RAPID COMMUNICATION
Selection and Expansion of Peripheral Blood CD34+ Cells in Autologous Stem Cell Transplantation for Breast Cancer

By Stephanie F. Williams, Wanda J. Lee, James G. Bender, Todd Zimmerman, Patricia Swinney, Mary Blake, Javier Carreon, Marta Schilling, Stephen Smith, Douglas E. Williams, Fred Oldham, and Dennis Van Epps

Cytopения after high-dose chemotherapy and autologous stem cell reinfusion is a major cause of morbidity. Ex vivo cultured expansion and differentiation of CD34+ peripheral blood progenitor cells (PBPC) to neutrophil precursors may shorten the neutropenic period further. We explored the use of these ex vivo cultured PBPCs in nine patients with metastatic breast cancer. All underwent PBPC mobilization with cyclophosphamide, VP-16, and G-CSF. Subsequently, they underwent four to five apheresis procedures. One apheresis product from each patient was prepared using the Isolene 300 Magnetic Cell Separation System (Baxter Immunotherapeutics, Irvine, CA) to obtain CD34+ cells. These cells were then cultured in gas permeable bags containing serum-free X-VIVO 10 (BioWhittaker, Walkersville, MD) medium supplemented with 1% human serum albumin and 100 ng/mL PIXY321. At day 12 of culture the mean fold expansion was 26x with a range of 6 to 64x. One patient’s cells did not expand because of a technical difficulty. The final cell product contained an average of 29.3% CD15+ neutrophil precursors with a range of 18.5% to 48.1%. The patients underwent high-dose chemotherapy with cyclophosphamide, carboplatin, and thiotepa. On day 0, the cryopreserved PBPCs were reinfused and on day +1 the 12-day cultured cells were washed, resuspended, and reinfused into eight of nine patients. One patient was not infused with cultured cells. The mean number of cultured cells reinfused was 44.6 x 10^6 cells/kg with a range of 0.8 to 156.8 x 10^6 cells/kg. No toxicity was observed after reinfusion. The eight patients have recovered absolute neutrophil counts >500/μL on a median of 8 days (range 8 to 10 days); the median platelet transfusion independence occurred on day 10 (range 8 to 12 days) and platelet counts >50,000/μL were achieved by day 12 (range 9 to 14) for the seven patients whose platelet counts could be determined. Expanded CD34+ selected PBPC can be obtained and safely reinfused into patients.

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MATERIALS AND METHODS
Patient selection and treatment protocol. Nine women with metastatic breast cancer were enrolled into this feasibility trial after obtaining written informed consent approved by the University of Chicago Institutional Review Board. Figure 1 is a schema of the trial design and treatment program. Table 1 is a summary of the patients’ prior therapy and the results of the mobilization treatment.

PB collection procedure. Patients underwent leukapheresis using the Fenwal CS3000 Plus blood cell separator (Baxter Fenwal, Deerfield, IL) per previously established procedures.1 The apheresis product from day 1, 2, or 3 was used for CD34+ selection and culture expansion. The apheresis product was assayed for total CD34+ cell content. The day of selection was chosen based on a minimum CD34+ cell content of 37 million cells/apheresis for patients no. 3000-3002 and 100 million cells/apheresis for all subsequent patients. The unselected apheresis products were cryopreserved in 10% dimethyl sulphoxide (DMSO) (Research Industries, Salt Lake City, UT) and Medium 199 (GIBCO, Grand Island, NY) using an automated programmable freezing system and stored until reinfusion in a liquid nitrogen refrigerator.

Enrichment of CD34+ cells by immunomagnetic selection.

