Optimization of Conditions for Ex Vivo Expansion of CD34+ Cells From Patients With Stage IV Breast Cancer

By Fred Shapiro, Tzy-Jyun Yao, George Raptis, Lilian Reich, Larry Norton, and Malcolm A.S. Moore

Multiple cycles of high-dose chemotherapy can be hematologically supported by repeated administration of peripheral blood progenitors obtained after mobilization using cytokine alone or in combination with chemotherapy. We have explored the quality of such cells and their potential to undergo ex vivo expansion. Twenty-five leukapheresis samples from 19 patients who had received extensive prior chemotherapy for stage IV breast cancer were subjected to CD34+ cell selection using immunoaffinity columns or immunomagnetic bead separation. Cells were cultured in suspension in the presence of c-*kit* ligand, interleukin-3, interleukin-6, erythropoietin, and granulocyte colony-stimulating factor. Ten experiments were performed using weekly exchange of media and cytokines (Delta assay). Median myeloid and erythroid progenitors expanded 15-fold at 7 days (range, 7 to 43), 40-fold at 14 days (range, 18 to 470), 46-fold at 21 days (range, 0 to 118), and 21-fold at 28 days (range, 0 to 61). In a system using gas-permeable bags without exchange of media or cytokine, median progenitors expanded 13-fold at 7 days (range, 7 to 36), 14-fold at 10 days (range, 4 to 61), 14-fold at 12 days (range, 3 to 46), and 10-fold at 14 days (range, 1 to 35). Progenitor expansion less than 10-fold occurred in 8% of experiments at day 7, in 17% at day 10, in 43% at day 12, and in 50% at day 14. When autologous plasma, autologous plasma processed (removal of cryoprecipitate, centrifugation, then filtration), or human serum were substituted for 20% fetal calf serum, the ratio of progenitor expansion at 7 days relative to 20% fetal calf serum for 10% human serum, 20% human serum, and 1% autologous plasma processed was 1.01 (range, 0.62 to 1.33), 0.88 (range, 0.61 to 1.20), and 0.96 (range, 0.55 to 1.64), respectively. These findings support the feasibility of ex vivo expansion in a system free of nonhuman proteins of CD34+-derived progenitors obtained from the peripheral blood of patients who have received prior chemotherapy.

HIGH-DOSE CHEMOTHERAPY (HDC) followed by hematologic rescue with autologous bone marrow (ABM) or peripheral blood mononuclear cells (PBMCs) provides superior remission rates and disease-free survivals compared with the historic experience in metastatic breast cancer.[1-3] A mathematical model of tumor kinetics,[4] supported by clinical trials,[5] has suggested that sequential chemotherapy, by delivering high dose-intensity, is superior to alternating regimens. High dose-intensity should result in greater tumor cell killing and more prolonged disease-free survival. This strategy can be applied to HDC with effective hematopoietic support. Multiple cycles of HDC, by delivering greater dose-intensity, may be superior to a single cycle of multiple agents. Trials applying this concept are ongoing at the Memorial Sloan-Kettering Cancer Center (New York, NY).[6]

Ex vivo expansion of PBMCs offers several potential advantages over conventional hematopoietic support. These include decreased number of leukaphereses, elimination of the need for large bore catheters and their related morbidity, the accomplishment of tumor purging via CD34+ selection, and decreased nadir and recovery of platelets and neutrophils. Because successive cycles of chemotherapy may compromise stem cell reserve,[7-9] PBMC collection early in the course of therapy and ex vivo expansion would be a logical strategy. Our laboratory has shown that syngeneic rescue with ex vivo expanded BM cells in a mouse model resulted in more rapid platelet and neutrophil recovery and improved survival as compared with that of BM alone[10]. Long-term repopulating potential of these ex vivo expanded cells was also maintained. We and others have shown that ex vivo expansion of PBMCs selected for CD34 cells can be achieved in liquid culture with various cytokine combinations, including c-*kit* ligand (KL; stem cell factor, mast cell growth factor, Steel factor), interleukin-1 (IL-1), IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage–CSF (GM-CSF), and erythropoietin (Epo).[11-13]

To make this strategy clinically feasible and safe, the elimination of nonhuman proteins may be required. A system that minimizes manipulation would also be desirable to avoid potential contamination. Because bovine serum infusions pose an allergic reaction risk,[14] replacement with medium free of nonhuman proteins that can maintain or improve progenitor expansion is needed. Few studies have explored bovine serum-free systems augmented with autologous plasma or heterologous serum for ex vivo expansion[13,15]. Here, we report ex vivo expansion of PBMCs selected for CD34 expression using the CellPro Ceprate System or immunomagnetic beads and explore bovine serum-free conditions with the use of either autologous plasma or autologous or heterologous serum. Using the Delta assay with weekly addition of fresh media and cytokines for up to 28 days as a measure of stem cell reserve and in gas-permeable bags without manipulation for up to 14 days (a system designed for clinical applications), this study reports ex vivo expansion of PB-derived CD34+ cells from patients with breast cancer who received prior chemotherapy.

