IN THE UNITED STATES DISTRICT COURT

FOR THE DISTRICT OF DELAWARE

THE JOHNS HOPKINS UNIVERSITY, a Maryland corporation, BAXTER HEALTHCARE CORPORATION, a Delaware corporation, and BECTON DICKINSON AND COMPANY, a New Jersey corporation,	)	
Plaintiffs,	)	Civil Action No. 94-105-RRM
v.	)	
CELLPRO, a Delaware corporation,	)	
Defendant.	)	

DECLARATION OF DR. ROBERT A. PRETI

Submitted by:

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Dated: April 28, 1997

DECLARATION OF DR. ROBERT A. PRETI

I, Robert A. Preti, Ph.D., hereby declare:

1. I am Director of Stem Cell Transplant Laboratories and Applied Research at Hackensack University Medical Center in Hackensack, New Jersey. I have co-authored 24 published scientific papers in the areas of CD34+ cell selection, bone marrow and stem cell transplantation, and related fields. A copy of my Curriculum Vitae is attached hereto as Exhibit A.

2. Prior to accepting my current position, I was employed at the New York Blood Center, where I was responsible for processing of blood and bone marrow for use in transplantation procedures. There, I became familiar with the capabilities of Baxter's Isolex® 300 Magnetic Cell Separator System, which we used in peripheral blood stem cell ("PBSC") transplantation. I am also familiar with Baxter's newer model, the 300i, which is being used at both the New York Blood Center and Hackensack Medical Center for PBSC transplants.

3. I was the Principal Investigator in two Baxter-sponsored, FDA-approved clinical trials using the Isolex® 300i, with the Isolex® 300 as a back-up, at New York Blood Center. One was a randomized breast cancer trial involving autologous transplants, the other was an allogeneic trial. I am also the Principal Investigator in a stage 4 breast cancer clinical trial underway at New York Blood Center under an IDE in my name. This trial originally used the Isolex® 300 and was subsequently modified to substitute the Isolex® 300i. Finally, I am involved as Laboratory Investigator in a recently opened multicenter IND trial which relates to the use of taxotere and other drugs for the treatment of metastatic breast cancer. In this trial, we are using the 300i for selection of CD34+ peripheral blood cells.

4. My experiences with the two Baxter devices have been entirely

satisfactory. We have seen no delayed engraftment following any of the procedures. Yields and purities of CD34+ cells have been very good with the exception of one procedure that produced very high yield (82%) at the expense of relatively poor purity (77.8%). In recent trials using the 300i with breast cancer patients mobilized with chemotherapy, we have achieved CD34+ purities in the range of 95-99.5%.

5. I am also familiar with CellPro's CEPRATE® SC stem cell concentrator, which has also been used in clinical procedures at Hackensack Medical Center. In comparing the Isolex®300i with the CEPRATE® SC, I would say, first, that the overall processing time is virtually *identical*. The advantage of the Baxter device is that it is more fully automated than the CellPro device, which requires some additional manual operations that must be performed by a technician. This difference in automation frees up an additional 2 hours of technician time, during which the operator is able to perform other laboratory functions. Further, the Baxter device in our hands has more consistently provided high purities and recoveries of CD34+ cells. For these reasons, my preference is to use the Baxter device rather than the CellPro device in future procedures.

6. I understand that CellPro has suggested that an advantage of its device is that in light of the device's recent FDA approval, clinicians can use it for "off-label" procedures. I am not completely comfortable with this suggestion, since the FDA's approval of the CellPro device was for a very narrow indication, and in my judgment, although precedent exists for the "off-label" use of approved drugs and devices, it is not yet clear that the FDA approves of the off-label use of this device for stem cell sources and/or indications for which it has not been approved.

I declare under penalty of perjury that the foregoing is true and correct. Executed this 25th day of April, 1997.

Robert Preti PhD

Robert A. Preti, Ph.D.

CURRICULUM VITAE

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Education

1978 B.S. Biology, Fordham University, Bronx, New York

1980 Certification for Secondary Level Instruction

1983 M.S. Biology, New York University, New York, NY
Thesis title: "The Effect of the Hepatic Erythropoietic Factor on the Anemia Related to Chronic Renal Failure.
Advisor: Albert S. Gordon, PhD

1987 Ph.D. Biology, New York University, New York, NY
Thesis title: "Studies on the Effect of Hepatic Erythropoietic Factor on the Anemia of Chronic Renal Insufficiency."
Advisor: A.S. Gordon, PhD

Experience

1996-present: Director, Hematopoietic Stem Cell Processing and Applied Research, Hackensack University Medical Center, Hackensack, NJ

1995-1997: Tissue Bank Director, Processing, Storage and Distribution
Director of Laboratories and Applied Research, Clinical Services Division, New York Blood Center, Valhalla, NY

