



Isolation of CD34⁺ cells from blood stem cell components using the Baxter Isolex system

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Summary:

The CD34 antigen is expressed by human hematopoietic progenitor and stem cells. These cells are capable of reconstituting marrow function after marrow-ablative chemo-radiotherapy. Several different technologies have been developed for the separation of CD34⁺ cells from bone marrow or peripheral blood stem cell (PBSC) components. We used an immunomagnetic separation technique to enrich CD34⁺ cells from PBSC components in anticipation of autologous transplantation for patients with B lymphoid malignancies. Twenty-nine patients enrolled on this study and received mobilization chemotherapy followed by G-CSF. Of these, 21 achieved a peripheral blood CD34⁺ cell level of at least $2.0 \times 10^4/l$ required by protocol for separation of the stem cell components. A median of three components per patient was collected for processing. The average CD34⁺ cell concentration in the components after apheresis was $1.0 \pm 1.2\%$. After the CD34⁺ cell selection, the enriched components contained $0.6 \pm 0.6\%$ of the starting nucleated cells. The recovery of CD34⁺ cells, however, averaged $58.4 \pm 19.2\%$ of the starting cell number, with a purity of $90.8 \pm 6.5\%$. Overall depletion of CD34⁻ cells was $99.96 \pm 0.06\%$. Nineteen patients were treated with marrow-ablative conditioning regimens and received an average of $6.2 \pm 2.0 \times 10^6$ CD34⁺ cells/kg body weight. These patients recovered to an ANC $>0.5 \times 10^9/l$ at a median of 11 days (range 8–14), and platelet transfusion independence at a median of 9 days (range 5–13). Four patients died of transplant-related complications or relapse before 100 days after transplantation. No patient required infusion of unseparated cells because of failure of sustained bone marrow function. These data demonstrate that peripheral blood-derived CD34⁺ cells enriched by use of an immunomagnetic separation technique are capable of rapid engraftment after autologous transplantation.

Keywords: CD34; peripheral blood stem cells; autologous stem cell transplantation; purging

Hematopoietic stem and progenitor cells can be identified by the presence of the CD34 antigen on the cell surface.^{1,2} It is believed that these cells are the only ones required to repopulate the hematopoietic system after myeloablative therapy. In a setting of autologous stem cell transplantation, enrichment of CD34-positive cells has the potential of reducing the number of contaminating tumor cells in the graft and reducing the side-effects from cryoprotectants used. Earlier studies have shown that engraftment of the hematopoietic system can be obtained with components enriched for CD34⁺ cells.

Use of progenitor cells from mobilized peripheral blood provides more rapid recovery of blood counts after myeloablative therapy as compared to transplanting cells harvested from bone marrow. It has been suggested that this rapid recovery of blood counts is related to the increased numbers of CD34⁺ cells obtained from peripheral blood stem cell (PBSC) components. Using a target of 5×10^6 CD34⁺ cells per kg patient weight, absolute neutrophil counts (ANC) greater than $0.5 \times 10^9/l$ and platelet transfusion independence can be obtained as early as day 9 post therapy.³ This rapid blood count recovery reduces the morbidity and, possibly, the mortality related to neutropenia and thrombocytopenia following therapy.

This study was initiated to investigate whether highly enriched CD34 cells from PBSC can provide the same rapid engraftment in a stem cell transplant as is observed from unmanipulated components. Earlier studies showed engraftment with enriched CD34⁺ cell fractions,⁴⁻¹² but these components were only 42–73% CD34⁺ cells. With more highly purified CD34⁺ cell fractions, it is necessary to assess whether or not a required accessory cell (or cells) is depleted from the components. It is also necessary to determine whether the methods used for purifying CD34⁺ cells might somehow reduce the function of the cells used for transplant.

The enrichment procedure was based on a magnetic microsphere technique to enrich the stem cells.^{13,14} Two different devices (manually-controlled and computer-controlled systems) were used to process the components. In addition, two different methods of releasing the cells from the beads (an enzymatic vs a competitive binding technique) were compared. This study was designed to validate the operating parameters that could affect the collection of sufficient numbers of functional CD34⁺ cells to

achieve rapid engraftment from PBSC in patients with B lymphoid malignancies.

Patients and methods

Twenty-nine subjects, 29–64 years of age were enrolled on this study between May, 1995, and December, 1996. Data were analyzed as of 1 May, 1997. Written informed consent for all patients was obtained using forms approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center. Patients were treated at five local hospitals, but apheresis, cell selection, and cryopreservation were performed at the Fred Hutchinson Cancer Research Center facilities. All patients had B cell malignancies as listed in Table 1. Patients with major cardiac, pulmonary, gastrointestinal, neurologic, renal, or hepatic disease; active infection; or Karnofsky score of less than 70% were excluded from enrollment.

Mobilization of PBSC and apheresis

All patients received chemotherapy mobilization (Table 1) followed by rhG-CSF (Neupogen, Amgen, Thousand Oaks,

CA, USA) administered subcutaneously at a daily dose of 10 µg/kg body weight starting on the day after the completion of the chemotherapy. For three patients who did not achieve the target level of 2.0×10^4 CD34⁺ cells/l in the peripheral blood required for PBSC processing, an increase of rhG-CSF to 32 µg/kg/day in divided doses was offered.

Apheresis was initiated after the peripheral blood white cell count exceeded 1.0×10^9 /l and the peripheral blood CD34⁺ cell count exceeded 2.0×10^4 /l. All collections were performed using the COBE Spectra (COBE BCT, Lakewood, CO, USA).³ In brief, 12 l of blood were processed after the interphase was established. Anti-coagulation consisted of a mixture of 10 units heparin per ml of acid-citrate-dextrose (ACD-A) at a ratio of anti-coagulation to blood of 1:30. An additional 40 ml of the heparin-ACD – A mixture was added to the collection bag before initiation of the apheresis procedure to prevent clumping of the component collected at this high blood to anti-coagulant ratio. Apheresis collections were performed daily until adequate numbers of CD34⁺ cells were collected for both the CD34⁺ cell separated primary components and unseparated reserve component(s) to be used in case of failure of engraftment of the CD34⁺ cell-enriched fraction.