MATERIALS AND METHODS

PB separation techniques. Aliquots of PBMCs were obtained from the heparinized leukapheresis product from patients with meta-

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static breast cancer who received prior chemotherapy for stage IV disease. Patients were enrolled on Institutional Review Board-approved protocols, and informed written consent was obtained previously. In the first protocol, PBMCs were mobilized (MSKCC IRB Protocol 92-77) by cyclophosphamide (CPA; 3 g/m^2) administered on day 3, after backup BM harvest (day 0), followed by G-CSF (5 μg/kg) on days 5 through 15, with leukapheresis collected on days 13 through 16. In the protocol that followed, PBMCs were mobilized twice, using two separate methods. (1) G-CSF (5 μg/kg) was administered on days 2 through 7, with leukapheresis collected on days 6 through 9. (2) CPA (5 g/m^2) was then administered on day 10, followed by G-CSF (5 μg/kg) on days 12 through 22, with leukapheresis collected on days 20 through 24 (MSKCC IRB Protocol 93-07). Low-density mononuclear cells (less than 1.077 g/mL) from the sample of leukapheresis product were separated over Ficoll-Paque (Pharmacia, Uppsala, Sweden). CD34$^+$ cells were enriched using the CellPro Ceprate System (kindly provided by CellPro, Bothell, WA) or with immunomagnetic beads (Dynal A.S., Oslo, Norway) following standard instructions provided. For separation using the CellPro Ceprate System, low-density mononuclear cells were washed twice with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), were resuspended in 1% BSA to a concentration of 1 to 2 × 10^8 cells/mL, and were incubated for 25 minutes with an IgM, biotinylated, mouse antihuman antibody to the CD34 antigen (12.8) at room temperature. The cells were washed with 1% BSA to remove excess antibody, then were resuspended in 5% BSA at a concentration of 1 to 2 × 10^8/mL, and were passed through an avidin column. Cells collected in the CD34$^+$ and CD34$^-$ fractions were counted (Coulter Counter ZM; Coulter, Hialeah, FL), and the percentage of cells in the CD34$^+$ fraction was determined. In experiments involving human serum or plasma, cells were suspended in 1% BSA or Iscove's modified Dulbecco's medium (IMDM) to avoid contamination with fetal calf serum (FCS). For CD34$^+$ separation using immunomagnetic beads, low-density mononuclear cells were washed twice with 0.1% BSA in PBS and were resuspended to 1 × 10^{8}cells/mL. A mouse IgG1 antihuman CD34$^+$ antibody developed in our laboratory (11.1.6; licensed to Oncogene Science, Uniondale, NY) was added to the cells at a concentration of 50 μg/mL for 30 minutes at 4°C. The cells were washed twice with 0.1% BSA in PBS and were resuspended to a concentration of 1 × 10^8 cells/mL, and 30 μL/mL of sheep antimouse IgG1(Fc) immunomagnetic beads, providing a 16:1 bead-to-cell ratio, were added for 30 minutes at 4°C. The bead-positive fraction was selected with a magnetic separator, resuspended in 20% FCS, and kept overnight at 37°C in 100% humidified air with 5% CO_2. The following day, the cells in the bead-negative fraction were recovered, washed twice in serum-free IMDM (GIBCO, Grand Island, NY) to remove residual FCS, and counted.

Preparation of human plasma and serum. Autologous plasma (AP) was obtained as a byproduct of leukaphereses anticoagulated with heparin, without citrate. AP was used without processing or with processing (APP). Processed plasma was prepared by freezing at −20°C and then thawed overnight at 4°C. Cryoprecipitate and residual cells and platelets were removed by centrifugation at 10,000*g* for 30 minutes at 4°C. The plasma was then filtered through a 0.2-micron filter (Nalgene, Rochester, NY).

Autologous serum (AS) or heterologous serum (HS) was obtained from PB before leukapheresis. The blood was left to clot over 2 hours and was centrifuged at 400*g* for 12 minutes. The supernatant was removed and filtered through a 0.2-micron filter.

Progenitor cell assays (primary colony-forming unit [CFU] agarose assay). A total of 1,000 cells from the CD34$^+$ fraction was suspended in 35-mm tissue culture dishes (Corning, Corning, NY), in triplicate, containing 20% FCS in IMDM supplemented with gentamicin 50 μg/mL (GIBCO) and monothioglycerol (7.3 × 10^{-5} mol/L; Sigma, St Louis, MO), 0.36% agarose (FMC Bioproducts, Rockland, ME), and a combination of five growth factors: human KL, 20 ng/mL (kindly provided by Immunex, Seattle, WA); human IL-3, 50 ng/mL (Immunex); mutein IL-6, 20 ng/mL (kindly provided by Imclone Systems Inc, New York, NY); Epo, 6 U/mL (Amgen, Thousand Oaks, CA); and human G-CSF, 100 ng/mL (Amgen). This combination is abbreviated as K36EG. Cultures were incubated at 37°C in 100% humidified 5% CO_2 in air and removed from the incubator at 14 days. Colonies were counted using an inverted microscope and defined as greater than 40 cells. Burst-forming unit erythroid (BFU-E), defined as multicentric hemoglobinized aggregates, and granulocyte-macrophage–CFU (CFU-GM) were observed using this assay.