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The beaddrosettes were retained in the disposable chamber and the primary magnet of the Isolex 300 Magnet Cell Separation System whereas the released cell suspension was passed over the secondary magnet to the product collection bag to capture any re-target cells were allowed to drain. A series of three washes was performed to remove the nontarget cells. Target cells were released (ChymoCell-T) as prompted by the Isolex 300 Magnetic Cell Separation System from the immunomagnetic beads by incubation with chymopapain at a cell ratio of 0.5: 1. After mixing at room temperature for 30 minutes, of CD34' cells were isolated from one of the patient's apheresis collections using the Baxter Isolex 300 Magnetic Cell Separation System and after the 12-day culture, by flow cytometry using a CD34 monoclonal antibody conjugated to phycoerythrin, HPCA2-PE (Becton Dickinson, San Jose, CA), and a FACScan flow cytometer (Becton Dickinson). In addition the CD15' cells were quantitated with FITC-Leu M1 (Becton Dickinson). The cellular morphology at day 12 of the culture (postharvest) was determined using Wright-Giemsa-stained cytopsins. Cultures were evaluated for the maturation of the granulocyte lineage and monocyte lineage by enumerating the presence of blasts, promyelocytes, myelocytes, banded neutrophils, segmented neutrophils, promonocytes, and monocytes.

Colonies were set-up in triplicate on day 0 (postselection) and day 12 of the culture (postharvest) using Iscove's methylcellulose medium obtained from Stem Cell Technologies (HCC 4330) (Vancouver, BC, Canada) supplemented with 300 U/mL IL-3 (R&D Systems, Minneapolis, MN), 40 ng/mL IL-6 (R&D Systems), 300 U/mL G-CSF (Amgen), and 300 Ul/mL GM-CSF (Prokine) (Immunex). The colony assays were incubated for 14 days at 37°C, 5% CO2 and high humidity. The data are reported as the total number of CD34' cells. Colony assays were set-up in triplicate on day 0 (postselection) and day 12 of the culture (postharvest) using Iscove's methylcellulose medium obtained from Stem Cell Technologies (HCC 4330) (Vancouver, BC, Canada) supplemented with 300 U/mL IL-3 (R&D Systems, Minneapolis, MN), 40 ng/mL IL-6 (R&D Systems), 300 U/mL G-CSF (Amgen), and 300 Ul/mL GM-CSF (Prokine) (Immunex). The colony assays were incubated for 14 days at 37°C, 5% CO2 and high humidity. The data are reported as the total number of G-M colony-forming units (CFU-GM), burst-forming unit erythroid (BFUe), and mixed colony-forming units (CFU-GEMM).

Culture of CD34+ cells. The CD34+ cells were initially seeded at a concentration of 0.5 to 1 × 10^6 cells/mL in the presence of PIXY321 (Immunex, Seattle, WA) in a modified prototype PL732 gas permeable bag containing serum-free X-VIVO 10 (BioWhittaker, Walkersville, MD). The bags were incubated for a total of 12 days in a high-humidity, 37°C incubator containing 5% CO2 and 95% air. For patients no. 3004-3008, the culture condition was modified to 5% CO2 and 5% O2. At day 7 of the culture, the cell concentration and viability was determined using a hemacytometer and the trypan blue dye exclusion method. An additional volume of culture medium containing PIXY321 at 100 ng/mL was supplemented to the culture on day 7 to return the cell concentration to ≤3 × 10^7 cells/mL. If no additional culture medium was required only 100 ng/mL of PIXY321 was added to the culture bag(s).

Phenotype, morphology, and colony assays of the culture-expanded CD34+ cells. The percent of CD34+ cells was determined, following selection with the Isolex 300 Magnetic Cell Separation System and after the 12-day culture, by flow cytometry using a CD34 monoclonal antibody conjugated to phycoerythrin, HPCA2-PE (Becton Dickinson, San Jose, CA), and a FACScan flow cytometer (Becton Dickinson). In addition the CD15 cells were quantitated with FITC-Leu M1 (Becton Dickinson). The cellular morphology at day 12 of the culture (postharvest) was determined using Wright-Giemsa-stained cytopsins. Cultures were evaluated for the maturation of the granulocyte lineage and monocyte lineage by enumerating the presence of blasts, promyelocytes, myelocytes, banded neutrophils, segmented neutrophils, promonocytes, and monocytes.