Table 1 Patient characteristics

Gender	
M	22
F	7
Diagnosis	
NHL	21
MM	6
ALL	1
CLL	1
Median age (range)	51 (29–64)
Mobilization regimen	
CyDex	1
CyVP16	9
CyVP16Dex	15
CyVP16Plat	3
Ara-CMtx	1
Average CD34 ⁺ cells $\times 10^6$ /kg (range)	
Collected	14.3 (5.1–48.4)
Recovered	7.7 (3.0–22.0)
Transplant conditioning regimen	
BuMelTT	9
CyVP16TBI	7
CyVP151131	2
MelTBI	1

Diagnoses are: NHL = non-Hodgkin's lymphoma; MM = multiple myeloma; ALL = acute lymphoblastic leukemia; CLL = chronic lymphocytic leukemia.

Mobilization chemotherapy regimens consisted of cyclophosphamide (Cy) 4 g/m² with concomitant VP-16 at a dose of 200 mg/m²/day for 3 days with or without concomitant cis-platinum (Plat), 35 mg/m²/day for 3 days, or dexamethasone (Dex), 10 mg four times a day for 4 days.¹⁵ One patient received methotrexate (Mtx), 1 g/m² followed by cytosine arabinoside (Ara-C), 3 g/m² twice a day for three days.

Transplant conditioning regimens consisted of busulfan (Bu), 1 mg/kg four times a day on days –8 to –6, followed by melphalan (Mel), 50 mg/m² daily on days –5 and –4, then thiotepa (TT), 250 mg/m² daily on days –3 and –2;¹⁶ total body irradiation (TBI), 1.5 Gy twice daily on days –8 to –5, followed by VP-16, 60 mg/kg on day –4, and Cy, 100 mg/kg on day –2;¹⁷ ¹³¹I-labelled B1 antibody (to achieve a maximum dose of 2000–2725 cGy to non-hematologic tissue) followed by VP-16, 60 mg/kg on day –4 and Cy, 100 mg/kg on day –2;¹⁸ and Mel, 140 mg/m² on day –5 and TBI, 150 cGy twice a day on days –4 to –1.

Separation of CD34⁺ cells

PBSC components were processed using either the Isolex 300SA or Isolex 300i device according to the manufacturer's guidelines. Separation of CD34⁺ cells was initiated only after the patient achieved a peripheral blood level of $\geq 2.0 \times 10^4$ CD34⁺ cells/l. In brief, for components processed using the Isolex 300SA, the cells were centrifuged at 1500 r.p.m. in a COBE 2991 blood cell washer (COBE BCT) to deplete platelets and washed once with a buffer solution of either RPMI 1640 (for components subsequently released using chymopapain; Gibco Laboratories, Grand Island, NY, USA) containing 1% (w/v) human serum albumin (HSA, Bayer Corporation, Elkhart, IN, USA), or with a buffer solution consisting of Ca⁺⁺ and Mg⁺⁺-free Dulbecco's phosphate-buffered saline (Bio-Whittaker, Walkersville, MD, USA) containing 1% HSA (w/v) and 0.36% (v/v) sodium citrate (Baxter Healthcare Corporation, Deerfield, IL, USA). The component volume was adjusted to 100 ml and 0.5 g of human immunoglobulin (IVIg, Gamimmune; Bayer Corporation) was added. Anti-CD34 antibody, clone 9C5, was also added to a final concentration of 0.5 µg/10⁶ nucleated cells. After a 15 min incubation, the cells were again washed once with the buffer solution using the COBE 2991. The antibody-stained cells were transferred to the Isolex separation chamber and the volume adjusted to 300 ml. An additional 0.5 g of IVIg was added. One vial of sheep-anti-mouse coated paramagnetic beads (Dynal, Great Neck, NY, USA) containing 4×10^9 beads was washed with the buffer solution and added to the chamber. After 30 min incubation with gentle rotation, the negative cells were drained from the chamber and the bead-cell rosettes washed three times with buffer solution. The cells were released from the beads by the addition of either 8000 pkat chymopapain (ChymoCell-T; Boots Pharmaceuticals, Lincolnshire, IL, USA) or 100 mg synthetic peptide (Baxter Healthcare Corporation). After

the chymopapain addition, the cells were incubated for 15 min with gentle rocking and the cells drained from the chamber for three washes and concentration by centrifugation; after the synthetic peptide addition, the cells were incubated for 30 min and similarly washed and concentrated.

For components processed using the Isolex 300i, the volume of the component was adjusted to 250–300 ml by the addition of a buffer solution consisting of Ca⁺⁺ and Mg⁺⁺-free Dulbecco's phosphate-buffered saline with the additives described above. IVIg, 0.5 g, was added and the cells incubated at room temperature without rocking for 15 min. The cells were filtered using a standard blood administration set with an inline 170 μm filter (Baxter Healthcare Corporation) before transfer into the Isolex 300i. All subsequent steps including a platelet depletion wash, 9C5 incubation, bead incubation, cell release, and cell concentration were accomplished using the automated features of the device. The duration of the antibody incubation was 15 min at room temperature, and the 4 × 10⁹ paramagnetic beads were washed once with buffer solution before addition. Cells processed on the 300i device were released from the magnetic beads only by the addition of synthetic peptide as described above.

