Enhanced Detection of Breast Cancer Cells Following CD34$^+$ Cell Selection Combined With Tumor Cell Purging

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ABSTRACT

Numerous studies have documented the presence of contaminating tumor cells in autologous stem cell grafts (autoSCGs) from breast cancer patients. It is presently unclear if the infusion of tumor cells in autoSCGs contributes to posttransplant relapse. Two studies using standard immunocytochemical (ICC) assays found no correlation with tumor cell contamination of autoSCG and posttransplant relapse in patients with metastatic disease. However, recent reports on high-risk breast cancer patients (>9 positive nodes) concluded that the infusion of tumor-contaminated autoSCGs was significantly correlated with relapse and disease-free survival at median follow-up of 42 and 21 months. Thus, infusion of tumor-contaminated autoSCGs may be associated with an increased risk of relapse in this patient population. Nexell Therapeutics is currently investigating tumor depletion of peripheral blood stem cells (PBSCs) in high-risk/metastatic breast cancer patients by CD34$^+$ selection (Isolex 300i Magnetic Cell Selection System, version 1.12, for positive selection) and by CD34$^+$ selection followed by additional tumor purging (Isolex 300i system, version 2.0, with investigational software for positive/negative selection). Peripheral blood stem cell apheresis collections (not for infusion) were shipped to Nexell for cell selection (positive or positive/negative) and analysis of tumor contamination. To document tumor cell presence and evaluate removal, we developed a bead-enriched, double-immunostaining ICC (dsICC) assay that is capable of detecting 1 tumor cell in 50 million hematopoietic cells in PBSCs. Use of the dsICC assay allowed us to detect the presence or absence of purging antibodies on cytokeratin-positive tumor cells. Validation studies indicate that the dsICC procedure provides improved tumor detection capabilities over standard immunocytochemical assays. We analyzed the preselected apheresis product, CD34$^-$ (waste), and CD34$^+$ fractions from both selection procedures using standard ICC. The CD34$^-$ and CD34$^+$ fractions were

processed and analyzed using the dsICC procedure. Preliminary results from patient specimens enrolled in clinical trials (n = 10) confirm that the dsICC assay is capable of enhanced detection of tumor contamination in $CD34^-$ and $CD34^+$ fractions from both selection procedures.

INTRODUCTION

The clinical use of autologous stem cell transplantation (autoSCT) as a means of hematopoietic reconstitution following high-dose chemotherapy (HDC) for the treatment of breast cancer has heightened the concern about tumor contamination of the autoSCG. Several studies have indicated that mobilization regimens used in HDC/autoSCT can contribute to tumor contamination of PBSC collections.[1–3] Although no study to date has demonstrated that infused tumor cells in contaminated autoSCG grafts are solely responsible for posttransplant relapse, the presence of gene-marked, infused tumor cells at sites of disease relapse has been documented in 3 malignancies.[4–6]

No comparable data exist using gene-marking techniques in breast cancer patients treated with HDC/autoSCT. However, 2 studies using immunocytochemical techniques to document tumor contamination of autoSCG found no correlation with tumor cell infusion and posttransplant relapse.[7,8] In contrast, others have reported that tumor contamination of autologous grafts in breast cancer patients approaches or achieves statistical significance in predicting poor posttransplant outcome.[9–11] Two recent studies using ICC and reverse transcriptase–polymerase chain reaction (RT-PCR) assays reported significant correlation of tumor-contaminated autoSCGs with posttransplant relapse and disease-free survival.[12,13]

As the above-mentioned studies illustrate, it is unclear if the infusion of tumor cells contributes to posttransplant relapse in breast cancer patients treated with HDC/autoSCT. However, tumor contamination of autoSCGs can be reduced or eliminated by in vitro pharmacological methods,[14,15] negative tumor depletion,[16] or positive progenitor cell selection.[17–19] Whereas pharmacological methods have been shown to adversely affect the hematopoietic reconstituting abilities of hematopoietic progenitor cells, immunomagnetic cell selection methods have been shown to be safe and effective for hematopoietic reconstitution in breast cancer patients treated with HDC/autoSCT.[17–19] Thus, positive selection for $CD34^+$ hematopoietic progenitor cells provides effective tumor purging without compromising hematopoietic reconstitution.