1991-1997:	Scientific Director, New York Blood Center, Clinical Services Division, Valhalla, NY
1990-1991:	Associate Investigator, Hudson Valley Blood Services, Division of the New York Blood Center, Valhalla, NY
1990-present:	Clinical Assistant Professor of Medicine, New York Medical College, Department of Medicine, Valhalla, NY
1989-1990:	Research Scientist, Marrow-Tech Incorporated, Elmsford, NY
1986-1989:	Post-Doctoral Fellow: City University of New York, Laboratory of Experimental Hematology, Immunology, and Tissue Culture.
1985 - 1988:	Adjunct Assistant Professor: York College, Health Professions and Occupational Therapy (Human Physiology in Health and Disease)
1987 - 1988:	Assistant Professor: Queensborough Community College, Department of Biological Sciences and Geology (Human Physiology and Anatomy) Department of Biological Sciences and Geology: Human Physiology and Anatomy.
1984 - 1989:	Adjunct Assistant Professor: Hunter College, Division of Medical Laboratory Sciences (Hematology, Histology, Immunology); Division of Nutrition and Food Science (Human Physiology). Adjunct Lecturer: Department of Physical Therapy (Physiology)
1978 - 1983	Elementary and Secondary Level Educator: Bronx, NY

Memberships:

1988-1990:	Society of Analytical Cytology
1992-present:	International Society of Hematotherapy and Graft Engineering
1993-present:	American Association for the Advancement of Science
1993-present:	American Society for Blood and Marrow Transplantation
1996-present:	American Association of Blood Banks

Committee Appointments/Activities:

1990-present:	Chair, Stem Cell Transplantation Research Team, New York Blood Center, New York Medical College

1991-1995:	Safety Committee - New York Blood Center Clinical Services
1992-1994:	Quality Management Board -NYBC
1992-1996:	Membership and Survey - International Society of Hematotherapy and Graft Engineering (ISHAGE) Constitution and By Laws - ISHAGE
1992-present:	Stem Cell Banking Committee - New York State Department of Health
1993-present:	Treasurer - International Society of Hematotherapy and Graft Engineering
1995-1996	Regional Director, North America, ISHAGE Education Committee
1994-present	Member - Scientific Advisory Board, Baxter Immunotherapy, Santa Ana, CA
1996-present:	Member- New York State Department of Health-Cord Blood Subcommittee
1996-present	Member - Advisory Board - Bloodline : On-line Hematology Resource - Kludge-Karden-Jennings, NY

Publications:

1. Zuckerman GB, Naughton BA, Gaito A, Preti RA, and Gordon AS (1984). The Effect of Methylcellulose on Extrarenal Erythropoietin Production. Proc. Soc. Exptl. Biol. Med. 176:197.

2. Naughton BA, Dornfest BS, Preti RA, Huie, ML, and Gordon AS (1984). Hepatic and Renal Erythropoietin Production During Various Intervals of Short-Term Hypoxia. J. Med 15:45

3. Huie MA, Naughton BA, Mirand EA, Leong S, Preti RA, and Gordon AS (1985). Effects of Interferon on Murine Erythropoiesis. Life Sciences 36:2469.

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9. Ahmed T, Preti RA, Wuest D. (1992) Refrigeration Storage of Bone Marrow. In: Bone Marrow and Stem Cell Processing: A Manual of Current Techniques. Areman, J Joachim Deeg, R A Sacher, eds.

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11. Preti RA, Collins N, Gee AP (1993). ISHAGE bone marrow processing survey: Report on an international information gathering process. J Hematotherapy 2:103-109.

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13. Preti RA, Farley TJ, Fan Y, Ahmed T, Rose M and Ciavarella D. (1994) The Combined Use of Soybean Agglutinin and Immunomagnetic Beads for T Lymphocyte Subset Depletion of Bone Marrow Allografts: A Laboratory Analysis. J Hematotherapy 3:111-120

14. Ahmed T, Preti RA, Razis E, Farley T, Lake DE, Beer M, Cook P and Ciavarella D. (1994) Peripheral Blood Mononuclear Mobilization with Sargramostim (GM-CSF): Prog. Clin. Biol. Res 389:457-462

15. Greenfeld J, Noizat-Pirenne F, Oluwole SF, Hesdorffer CA, Preti RA, Hardy MA, De Groote D and Franchimont P (1994) The Effect of UVB Irradiation on Proliferative Activity and Cell Growth Potential of Cord Blood. Transplantation 58:504-510.

16. Farley TJ, Preti RA, Ahmed T, Ciavarella D. (1994) A Two-Phase Approach to B Lymphocyte Purging of Autologous Bone Marrow Grafts for Patients with Malignant Lymphoma Contaminated Bone Marrow: Advances in Bone Marrow Purging and Processing, 105-109.

17. Fan Y, Preti RA, Ahmed T, Ciavarella D. (1994) Selection and Purification of CD34+ Cells Using Monoclonal Antibody and Ferrofluids: Advances in Bone Marrow Purging and Processing, 309-315.

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20. Ahmed TA, Lake D, Feldman E, Seiter K, Helson L, Mittelman A, Puccio C, Chun H, Grima K, Akhtar T, Perchick J, Niazi Z, Preti RA, et al. Factors Influencing Prognosis After Dose Intensive Therapy for Recurrent or Refractory Hodgkins Disease: Results of Sequential Trials: A Case for Treating Patients with Resistant Disease. Annals of the New York Academy of Science, 1-12, 1995.

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Current Interests:

1. The use of cell-specific surface antigens for positive and negative selection of hematopoietic populations; particularly in Breast Cancer.

