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Bertolini F, Thomas T, Battaglia M, Gibelli N, Pedrazzoli P, Robustelli della Cuna G

Division of Medical Oncology, IRCCS Maugeri Foundation, Pavia Medical Center, Italy.

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MeSH Terms:

- Antigens, CD34/immunology
- B-Lymphocytes/pathology*
- Bone Marrow Purging/methods*
- Breast Neoplasms/pathology
- Breast Neoplasms/immunology
- Flow Cytometry
- Hematopoietic Stem Cell Transplantation*
- Human
- Lymphocyte Count
- Lymphocyte Depletion*
- Support, Non-U.S. Gov't
- T-Lymphocytes/pathology*
- Transplantation, Autologous
- Transplantation, Homologous
- Tumor Cells, Cultured

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- Antigens, CD34

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A new 'two step' procedure for 4.5 log depletion of T and B cells in allogeneic transplantation and of neoplastic cells in autologous transplantation

F Bertolini[1], T Thomas[2], M Battaglia[1], N Gibelli[1], P Pedrazzoli[1] and G Robustelli della Cuna[1]

[1]*Division of Medical Oncology, IRCCS Maugeri Foundation, Pavia Medical Center, Pavia, Italy; and* [2]*StemCell Technologies Inc, Vancouver, Canada*

Summary:

To evaluate a new 'two step' method for purging T, B and neoplastic cells from hematopoietic progenitor cells (PC), PCs were collected by apheresis and some suspensions were deliberately contaminated with 2–5% breast cancer cells. PCs were first processed through CellPro columns for positive selection of cells that express CD34. After this first step, the mean $CD34^+$ cell recovery was 68 ± 12%, and $CD34^-$ cell purity was 61 ± 11%; $CD3^+$ and neoplastic cell depletion were 2.1 ± 0.4 and 1.9 ± 0.4 logs, respectively. Cells were further processed through the StemSep device for direct depletion of T and B cells or of breast cancer cells. After the first and the second step, overall $CD34^+$ cell recovery was 50 ± 7%, T and B cell removal was 4.7 ± 0.4 log and neoplastic cell purging was 4.4 ± 0.3 log, ie significantly superior to methods described in the past.

Keywords: stem cell; progenitor cell; transplantation; purging; processing

In HLA-mismatched or haploidentical allogeneic progenitor cell (PC) transplantation, T cell removal can avoid graft-versus-host disease,[1] and B cell removal might reduce the incidence of some viral diseases.[2] In autologous transplantation, purging of contaminating neoplastic cells might reduce the incidence of relapse.[3] In the clinical field, currently available strategies for T cell or $CD34^-$ neoplastic cell removal from bone marrow (BM) or peripheral blood progenitor cells (PC) by means of immunoaffinty include purification of $CD34^+$ cells by positive selection or direct cell removal (negative selection) by means of anti-T or anti-neoplastic cell antibodies. However, most of these procedures do not consistently achieve more than 3.5–4 logs of target cell removal.[4,5] Aggressive T cell removal with soybean agglutination and E-rosetting results in a 3–3.5 log depletion,[1] but these procedures are cumbersome and quite difficult to reproduce. Here we report the use of a new 'two step' procedure combining both positive and negative immunoaffinity selection which allows higher than 4-log depletion of T, B or neoplastic cells from PC collected by apheresis.

Correspondence: Dr F Bertolini, Division of Medical Oncology, IRCCS Maugeri Foundation, Pavia Medical Center, viale Boezio 26, 27100 Pavia, Italy
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Materials and methods