In a recent study of high-risk stage II/III and metastatic stage IV breast cancer patients, Umiel et al.[20] used an enriched ICC assay to document tumor purging in $CD34^+$-selected autologous PBSC grafts. This enhanced assay was capable of increasing tumor cell detection sensitivity ~50-fold over their standard ICC assay. Their preliminary findings on patient specimens indicated that tumor contami-

nation of the grafts may be substantially higher than previously reported.[21–23] Further, Umiel et al. reported that $CD34^+$ selection using the Isolex 300i Magnetic Cell Selection System reduced tumor cell contamination from 8 of 31 (26%) to 5 of 31 (16%) in paired PBSC specimens. However, tumor cell contamination was still present in some $CD34^+$-selected specimens. In such instances, additional tumor purging may be accomplished by adding a negative tumor depletion step during $CD34^+$ cell selection with the Isolex 300i system.[24,25]

The purpose of this study was to evaluate the potential additional tumor purging capability of the Isolex 300i positive/negative procedure in breast cancer patient PBSC specimens. To assess tumor cell contamination, we developed a unique bead-enriched, double-staining immunocytochemical assay. This assay was used to compare tumor contamination of unmanipulated PBSCs, $CD34^-$ (waste), and $CD34^+$ fractions from breast cancer patient PBSC specimens processed with the Isolex 300i CD34 positive or positive/negative procedures.

MATERIALS AND METHODS

Tumor Cell Enrichment Procedure: Validation Experiments

Tumor cell seeding experiments were performed to validate the dsICC procedure (Figure 1). This procedure was modified from that originally developed by Brockmeyer et al.[24] Briefly, CAMA-1 cells (ATCC, Manassas, VA), a breast cancer tumor cell line maintained in culture, were added to mononuclear cells (MNCs) obtained from a normal donor. The percent of CAMA-1 cells seeded into the MNCs ranged from 0.0001% ($1{:}1.0\times10^6$) to 0.000002% ($1{:}5.0\times10^7$). Unseeded MNC specimens were used as the negative control. Two separate aliquots of MNCs were used for the dsICC procedure at each seeding level of CAMA-1 cells, one containing 2.0×10^9 cells and the other containing 1.0×10^8 cells, to approximate the expected number of cells in the CD34-negative and -positive fractions, respectively, obtained from breast cancer patient leukapheresis products. Cell suspensions were incubated separately with Immune Globulin Intravenous (Gammagard; Baxter Healthcare, Hyland Division, Glendale, CA) at room temperature for 15 minutes. The cells were then incubated with a cocktail of 3 murine anti-human breast cancer antibodies (9184, 9187, and 9189; Nexell Therapeutics, Irvine, CA) at a concentration of 2.5 μg/mL each for 30 minutes, rotating to mix. The cells were washed twice with buffer to remove unbound antibody. Sheep anti-mouse (SAM)-coated paramagnetic beads (Dynabeads M-450, sheep anti-mouse immunoglobulin G; Dynal ASA, Oslo, Norway) were added at a ratio of 1 bead per 100 cells and incubated with the cells for 30 minutes to capture the tumor cells. While holding the tube against a magnet, unbound cells