2. Implementation of Molecular Diagnostic capabilities for detection of minimal residual disease in stem cell grafts.

3. Practical matters of hemopoietic stem cell collection, processing and storage.

4. Continuous development of Standard Operating Procedures for Operation, Quality Control and Quality Assurance for GMP implementation in the stem cell processing, purging and storage laboratory. Instituting mechanisms for compliance with CGMP principles in the cell processing lab.

5. Examination of the role of HER-2/*neu* overexpression and amplification in relapse following PBSC and BM transplantation for advanced Breast Cancer.

6. Alternatives to cryopreservation for short-term storage of hemopoietic stem cells for transplantation, including combinations and timing of growth factor administration/exposure in vitro to enhance transplantability of hemopoietic stem cells.

7. Development of an on-line, integrated network computer system for the stem cell laboratory and apheresis activities.

8. Advisory and analysis for FDA activities and opinions regarding the field of Hematopoietic Stem Cell Transplantation, particularly with respect to FDA's interpretation of minimal manipulation and its implications.

Manuscripts in progress:

1. Farley, TJ, Ahmed, TA, Preti, RA. Use of Anti-CD33 Monoclonal Antibody and Immunomagnetic Beads for Ex Vivo Tumor Cell Depletion of Bone Marrow in Acute Myeloid Leukemia. In preparation.

2. Farley, TJ, Ahmed, TA, Kuhns, RE, Preti, RA. Comparison of Three Methods for CD34 Enumeration. In preparation.

3. Preti, RA, Farley, TJ, Rooney, W, Palesi, C, Sheehy, J, Nadasi, S. Bone Marrow Buffy Coat Concentration Using the Fresenius AS104 Apheresis System. In preparation

4. Preti, R.A., Zahos, K, Jennis, A.A., Pecora, A.L Effect of Interface/Offset (I/O) Adjustment on Collection Efficiency Using the Fenwal CS3000 Plus Blood Cell Separator for Peripheral Blood Progenitor Cell (PBPC) Collection. Submitted for publication.

JOURNAL OF HEMATOTHERAPY 6:53–60 (1997)
Mary Ann Liebert, Inc.

Optimization of CD34+ Cell Selection Using Immunomagnetic Beads: Implications for Use in Cryopreserved Peripheral Blood Stem Cell Collections

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ABSTRACT

Isolation of CD34+ cells from bone marrow, umbilical cord blood, and mobilized peripheral blood stem cell (PBSC) collections has many potential clinical benefits. The aim of this study was to evaluate the use of the ISOLEX 300 system to select hematopoietic precursors and determine the effectiveness at depleting contaminating tumor cells from cryopreserved/thawed PBSC. Median recovery of CD34+ cells and CFU-GM colonies was 71% and 51.6%, respectively, using a protocol optimized for our laboratory. A mean 2.9 log_{10} decrease in contaminating breast carcinoma cells was seen after the selection process. Selected CD34+ cells underwent a second round of cryopreservation/thawing while retaining 85.6% viability and 72.3% recovery of CFU-GM colonies.

INTRODUCTION

A great deal of interest has been focused recently on developing methods to isolate the CD34+ cell population from bone marrow, cord blood, and peripheral blood leukapheresis products. These efforts arose from the initial identification of the CD34 antigen and its presence on the surface of cells in the early stages of hematopoietic development (1). The majority of isolation methodologies rely on the use of anti-CD34 monoclonal antibodies (mAb) in combination with a unique means of capturing the hematopoietic precursors. Examples include immunomagnetic particles (2,3), avidin-biotin immunoadsorption (4), immobilization on solid surfaces (5), and fluorescence-activated cell sorting (6). One of the promises of CD34+ selection technologies is the passive depletion of contaminating tumor cells from the stem cell product (7,8). This is of particular importance following the demonstration that disease relapse after autologous transplantation of bone marrow has been associated with the infusion of tumor cells in the graft (9,10). Mobilized peripheral blood is also often contaminated with tumor cells (11), although to a lesser degree. Transplantation of CD34+ selected cells has been demonstrated previously to result in successful hematopoietic engraftment in humans (12). In this report, optimization of a CD34+ immunomagnetic selection method using the ISOLEX 300 was undertaken, with the goal of achieving maximum recovery and purity of the CD34+ population. In addition, the tumor depletion capabilities of the methodology were evaluated.

MATERIALS AND METHODS

Specimens

For the series of experiments designed to optimize the ISOLEX system (Baxter Healthcare, Chicago, IL) for our laboratory operation, small aliquots of freshly collected peripheral blood apheresis (PBSC) products were employed. All products were collected via the Spectra (COBE BCT, Lakewood, CO) from patients diagnosed with non-Hodgkin's lymphoma (NHL) or breast carci-

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noma. To determine if the optimized procedure could be scaled up for use in a clinical setting, previously frozen PBSC collections from patients with a history of NHL were processed using the ISOLEX 300 device. These products were also collected via the COBE BCT Spectra, cryopreserved in medium containing a final concentration of 4% human serum albumin (HSA, Alpine Biologics, Bern, Switzerland), 5% DMSO (Research Industries Corp. Salt Lake City, UT), and 6% hydroxyethyl starch (McGaw Labs, Irvine, CA) and stored at −95°C (13).