PC collection and processing

For PC collection, patients undergoing PBPC autologous transplantation were treated with 7 g/m^2 cyclophosphamide and subsequent administration of 10 μg/kg/day recombinant human G-CSF. PBPCs were collected by apheresis the day on which the postnadir $CD34^+$ cell count was greater than 20/μl. The approval for use of human subjects was obtained by the Institutional Review Board, and cells were obtained after informed consent. An aliquot of <5% of the apheresis product was processed by Ficoll (1.077 g/dl; Cedar Lane, Ontario, Canada) and the mononuclear cell (MNC) fraction was incubated with anti-CD34 monoclonal antibodies (CellPro, Bothell, WA, USA). Cells were processed through CellPro LC34 immunoaffinity columns for positive selection of target $CD34^+$ cells according to the manufacturer's instructions. After $CD34^+$ positive selection, cells were washed, resuspended in PBS without Ca^+Mg^+ supplemented by 5% autologous serum and further incubated in ice with a mixture of anti-CD3 and anti-CD19 tetrameric antibody complexes (StemCell Technologies, Vancouver, Canada) or with anti-breast cancer tetrameric antibody complexes (StemCell Technologies, anti-breast cancer clone 5E11) at 3 μg/ml concentration. These tetrameric antibody complexes are bispecific cross-linkers which bind the described antigen and dextran. After 30 min incubation, 20 nm magnetic colloidal iron/dextran particles were added, and after 30 min incubation cells were processed through the StemSep device for depletion of targeted cells according to the manufacturer's instructions (StemCell Technologies). A total of 19 samples were processed. On eight occasions, T and B cells were depleted; on seven occasions, MNC were deliberately contaminated with 2–5% breast cancer cells from CG5 and CAMA-1 cell lines to evaluate the efficiency of breast cancer cell removal. In addition, breast cancer cell removal was studied in four apheresis samples from breast cancer patients known to have neoplastic cells contaminating the apheresis product.

PC, T, B and neoplastic cell evaluation

PE- or FITC-labeled anti-CD3, CD4, CD8, CD19, CD20, CD33, CD34 and CD38 monoclonal antibodies manufactured by Becton Dickinson (Mountain View, CA, USA), PharMingen (San Diego, CA, USA) and Immunotech (Marseille, France) were used to enumerate T, B and PC. A total of $100–500 \times 10^3$ cells were incubated at 22°C for 30 min in PBS-1% BSA with monoclonal antibodies. By means of flow cytometry (FACScan; Becton Dickinson), the percentage of stained cells was determined as compared to PE- or FITC-conjugated mouse isotypic control. A portion of each sample was incubated with the appropriate isotype control antibodies to establish the background level of non-specific staining, and positivity was defined as being greater than non-specific background staining. Cell viability was evaluated by staining cells with ethidium bromide and acridine orange. Dead cells were gated out based on orange fluorescence. For neoplastic cell evaluation, pre- and post-processing cytospin slides of 2.5×10^6 cells were stained with a commercially available APAAP[6] based kit for cytokeratin-positive breast cancer cell detection (Micromet, Planegg, Germany). At least 1×10^7 cells per sample were analyzed by microscopy for enumeration of breast cancer cells. Using breast cancer lines in limiting dilution assays, the sensitivity of our immunohistochemistry procedure for breast cancer cell enumeration was found to be of one cytokeratin-positive cell out of 500×10^3 evaluated cells.

The frequency of cobblestone area-forming cells (CAFC, or long-term culture-initiating cells, LTC-IC) was evaluted before and after cell processing. We followed the LDA assay recently described by Pettengell *et al*[7] with few modifications. Briefly, a layer of genetically engineered M2–10B4 murine stromal cells was generated in 96-well plates in the presence of Long Term Myelocult Medium (Stem-Cell Technologies). The layer was irradiated (80 Gy), a minimum of 0.5×10^3 and maximum of 30×10^3 nucleated cells/ml were seeded in five different dilutions in 10 replicate wells each. Cultures were fed weekly with Myelocult medium. At the end of 5 weeks culture, wells were evaluated as positive or negative for the presence of cobblestone areas, defined as clusters of small, tightly packed cells that were nonrefractory when viewed under a phase contrast microscope and originated from a CAFC. At week 5, wells with cobblestone areas of greater than 15 cells or three separate foci of more than five cells were scored as positive.

Data analysis

Statistical comparisons were performed by Primer (McGraw Hill, New York, NY, USA) and StatWiew (Abacus Concepts, Berkeley, CA, USA) software using the non-parametric analyses of Mann–Whitney, Wilcoxon and Kruskal–Wallis. Values of P lower than 0.05 were considered as statistically significant.