Enumeration of CD34⁺ cells, lymphocytes and clonal cells

Samples of peripheral blood before apheresis, the component before separation, and the CD34⁺ cell-enriched and CD34⁺ cell-depleted fractions were obtained for enumeration of CD34⁺ cells. Erythrocytes were lysed from a sample of 10⁶ cells using ammonium chloride lysis with serial washing. Phycoerythrin-conjugated anti-CD34 stain (HPCA-2; Becton Dickinson, San Jose, CA, USA) and fluorescein isothiocyanate-conjugated antibody to CD14 (Leu-M3; Becton Dickinson) were added at the concentrations recommended by the manufacturer and the cells incubated on ice for 30 min. Control samples consisted of the same cell quantity incubated with phycoerythrin-conjugated IgG₁ (Becton Dickinson) in conjunction with the CD14 stain. After incubation and washing, the cells were run on a FACScan or FacsCalibur flow cytometer (Becton Dickinson) and 100 000 events acquired. Data were analyzed using Winlist software (Verity Software House, Topsham, ME, USA). 'Bright' CD34⁺ cells were defined with histogram analysis using the gates first established on nucleated cells (defined by forward and orthogonal light scatter characteristics) and CD14-negative cells (defined by low orthogonal light scatter and lack of CD14 staining). The percentage of CD34⁺ cells was calculated after subtraction of the proportion of cells showing non-specific staining. The absolute number of CD34⁺ cells was obtained by multiplying the percentage of CD34⁺ cells by the cell count and volume of the specimen.

Lymphocytes were enumerated using a similar technique except using phycoerythrin or fluorescein isothiocyanate-conjugated monoclonal antibodies, but without co-staining with antibodies directed against the CD14 antigen. Usually, 10 000 events were obtained for analysis, although for some analyses, 100 000 events were obtained. CD19⁺ cells were

defined with histogram analysis using a gate first established on nucleated cells (defined by forward and orthogonal light scatter characteristics). The percentage of CD19⁺ cells was calculated after subtraction of the proportion of cells showing non-specific staining. The absolute number of CD19⁺ cells was obtained by multiplying the percentage of CD19⁺ cells by the cell count and volume of the specimen. For some components, CD3⁺ cells were quantified using this same technique. Antibodies for lymphocyte analysis were obtained from Becton Dickinson and used at the manufacturer's recommended concentration.

Samples of the PBSC component before separation and of the CD34⁺-enriched and CD34⁺-depleted components after separation were cultured for hematopoietic progenitor cells using techniques previously described.¹⁹ For patients enrolled after 20 May, 1996 (10 patients), we cultured the cells in commercially prepared methylcellulose-based medium with rhSCF, rhIL-3, rhGM-CSF and rhErythropoietin as growth factors (Stem Cell Technologies, Vancouver, Canada), after first establishing the similarity of this medium to that previously used by this laboratory. Unseparated cells were plated at a density of 3 × 10⁴ and 1 × 10⁵ cells/ml, CD34⁺-enriched samples were plated at 5 × 10² and 1 × 10³ cells/ml, and CD34⁺-depleted components were plated at 1 × 10⁵ and 3 × 10⁵ cells/ml. Four 1 ml plates at each seeding density were maintained in a humidified 5.5% CO₂ in air atmosphere at 37°C and colonies enumerated using an inverted microscope after 14–17 days of culture.

All cell counts during processing were obtained using a Coulter ZM particle counter (Coulter Electronics, Hialeah, FL, USA).

Tumor cell analysis by flow cytometry and molecular techniques

Lymphoma cells were detected by multidimensional flow cytometry using a panel of B lymphoid antibodies selected to detect abnormal phenotypes.²⁰ Briefly, lymphocytes were defined using CD45–peridinin chlorophyll protein complex (PerCp; Becton Dickinson) in combination with right angle light scatter.²¹ CD19 and CD20 (PE; Becton Dickinson) were used to identify B lymphoid cells. In combination with CD5 and CD10 (FITC; Becton Dickinson) small, homogeneous populations of B lymphoid cells could be identified that are not detected in normal PBSC. Confirmation of clonality was made using CD19 (or CD20) paired with FITC-conjugated anti-kappa and anti-lambda immunoglobulin light chain reagents (Becton Dickinson). The proportion of monoclonal B lymphoid cells was determined from the analysis of 50 000 cells on an Ortho Cytoron Absolute (Ortho Diagnostics, Raritan, NJ, USA). Analysis of the list mode data was made using Winlist (Verity Software House). The lower limit of detection using this technique is approximately 0.1%.

Plasma cells in myeloma patients were identified by high expression of CD38 (PE; Becton Dickinson).²² Clonality of these plasma cells was assessed by cytoplasmic staining of anti-kappa and anti-lambda immunoglobulin light chain reagents using PermeaFix (Ortho Diagnostics). Again, the

lower limit of detection of monoclonal plasma cells is 0.1%.

PCR amplification of the Bcl2-IgH gene rearrangement

DNA was extracted from bone marrow cells using the Pure-gene kit (Gentra, Research Triangle Park, NC, USA). PCR amplification of the Bcl2-IgH gene rearrangement was performed by a modification of the single-tube nested method.²³ One microgram of DNA was PCR amplified in 50 μ l of buffer (0.1 M Tris pH 8.3, 0.5 M KCl, 15 mM MgCl₂, 0.1% gelatin), 200 μ M deoxynucleotide triphosphates, 3.75 picomoles of primers 14;18-5'(5'GGTGGTTTGACCTTTAGA3') and 14;18-3'(5'TGAGGAGACGGTGACCAGGGT3'), and 1.25 units of Taq polymerase. The amplification conditions for the first PCR were 95°C \times 5 min, followed by 15 cycles of 94°C \times 30 s, 55°C \times 1 min, and 72°C \times 30 s. After the completion of the first PCR 15 picomoles of primers 14;18-3' and 14;18-nest (5'TTTAGAGAGTTGCTTTACGTGGC3'), and 1.25 units of Taq polymerase were added. The cycle conditions for the second PCR were 94°C \times 5 min, followed by 30 cycles of 94°C \times 30 s, 65°C \times 1 min, and 72°C \times 30 s, and 72°C \times 7 min. The PCR products were electrophoresed through a 5% polyacrylamide gel. The sensitivity of this assay can detect one Bcl2-IgH bearing cell in a background of 10 000 normal cells. Precautions to eliminate PCR carry-over contamination included separate rooms for pre-PCR and amplification procedures, filter-containing disposal pipette tips, and no-DNA PCR reactions as negative control in all PCR amplification reactions.²⁴ The integrity of the extracted DNA was tested by PCR amplification of N-ras exon 1.²⁵