Sample preparation

Frozen PBSC products were thawed at 37°C in a waterbath immediately before use. All PBSC specimens, fresh or frozen, were prepared for the subsequent studies by expanding the sample volume approximately 1:5 with a wash buffer consisting of Dulbecco's phosphate-buffered saline (D-PBS, without Ca^{2+}, Mg^{2+}, BioWhitaker, Walkersville, MD), 1% HSA (Alpine Biologics), and 2% citrate dextrose (Baxter). The samples were washed twice by centrifugation at 250*g* (with no braking) for 10 min at ambient temperature. The supernatant was removed using a plasma expressor. Before antibody staining, the nucleated cell (NC) concentration was brought to 1×10^7/ml by addition of wash buffer. The resuspended specimen was incubated with human immunoglobulin (Gamimmune, Miles Inc, Elkhart, IN) at a final concentration of 0.5% for 15 min at ambient temperature.

Monoclonal antibody optimization

The mAb used for CD34+ cell selection was the 9C5 clone. Fresh PBSC samples were used during the optimization procedure and prepared as previously described. The number of CD34+ cells in each specimen was determined by flow cytometry. Cell suspensions containing 1×10^6 CD34+ cells were added to each of eight tubes. Median purity of CD34+ cells in the specimens was 3.3% (range 2.5%–10.8%) of the NC population. Decreasing amounts of the 9C5 clone (50,000–25 ng) were added to each tube. Samples were incubated at 4°C for 30 min. The tubes were then washed three times by addition of wash buffer and centrifuged at 500*g* for 5 min, and the cells were resuspended in 0.1 ml of buffer. Next, approximately 50 pg of goat antimouse IgG-FITC Fab'_2 antiserum (Accurate, Westwood, MA) was added to each tube and incubated at 4°C for 30 min. All tubes were washed once more as described and resuspended in 0.5 ml of buffer. The samples were analyzed immediately by flow cytometry, with fluorescence analysis restricted to the lymphocyte region based on light scattering. The mean green fluorescence (MGF) of the gated population was measured for each test sample. Maximum binding to surface epitopes by the 9C5 antibody was gauged by comparing the MGF collected from each sample with the MGF obtained from the initial tube (50,000 ng antibody).

CD34+ cell selection

Before use in the selection process, the sheep anti-mouse IgG immunomagnetic beads (MagB, Dynal AS, Oslo, Norway) were washed three times with wash buffer. The amount of MagB required for the process, based on a desired bead/target ratio, was placed in a 50 ml conical test tube (Falcon, Becton Dickinson, San Jose, CA). The tube was placed in a hand-held magnetic particle concentrator (MPC-1, Dynal), and the MagB were allowed to pellet for 3 min. The supernatant was discarded, and the MagB were resuspended in wash buffer. After three washes, the MagB were resuspended to their original volume.

For separation of the CD34+ cells, MagB were added to the prepared PBSC specimen and incubated with rocking for 30 min at ambient temperature. The median CD34+ purity of the fresh PBSC used in this experiment was 2.9% (range 2.1%–4%). The specimens were then exposed to a magnetic field for 3 min. After cell/MagB rosettes had deposited near the magnet, the supernatant was drained off and designated the CD34− fraction. The magnetic field was removed, and the cells/MagB were resuspended in buffer and washed in this manner twice more, pooling the supernatant from each wash into the CD34− fraction.

CD34+ cell release from magnetic beads

After the washing steps, the cell/MagB rosettes were resuspended in buffer. To this volume was added the chymopapain releasing enzyme. For the reagent optimization experiments, 100 pKat of chymopapain was used to detach the cells from the MagB. For the clinical scale selections, 8000 pKat of chymopapain was required. Following addition of the appropriate amount of chymopapain, the specimens were incubated with rocking at ambient temperature for 15 min. Samples were reexposed to the magnetic field for 3 min, and the collected supernatant was designated as the CD34+ fraction. The MagB were washed once more as described, and the supernatants were pooled into the CD34+ fraction.

Laboratory analysis

All nucleated cell counts were determined using a Coulter MAX-M Hematology analyzer (Coulter Corp, Hialeah, FL). Flow cytometric analysis was performed using the EPICS Profile II (Coulter). The following reagents were used for testing of aliquots from all stages of processing: IgG1-FITC, IgG1-PE, CD34-FITC, CD38-PE, CD45-PerCP (Becton Dickinson, San Jose, CA). Viability of CD34+ cells was determined using propidium iodide (Sigma Chemical, St. Louis, MO). Samples were stained with 20 μl of the monoclonal reagents at 4°C for 30 min. Propidium iodide (5 μg) was added 5 min before completion of the antibody incubation. Erythrocytes in all samples were lysed with a 0.87% NH_4Cl solution, and the cells were then centrifuged, resuspended in D-PBS, and analyzed immediately by flow cytometry.

Hematopoietic colony assays

Colony-forming assays were performed using 0.9% methylcellulose (StemCell Technologies, Vancouver, Canada) in duplicate in 35 mm dishes. Media were supplemented with 400 U/ml of GM-CSF (Immunex Corp, Seattle, WA) and 1.25 U/ml of erythropoietin (Amgen Biologicals, Thousand Oaks, CA). Viability of the specimens was determined by exclusion of trypan blue dye (Sigma) as measured by light microscopy. Preselection and CD34− specimens were plated using 4×10^4 viable cells, and the CD34+ fraction was plated with 4×10^4 viable cells. Cultures were maintained at 5% CO_2 and 37°C. On day 14, CFU-GM colonies were scored if they consisted of more than 50 cells, and BFU-E colonies were counted if they consisted of more than 200 cells.