Liquid culture (Delta assay). A total of 4 × 10^4 CD34$^+$ cells was placed in a liquid culture system in 24-well plates (Costar, Cambridge, MA), to a final volume of 1 mL using 20% FCS in IMDM supplemented with gentamicin, monothioglycerol, and the five factor-cytokine combination (K36EG) at the same concentrations described for the progenitor assay. FCS was substituted by either AP, APP, or HS in various concentrations. Cultures were incubated at 37°C in 100% humidified 5% CO_2 in air. All the cells were retrieved every 7 days for up to 28 days, counted, then washed in IMDM with or without FCS (depending on starting conditions), recounted, and evaluated for progenitors. The cells were reseeded in liquid culture at the same starting concentration of 4 × 10^4 cells/mL with fresh media and cytokines. On day 0 and day 7, only 1 well was required. For day 14, 4 wells were required, and on day 21, 6 wells were required to ensure enough cells were harvested. Excess cells were discarded.

Liquid culture (gas-permeable bags). To use a container applicable to clinical trials, gas-permeable Teflon bags (American Fluroseal, Columbia, MD) were used. For experimental use, a bag with a 7-mL capacity was used. As described in the Delta assay, 4 × 10^4 CD34$^+$ cells/mL (2 × 10^5 total cells) were placed in 5 mL of the media and cytokine combination. On days 7, 10, 12, and 14, the bags were gently agitated, and approximately 200 μL was removed (this method preserved the cell concentration, though slightly altered the volume-to-surface area ratio). There was no addition of cytokine or media to this system. The cells were counted, washed with 20% FCS in IMDM, and recounted before secondary plating. No experiments were performed beyond 14 days.

Secondary CFU. Cells harvested on days 7 through 28 were evaluated for progenitors in triplicate using the identical method as for the primary assay, except that escalating cell concentrations were used (ranging between 2.5 × 10^3 to 4 × 10^4 cells per plate).

Flow cytometry. PBMCs were analyzed for CD34 expression by our clinical laboratory. The pheresis product was incubated with anti-CD34$^+$ antibody HPCA-2 (Becton Dickinson, San Jose, CA). After incubation, red blood cells were lysed using ammonium chloride. The remaining cells were diluted 1:10 and then analyzed on a Coulter EPICS Profile 2 (Coulter). The percentage of CD34$^+$ cells from the pheresis product is represented.

Calculations. For the Delta assay, the number of cells per well on day 7 (generated from 4 × 10^4) was divided by 4 × 10^4. For each week, cumulative total cells were calculated based on the amplification of the input population of 4 × 10^4 cells. Cumulative expansion was determined for 4 weeks.

To determine baseline progenitors, the number of colonies generated at day 0 was adjusted to 4 × 10^4 CD34$^+$ cells. In the following 4 weeks, the cumulative number of colonies generated was calculated using the following formula: (no. of colonies/no. of cells plated × total cumulative cells). Expansion was determined by dividing the cumulative number of colonies by the baseline number of colonies.

Statistical analysis. To test the hypothesis that the ratio of progenitor expansion relative to FCS is equal to 1, the signed rank test was applied (ratio − 1).

Table 1. Patient Information

Exp No.	No. of Cycles of Prior Chemotherapy	Prior GF	Prior RT	Sep Proc	%CD34+ by FACS (PBMC)	%CD34+ by Sep	Mobilization (g/m²)
1	12	No	No	C	0.3	0.4	CPA(3) + G
2*	13	No	No	C	0.1	0.2	G
3	6	PIXY	No	C	0.0	1.3	G
4	15	No	CW	C	0.2	0.5	G
5	6	No	No	C	NA	1.2	CPA(3) + G
6	15	G	No	C	0.8	0.7	CPA(3) + G
7	4	G	No	B	1.5	1.1	CPA(5) + G
8	8	PIXY	CW	B	0.0	0.1	G
9†	5	No	No	C	0.2	0.2	G
10*	13	No	No	C	1.7	2.8	CPA(5) + G
11	4	No	No	C	1.9	1.3	CPA(3) + G
12	12	No	No	B	1.8	1.0	CPA(5) + G
13*	13	No	No	C	1.7	2.8	CPA(5) + G
14	6	No	No	B	0.3	0.4	G
15†	5	No	No	B	0.1	0.3	G
16	14	G	No	C	0.3	0.5	G
17	12	G	CW + SCLN	C	0.1	0.2	G
18†	5	No	No	B	0.3	0.5	CPA(5) + G
19‡	4	No	No	C	0.1	0.4	G
20‡	4	No	No	B	NA	2.0	CPA(5) + G
21	12	No	No	B	0.4	2.1	G
22	23	No	No	B	0.2	0.5	G
23§	4	No	No	C	0.4	0.7	G
24	14	No	No	C	0.2	0.4	G
25§	4	No	No	B	1.7	1.2	CPA(5) + G

The percentage of CD34+ by separation is relative to the starting PBMC after Ficoll.