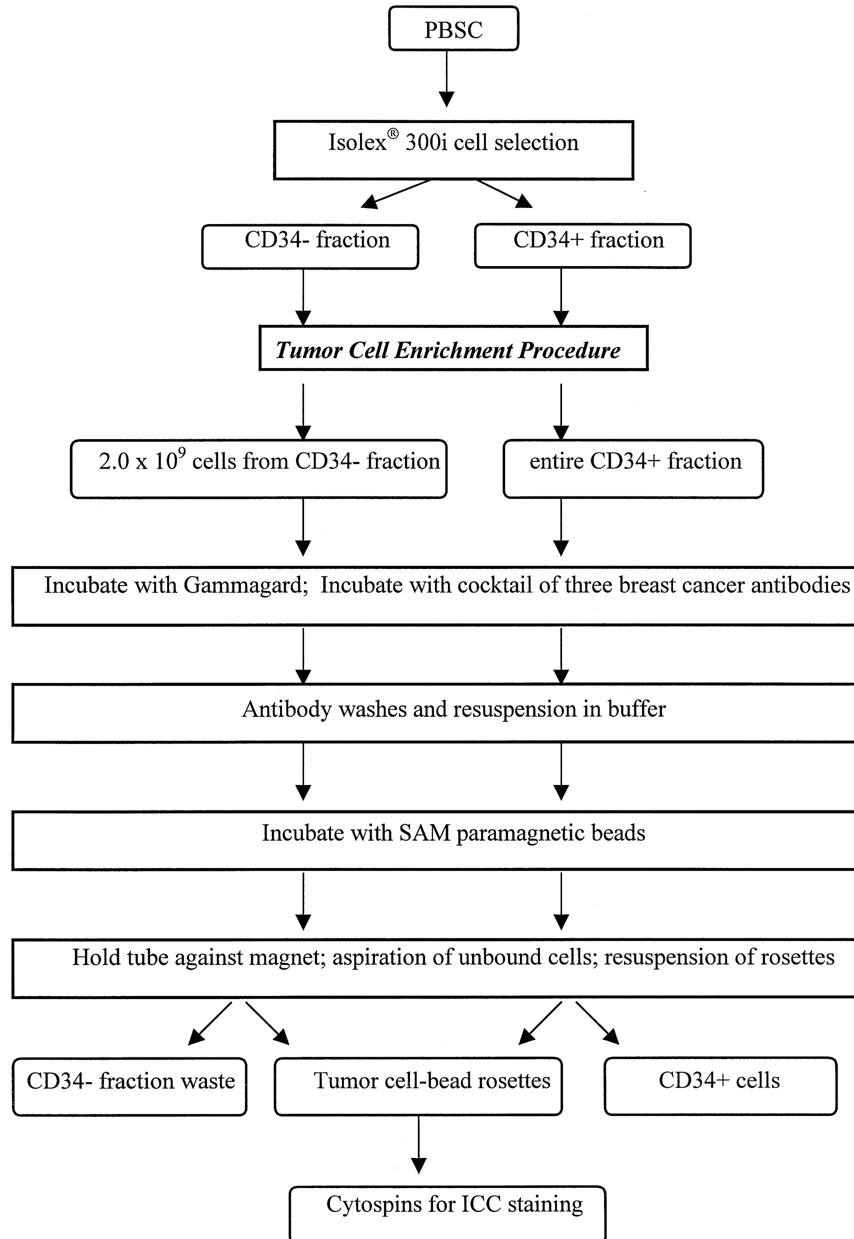

Figure 1. *Tumor enrichment procedure. ICC, immunocytochemical; PBSC, peripheral blood stem cell; SAM, sheep anti-mouse.*

were aspirated. The tumor cell–bead rosettes from each fraction were resuspended in buffer, centrifuged onto positively charged slides, and stained using the Nexell Cytonex ICC kit, in conjunction with the double-staining method described below.

Tumor Cell Detection: Double-Staining of Tumor Cell–Bead Rosettes

Twelve slides were prepared using tumor cell–bead rosettes obtained from all CAMA-1 cell MNC samples. Slides were stained using a double-staining method for dual detection of cytoplasmic cytokeratin antibodies and the membrane-bound breast cancer antibody cocktail (BCAC). Briefly, immunoperoxidase staining with 3,3-diaminobenzidine (DAB) was initially performed to develop a brown color reaction to the BCAC on the cell surface. Immunoalkaline phosphatase staining was subsequently performed on the cytospin preparation with the Cytonex ICC kit according to manufacturer's instructions.

Patient Specimens

PBSCs were obtained by leukapheresis from female patients with high-risk (stage II with >10 positive nodes, stage III) or metastatic (stage IV) adenocarcinoma of the breast. Patients were assigned a Nexell patient study number and randomization assignment for either CD34 positive cell selection or CD34 positive/negative cell selection. All patients rendered their informed written consent under a US Food and Drug Administration (FDA)/institutional review board (IRB)–approved protocol. Patients were mobilized with either granulocyte colony-stimulating factor (G-CSF) alone (10 μg/kg per day) or G-CSF in combination with chemotherapy (cyclophosphamide 4 g/m^2 followed by G-CSF 5 μg/kg per day). Patients began apheresis collections when the mobilization regimen was deemed successful (>20 CD34 cells/μL peripheral blood). When sufficient cells for all clinical target collections (including an unselected back-up) had been obtained ($>2.0\times10^6$ CD34 cells/kg), an additional leukapheresis product was collected for this laboratory study. No cells from the additional leukapheresis product collected for this laboratory study were infused into patients.