Evaluation of passive tumor cell removal by CD34+ cell selection

A breast tumor cell line (SK-BR3) was cultured and maintained in T-250 flasks (Corning Ware, Corning, NY). Tumor cells were originally seeded at approximately 2×10^6 cells per flask in 10 ml of McCoy's 5A medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Sigma) and 200 μm of glutamine (Sigma). Cultures were incubated at 5% CO_2 and 37°C and fed every 2 days with fresh medium. Cells were passaged approximately every 7–10 days when the adherent cell layer had become confluent by removing the medium and incubating with 5 ml of 1× trypsin-EDTA (GIBCO) solution at 37°C for 15 min to detach the cells from the flask. Trypsin was inactivated by the addition of 5 ml of FBS, and the suspension was transferred to a 50 ml conical tube. The cells were washed by centrifugation at 500 × g for 5 min, and the supernatant was discarded. Three washes were performed with the addition of culture medium, and the cells were used in the CD34+ cell selection study or reseeded as described previously.

SK-BR3 cells were added to thawed PBSC products at a concentration of 1 tumor cell/1000 nucleated cells. Preselection, CD34−, and CD34+ cell suspensions were set up in an assay system optimized to grow SK-BR3 colonies. One million cells from each sample were plated on 0.9% methylcellulose (Stem Cell Technologies) in duplicate in 35 mm dishes. The plating medium was supplemented with 10% FBS and 200 μm of glutamine but no hematopoietic growth factors. Cultures were incubated at 5% CO_2 and 37°C for 1 week. SK-BR3 colonies were characterized by compact clusters of greater than 100 cells. No colonies of hematopoietic origin were observed in these assays. The total number of SK-BR3 cells contaminating any product was determined by multiplying the average number of SK-BR3 colonies on the plates by the formula: Total nucleated cells in PBSC/ 1×10^6.

RESULTS

The results of the optimization experiments with the anti-CD34 mAb clone 9C5 are shown in Figure 1. Saturation of the surface-expressed CD34 epitopes was achieved at levels equal to or exceeding 25 pg of antibody per CD34+ cell. A significant decrease ($p = 0.004$) in epitope saturation was seen between concentrations of 2.5 and 0.5 pg/CD34+ cell. At the 2.5 pg/CD34+ cell

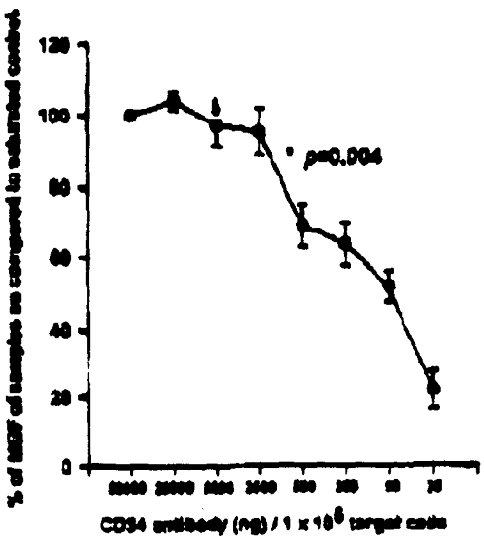

FIG. 1. Titration curve for the anti-CD34 monoclonal antibody 9C5. Measurement of the mean green fluorescence (MGF) indicated the degree of cell surface antigen saturation. Cells from PBSC samples ($n = 6$) were treated with decreasing amounts of the 9C5 antibody, followed by an FITC-tagged goat antimouse IgG antiserum. Results are expressed as the percentage of mean (± 1 SD) MGF compared with a 9C5-saturated control sample (50,000 ng). A significant (*$p = 0.004$) decrease in MGF was seen below the 2.5 pg antibody:CD34+ cell as compared with saturated control.

level, approximately 90% of the available CD34 epitopes in a sample had bound 9C5 molecules. Based on these data, a concentration of 2.5 pg/CD34+ cell was subsequently used in all optimization schemes and clinical scale selection procedures.

Data from the experiments designed to optimize the use of MagB in the selection process are shown in Figure 2. As the MagB/target cell ratios increased, there was a corresponding increase in the overall yield of CD34+ cells. At a ratio of 500:1, the mean (± 1 standard deviation, SD) CD34+ cell yield in the final product was 70.4% ± 13.5%. However, at this same ratio, the average purity of the selected products (61.7% ± 12.4) was the lowest observed in the experiment. The highest product purity was seen in those specimens tested with the lowest MagB/target cell ratios (1:1). Based on these data, a MagB/target cell ratio of 100:1 was used in the clinical scale experiments.

The aims of the clinical scale procedures were to (i) demonstrate the efficacy of the newly optimized laboratory-scale CD34+ cell selection procedure, (ii) determine the capacity of the procedure to remove contaminating breast carcinoma cells, and (iii) evaluate the use of CD34+ cell selection from cryopreserved PBSC. Thawed PBSC collected from patients with a history of NHL were spiked with SK-BR3 tumor cells and used as the source of CD34+ cells for these series of selections. The average (± 1 SD) plating efficiency of the SK-BR3 cells in these experiments was 2.8% (± 1.3%).