Cryopreservation, storage and infusion

The CD34⁺ cell-enriched component was concentrated by centrifugation. Aliquots of 2.25 ml were placed in 5 ml cryovials (Nalge, Rochester, NY, USA). An equal volume of cryopreservation solution consisting of 20% dimethylsulfoxide (Cryoserv, Research Industries Corporation, Salt Lake City, UT, USA) and 8% human serum albumin in Normosol-R (Abbott Laboratories, North Chicago, IL, USA) was added to achieve a final DMSO concentration of 10%. The cells were cooled at 1°C/min to -40°C, and then at a rate of 10°C/min to -80°C before storage at -135°C in mechanical freezers. Unseparated PBSC components saved for use in case of engraftment failure were cryopreserved and stored as previously described.¹⁹

On the day of infusion, the vials were thawed in a 37°C water bath at the patient's bedside. The vial contents were aspirated into a syringe and injected into a central venous catheter. The vials were rinsed with normal saline that was also administered to the patient.

Conditioning regimen for transplantation

Four different conditioning regimens were used for this study as described in Table 1. Patients were admitted to a local hospital as necessary for conditioning or for treatment of neutropenic fevers or regimen-related toxicities,

although some patients received their conditioning regimen and cell infusion as an outpatient. Platelet transfusions were administered when the platelet count fell below $20.0 \times 10^9/l$. Red blood cell components were transfused for a hematocrit below 26%. Antibiotics were administered as dictated by clinical circumstances. Seven patients received post-transplant rhG-CSF in accordance with preference of the transplant team responsible for the care of the patient. Day 0 was defined as the day of cell thawing and infusion.

Statistical analysis

Data shown are means and standard deviations unless otherwise described. Examinations of the relationships between the quantity of cells loaded into the Isolex devices and the percent recovery and purity of the enriched fractions, and between the logarithm of the dose of cells used for transplant and the speed of hematologic recovery after transplantation were performed using analysis of Pearson's product-moment correlation. No adjustment was made for multiple comparisons and *P* values between 0.01 and 0.05 should be viewed only as suggestive of significance. All *P* values reported are two-sided.

Results

Mobilization, collection and processing of PBSC

Patients enrolled on this study underwent mobilization of PBSC using cyclophosphamide and rhG-CSF-based regimens as shown in Table 1. Two patients, not listed in Table 1, were removed from the study before apheresis. The first exhibited allergic reactions to rhG-CSF and was switched to rhGM-CSF, and the second recovered from the mobilization regimen with circulating tumor cells. Six other patients (three of whom received increased doses of G-CSF) failed to achieve a peripheral blood CD34⁺ cell level of $\geq 2.0 \times 10^4/l$ so that components collected by apheresis were not enriched for CD34⁺ cells. These patients were excluded from further study. Twenty-one patients achieved adequate CD34⁺ cell levels in the peripheral blood to proceed to isolation of CD34⁺ cells.

A total of 59 components were enriched for CD34⁺ cells for these 21 patients. One component was divided into two fractions for simultaneous processing with both the Isolex 300SA and the Isolex 300i. Each was analyzed for cell recovery and enrichment, giving a sample size of 60 components. A median of three components (range 1-4) were processed for each patient in an attempt to reach a target dose of at least 5×10^6 CD34⁺ cells/kg patient weight.

Three different processing techniques were used (Table 2). The Isolex 300SA with chymopapain release was used for the first two patients enrolled on the study; for an additional two patients, the components were enriched using the Isolex 300SA device, but alternated between chymopapain and synthetic peptide release mechanisms. For these four patients, a total of 10 components was treated with chymopapain. Including the two patients whose

Table 2 Processing results

	<i>Isolex 300SA</i> <i>Chymopapain release</i>	<i>Isolex 300SA</i> <i>Peptide release</i>	<i>Isolex 300i</i> <i>Peptide release</i>
No. components	10	17	33
Start ^a			
Cell load ($\times 10^{10}$)	4.9 \pm 1.0 (2.8–6.4)	4.6 \pm 2.0 (1.5–8.6)	4.4 \pm 1.7 (1.6–9.0)
CD34 ⁺ (%)	1.3 \pm 2.2 (0.3–7.3)	1.1 \pm 1.0 (0.3–4.4)	0.8 \pm 0.8 (0.2–3.6)
CFU-GM (per 3×10^4)	35.2 \pm 17.1 (12.2–62.8)	55.2 \pm 38.3 (13.8–128.8)	40.0 \pm 20.3 (15.0–82.0)
CD34 ⁺ enriched ^a			
Cell No. ($\times 10^{-8}$)	2.0 \pm 1.7 (0.8–6.6)	3.3 \pm 3.6 (0.6–14.3)	2.2 \pm 1.6 (0.6–6.4)
Cell recovery (%)	0.5 \pm 0.7 (0.2–2.3)	0.7 \pm 0.6 (0.2–2.6)	0.6 \pm 0.5 (0.1–2.5)
CD34 ⁺ purity (%)	94.5 \pm 3.2 (87.8–98.0)	93.6 \pm 8.0 (68.9–98.4)	88.1 \pm 10.8 (48.0–98.7)
CD34 ⁺ recovery (%)	50.9 \pm 11.3 (31.3–70.0)	54.1 \pm 13.2 (26.8–76.6)	62.8 \pm 22.6 (18.3–108.5)
CFU-GM (per 1×10^3)	29.9 \pm 15.6 (13.0–59.0)	77.4 \pm 46.4 (27.5–193.5)	95.0 \pm 42.6 (28.5–197.5)
CFU-GM recovery (%)	12.4 \pm 11.0 (1.3–38.7)	30.5 \pm 32.8 (11.5–154.5)	32.9 \pm 24.6 (5.9–121.6)
CD34 ⁺ depleted ^a			
Cell No. ($\times 10^{10}$)	4.4 \pm 1.0 (2.1–5.6)	4.6 \pm 2.1 (1.4–8.6)	4.0 \pm 1.7 (1.4–8.8)
Cell recovery (%)	89.2 \pm 5.8 (75.6–94.6)	98.6 \pm 8.2 (87.2–116.8)	91.3 \pm 12.1 (70.4–127.7)
CD34 purity (%)	0.8 \pm 1.6 (0.05–5.3)	0.4 \pm 0.4 (0.1–1.6)	0.2 \pm 0.2 (0.1–1.0)
CD34 ⁺ recovery (%)	36.5 \pm 15.9 (17.3–66.2)	36.0 \pm 18.2 (10.0–77.3)	28.6 \pm 19.8 (2.4–90.0)
CFU-GM (per 1×10^5)	13.1 \pm 10.4 (1.0–28.8)	18.7 \pm 20.2 (1.0–68.0)	10.4 \pm 8.6 (0.8–35.8)
CFU-GM recovery (%)	9.7 \pm 5.9 (2.1–16.8)	8.1 \pm 5.0 (1.2–21.1)	7.2 \pm 5.9 (1.1–29.9)
Total cell recovery (%) ^b	89.7 \pm 5.3 (77.9–94.8)	99.3 \pm 8.7 (87.7–119.3)	91.9 \pm 12.0 (70.6–128.2)
Total CD34 ⁺ cell recovery (%) ^b	87.4 \pm 12.0 (71.7–113.8)	90.1 \pm 22.5 (51.6–132.1)	91.5 \pm 20.6 (38.9–138.3)
Total CFU-GM recovery (%) ^b	19.2 \pm 8.3 (7.0–28.7)	30.9 \pm 9.8 (17.5–52.4)	40.2 \pm 24.0 (12.5–122.7)

