Collection of Pluripotential Hematopoietic Stem Cells by Cytapheresis

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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 CS3000 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 N_2 was 54% ± 8% (n = 8). Cytapheresis collection of large numbers of pluripotent 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-mono- and megakaryocyte colony-forming units (CFU-GM) and/or erythrocyte burst-forming units (BFU-E) have been assayed in cytapheresis products collected with several different pheresis devices.\(^1\) Nonleukemic hematopoietic stem cells collected from peripheral blood have rescued animals from marrow ablation following radiation and chemotherapy.\(^4\) Successful hematopoietic reconstitution has yet to be achieved in humans.\(^5\),\(^6\)

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\(^7\) have described an in vitro culture for a pluripotent stem cell measured by growth in single colonies of granulocytic, erythrocytic, monocytic, 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\(^8\) and by CFU-GM in stored marrow\(^9\) and peripheral blood concentrate.\(^10\) (3) A computer controlled blood cell separator, the Fenwal CS3000, has been introduced and evaluated for collection of platelets and granulocytes\(^11\) 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 cell 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,\(^14\) was used in all subjects. Sixteen-gauge Terumo needles were inserted in
antecubital 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 mononuclear 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 placed in a conical tube for washing with media. The wash was performed at 250 g for 10 min and repeated 3 times. For sheep rosette determinations and immunofluorescence (see below), two drops of latex particles/tube were added and the tubes were rocked for 15 min at 37°F in water in an incubator.

For sheep erythrocyte binding, sheep cells were obtained commercially or collected from sheep, defibrinated, and stored in Alsevers solution. One-tenth milliliter of packed sheep red blood cells was resuspended in 100% absorbed newborn calf serum. Mononuclear cells (10^6) in 0.25 ml of media was mixed with 0.25 ml of sheep red blood cells. This was incubated for 5 min at 37°C in water in an incubator. The mixture was then spun in a serofuge for 3 min. It was incubated at 4°C for 1 hr. Duplicate samples were read. Two-hundred white cells each were counted. Three or more sheep red blood cells around a lymphocyte were defined as a positive sheep erythrocyte rosette.

To assess the presence of surface immunoglobulin, the cells were stained using tetramethylrhodamine isothiocyanate goat anti-human immunoglobulin (Cappel Laboratories, Cochranville, Pa.) at a 1:8 dilution for 30 min at 4°C. Cells were then washed 3 times in buffer, mounted under glass cover slips, and examined for fluorescence using a Zeiss fluorescent microscope equipped with a Poelme-epiillumination.

Recently, monoclonal anti-T-cell antibodies, which are much more specific than antihymocyte globulin for immunoreactive thymocytes, and monoclonals versus other leukocyte antigens have been developed. These monoclonal antibodies have been purified from the ascites of hybridoma-bearing mice. The OKT3 antibody identifies human T lymphocytes. OKT4 recognizes inducer-helper T cells. OKT8 can be used to identify suppressor/cytotoxic T cells. The BA-1 antibody, a monoclonal antibody directed against the NALM-6-M1 acute lymphoblastic leukemia (ALL) cell line, reacts with peripheral blood B lymphocytes, chronic lymphocytic leukemias, pre-B-cell ALL, most non-Hodgkin lymphomas, and most non-T, non-B ALLs. Monoclonal antibody J5 is directed against the common ALL antigen. This antibody was kindly supplied by Dr. Jerome Ritz, Sidney Farber Cancer Center, Boston. BA-2 antibody is directed against cells early in the lymphocyte line. All of these monoclonal antibodies have been used to characterize subpopulations of mononuclear cells.

Monoclonal antibodies BA-1, BA-2, J5, OKT3, OKT4, and OKT8 were used to assess the presence of these antigens using the following technique of indirect immunofluorescence. Target cells were washed and resuspended to a concentration of 1.5 x 10^7/ml in phosphate-buffered saline (PBS, pH 7.2) containing 2.5% fetal calf serum and 0.2% sodium azide. Appropriately diluted monoclonal antibody or control ascitic fluid were then mixed with an equal volume of target cells incubated for 40 min on ice. After washing twice in buffer, cells were stained with fluorescein isothiocyanate goat anti-mouse immunoglobulin (Meloy Laboratories, Inc., Springfield, Va.) in a dilution of 1:8. Incubation was continued for 40 min on ice, and cells were washed 3 times in buffer and mounted under glass cover slips. The cells were examined for fluorescence as was done for surface immunoglobulin.