A summary of the results of the clinical scale runs is shown in Table 1. The initial five selection procedures followed recommended guidelines for reacting reagents with the specimen. Most importantly, the MagB incubation step occurred at an NC concentration of 2–5 × 10^7/ml. The data from this series of procedures were pooled and designated as Group 1. Subsequently, another five clinical-scale procedures were undertaken in which the MagB incubation step proceeded at higher NC concentrations ranging from 8 to 18 × 10^7/ml. The data from these five procedures were designated as Group 2. There was no significant difference between the groups in terms of CD34% or MNC% of the specimens. However, following the selection process, there was a significantly (p = 0.04) higher yield of CD34+ cells in the Group 2 procedures as compared with Group 1. The median recovery of CFU-GM colonies was also significantly higher (p = 0.04) in Group 2 as compared with Group 1 (51.5% versus 27%). However, there was no significant difference (p = 0.30) in the resulting purity of the selected products between the groups. The median purity of the Group 1 products was 85.3% (34.8%–91.8%), and for Group 2, the median purity was 85.2% (59.4%–94.7%).

An examination of the tumor-depletion abilities of this optimized CD34+ selection system is shown in Figure 3. An approximately 3.0 $\log_{10}$ decrease in the number of contaminating SK-BR3 cells was observed following the selection procedure. Performing the selection within the higher range of NC concentrations (Group 2 versus Group 1) had no significant effect (p = 0.24) on the depletion of breast tumor cells. There was a direct correlation between the final purity of the CD34+ selected products and the amount of tumor depletion achieved during the selection process (r = 0.63, p = 0.05). However, there was no significant correlation between CD34+ yield and tumor depletion (r = 0.38, p = 0.26).

During the entire procedure, some NC cell loss occurred because of the formation of cellular aggregates. Results of both NC and CD34+ cell recovery at several stages of the selection process are shown in Figure 4. All results were compared with data collected immediately before the cryopreservation of the PBSC. The mean NC recovery postthaw was 91.8% (± 6.8%), and after all washing steps, it had further decreased to 67.7% (± 9.3%). On completion of the selection procedure, only 59.3% (± 10.1%) of the precryopreserved NC population could be accounted for between both the CD34+ and CD34− fractions. However, the loss of the CD34+ cell population through the various stages of the selection was not as severe. A mean 79.6% (± 11.0%) of the CD34+ cell population before cryopreservation was re-

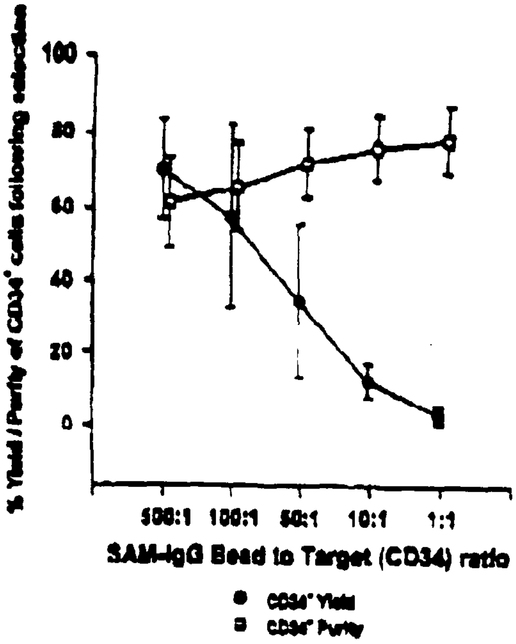

FIG. 2. Effect of different magnetic bead/target cell (CD34+) ratios on subsequent recovery and purity of the CD34+ population in the selected fraction. Results from studies on fresh PBSC samples (n = 6) are expressed as an average (± 1 SD).

TABLE 1. SUMMARY OF CLINICAL SCALE CD34+ SELECTIONS*

	Group 1	Group 2	Significance
n	5	5	
Starting product			
Purity	0.5% (0.2%–0.5%)	0.5% (0.2%–0.6%)	
NC concentration	4.5 × 10^7/ml (2–5.4 × 10^7/ml)	11 × 10^7/ml (7.9–18 × 10^7/ml)	
CD34+ fraction			
Yield	50.4% (38.3%–56.9%)	71.0% (50%–75.2%)	$p = 0.04$
Purity	85.3% (34.8%–91.8%)	85.2% (59.4%–94.7%)	NS
CFU-GM recovery	27.0% (14.8%–45.3%)	51.5% (43.6%–90%)	$p = 0.04$
SK-BR3 depletion ($\log_{10}$)	2.93 (2.88–3.10)	3.08 (2.83–3.20)	NS
CD34− fraction			
Yield	49.1% (35.2%–66.4%)	40.7% (22.7%–50.4%)	NS

*Results indicate median, with high–low ranges in parentheses.