^aShown are mean \pm standard deviation (range) for the apheresis component and for the CD34⁺ cell-enriched and CD34⁺ cell-depleted fractions according to processing technique used. Purity refers to the proportion of cells in the fraction that are CD34⁺. CFU-GM-derived colony numbers are for the seeding density stated.

^bProportion of starting cells recovered in the enriched and depleted fractions combined.

components were alternated between the two release mechanisms, a total of 17 components from 9 patients was processed using the Isolex 300SA and synthetic peptide release. Thirty-three components from 12 patients were processed using the Isolex 300i with synthetic peptide release. One of two components collected from a single patient was split into two equal portions with half the cells being processed using the Isolex 300SA and half using the Isolex 300i. For all three processing techniques combined, the average number of nucleated cells loaded onto the columns was $4.5 \pm 1.7 \times 10^{10}$ cells (\pm standard deviation). CD34⁺ cells comprised $1.0 \pm 1.2\%$ of these cells, or a total of $4.2 \pm 4.5 \times 10^8$ CD34⁺ cells (range 7.0×10^7 – 2.4×10^9). After enrichment, an average of $0.6 \pm 0.6\%$ of the nucleated cells were recovered in the adsorbed fraction, and $90.8 \pm 6.5\%$ (range 48.0–98.7%) of these cells were CD34⁺. Recovery of CD34⁺ cells in the adsorbed fraction was $58.4 \pm 19.2\%$ of the starting number (range, 18.3–108.5%). Most of the remaining CD34⁺ cells were in the non-adsorbed fraction. A total of $90.4 \pm 19.8\%$ of the starting CD34⁺ cells could be accounted for in these two fractions. There were no differences in CD34⁺ cell recovery or purity between the three techniques used.

Hematopoietic progenitor colony-forming cells were also enriched in the adsorbed fraction (Table 2). Overall, $28.5 \pm 26.5\%$ of myeloid progenitor cells (CFU-GM) were recovered in the CD34⁺ cell-enriched fraction, and only $7.9 \pm 5.6\%$ in the CD34⁺ cell-depleted fraction. We believe that the low recovery of myeloid progenitor cells in the enriched fraction is a consequence of the high purity of the CD34⁺ cells in this fraction. In testing of cells obtained

from normal donors and similarly separated using the Isolex 300i, we found the addition of an irradiated cell feeder layer greatly enhanced both the number and size of colonies cultured from the CD34⁺ cell fraction (data not shown), demonstrating that the culture conditions used were not optimal to support the growth of these highly enriched CD34⁺ cells. The recovery of erythroid and mixed erythroid and myeloid colonies paralleled that of the myeloid colonies in the adsorbed fraction, although a higher proportion ($32.5 \pm 19.8\%$, compared to starting numbers) of erythroid colonies was recovered in the CD34⁺ cell-depleted fraction.

We examined the relationship between the number of nucleated cells and the number of CD34⁺ cells loaded onto the Isolex devices and the purity and recovery of CD34⁺ cells after separation (Table 3). A negative correlation was found between the number of nucleated cells loaded onto the Isolex SA and the purity of the enriched fraction (percent of cells that are CD34⁺) but not the recovery of CD34⁺ cells in the enriched component. This relationship was weak with a correlation coefficient of only -0.42 . Similarly, a weak negative correlation ($r = -0.45$) was found between the number of nucleated cells loaded into the Isolex 300i and recovery of CD34⁺ cells after processing but not with the purity of the CD34⁺ cell-selected component. A weak positive correlation between the number of CD34⁺ cells loaded into the Isolex 300i and purity of CD34⁺ cells after processing was also found ($r = 0.40$). However, this relationship was in the opposite direction of that made for total nucleated cells loaded, with increasing purity found with higher CD34⁺ cell loads. The weakness of the correlation coefficients and the inconsistent results for these

