells may be possible. Such reconstitution has been achieved in animals. Stem cells collected from the blood of individuals with chronic granulocytic leukemia have also been utilized successfully.

Previous studies have documented the presence of circulating committed granulocyte monocyte (CFU-GM) and erythrocyte (BFU-E) precursors

<table>
<thead>
<tr>
<th>Table 1. Cells Collected in 1–2 hr</th>
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<tr>
<td><strong>Produced at:</strong></td>
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<tr>
<td>800 rpm  1000 rpm  1400 rpm</td>
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<tr>
<td>Number of procedures</td>
</tr>
<tr>
<td>Mean collection volume (ml)</td>
</tr>
<tr>
<td>Mean total white count × 10³/ml</td>
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<tr>
<td>Mean percent lymphocytes</td>
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<tr>
<td>Mean percent mononuclear cells</td>
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<tr>
<td>Mean percent granulocytes</td>
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<tr>
<td>CFU-GEMM per ml</td>
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<tr>
<td>CFU-GEMM per 10⁶ NC</td>
</tr>
<tr>
<td>Peripheral Blood*</td>
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<tr>
<td>Bone Marrow*</td>
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<tr>
<td>CFU-GEMM per 10⁶ MNC (blood)</td>
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<tr>
<td>or NC (marrow)</td>
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<td>MNC, mononuclear cell; NC, nucleated cell.</td>
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* Mean values for healthy adult men (bone marrow figure from reference 21).

18.3 × 10⁴/ml for seven 1000-rpm procedures, was 9.6 for a single 800-rpm procedure, and averaged 18.7 for two 1400-rpm procedures. Average differential values, expressed as percentages, were 76, 90, and 10 for lymphocytes, MNC, and granulocytes, respectively, for seven 1000-rpm runs; 63, 83, and 17 for the 800-rpm procedure; and 83, 100, and 0 for two 1400-rpm procedures.

Collection efficiency, defined as the number of cells processed (or the mean number of cells/ml blood times the number of ml of blood processed) divided into the number of cells collected, expressed as a percentage, averaged 47 and 45 for lymphocytes and MNC, respectively, for seven procedures performed at 1000 rpm. These values were 25 and 26 for a single procedure performed at 800 rpm, and averaged 83 and 78 for two procedures performed at 1400 rpm.

Surface marker studies performed on the MNC collected during six 1000-rpm phereses showed that the E-rosette-positive percentage (69.5% ± 5.6%, mean ± SE) closely approximated the OKT3-positive percentage (61.6% ± 5.9%). The collection efficiency for these lymphocyte subpopulations (34.5% ± 6% for E-rosette and 31.5% ± 3.6% for OKT3-positive cells) approximated that for morphological lymphocytes overall (37.6% ± 7.5%). Efficiency of collection of surface immunoglobulin-positive B cells (26.8% ± 7.6%) also did not to differ significantly from the lymphocyte collection efficiency. The efficiency of collection of other surface marker positive cells, including J5, OKT4, OKT8, BA-1, and BA-2, showed no consistent relationship to the lymphocyte or stem cell (as described below) collection efficiencies.

<table>
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<tr>
<th>Table 2. Percent Freezing Yield for Eight Mononuclear Cell Apheresis Product Samples Stored in Small Tubes</th>
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<tr>
<td><strong>Mean ± SE</strong></td>
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</tr>
<tr>
<td>CFU-GEMM</td>
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<tr>
<td>BFU-E</td>
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<td>CFU-C</td>
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in peripheral blood\textsuperscript{22} and cytophoretic concentrates\textsuperscript{1,3} from humans. Despite the presence of these committed precursors, complete hematopoietic reconstruction in man using stem cells isolated solely from nonleukemic peripheral blood has yet to be reported. Two published reports have documented unsuccessful attempts to achieve this.\textsuperscript{5,6} Both discuss only the committed colony-forming unit (CFU-GM) content of the stem cell concentrate. Failure in each case may have been related to inability to evaluate the “stem cell” preparation for pluripotential stem cells. In this article we have documented the presence of the more primitive and less committed mixed cell colony forming unit (CFU-GEMM) in products of cytophoresis specifically designed for the stem cell collection.

The CFU-GEMM assay utilized in this study identifies a class of human hematopoietic progenitors that exhibit at least some features that characterize the pluripotential stem cell of the mouse (CFU-S).\textsuperscript{25} Since such pluripotential progenitors are those cells from which both committed progenitors, and ultimately, all of the several mature blood cell types derive, we believe that this type of study may provide a better quantitative assessment of the “stem cell” content of cytophoretic products for hematopoietic reconstitution.

The CFU-GEMM is functionally identified by ability to form mixed hematopoietic colonies in which differentiated granulocytic, erythroid, monocyte-macrophage, and megakaryocytic elements can be recognized. That such colonies are the clonal products of single cells has been demonstrated by karyotype analysis of single colonies formed in coculture experiments\textsuperscript{8} and supported by sedimentation velocity studies showing that these “colony-forming units” have size characteristics of single cells similar to BFU-E and CFU-GM. This has also been shown by the linear relationship, extrapolating to zero, between cells plated and colonies detected.\textsuperscript{21} Recloning experiments\textsuperscript{21} also support the concept that CFU-GEMM have “stem cell” properties not possessed by the committed progenitors, in that CFU-GEMM have a capacity for giving rise to secondary colonies of differentiated progenitor type and for at least limited self-renewal in vitro.\textsuperscript{21} We therefore believe that such studies as this offer additional information about the hematopoietic capacity of peripheral MNC.