Results

On 19 occasions, $2.1 \pm 3.3 \times 10^9$ cells were processed through CellPro (first step) and StemSep (second step). Before processing, a mean of $1.1 \pm 0.8\%$ of nucleated cells were $CD34^+$, and average CAFC frequency was 1:11 270 nucleated cells. As shown in Table 1, after the positive selection step about 35% of $CD34^+$ cells were lost, and after the second step the final $CD34^+$ cell recovery was $50 \pm 7\%$. After 'two step' T and B cell removal, CAFC recovery was $48 \pm 9\%$ ($n = 5$); after 'two step' neoplastic cell removal, CAFC recovery was $53 \pm 11\%$ ($n = 5$). Recovery of immature progenitors was also supported by the observation that the percentage of $CD34^+$ cells which were CD33 and CD38 negative (lineage−) was not significantly reduced after the purging procedure (7–18% before and 12–20% after the 'two step' purging procedure). The first step allowed 1.9–2.1 log depletion of T, B and neoplastic cells, and after the final negative depletion T and B cell removal was 4.7 ± 0.4 log and neoplastic cell purging was 4.4 ± 0.3 log (significantly increased level of purging compared to the 'first step' only, $P < 0.001$). In unmanipulated samples from breast cancer patients, as expected, the frequency of contaminating breast cancer cells was low (1–50 cells out of 10×10^6 WBC evaluated before depletion and absence of breast cancer cells in 10×10^6 WBC evaluated after two step depletion); given the sensitivity of our procedure, in these studies efficiency of neoplastic cell removal was reported as >4 log (Table 2). Since anti-CD3 and anti-CD19 tetramers could partially block the sites for CD3 and CD19 staining, on three occasions T and B cell removal was further confirmed to be in the range of 4.7 ± 0.4 by enumeration of $CD4^+$, $CD8^+$ and $CD20^+$ cells. Figure 1 shows a representative flow cytometric analysis of cells before and after processing. To assess whether breast cancer cells used in this study were expressing the CD34 antigen as previously reported for some patient samples,[8] CG5 and CAMA-1 cells were evaluated by flow cytometry and found to be CD34 negative.

Discussion

A number of procedures including T cell depletion, neoplastic cell purging and *ex vivo* PC expansion are currently under evaluation to improve the clinical outcome of PC transplantation. In the present study we have investigated a novel 'two step' procedure which can be utilized for both allogeneic and autologous PC transplant. In our system, the main beneficial effect of the $CD34^+$ cell enrichment step is to debulk the graft, and 4.5 log depletion of target cells is obtained by the second negative depletion procedure. By means of flow cytometry and long-term cultures, we have estimated that about 50% of PC were recovered after the 'two step' purging. Moreover, the procedure did not selectively remove more immature progenitors such as $CD34^+$lineage− cells and CAFC. These assays are currently thought to be among the best surrogate for early hematopoietic progenitor enumeration.[7,9,10]

Very high doses of T cell-depleted PC collected from BM and peripheral blood of HLA-mismatched related donors have been recently used with success to engraft patients who lacked an HLA-matched donor.[1,11] Since 3 to 4 log depletion of donor T cells is needed to avoid severe GVHD in these patients, the new 'two step' procedure is a

Table 1 T and B cell and breast cancer cell removal

Exp	*After 'first step' positive CD34⁺ selection*				*After negative depletion of T and B cells*			
	CD34⁺ cell		*CD3⁺ cell*	*CD19⁺ cell*	*CD34⁺ cell*		*CD3⁺ cell*	*CD19⁺ cell*
	% recovery	*% purity*	*log depletion*		*% recovery combined-both steps*	*% purity combined-both steps*	*log depletion combined-both steps*	
T and B cell removal								
1	53.4	52.8	1.4	1.5	47.3	90.8	4.0	3.7
2	66.0	75.9	2.4	2.4	57.1	82.7	5.1	4.1
3	87.1	49.4	2.3	2.2	49.7	99.1	5.1	5.3
4	56.4	83.4	2.2	2.3	36.8	94.6	4.9	5.1
5	59.5	48.7	1.9	1.8	45.0	98.2	4.7	4.6
6	71.0	59.3	2.5	2.4	49.3	93.7	4.7	4.4
7	80.1	82.8	2.1	2.3	48.1	96.2	4.8	4.9
8	76.2	54.0	2.7	2.6	55.9	98.2	4.9	5.0
Mean (± 1 s.d.)	68 ± 12	63 ± 15	2.1 ± 0.4	2.1 ± 0.3	48 ± 6	94 ± 5	4.7 ± 0.3	4.6 ± 0.5