Table 3 Relationship between cell load and CD34⁺ cell recovery and purity

Device	<i>r</i>	<i>P</i>
Isolex 300SA (<i>n</i> = 27)		
Nucleated cell load vs CD34 ⁺ cell recovery	0.14	0.50
Nucleated cell load vs CD34 ⁺ cell purity	-0.42	0.03
CD34 ⁺ cell load vs CD34 ⁺ cell recovery	-0.26	0.20
CD34 ⁺ cell load vs CD34 ⁺ cell purity	-0.08	0.68
Isolex 300i (<i>n</i> = 33)		
Nucleated cell load vs CD34 ⁺ cell recovery	-0.45	0.008
Nucleated cell load vs CD34 ⁺ cell purity	-0.12	0.51
CD34 ⁺ cell load vs CD34 ⁺ cell recovery	-0.29	0.10
CD34 ⁺ cell load vs CD34 ⁺ cell purity	0.40	0.02

Shown is the Pearson's correlation coefficient (*r*) and the significance of the slope (*P*) for the relationship between the number of nucleated cells or the number of CD34⁺ cells loaded onto the separation device and the percent recovery and purity of CD34⁺ cells in the enriched fraction after processing.

techniques call into question the clinical relevance of these observed relationships. Further study to determine the optimal cell load for the Isolex system must be conducted.

The recovery of CD19⁺ cells in the adsorbed fraction was approximately equal to the recovery of nucleated cells, with less than 0.3 ± 0.7% of the starting number of these lymphocytes remaining in the enriched fraction (Table 4). The limited number of CD19⁺ cells in these peripheral blood stem cell components before and after the separation steps hindered analysis of lymphocyte contamination after separation because many samples were below our limits of detection. We also examined the recovery of CD3⁺ cells in the enriched fraction (Table 4). A larger number of these cells persisted in the enriched fraction allowing for more accurate measurement. The percent recovery of CD3⁺ cells,

which averaged 0.03 ± 0.03% for all separations combined, was in the range expected if non-specific cell trapping accounted for the persistence of these cells in the adsorbed fraction. Approximately 5–10% of the 0.6% of the initial nucleated cells recovered in the adsorbed fraction were CD34⁻. Thus, the percent depletion of CD34⁻ cells averaged 99.96 ± 0.06% (range 99.61–99.99%).

All patients but one had at least one component tested by flow cytometric analysis for the presence of tumor cells. Tumor was detected in the pre-separation sample(s) for five patients (Table 5a). For one component, tumor was also detected in the CD34⁺ cell-enriched component, although at a much lower frequency. The presence of tumor in the other components was below the limits of detections of this technology (<0.1% of the cell population studied). We

Table 4 Depletion of lymphoid cells

	<i>Isolex 300SA</i> <i>Chymopapain release</i>	<i>Isolex 300SA</i> <i>Peptide release</i>	<i>Isolex 300i</i> <i>Peptide release</i>
No. components analyzed			
CD19 ⁺ cells	5	15	31
CD3 ⁺ cells	10	6	21
Start ^a			
CD19 ⁺ (%)	1.0 ± 1.0 (0.1–2.7)	0.2 ± 0.2 (0.0–0.5)	0.4 ± 0.7 (0.0–2.7)
CD19 ⁺ cell load (×10 ⁻⁸)	4.7 ± 4.9 (0.5–12.5)	1.1 ± 1.4 (0.0–4.3)	1.5 ± 2.5 (0.0–11.8)
CD3 ⁺ (%)	5.7 ± 4.0 (1.2–12.0)	5.1 ± 2.6 (2.7–9.3)	9.1 ± 9.1 (0.3–36.1)
CD3 ⁺ cell load (×10 ⁻⁹)	2.7 ± 1.8 (0.8–5.4)	2.2 ± 0.6 (1.5–3.1)	3.7 ± 3.3 (0.1–9.4)
CD34 ⁺ enriched ^a			
CD19 ⁺ (%)	0.1 ± 0.1 (0.0–0.2)	0.1 ± 0.1 (0.0–0.5)	0.1 ± 0.1 (0.0–0.5)
CD19 ⁺ cell recovery (%)	0.2 ± 0.2 (0.02–0.5)	0.3 ± 0.7 (0.0–2.4)	0.4 ± 0.8 (0.0–3.1)
CD19 ⁺ cell No. (×10 ⁻⁵)	3.6 ± 1.6 (1.0–5.2)	1.7 ± 2.4 (0.0–6.4)	2.0 ± 2.6 (0.0–10.1)
CD3 ⁺ (%)	0.5 ± 0.5 (0.04–1.4)	0.3 ± 0.5 (0.0–1.2)	1.1 ± 1.3 (0.0–4.8)
CD3 ⁺ cell recovery (%)	0.03 ± 0.02 (0.0–0.8)	0.01 ± 0.02 (0.0–0.4)	0.04 ± 0.04 (0.0–0.2)
CD3 ⁺ cell No. (×10 ⁻⁵)	9.1 ± 1.2 (0.4–38.9)	3.3 ± 3.8 (0.0–10.4)	10.3 ± 11.6 (0.0–38.4)
CD34 ⁺ depleted ^a			
CD19 ⁺ (%)	1.5 ± 0.8 (0.9–2.5)	0.3 ± 0.3 (0.1–1.6)	0.2 ± 0.3 (0.0–0.9)
CD19 ⁺ cell recovery (%)	92.6 ± 15.3 (83.2–110.2)	200.4 ± 110.8 (14.5–403.0)	98.6 ± 89.8 (0.0–393.7)
CD19 ⁺ cell No. (×10 ⁻⁸)	6.5 ± 3.5 (3.9–10.5)	1.7 ± 2.5 (0.0–9.1)	0.9 ± 1.4 (0.0–5.7)
CD3 ⁺ (%)	7.6 ± 5.9 (1.8–19.1)	5.7 ± 3.9 (1.7–10.5)	6.4 ± 5.5 (0.6–23.0)
CD3 ⁺ cell recovery (%)	116.7 ± 19.2 (90.9–146.9)	110.2 ± 34.2 (54.9–142.0)	86.7 ± 45.8 (30.8–205.2)
CD3 ⁺ cell No. (×10 ⁻⁹)	3.2 ± 2.5 (1.0–7.9)	2.3 ± 1.2 (0.8–3.7)	2.4 ± 2.1 (0.2–6.4)

^aShown are mean ± standard deviation (range) for the apheresis component and for the CD34⁺ cell-enriched and CD34⁺ cell-depleted fractions according to processing technique used. Not all fractions for all components were analyzed for lymphocyte content, accounting for the different number of components that underwent processing with each technique.