Abbreviations: Exp, experiment; GF, growth factor; RT, radiation therapy; Sep Proc, separation procedure; CW, chest wall; SCLN, supraclavicular lymph node; NA, not available; C, CellPro Ceprate Column; B, immunmagnetic beads; G, G-CSF.

* Experiments no. 2, 10, and 13 were performed on the same patient.

† Experiments no. 9, 15, and 18 were performed on the same patient.

‡ Experiments no. 19 and 20 were performed on the same patient.

§ Experiments no. 23 and 25 were performed on the same patient.

RESULTS

Patient information is shown in Table 1. Twenty-five experiments were performed with PBMCs from 19 different patients. In two instances, three experiments were performed from the same patients. Experiments no. 9, 15, and 18 involved 1 patient, and experiments no. 2, 10, and 13 involved another. In addition, 4 patients had PBMCs mobilized by two separate methods, G-CSF alone then CPA plus G-CSF (experiments no. 2 and 10, no. 15 and 18, no. 19 and 20, and no. 23 and 25). All patients had received prior chemotherapy for stage IV disease, with a median of 12 prior cycles of chemotherapy (range, 4 to 23). A total of 6 received prior cytokines (4, G-CSF; 2, PIXY 321), and 3 received radiation therapy (2 to the chest wall and 1 to the chest wall plus supraclavicular lymph nodes). No patient received pelvic irradiation. $CD34^+$ selection was performed using the CellPro Ceprate System in 15 experiments and immunomagnetic beads in 10. $CD34^+$ analysis of cells from the leukaphereses product using a fluorescence-activated cell sorter (FACS) ranged from 0% to 1.9%. The percentage of cells collected in the column or bead $CD34^+$ fraction ranged from 0.1% to 2.8% (relative to the starting PBMCs after Ficoll separation). Statistically, no correlation between the number of cycles of prior chemotherapy and the percentage of CD34 as determined by either method was shown.

In 10 Delta assay experiments involving 20% FCS in IMDM supplemented with K36EG, cells continued to expand for up to 28 days (Fig 1). Median cellular expansion at 7 days was 25-fold (range, 4- to 35-fold); at 14 days, 501-fold (range, 87- to 1,513-fold); at 21 days, 3,250-fold (range, 398- to 6750-fold); and at 28 days, 9,500-fold (range, 708- to 18,650-fold; see Table 2). Cellular expansion was significantly less than average in 2 patients (experiments no. 3 and 8). Both patients had received rapidly cycled standard doses of adriamycin (60 mg/m²) plus higher than standard doses of thiotepa (20 to 40 mg/m²) with PIXY 321 (GM-CSF–IL-3 fusion protein; Immunex) rescue on a prior Institutional Review Board-approved protocol.

In the primary clonogenic assay of $CD34^+$ cells, the total CFU-GM/BFU-E was 3,674 (range, 933 to 6360) per 4×10^4 cells (cloning efficiency, 9.2%). Median expansion of progenitors was 15-fold at day 7 (range, 7- to 43-fold), 40-fold at day 14 (range, 18- to 470-fold), 46-fold at day 21 (range, 0- to 118-fold), and 21-fold at day 28 (range, 0- to 34-fold; see Fig 2). $CD34^+$ cells from patient no. 3 had limited progenitor expansion, whereas $CD34^+$ cells from pa-

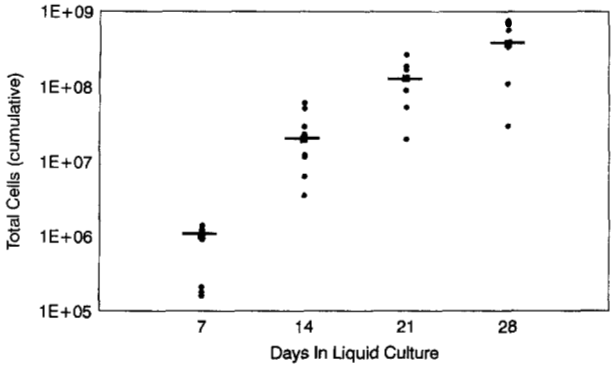

Fig 1. Ex vivo expansion of CD34⁺ cells using the Delta assay. A total of 4 × 10⁴ CD34⁺ cells were placed in 20% FCS in IMDM supplemented with KL, IL-3, IL-6, Epo, and G-CSF. Weekly passages of 4 × 10⁴ cells were performed with fresh media and cytokines. Cells were evaluated for progenitors using the agarose assay with the same cytokine combination. Cumulative cell counts were calculated and are shown. The bar represents the median.