CD34+ cells were isolated from one of the patient’s apheresis collections using the Baxter Isolex 300 Magnetic Cell Separation System (Baxter Immunotherapy, Irvine, CA). Sensitization of the mononuclear cells (MNCs) was performed using 9069 (9 C5) anti-CD34 monoclonal antibody (Baxter Immunotherapy) at a cell concentration of 0.5 μg/10^6 MNCs for 30 minutes at 4°C. After the sensitization, the cells were washed to remove excess/unbound antibody. Freshly prepared Dynal (Oslo, Norway) paramagnetic microspheres [SAM IgG, (Fc) ST] were then added to the washed, sensitized cells at a final bead/ cell ratio of 0.5:1. After mixing at room temperature for 30 minutes, the beads/rosettes were separated from the unbound cells using the primary magnet of the Isolex 300 Magnet Cell Separation System. The beads/rosettes were retained in the disposable chamber and the apheresis bags were allowed to drain. A series of three washes was performed to remove the non-target cells. Target cells were released from the immunomagnetic beads by incubation with chymopapain (ChytoCell-T) as prompted by the Isolex 300 Magnetic Cell Separator. After the release step, the beads were retained by the primary magnet whereas the released cell suspension was passed over the secondary magnet to the product collection bag to capture any remaining beads.

Culture of CD34+ cells. The CD34+ cells were initially seeded at a concentration of 0.5 to 1 × 10^6 cells/mL in the presence of PIXY321 (Immunex, Seattle, WA) in a modified prototype PL732 gas permeable bag containing serum-free X-VIVO 10 (BioWhittaker, Walkersville, MD). The bags were incubated for a total of 12 days in a high-humidity, 37°C incubator containing 5% CO2 and 95% air. For patients no. 3004-3008, the culture condition was modified to 5% CO2 and 5% O2. At day 7 of the culture, the cell concentration and viability was determined using a hemacytometer and the trypan blue dye exclusion method. An additional volume of culture medium containing PIXY321 at 100 ng/mL was supplemented to the culture on day 7 to return the cell concentration to ≤3 × 10^7 cells/mL. If no additional culture medium was required only 100 ng/mL of PIXY321 was added to the culture bag(s).

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Table 1. Patient Characteristics and Mobilization

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Patient Age</th>
<th>Prior Chemotherapy</th>
<th>Prior Radiotherapy</th>
<th>Total Cryo MNC/kg × 10^6</th>
<th>Total Cryo CD34/kg × 10^6</th>
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<tbody>
<tr>
<td>3000</td>
<td>39</td>
<td>CMF, CAF</td>
<td>Yes</td>
<td>2.93</td>
<td>3.73</td>
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<tr>
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<td>CMF, Ctx, Adria</td>
<td>No</td>
<td>4.23</td>
<td>5.08</td>
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<td>3002</td>
<td>52</td>
<td>CAF</td>
<td>No</td>
<td>3.22</td>
<td>9.38</td>
</tr>
<tr>
<td>3003</td>
<td>33</td>
<td>CMF, CA</td>
<td>No</td>
<td>2.77</td>
<td>5.13</td>
</tr>
<tr>
<td>3004</td>
<td>41</td>
<td>CAF, MTX L-PAM, 5FU</td>
<td>No</td>
<td>3.80</td>
<td>45.30</td>
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<tr>
<td>3005</td>
<td>49</td>
<td>CMF, CAF</td>
<td>Yes</td>
<td>1.59</td>
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<tr>
<td>3006</td>
<td>47</td>
<td>CMF, Tamox</td>
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<td>3.03</td>
<td>8.61</td>
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<tr>
<td>3007</td>
<td>51</td>
<td>CMF, Tamox</td>
<td>No</td>
<td>2.63</td>
<td>10.09</td>
</tr>
<tr>
<td>3008</td>
<td>39</td>
<td>CMF, CAF, FAC</td>
<td>No</td>
<td>4.06</td>
<td>18.67</td>
</tr>
<tr>
<td>Average</td>
<td>43</td>
<td></td>
<td></td>
<td>3.30</td>
<td>12.41</td>
</tr>
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</table>