Table 5 Tumor detection by flow cytometric analysis

UPN	Component tested	Marker	Harvest %	CD34 ⁺ cell enriched %	CD34 ⁺ cell depleted %
9238	1	CD5 ⁺ CD20 ⁺	1.2	<0.1	ND
10582	1	CD5 ⁺ CD19 ⁺	1.3	<0.1	ND
10892	1	CD5 ⁺ CD20 ⁺	2.3	0.14	2.8
	2		2.7	<0.1	ND
10992	1	CD5 ⁺ CD20 ⁺	0.45	<0.1	0.5
	2		0.12	<0.1	0.17
	3		0.11	<0.1	0.11
10180	1	CD5 ⁺ CD19 ⁺ CD20 ⁻	0.43	<0.1	0.34
	2		0.26	<0.1	0.18
	3		0.10	<0.1	<0.1
			<0.1	<0.1	<0.1

Shown is the percent of cells in the component before and after separation that were found to express the phenotype described. Cells expressing these phenotypes are believed to be malignant. Malignant cells were detected by flow cytometry in the components of only the patients listed in this table.

similarly tested components using PCR analysis for Bcl2-IgH gene rearrangement for eight patients (Table 6). We were able to detect Bcl2-IgH gene rearrangement in the pre-separation samples for two patients. For both patients, Bcl2-IgH gene rearrangements were also detected in the CD34⁺ cell-enriched component. The molecular analysis performed was not quantitative, so the effectiveness of tumor depletion could not be determined by this analytic technique.

All but one of the CD34⁺-enriched components were cryopreserved in vials. The average cell concentration was $1.9 \pm 0.6 \times 10^7$ nucleated cells/ml (\pm standard deviation, range $1.2\text{--}4.0 \times 10^7$) in a volume of 11.2 ± 7.3 ml (\pm standard deviation, range 4.5–36.0). One component was cryopreserved in 80 ml total volume in two polyolefin bags at a cell concentration of 8.9×10^6 nucleated cells/ml. Cell samples were not obtained after thawing for cell counts, cytometric analysis, or hematopoietic progenitor cell cultures.

Two patients did not proceed to transplantation after collection of CD34⁺ cells. The other 19 patients received CD34⁺-enriched cells after receiving transplant conditioning regimens described in Table 1. No adverse events occurred during or immediately after the infusion of cells. All patients achieved the targeted endpoints of granulocyte

and platelet recovery. The median time to achieve an ANC $>0.5 \times 10^9/l$ was 11 days; the median time to achieve independence of platelet transfusions was 9 days. These patients were transfused a median of 8 units of red cells and 24 units of platelets. None of the patients required infusion of reserve components cryopreserved without enrichment of CD34⁺ cells. For six of the patients, only a portion of the CD34⁺-enriched components was infused. An average (\pm standard deviation) $6.9 \pm 2.4 \times 10^6$ nucleated cells per kilogram patient weight containing an average of $6.2 \pm 2.0 \times 10^6$ CD34⁺ cells/kg was infused. No relationship between either the number of nucleated cells or CD34⁺ cells (after enrichment) given per kilogram patient weight and time to granulocyte or platelet recovery was found. In contrast, when adjusted for the fraction of the components infused for the six patients who did not receive the entire components, we found weak but significant negative correlations between the log of the quantity of CD34⁺ cells harvested (before enrichment) and the times to last platelet transfusion ($r = -0.53$, $P = 0.04$), and to achieve peripheral blood platelet counts exceeding $20.0 \times 10^9/l$ ($r = -0.45$, $P = 0.09$) and $50.0 \times 10^9/l$ ($r = -0.63$, $P = 0.01$).

Four patients expired of post-transplant complications or relapse within 100 days of transplantation. One patient died on day 24 of idiopathic interstitial pneumonitis, a second

Table 6 Tumor detection by molecular analysis

UPN	No. components tested	Marker	Harvest	CD34 ⁺ cell enriched	CD34 ⁺ cell depleted
8036	1	Bcl2-Igh	POS	POS	ND
10180	4	Bcl2-Igh	NEG	ND	ND
10571	2	Bcl2-Igh	NEG	NEG	ND
10970	1	Bcl2-Igh	POS	POS	POS
10992	4	Bcl2-Igh	NEG	NEG	NEG
11216	3	Bcl2-Igh	NEG	NEG	NEG
11641	2	Bcl2-Igh	NEG	ND	ND
11865	4	Bcl2-Igh	NEG	ND	BD

Shown is a description of those components that were tested for Bcl2-IgH gene rearrangement. All patients tested are included in this table. Testing of samples was before separation (Harvest) and after separation (CD34⁺ cell enriched and CD34⁺ cell depleted). POS = positive for this marker; NEG = negative; ND = not done.

died on day 40 of relapsed disease, a third died of CMV pneumonia on day 52, and a fourth died on day 59 of hepatic veno-occlusive disease. Two patients subsequently succumbed to progressive disease after transplantation. At present, 13 patients are alive at 52+ to 362+ days after transplantation.

Discussion

In this study, we demonstrated that transplantation of PBSC components enriched for CD34⁺ cells using an immunomagnetic separation technique are capable of rapid engraftment. The median time to achieve a peripheral blood neutrophil count of over $0.5 \times 10^9/l$ was 11 days; to achieve platelet transfusion independence, 9.0 days. The rate of rise of blood counts was also rapid with median times to achieve $>1.0 \times 10^9$ neutrophils/l and $>50.0 \times 10^9$ platelets/l of 11 and 15 days, respectively. These times are comparable to those observed after transplantation of unseparated PBSC components containing similar numbers of CD34⁺ cells.³ Two patients received only components released from the beads using chymopapain, and both reached an ANC $\geq 0.5 \times 10^9/l$ by 10 days and platelet transfusion independence by 11 days after transplantation, indicating that the enzymatic treatment of the cells did not materially interfere with cell homing or proliferation.