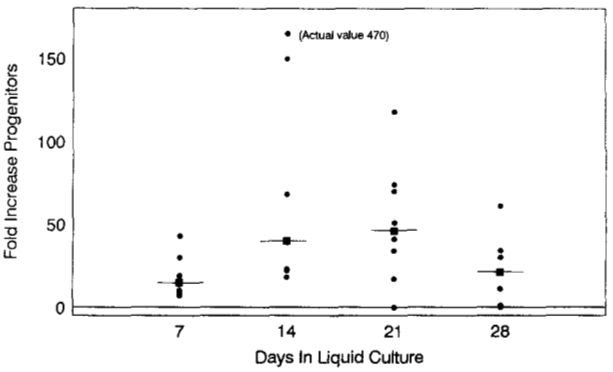

Fig 2. Fold increase of progenitors over baseline after ex vivo expansion using the Delta assay. Cells retrieved from liquid culture were placed in 0.36% agarose supplemented with KL, IL-3, IL-6, G-CSF, and Epo and then incubated for 14 days. CFU-GM/BFU-E were counted and cumulative progenitors were calulated and compared with baseline. The bar represents the median.

tient no. 8 had extensive early progenitor expansion but no detectable CFUs on day 21, despite less than average cellular expansion (eliminating dilutional effect; see Table 3).

Liquid culture systems requiring exchange of media and cytokine involve additional manipulation and increased contamination risk. Therefore, we focused on a system requiring minimal manipulation. Cells, media, cytokines, and FCS or its substitute were placed in gas-permeable bags. In 15 experiments performed under these conditions, cells continued to expand for up to 14 days (Table 4). Starting with 4×10^4 cells/mL, median total cells recovered from 4×10^4 input CD34⁺ cells reached 4.2×10^6/mL on day 14 (104-fold; range, 81- to 127-fold; see Fig 3). In the primary clonogenic assay of CD34⁺ cells, the total CFU-GM/BFU-E was 3,507 (920 to 8,240) per 4×10^4 cells (cloning efficiency, 8.8%). Progenitors expanded a median of 13-fold (range 7- to 36-fold) on day 7, 14-fold (range, 4- to 61-fold) on day 10, 14-fold (range, 3- to 46-fold) on day 12, and 10-fold (range, 1- to 35-fold) on day 14 (Fig 4 and Table 5). The proportion of patients who had less than 10-fold progenitor expansion was 1 for day 7 (8%), 2 for day 10 (17%), 6 for day 12 (43%), and 7 for day 14 (50%). In the 10 experiments where all 4 days were evaluated for progenitors, peak expansion occurred 4 times at day 7, 4 times at day 10, 2 times at day 12, and 0 times at day 14 (Table 5).

In experiments comparing FCS with AP, APP, or HS, the median ratio of progenitor expansion relative to FCS was calculated from pooled data at day 7, including plastic-well and gas-permeable–bag experiments (Table 6 and Fig 5). With concentrations of 1% AP, the ratio relative to FCS was 0.72 (range, 0.41 to 1.29), and, with concentrations of 5% AP, the ratio was 0.66 (range, 0.0 to 1.45). AS was used in all experiments except 1, where HS was used. For experiments using 1% HS, the ratio was 0.66 (range, 0.13 to 0.72);

Table 2. Ex Vivo Expansion of CD34⁺-Derived Cells Using the Delta Assay

Experiment No.	Day 7 (×10⁵)	Day 14 (×10⁶)	Day 21 (×10⁸)	Day 28 (×10⁸)
1	9.1	50.8	1.9	5.7
2	1.8	6.3	0.5	1.1
3	1.6	3.5	0.2	0.3
4	2.1	20.1	1.3	ND
5	14.0	12.3	0.9	3.8
6	10.3	23.3	1.9	7.3
7	10.3	29.6	2.7	7.5
8	9.8	11.5	0.2	ND
9	12.4	60.5	1.7	3.4
10	11.9	ND	ND	ND
Number	10	9	9	7
Median	10.1	20.1	1.3	3.8

The initial cell count was 4×10^4 and was cultured in 20% FCS supplemented with KL, IL-3, IL-6, G-CSF, and Epo. Cumulative cell counts were calculated.

Abbreviation: ND, not done.

Table 3. Fold Increase of Progenitors After Ex Vivo Expansion of CD34⁺ Cells Placed in Liquid Culture Using the Delta Assay Supplemented With KL, IL-3, IL-6, G-CSF, and Epo

Experiment No.	Day 7	Day 14	Day 21	Day 28
1	10	39	51	11
2	15	22	74	21
3	16	18	17	1
4	10	40	118	ND
5	7	23	TE	30
6	8	68	41	34
7	14	150	34	ND
8	43	470	0	0
9	19	40	70	61
10	30	ND	ND	ND
Number	10	9	8	7
Median	15	40	46	21

Progenitors were determined in an agarose assay in the presence of KL, IL-3, IL-6, G-CSF, and Epo.

Abbreviation: TE, technical error.