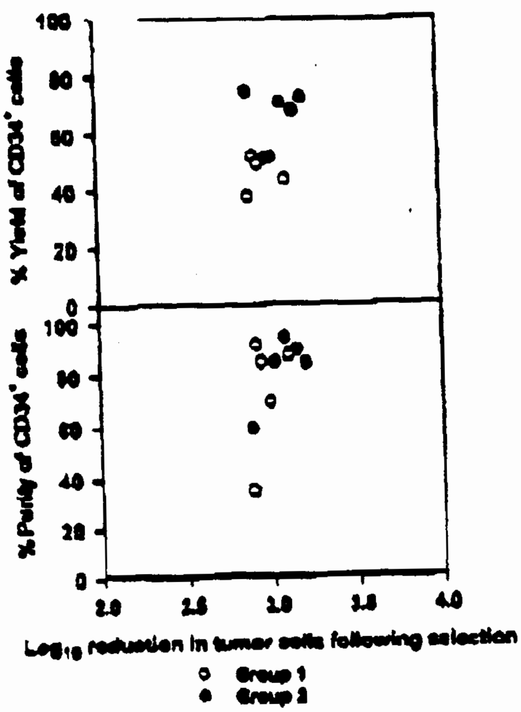

FIG. 3. Comparison of the reduction in tumor contamination (SK-BR3) with CD34+ fraction yield and purity for each group ($n = 5$) following clinical-scale CD34+ selections.

covered between the CD34+ and CD34− fractions. Again, the greatest loss of CD34+ cells occurred during the washing steps required to reduce platelet contamination and remove unbound anti-CD34 mAb (Fig. 4).

Of particular interest was the viability of the CD34+ cells isolated from the thawed PBSC collections. Using a propidium iodide dye exclusion test to measure cell membrane integrity revealed that the average viability for the CD34+ cell population postthaw was 100% and remained at 100% throughout the entire selection procedure (Table 2, $n = 10$). In contrast, there was a mean 20.2% (±13.3%) loss in viability among the remaining NC population.

Cryopreservation of the CD34+ cells selected from the thawed PBSC resulted in a mean (±1 SD) CD34+ cell viability of 85.6% (±4.7%) (Table 3). The mean viability of the NC population was 74.2% (±12.1%) at 5 min postthaw and slightly decreased to 70.9% (±16.7%) during the 30 min incubation required for CD34 analysis. At this point, 98.9% (± 0.8%) of non-CD34+ leukocytes (CD45+) had become nonviable.

Analysis of the hematopoietic potential of the CD34+ selected fractions after a second thaw is summarized in Table 4. Plating of the thawed samples in the methylcellulose assay resulted in a mean recovery of 72.3% (±10.9%) of the original CFU from the CD34+ selected products.

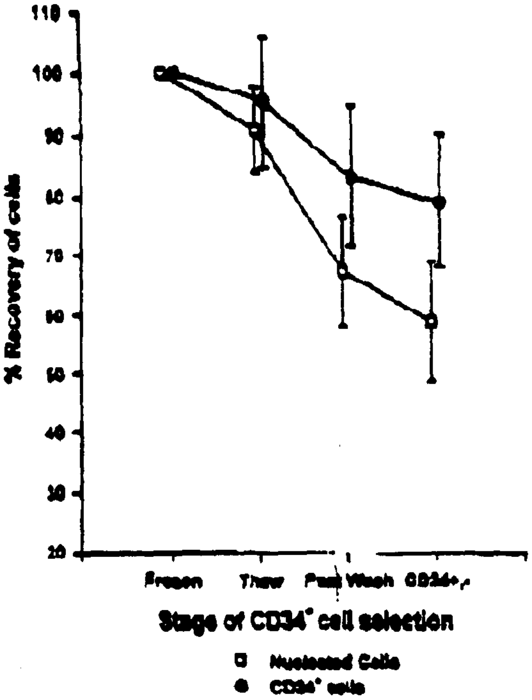

FIG. 4. Summary of the recovery of nucleated cell and CD34+ cell populations through each stage of the selection process. Results are expressed as a percentage of average recovery (±1 SD) for all clinical-scale procedures as compared with the original precryopreservation data for each PBSC (n = 10).

DISCUSSION

The purpose of this study was to determine the optimal conditions under which the ISOLEX 300 system may achieve maximum recovery and purity of the CD34+ cells in the selected fraction. The optimal amount of MagB required for a selection process is dependent on whether the final purity or overall recovery of CD34+ cells in the selected fraction is more critical for the intended use of the cells. Ideally, both target cell recovery and product purity could be maximized to provide a high-quality product for use in any application. From this standpoint, a MagB/CD34+ cell ratio of 100:1 offered the best compromise between the two goals. In terms of target cell capture, similar results were observed with regard to using similar types of MagB in negative purging situations (14). There was a direct correlation between MagB/target cell ratio and the amount of B lymphocyte depletion. However, in the setting of negative purging methods, the capture of nontarget cells is a less critical consideration. The presence of non-CD34+ cells in the selected product, particularly if tumorigenic, may contribute to disease relapse posttransplant (9–11).

Acquiring sufficient numbers of CD34+ cells to obtain rapid multilineage engraftment is a desirable goal. However, it may be possible to achieve this goal while also emphasizing the purity of the selected product. Transplant doses of CD34+ cells may be attained using selected cells expanded many fold *ex vivo* (15). In the event that these expanded cells are capable of inducing a prompt and durable engraftment, maximization of tumor cell depletion at the expense of cell recovery is acceptable.