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°/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 2 times in Hanks with albumin and cultured.

Culture Techniques

A modification of the CFU-GEMM (granulocyte, erythrocyte, megakaryocyte, monocyte precursor) assay of Messner 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 coefficient of variation, both within and between normal individuals, is also smaller (as is the extent of the "normal range"). Phytomhemagglutinin (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 anemia patients. Mononuclear cells were mixed with 30% fetal calf serum, 5 x 10^-5 M 2-mercaptoethanol, 5% percent PHA-lymphocyte 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 the basis of these colonies.

RESULTS

The volume of the products ranged from 180 to 200 ml (Table 1). The total product white count averaged
Table 1. Cells Collected in 1–2 hr

<table>
<thead>
<tr>
<th>Produced at:</th>
<th>800 rpm</th>
<th>1000 rpm</th>
<th>1400 rpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of procedures</td>
<td>1</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Mean collection volume (ml)</td>
<td>190</td>
<td>190</td>
<td>190</td>
</tr>
<tr>
<td>Mean total white count x 10^9/ml</td>
<td>9.8</td>
<td>18.3</td>
<td>18.7</td>
</tr>
<tr>
<td>Mean percent lymphocytes</td>
<td>63</td>
<td>76</td>
<td>83</td>
</tr>
<tr>
<td>Mean percent mononuclear cells (MNC)</td>
<td>83</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>Mean percent granulocytes</td>
<td>17</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>CFU-GEMM per ml</td>
<td>358</td>
<td>392</td>
<td>802</td>
</tr>
<tr>
<td>CFU-GEMM per 10^6 NC</td>
<td>45</td>
<td>32</td>
<td>43</td>
</tr>
<tr>
<td>CFU-GEMM Peripheral Blood*</td>
<td>23</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>CFU-GEMM Bone Marrow*</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-GEMM CFU-C (days)</td>
<td>54±8</td>
<td>65±8</td>
<td>64±22</td>
</tr>
<tr>
<td>BFU-E (days)</td>
<td>30±3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| MNC, mononuclear cell; NC, nucleated cell.

*Mean values for healthy adult men (bone marrow figure from reference 21).

18.3 x 10^9/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.

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>Storage Time (days)</th>
<th>CFU-GEMM</th>
<th>BFU-E</th>
<th>CFU-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SE</td>
<td>54 ± 8</td>
<td>65 ± 8</td>
<td>64 ± 22</td>
</tr>
<tr>
<td>Range</td>
<td>33–112</td>
<td>40–118</td>
<td>12–221</td>
</tr>
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Table 2. Percent Freezing Yield for Eight Mononuclear Cell Apheresis Product Samples Stored in Small Tubes

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in peripheral blood\textsuperscript{22} and cytapheresis 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 cytapheresis specifically designed for the stem cell collection.

The CFU-GEMM assay utilized in this study identifies a class of human hemopoietic progenitors that exhibit at least some features that characterize the pluripotent stem cell of the mouse (CFU-S).\textsuperscript{23} 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 cytapheresis products for hemopoietic reconstitution.

The CFU-GEMM is functionally identified by ability to form mixed hemopoietic 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{4} 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{8} bone marrow mononuclear cells are necessary for a BMT in a 70-kg human. This contains about 9 × 10\textsuperscript{8} CFU-GEMM. If a 4-hr CS3000 MNC cytapheresis will produce 2 × 10\textsuperscript{8} CFU-GEMM, and if they are qualitatively identical to those from bone marrow in their repopulating ability, then about 4 or 5 cytaphereses would be necessary for an allogenic 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 cytapheresis 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{8,28,30} 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 Körbling 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 inocu-
similar work involving barriers. The stem cell and minor histocompatibility becomes tolerant to 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 cytopheresis 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 pluripotential stem cells in large quantity in cytopheresis 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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Collection of pluripotential hematopoietic stem cells by cytapheresis

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