Exp	*After 'first step' positive CD34⁺ selection*			*After negative depletion of breast cancer cells*		
	CD34⁺ cell		*Cytokeratin + cell*	*CD34⁺ cell*		*Cytokeratin + cell*
	% recovery	*% purity*	*log depletion*	*% recovery combined-both steps*	*% purity combined-both steps*	*log depletion combined-both steps*
Breast cancer cell removal						
1	72.6	62.3	1.9	66.9	90.6	4.9
2	87.3	55.6	1.5	49.5	69.5	4.3
3	54.2	68.4	1.2	39.1	55.3	4.2
4	60.7	64.9	2.2	48.9	75.9	4.4
5	56.1	51.0	2.1	54.5	76.1	4.0
6	68.5	54.9	2.0	53.2	78.0	4.5
7	71.1	64.8	2.4	57.1	69.9	4.7
Mean (± 1 s.d.)	67 ± 11	60 ± 6	1.9 ± 0.4	52 ± 8	73 ± 10	4.4 ± 0.3

CD34⁺ cell recovery and cell purging after the first (positive selection) and the second (negative selection) procedure for immunoaffinity removal of T, B and breast cancer cells. In the first step, hematopoietic progenitor cells (PC) collected from cancer patients by apheresis were processed through CellPro columns for positive selection of cells which express CD34. In the second step, PC were further processed by the StemSep 'negative depletion' device for immunodepletion of T and B cells targeted with anti-CD3 and anti-CD19 antibodies or of breast cancer cells targeted with the 5E11 antibody. In the study of breast cancer cell removal the start cell suspensions were mixed with 2–5% breast cancer cells from the CG5 and CAMA-1 cell lines.

Table 2 Breast cancer cell removal in clinical samples

Exp	*Before processing*			*After 'first step' positive CD34⁺ selection*			*After negative depletion of breast cancer cells*			
	Total WBC	*Total CD34⁺*	*Total cytokeratin+*	*CD34⁺ cell*		*Total cytokeratin+*	*CD34⁺ cell*		*Total cytokeratin+*	*log depletion (combined)*
	×10⁶		*×10³*	*% recovery*	*% purity*	*×10³*	*% recovery combined-both steps*	*% purity combined-both steps*	*×10³*	
1	1150	12	0.2	68.4	71.5	0.1	47.6	78.7	0.0	>4
2	870	16	0.9	64.5	68.7	0.1	53.8	69.6	0.0	>4
3	1220	8	0.2	78.2	58.3	0.0	57.1	64.7	0.0	>4
4	1170	10	3.3	63.9	68.2	0.3	51.0	72.3	0.0	>4
Mean ± 1 s.d.	1100 ± 150	11 ± 3	1.1 ± 1.5	68 ± 6	66 ± 5	0.1 ± 0.1	52 ± 4	71 ± 6	0.0	>4

Breast cancer cell removal in four apheresis samples from breast cancer patients. Cells underwent positive CD34⁺ cell selection (first step) and negative depletion of breast cancer cells (second step). An aliquot of <5% of the apheresis product was processed.

good alternative to the more cumbersome and less effective soybean agglutination plus E-rosetting method used in these studies. Moreover, the new method has the advantage of the selective removal of B cells, which can carry harmful viruses like EBV, and could be used in the future for selective removal of CD4⁺, CD8⁺, natural killer or dendritic cells, ie cell subsets that might play a pivotal role in the onset of GVHD. However, it must be remembered that T