Summary of the patients’ treatment history and the CD34+ and mononuclear cell content of the cryopreserved apheresis products.
using the CS3000 Plus Cell Separator and resuspended in 150 to 250 mL of a reinfusion solution consisting of Plasma-Lyte A (Baxter Fenwal, Deerfield, IL) (code 2B2543) supplemented with 1% human serum albumin (Baxter Hyland, Glendale, CA). The cells were kept at room temperature and evaluated for cell recovery and percent viability by trypan blue dye exclusion. The cells were then rapidly reinfused into the patient through an indwelling central venous catheter. Vital signs and toxicity observations including cardiopulmonary, neurological and gastrointestinal abnormalities, anaphylaxis, and myalgias were made and recorded for 24 hours after administration.

**Hematologic recovery.** Patients were observed daily for evidence of hematologic recovery defined as the day the absolute neutrophil count (ANC) was greater than or equal to 500/μL. Platelet independence was quantified as the day the platelet count was greater than 50.000/μL with transfusion independence and a platelet count greater than 20.000/μL at day 9. Platelets greater than 20.000/μL with transfusion independence at day 10 (range 8 to 12) and platelet recovery greater than 50,000/μL was achieved on day 12 (range 9 to 14 days) (Fig 3A).

In the seven patients who had platelet recovery, platelets were greater than 20,000/μL with transfusion independence at day 10 (range 8 to 12) and platelet recovery greater than 50,000/μL was achieved on day 12 (range 9 to 14 days) (Fig 3B). Platelet recovery could not be determined in one patient because of necrotizing fascitis and need for platelet transfusions because of multiple surgical procedures. Patient no. 3001, who did not receive expanded cells recovered her neutrophils >500/μL at day 9. Platelets greater 20,000/μL with transfusion independence and a platelet count greater than 50,000/μL were achieved on day 10.

**RESULTS**

**Proliferation of CD34+ cells in culture.** These positively selected CD34+ PBPC were capable of proliferation in vitro. Table 2 summarizes the results. Eight patients’ CD34+ PBPC showed an average proliferation of 26-fold, with a range of 6- to 64-fold. One patient’s PBPC did not expand because of a technical error and subsequently she did not receive in vitro culture expanded CD34+ PBPC. The mean viability of these procured cultured cells before reinfusion was 76.9% (range 54% to 92%). The average total number of cells after 12 days of culture was 335×10^6 (range 5.6 to 1,210×10^6 cells).

**Morphology and flow cytometric phenotype.** Morphologically these expanded cells averaged 70% granulocytic precursors including promyelocytes, myelocytes, and metamyelocytes as observed from the cytospin preparations. Figure 2 is a photomicrograph of representative day 12 cultured cells showing the presence of promyelocytes and blast cells. The mean percentage of CD15+ cells in the final product infused into the patient was 29.3% (range 18.5% to 48.1%).

**Colony assays.** Only six patients were evaluated for colony-forming cells. The results of the six patients’ samples are summarized in Table 3. The average total number of CFUs that were present in the initial culture was 6.36×10^6 (range 1.65×10^6 to 1.82×10^6 CFU). At day 12 the total number of CFU was increased an average of 4.7-fold (range 1.0 to 11.60) to give an average total CFU of 1.22×10^7 (range 8.75×10^6 to 2.90×10^7). The cloning efficiency of the CD34+ selected population decreased 7.8-fold from 22% to 2.8% after 12 days in culture.

**Toxicity and hematologic recovery.** Eight patients received in vitro culture expanded CD34+ PBPC 24 hours after infusion of cryopreserved PBPC. There were no adverse events associated with the reinfusion of these cultured cells. Hematopoietic recovery was prompt in the eight patients that received cultured cells with a median neutrophil recovery greater than 500/μL at day 8 (range 8 to 10 days) (Fig 3A).