Table 4. Ex Vivo Expansion of CD34+-Derived Cells Using Gas-Permeable Bags

Experiment No.	Day 7 (×10⁵)	Day 10 (×10⁶)	Day 12 (×10⁶)	Day 14 (×10⁶)
11	8.6	ND	3.5	4.1
12	17.0	ND	3.9	4.7
13	10.6	ND	ND	ND
14	11.0	3.4	3.7	4.6
15	7.5	2.5	3.6	4.2
16	ND	2.7	3.4	4.1
17	ND	2.6	4.2	4.3
18	5.8	1.9	3.1	3.6
19	7.8	2.6	3.9	4.3
20	5.2	1.8	2.7	3.2
21	21.8	3.8	4.0	5.1
22	15.0	2.9	3.6	4.0
23	8.3	2.7	3.6	3.9
24	13.4	3.3	4.6	3.9
25	23.5	4.0	4.6	4.5
Number	13	12	14	14
Median	10.6	2.7	3.7	4.2

The initial cell count was 4 × 10⁴ and was cultured in 20% FCS supplemented with KL, IL-3, IL-6, G-CSF, and Epo.

using 5% HS, 0.72 (range, 0.25 to 0.82); using 10% HS, 1.01 (range, 0.62 to 1.33); and using 20% HS, 0.88 (range, 0.61 to 1.20). With 1% APP, the median ratio was 0.96 (range, 0.55 to 1.64), and with 5% APP, it was 0.84 (range, 0.0 to 1.29). Only 1% AP, 1% HS, and 5% HS were statistically less effective ($P < .05$) as compared with 20% FCS. All other substitutes (5% AP, 10% HS, 20% HS, 1% APP, and 5% APP) lacked statistical evidence to support either an advantage or disadvantage in supporting ex vivo expansion as compared with 20% FCS. Given the small sample size, an insignificant result of the signed rank test does not imply equivalence between 20% FCS and the tested substitute.

DISCUSSION

We here confirmed the feasibility of ex vivo expansion of total cells and progenitors starting with a population en-

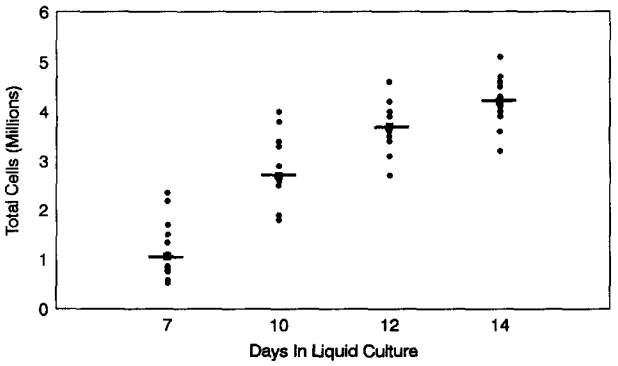

Fig 3. Ex vivo expansion of CD34+ cells using gas-permeable bags. A total of 2 × 10⁵ CD34+ cells (4 × 10⁴ /mL) were placed in gas-permeable bags in 20% FCS in IMDM supplemented with KL, IL-3, IL-6, Epo, and G-CSF. Aliquots were removed at 7, 10, 12, and 14 days without the addition of cytokines or media exchange. Actual cell counts are represented. The bar represents the median.

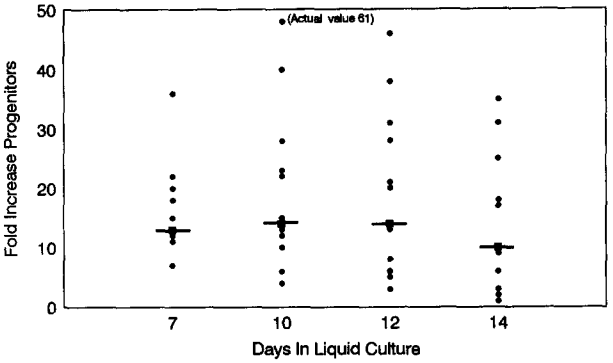

Fig 4. Fold increase of progenitors over baseline after ex vivo expansion using gas-permeable bags. Cells retrieved from liquid culture were placed in 0.36% agarose supplemented with KL, IL-3, IL-6, G-CSF, and Epo and then incubated for 14 days. CFU-GM/BFU-E were counted and compared with baseline. The bar represents the median.

riched for CD34 expression, which has been accomplished by several other groups[11-13]. However, there has been a lack of information on the extent and cause of the variability of expansion potential of CD34-derived progenitors. This report describes a simple system, which is clinically applicable, that uses rapid CD34+ separation procedures. We describe one useful cytokine combination, test a simple container system, and evaluate various replacements for FCS.

The enrichment of CD34+ cells can be accomplished by various techniques. The CellPro Ceprate system offers a quick and contained method for large scale clinical separa-

Table 5. Fold Increase of Progenitors After Ex Vivo Expansion of CD34+ Cells Placed in Liquid Culture in Gas-Permeable Bags Supplemented With KL, IL-3, IL-6, G-CSF, and Epo

Experiment No.	Day 7	Day 10	Day 12	Day 14
11	ND	ND	38	17
12	12	ND	13	2
13	13	ND	ND	ND
14	22	40	20	25
15	12	15	14	10
16	ND	12	8	6
17	ND	61	31	31
18	20	28	46	35
19	15	23	21	18
20	11	22	28	9
21	36	13	6	3
22	13	4	3	1
23	7	6	3	9
24	18	10	6	10
25	11	13	5	1
Number	12	12	14	14
Median	13	14	14	10
Number <10×*	1 (8%)	2 (17%)	6 (43%)	7 (50%)

Progenitors were determined in an agarose assay in the presence of KL, IL-3, IL-6, G-CSF, and Epo. Underscoring indicates maximal expansion in experiments with all data points.