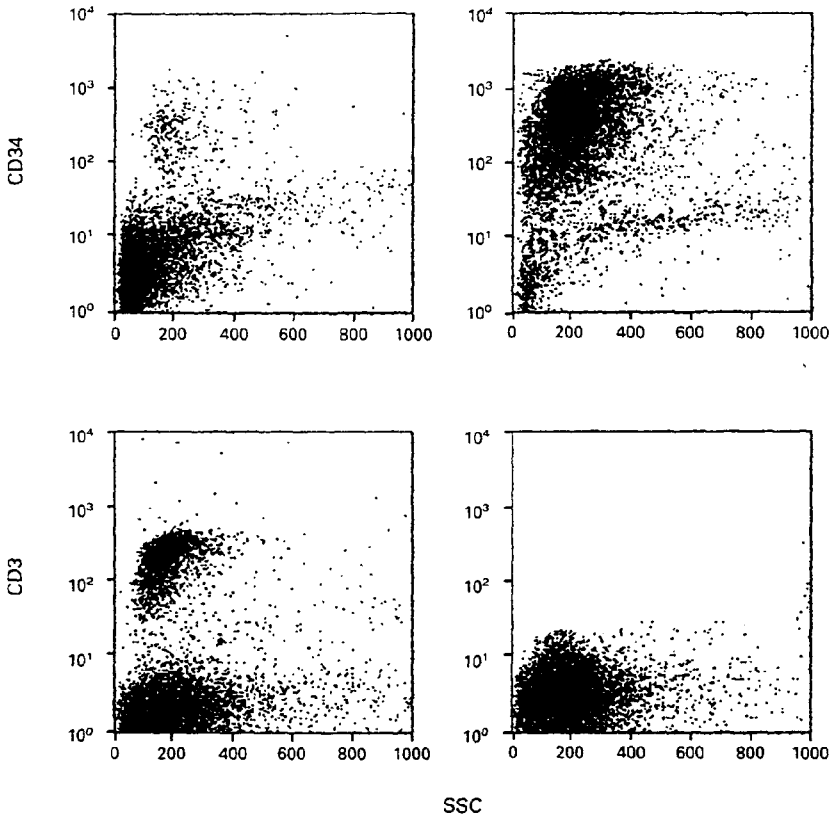

Figure 1 Fluorescent activated cell sorting analysis of cells before (left) and after (right) 'two step' depletion of T cells. This 'two step' procedure includes CellPro-positive selection followed by StemSep negative depletion. The top two dot plot profiles are of CD34 fluorescence *vs* side-scatter (SSC) and the bottom two plots are CD3 fluorescence *vs* SSC.

cells exert a graft-versus-leukemia effect, and that extensive T cell depletion has led in the past to graft failure in BM transplant recipients. Clinical studies are now warranted to evaluate the engraftment potential and the residual anti-leukemia effect of 4.5 log T cell-depleted PC.

For the autologous PC transplantation setting, the new 'two step' approach is associated with a purging efficiency significantly superior to methods described in the past,[12–15] and can also be used for removal of clonal $CD34^+19^+$ BCL2-IgH-positive progenitors from PC autografts of follicular lymphoma patients.[16] Regarding the 5E11 monoclonal antibody used to remove breast cancer cells, in previous studies[17] it was found to be the most suitable for breast cancer cell removal among a panel of monoclonal antibodies reacting with epithelial antigens expressed on breast cancer cells and absent on hematopoietic cells. The 5E11 hybridoma was raised from a fusion of Sp2 cells and the spleen cells from a mouse immunized with cells from the human breast carcinoma cell line T47D. The 5E11 antigen has not been fully characterized, but the 5E11 antibody binding is not blocked by antibodies to the protein of human polymorphic epithelial mucin, the BCA-225 glycoprotein, or erb-2 (T Thomas, unpublished observations). It should be remembered that the CD34 antigen has been reported to be expressed in 5–14% of fresh breast cancer cells.[8,18,19] In these patients, PC purging solely by positive selection of $CD34^+$ cells is clearly not indicated, but a second negative selection step would ensure removal of neoplastic cells. Phase I–II trials based upon this new technology will evaluate the clinical benefit of extensive neoplastic cell removal.

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