The efficiencies of separation did not differ according to the device or releasing agent used. The average CD34⁺ cell recovery, in the range of 50–60%, was similar to what has been previously described for the biotin–avidin separation system, but the purity of CD34⁺ cells in the separated components averaged over 90%, consistently much higher than with the other separation system.^{4–12} This higher purity of CD34⁺ cells results in about a 10-fold greater depletion of CD34[−] cells from the component. We did not achieve this level of purity for all components, and the causes of lower purities are difficult to discern. In one separation, a failure of the disposable set required reconcentration of the cells and a second processing procedure. There is a suggestion in our data that the quantity of cells loaded onto the column may affect the purity of the CD34⁺ cells. High cell numbers are usually a result of large quantities of granulocytes collected into the component, and it is possible that clumping of these fragile cells could account for a poorer selection result. We found a significant negative correlation between the numbers of cells loaded into the Isolex 300i and the recovery of CD34⁺ cells. This was a modestly weak correlation with a correlation coefficient of only -0.45 . Seven components processed using this device had CD34⁺ recoveries of less than 50%, the lowest being 18.3% for a component with a starting cell quantity of 4.2×10^{10} nucleated cells. However, in this series, the highest cell load of 9.0×10^{10} nucleated cells resulted in a 59% recovery of CD34⁺ cells. The manufacturer currently recommends that the cell load not exceed 5×10^{10} nucleated cells. This parameter must be defined with additional experience using this device.

The average CD34⁺ cell purity of 90.8%, with a range of 48.0–98.7%, translates into a 99.96% (range 99.61–99.99%) depletion of CD34[−] cells in the adsorbed fraction

compared to the starting component. We studied the persistence of B (CD19⁺) and T (CD3⁺) lymphocyte populations in the adsorbed fraction. CD19⁺ cell quantities from these patients mobilized with chemotherapy were often lower than the quantity of CD34⁺ cells in the harvested component, frequently below our level of detection by flow cytometry, which complicated this analysis. In contrast, CD3⁺ cells were found at a much higher level and may serve as a surrogate marker for B lymphocyte depletion. The average recovery of CD3⁺ cells of 0.03% in the adsorbed fraction is comparable to the 0.04% persistence of CD34[−] cells in this fraction, probably resulting from non-specific trapping of these cells. This study was designed to explore the feasibility of the immunomagnetic separation technology, not to demonstrate clinical utility for purging of tumor cells. Samples were analyzed by flow cytometry and PCR for the presence of malignant cells. Despite the relatively high enrichment of the CD34⁺ cells, we were able to detect persistence of the PCR signal after separation for two patients. This suggests that additional steps such as combination of CD34⁺ cell enrichment with tumor cell depletion will be necessary for optimal depletion of tumor cells from the PBSC component. Pre-clinical studies demonstrating the feasibility of the combined approach are in progress in this laboratory.

The study parameters required that patients achieve a peripheral blood level of $>2.0 \times 10^4$ CD34⁺ cells/l before the collected cells could be processed using the Isolex devices. This is recommended by the manufacturer because of pre-clinical data showing less consistent yield and purities for components with starting levels of CD34⁺ cells $<0.5\%$. In this series, the number of CD34⁺ cells in the starting component did not predict for the recovery of CD34⁺ cells in the adsorbed component. It may be possible that patients with low levels of CD34⁺ cells in the peripheral blood could undergo extended apheresis collections in order to collect similar quantities of CD34⁺ cells and, thereby, reduce the number of separation procedures required. The limited range of CD34⁺ cells infused does not answer the question of what dose of CD34⁺ cells is required, before or after selection, for rapid and complete hematologic reconstitution. This center uses a target dose of 5×10^6 CD34⁺ cells/kg for recipients of unprocessed components. Others reported hematologic recovery after transplantation of lower quantities of CD34⁺-enriched components.^{4–7} A larger clinical experience will be required to determine if the dose of CD34⁺ cells after separation remains a predictor of speed of hematologic recovery as found in transplantation of unseparated PBSC.²⁶ The possibility that the quantity of CD34⁺ cells before separation is a better predictor for engraftment than the quantity after separation is an interesting suggestion from our analysis. This deserves further consideration as it may simplify patient and laboratory management.

Three patients died of regimen-related toxicity and a fourth of relapse after transplantation. The causes of death in the three patients, each documented by autopsy, differed. Of concern is the development of CMV pneumonia in one patient. It is conceivable that the depletion of lymphocytes from these components may predispose the recipient to opportunistic infectious complications. This patient was not

screened for the advent of CMV antigenemia post transplant,^{27,28} and the possibility of developing CMV pneumonia after autologous marrow transplantation is about 5% in populations of patients not prophylactically managed for this complication.^{28,29} The rapid relapse of one patient could, conceivably, also be related to transplant-related immune suppression. Regardless, future studies of transplantation with highly enriched components should include analysis of the reconstitution of immunologic function.

In summary, these data demonstrate that the Baxter Isolex system is capable of achieving consistently high levels of CD34⁺ cell purity, and that recipients of these cells will engraft rapidly. The value of this approach in preventing relapse is not evident from these data. A role in purging tumor cells from the cell inoculum before autologous transplantation will require studies designed to specifically answer that question. This laboratory is also conducting pre-clinical studies of CD34⁺ cell enrichment in combination with tumor cell depletion (an approach for which the Isolex system is easily adapted) to achieve even higher levels of purging. The level of CD34⁺ cell enrichment demonstrates the feasibility of this system for obtaining PBSC that may then be used for autologous transplantation, or, conceivably, allogeneic transplantation, progenitor cell expansion, or gene therapy.

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