* Number of experiments with less than 10-fold expansion.

Table 6. Comparison of AP, APP, or HS With FCS

Conditions	No. of Experiments	Median Ratio to 20% FCS (Range)
1% AP	9	0.72 (0.41-1.29)
5% AP	7	0.66 (0.00-1.45)
1% HS	6	0.66 (0.13-0.72)
5% HS	6	0.72 (0.25-0.82)
10% HS	5	1.01 (0.62-1.33)
20% HS	9	0.88 (0.61-1.20)
1% APP	9	0.96 (0.55-1.64)
5% APP	8	0.84 (0.00-1.29)

The median ratio of progenitor expansion using FCS substitute to 20% FCS after 7 days in liquid culture is shown.

tions. The purity of the $CD34^+$ fraction ranged between 56% to 92% (data not shown).

The Delta Assay places major proliferative demand on the stem cell pool. At 4 weeks, there is probably a depletion of the stem cell pool. Early in culture (7 to 14 days), maintainence or minimal expansion of long-term culture-initiating cells (LTCIC) occurs.[16] For this reason, clinical use of expanded cells should be performed with the first passage, where there is significant increase in the progenitor population and no exhaustion of LTCIC. The usual decrease in progenitor expansion that occurred during days 14 through 21, despite an increase in total cells, may be partially explained by a dilution effect. The frequency of $CD34^+CD38^-$ cells (a population highly enriched for stem cells) in the $CD34^+$ fraction is approximately 1%,[17] and that of the LTCIC is less than 1% of total $CD34^+$ cells.[18] Starting with 4×10^4 cells, we conservatively estimate that fewer than 400 primitive stem cells are present. If stem cell numbers are maintained but not expanded, then by day 21 all available stem cells would have been diluted by the 10^3- to 10^4-fold increase in total nucleated cells. Furthermore, if primitive cells divide every 12 hours under exponential expansion conditions, greater than 50 population doublings could be achieved over the 28 to 35 days of culture and could lead to their exhaustion.

There remains disagreement as to the optimal combination of cytokines offering the greatest progenitor expansion. Haylock et al[12] reported that KL, IL-1, IL-3, IL-6, G-CSF, and GM-CSF to be their optimal regimen; whereas Brugger et al[13] found KL, IL-1, IL-3, IL-6, and Epo to be optimal, and the addition of G-CSF or GM-CSF was inhibitory. We have failed to confirm the findings of Brugger and colleagues that the addition of Epo expands CFU-GM, and we were unable to confirm a role for Epo in myeloid or erythroid progenitor expansion (Shapiro, Schneider, and Moore, unpublished data). We have observed that a seven-factor combination (IL-1, KL, IL-3, IL-6, G-CSF, GM-CSF, and Epo) was superior to IL-1, KL, IL-3, IL-6, and Epo or to the five-factor combination used in this study (Shapiro and Moore, unpublished data). The cause of the discrepancy remains unresolved. However, adequate rather than maximal expansion is our clinical goal, and any of the combinations reported in the literature appear be adequate for clinical trials. The five-factor combination described in this paper (K36EG) was tested as a practical matter, because these may be the most readily available cytokines for clinical trials. Because all of the published studies used different methods of expansion, a direct comparison of progenitor expansion cannot be used. Variables such as initial cell concentration can alter cumulative progenitor expansion. For example, reducing cell concentrations from 4×10^4 to 2×10^4cells/mL increased the expansion potential by almost twofold ((Bengala, Shapiro, and Moore, unpublished data). Maximal progenitor expansion occurred most often on days 7 and 10. In most, but not all, experiments progenitor (but not total cell production) decreased by day 12 in gas-permeable bags. Therefore, expansion should be performed for 7 to 10 days using this system.

The use of gas-permeable bags offers potential advantages over other systems. Because the system is closed, this may reduce the likelihood of contamination and/or technical processing errors. Small aliquots can easily be removed for monitoring. Transfer of cells uses techniques already in use and approved for other blood products.

The infusion of fetal bovine serum into patients potentially could induce allergic reactions including serum sickness or anaphylaxis.[14] In one clinical trial, FCS was used for ex vivo expansion of BM in a bioreactor system with a washout before infusion[19-20]. However, multiple rescues with expanded progenitors risks infusion of trace amounts of FCS and could elicit an anamnestic response. To avoid this potential complication, we explored ex vivo expansion of $CD34^+$ cells under FCS-free conditions supplemented with AP, AS, or HS. However, this experimental system was not totally free of nonhuman protein. BSA was used because it was supplied for the separation kits. In the clinical scale separation, human serum albumin will replace BSA, making this system totally free of nonhuman protein.