Even in this case, and until such time that the functional activity of these expanded cells is adequately documented, our aim was to optimize both maximal CD34+ recovery and purity. The MagB were incubated with a higher NC concentration than recommended by the manufacturer. To test the efficacy of this method, several clinical-scale procedures were performed using frozen PBSC. The ISOLEX system had been shown previously to be effective when using previously frozen PBSC as the starting material (15,16). Two groups of five procedures each were used to compare the effect cell concentration has on CD34+ selection. There was a significant (p = 0.04) improvement in recovery of CD34+ cells when MagB were incubated with higher NC concentrations (1–2 × 10^8/ml). Interestingly, there was no significant change in the purity of the selected products from either group. A possible explanation for increased CD34+ cell yields is that increasing the NC concentration (decreasing incubation volume) forces the MagB into closer proximity with the target cells, thereby improving the capture efficiency of the MagB.

TABLE 2. VIABILITY OF THAWED PBSC THROUGH CD34+ SELECTION

	Postthaw	*Postwash*	*CD34+ fraction*	*CD34− fraction*
NC[a] Mean	79.3%	83.4%	100%	98.2%
Range	(± 13.3%)	(± 12.9%)	–	(± 0.9%)
CD34[b] Mean	100%	100%	100%	100%
Range	–	–	–	–

[a]Nucleated cell population as determined by CD45+.
[b]CD34+ population in sample.

TABLE 3. VIABILITY OF CELLS FOLLOWING RECRYOPRESERVATION/THAWING OF CD34+ FRACTION[a]

	Pre[b]	At 5 min	At 30 min
NC	100%	74.2% (± 12.1%)	70.9% (± 16.7%)
CD34+	100%	ND[c]	85.0% (± 4.7%)
CD45+CD34−	100%	ND	1.1% (± 0.8%)

[a]Mean of samples (n = 10) ± 1 SD.
[b]Data from immediately postselection/pre-2nd cryopreservation.
[c]ND, not done.

Spiking the thawed PBSC products with a breast carcinoma cell line (SK-BR3) provided a means of evaluating the tumor-depletion capabilities of this selection method. The approximately 3.0 $\log_{10}$ level of SK-BR3 depletion reported in this study is similar to results reported from studies using a CML cell line (7). Nearly 4.0 $\log_{10}$ removal of contaminating tumor cells also has been reported (2,8). However, there is evidence to suggest that the degree of tumor cell depletion is not related to the lineage of the tumor cell but instead correlates to the final purity of the CD34+ selected product (2). A significant correlation (p = 0.05) was seen between the level of tumor cell depletion and the purity of the selected CD34+ fraction (Fig. 4).

The concentration (1:1000) of SK-BR3 cells spiked into the thawed PBSC, although within the range of contamination seen in the clinical setting, is higher than normally seen in the majority of PBSC samples (17). This level of seeding was used in these experiments to ensure that sufficient cells would be present to demonstrate the passive tumor-depletion capabilities of the selection process adequately. Tumor clonogenic assays have been shown previously to be as effective as detecting breast tumor contamination as immunocytochemical assays and may also provide an in vitro correlate of a tumor cell's growth potential (18).

Performing CD34+ cell selections on frozen PBSC collections revealed several interesting results. Minor cellular aggregates formed during the washing steps, which were removed either by syringe or lost during processing, resulted in a loss of ~40% of the NC population. However, this was accompanied by a loss of only about 20% of the CD34+ cell population. A possible reason is that clumping of myeloid cells was primarily responsible for the formation of cell aggregates.

Analysis of the viability of the CD34+ population during the selection process revealed the maintenance of membrane integrity in the population. Even when this fraction was subjected to a second round of cryopreservation/thawing, the majority (85%) of the CD34+ cells remained viable. The hematopoietic potential of the CD34+ cells was also maintained despite the almost complete loss of viability among the rest of the leukocytes. These intriguing results suggest that frozen PBSC may successfully undergo CD34+ selection. Furthermore, the selected fraction may be cryopreserved a second time, with only marginal loss in hematopoietic potential.

In summary, the ISOLEX system was optimized for both maximal CD34+ cell recovery and purity. Significantly increased recoveries of CD34+ cells, but no significant change in the resulting CD34+ purity of the final product, resulted when greater cell concentrations were used in the procedure. Additionally, a mean 2.9 $\log_{10}$ depletion of contaminating breast tumor cells was seen with the selection process. Frozen PBSC were shown to be useful products for selecting CD34+ cells, with only small losses of cells caused by the formation of cellular aggregates. Indeed, selected CD34+ cells from the thawed PBSC were successfully frozen and thawed with little loss in the proliferative potential of the hematopoietic precursors.

TABLE 4. HEMATOPOIETIC PRECURSOR RECOVERY FOLLOWING RECRYOPRESERVATION/THAWING OF CD34+ SELECTED FRACTION[a]

	Post-1st thaw	Post-2nd thaw	Recovery
CFU-GM	1.6×10^4 ($\pm 1.6 \times 10^4$)	1.1×10^4 ($\pm 0.8 \times 10^4$)	72.3% (± 10.9%)
BFU-E	1.6×10^4 ($\pm 1.9 \times 10^4$)	1.2×10^4 ($\pm 1.4 \times 10^4$)	73.9% (± 12.9%)

[a]Mean no. of colonies from samples (n = 10) (± 1 SD).

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