The numbers of CFU-GM and BFU-E isolated in the MNC products reported here are comparable to those reported in previous studies.\textsuperscript{1,3,26-28} In a number of other reports, the normal peripheral blood colony count is not available for comparison. When it is, the ratio of stem cell numbers in the product versus that in peripheral blood are within an order of magnitude of those that we find in our MNC products.

The assay that we have used measures about 150 [actually 146 ± 58 (mean ± SE, n = 12)] CFU-GEMM/10\textsuperscript{6} MNC in normal bone marrow. If one assumes that 3 × 10\textsuperscript{6} nucleated cells/kg recipient body weight are necessary for an allogeneic bone marrow transplant (BMT) (this is the figure used for collection purposes at the University of Minnesota), and that 30\% of these are “mononuclear” cells (morphological lymphocytes and monocytes), then at least 6 × 10\textsuperscript{6} bone marrow mononuclear cells are necessary for a BMT in a 70-kg human. This contains about 9 × 10\textsuperscript{6} CFU-GEMM. If a 4-hr CS3000 MNC cytophoresis will produce 2 × 10\textsuperscript{6} CFU-GEMM, and if they are qualitatively identical to those from bone marrow in their repopulating ability, then about 4 or 5 cytophoreses would be necessary for an allogeneic BMT using only fresh peripheral stem cells. A freezing and storage loss of 60\% (pessimistically) would make 9–12 procedures necessary, relying only on frozen-stored products. Since the recipient’s residual immune system would tend to tolerate the cells for autologous hematopoietic reconstruction, less (perhaps one-third as many) CFU-GEMM, and hence cytophoresis products, would be necessary.

Bone marrow has been successfully frozen, generally with dimethylsulfoxide (DMSO) as a cryoprotectant, and after thawing, transfused, producing successful bone marrow engraftment, both in animals and humans.\textsuperscript{8,29,30} A so-called “closed system” for collection and freezing of stem cells from peripheral blood using the Aminco blood cell separator has even been proposed—although not tested through the transfusion stage.\textsuperscript{11} Using our method of mixture with 10\% DMSO, controlled rate freezing, and storage in liquid nitrogen in small tubes, the recovery of CFU-GEMM has averaged 54\%. The recovery of BFU-E and CFU-GM has been 63\% and 64\%. The latter are somewhat less than the 95.7\% (range 67\%-132\%) recovery described by Köhring et al.\textsuperscript{11} for CFU-GM storage in 10\% DMSO. It is possible that better stem cell yields can be achieved if the cells are stored in bags. This would allow more immediate dilution upon thawing, either with protein and heparin containing media for in vitro studies or with the recipient’s blood in vivo.

The “T-cell” lymphocyte population collection efficiency for the MNC products approximated both the “B-cell” and the lymphocyte collection efficiency. Knowledge of these relative efficiencies may be helpful in the future prevention of graft-versus-host disease (GVHD) in the case of allogeneic reconstitution. Recent work has demonstrated that treatment of mouse cells with monoclonal antibody directed against T lymphocytes (anti-Thy) and complement can be used to treat a mouse marrow and lymphocyte inco-
lum to prevent graft-versus-host disease across major\textsuperscript{11} and minor\textsuperscript{12} histocompatibility barriers. The stem cell proliferative capability of the cells is not destroyed by this treatment. Similar work involving barriers. The stem cell and minor histocompatibility to stem cells becomes tolerant T lymphocytes in the BMT inoculum determines the development of GVHD and suggests that the lymphocyte population growing from the transplanted stem cells becomes tolerant to the host. Thus, minimization of the numbers of T cells collected by future manipulation of the collection procedure may help prevent GVHD, and being aware of the relative lymphocyte subpopulation numbers collected will aid in possible immunologic treatment of the MNC concentrates before infusion.

The use of the CS3000 microcomputer-controlled blood cell separator offers several advantages over other cytapheresis devices for the collection of peripheral stem cells. The computer control allows reproducibility from donor to donor, since the same program can be used for each one. Operator variation in identification of the buffy coat layer is eliminated. Separation and collection chambers can be changed easily and reproducibly to maximize cell yields. The system, unlike any previously introduced, has the potential to be completely closed, since there is no rotating seal. This would vastly decrease the potential for bacterial or viral contamination, a very important feature when considering injection into immunocompromised recipients.

This study demonstrates the presence of pluripotent stem cells in large quantity in cytapheresis products collected from normal donors. We show that these cells can successfully be stored in liquid nitrogen using DMSO as a cryoprotectant. Further studies that lead to maximization of the stem cell yield, that document the viability of the cells when stored in plastic bags, and that explore ways to increase the yield of stem cells are necessary before complete hematopoietic reconstitution using these cells can and should be tried in humans.

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REFERENCES

21. Ash RC, Detrick RA, Zanjani ED: Studies of human pluripo-


Collection of pluripotential hematopoietic stem cells by cytapheresis

LC Lasky, RC Ash, JH Kersey, ED Zanjani and J McCullough