In the seven patients who had platelet recovery, platelets were greater than 20,000/μL with transfusion independence at day 10 (range 8 to 12) and platelet recovery greater than 50,000/μL was achieved on day 12 (range 9 to 14 days) (Fig 3B). Platelet recovery could not be determined in one patient because of necrotizing fascitis and need for platelet transfusions because of multiple surgical procedures. Patient no. 3001, who did not receive expanded cells recovered her neutrophils >500/μL at day 9. Platelets greater 20,000/μL with transfusion independence and a platelet count greater than 50,000/μL were achieved on day 10.
Table 3. Colony Assay Data Summary

<table>
<thead>
<tr>
<th>Day 0 Culture</th>
<th>Day 12 (Postharvest)</th>
<th>Fold Increase (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Cloning Cloning</td>
<td>CFU-GM/ BFUei Mixed/ Increase</td>
<td>Total CFU</td>
</tr>
<tr>
<td>ID</td>
<td>Total Cells</td>
<td>Efficiency</td>
</tr>
<tr>
<td>3000</td>
<td>1.84E + 07</td>
<td>9%</td>
</tr>
<tr>
<td>3004</td>
<td>7.28E + 08</td>
<td>25%</td>
</tr>
<tr>
<td>3005</td>
<td>1.94E + 07</td>
<td>9%</td>
</tr>
<tr>
<td>3006</td>
<td>1.93E + 08</td>
<td>31%</td>
</tr>
<tr>
<td>3007</td>
<td>1.70E + 08</td>
<td>35%</td>
</tr>
<tr>
<td>Avg</td>
<td>2.26E + 08</td>
<td>22%</td>
</tr>
</tbody>
</table>

DISCUSSION

After high-dose chemotherapy severe pancytopenia develops despite the use of an ASCR and/or the use of myeloid growth factors. There appears to be an obligate period of neutropenia where patients are susceptible to febrile episodes and infections. Aggressive use of antibiotics both prophylactically and therapeutically is necessary during this time. In vitro culture expansion and differentiation of selected CD34+ progenitor cells may shorten this period of severe neutropenia, thus decreasing the febrile episodes and need for antibiotics. Preliminary studies of long-term reconstitution have been performed in mice and show the efficacy of this approach after lethal irradiation.5

We have previously reported our results with in vitro expansion of neutrophil precursors and progenitors in suspension cultures of CD34+ cells selected from bone marrow.3 There we showed the ability to obtain extensive proliferation of CD34+ bone marrow cells in this system. Preclinical full-scale experiments with PB CD34+ cells cultured in PIXY3216 paralleled the small-scale studies.3 Haylock et al8 have also demonstrated substantial expansion of myeloid precursors cultured in a combination of growth factors. Another group has also shown up to a median 40-fold increase in myeloid PB progenitors through ex vivo expansion in breast cancer patients using different growth factors in serum supplemented medium.8

In this report we demonstrate that this approach is feasible. In addition, the reinfusion of these expanded myeloid progenitors did not lead to acute toxicity. There were no signs nor symptoms of leukagglutination reactions. Hematopoietic recovery, especially neutrophil recovery, was prompt; however, at the dose of cells infused it did not appear to be shortened in this preliminary trial. However, it should be pointed out that the dose of cultured cells reinfused covered a 2-log range. Interestingly, the higher cultured cell doses were associated with patients showing the most efficient cell recovery, although the database is too small to draw a final conclusion.

Significant large-scale culture expansion and differentiation of CD34+ selected PBPC can be achieved in vitro. These cells can be safely reinfused into patients. Further studies will be needed to determine its full impact on hematologic recovery after high-dose chemotherapy. Further studies will determine appropriate dosing of these cells. In addition, the system described here is amenable to the use of additional growth factors, which may enhance or redirect CD34 cell differentiation. This methodology provides a means of full-scale expansion of CD34 cells to address a variety of problems related to the cytopenias associated with antineoplastic therapy.

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REFERENCES


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