Media supplemented with 1% APP would be the preferred substitute as compared with the other supplements tested in this study. Statistical analysis failed to show any advantage to the use of 20% FCS as compared with 1% APP. At AP or APP concentrations of 5%, the results were quite variable.

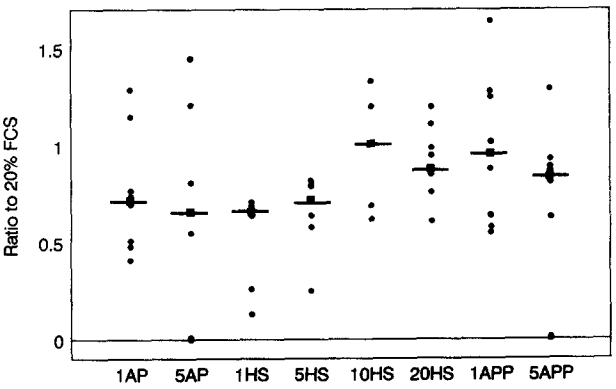

Fig 5. Ratio of progenitor expansion using FCS-free conditions to 20% FCS. Progenitor expansion determined at 7 days from experiments performed using either AP, HS, or APP was compared with that of 20% FCS in the same experiments, and the ratio was calculated. The bar represents the median.

In two experiments, 5% AP or 5% APP were inhibitory. There are several possible explanations. The presence of negative regulators, such as transforming growth factor-β, tumor necrosis factor, or interferons could adversely influence expansion, as could high concentrations of lipids. Brugger et al[13] reported that 1% AP was as effective as FCS. Their collection of plasma was from PB anticoagulated with heparin; we used the byproduct from leukapheresis. Because this is a waste product after leukapheresis, no additional phlebotomy would be required, and several hundred milliliters would be available. To determine if only centrifugation and filtration are required, studies are ongoing to determine the need for removal of cryoprecipitate.

Both 10% and 20% HS appear to be adequate substitutes for FCS and have less variability than 1% APP. However, considerable phlebotomy would be required to obtain enough AS to support ex vivo expansion clinically (approximately 200 to 400 mL to accommodate two 1-L bags). Therefore, carefully screened pooled HS could be a useful alternative and has previously been used in a clinical trial involving ex vivo expansion of $CD8^+$ cells.[15] Serum obtained in this fashion is commercially available and screened for infectious material. This serum could be tested for expansion potential and could reduce the intrapatient variability of AP. However, despite all available testing, no product could be guaranteed to be totally safe. Should one pooled batch be contaminated with an infectious agent, then every patient receiving that product would be at risk. For this reason, we believe the potential risks outweigh the benefit.

The expansion of progenitors from one leukapheresis is approximately 10- to 20-fold in 7 to 10 days without exchange of media or cytokine. This expansion should be adequate to compare unexpanded PBMCs and ex vivo expanded $CD34^+$ cells to determine both short- and long-term engraftment. Because LTCIC are either maintained or minimally expanded in 7 to 10 days of liquid culture and progenitors expand to a greater degree, this may result in more equivalent short-term engraftment; however, adequate LTCIC need to be infused to ensure long-term engraftment. Should equivalent efficacy be shown, not necessarily on a cell-to-cell basis, then the expansion of one PBMC collection should be capable of rescuing multiple cycles of HDC. Clinical trials will be needed to determine the threshold number of ex vivo expanded cells as compared with that of unexpanded cells to provide equivalent hematopoietic rescue. Mathematical models and preliminary results from our clinical trials suggest an advantage to multiple cycles of HDC. Hence, it would be desirable to collect PBMCs at the earliest possible consideration of high-dose therapy.[7-9] Ex vivo expanded progenitors without cryopreservation could be available 7 to 10 days after pheresis. Experiments involving cryopreservation of $CD34^+$ ex vivo expanded cells are underway to determine the viability and expansion potential of the progenitors.

The use of $CD34^+$ selection may have an additional benefit in that it provides a means of tumor cell purging.[21] In one study BM micrometastasis (BMM) correlated with distant relapse.[22] PBMCs have been found to contain fewer tumor cells as compared with BM, and $CD34^+$ selection further improves depletion.[20]

Two patients in our study had previously received multiple cycles of standard-dose adriamycin plus higher than standard-dose thiotepa followed by PIXY-321. These patients showed exhaustion of progenitors. One possible explanation is that an earlier acting cytokine, in this case IL-3, keeps more primitive progenitors in cycle longer, rendering them more susceptible to damage in subsequent cycles of therapy. Assays that measure committed progenitors, such as CFU-GM, lack the capacity to identify this deficit.

The use of ex vivo expansion of $CD34^+$ cells offers the potential for therapeutic benefit in hematologic as well as solid tumor malignancies. For example, in chronic myeloid leukemia, normal stem cells have been separated from malignant clones in the $CD34^+HLA\text{-}DR^-$ fraction in some patients.[23] Ex vivo expansion of this population could result in earlier engraftment as compared with that for selected stem cells alone. These new approaches offer clinical strategies for the future.

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