Processing of Specimens: Cell Selection

Patient PBSC collections were processed using the Isolex 300i Magnetic Cell Selection System (Nexell Therapeutics), either CD34 positive selection (version 1.12) or CD34 positive/negative selection (version 2.0 with investigational software), depending on the randomization assignment. The Isolex 300i for positive selection consists of a device, disposable set, and reagents that are designed, through a serious of automated steps, to select $CD34^+$ cells from PBSCs.

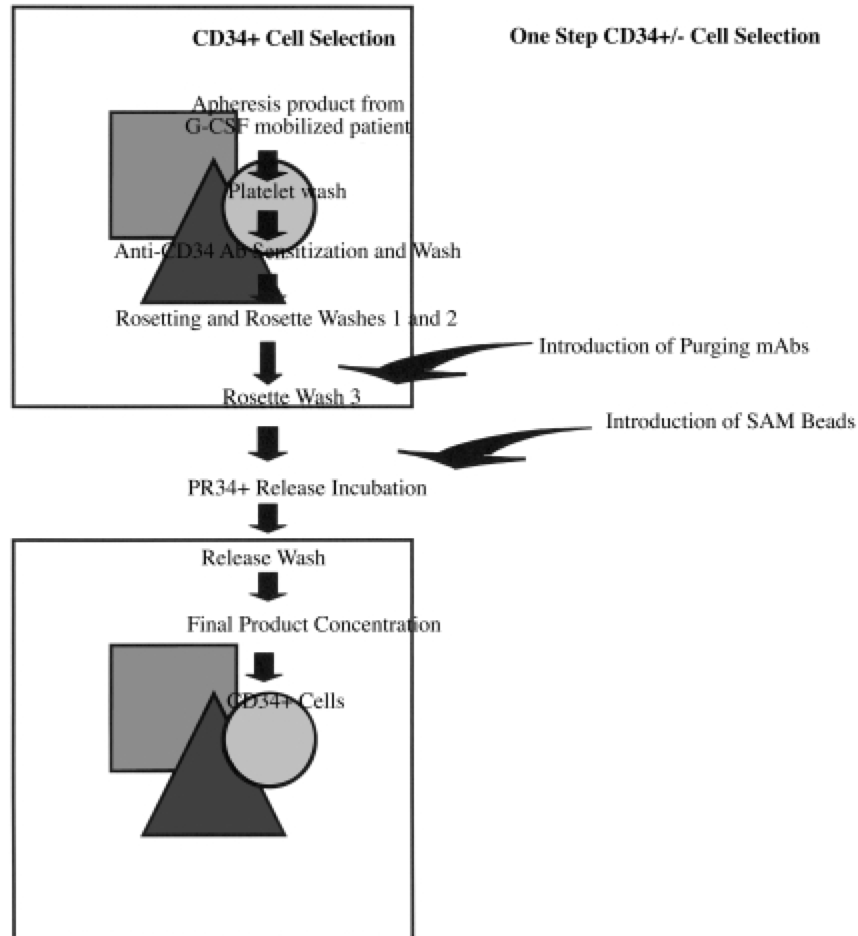

Figure 2. *Isolex 300i magnetic cell selection procedures. mAb, monoclonal antibody; SAM, sheep anti-mouse.*

Briefly, the procedure (Figure 2) involves a platelet wash, followed by anti-CD34 antibody sensitization and antibody wash. Sensitized cells are next incubated with SAM-coated paramagnetic beads, which bind to the murine CD34 antibody on the cell. Cell rosettes are washed to remove nontarget cells (negative fraction), which are diverted into a separate bag. A nonenzymatic stem cell releasing agent ($PR34^+$) is used to separate the targeted $CD34^+$ cells from the paramagnetic beads. The released $CD34^+$ cells are collected in a separate sterile bag. The Isolex 300i for positive/negative selection consists of all the components and steps described above plus 2 additional steps (Figure 1): (1) purging of breast tumor cells with the

use of 3 murine anti–breast cancer monoclonal antibodies (9184, 9187, and 9189; Nexell Therapeutics) and (2) the introduction of a second vial of SAM-coated paramagnetic beads just before the $PR34^+$ release step. This procedure allows for the simultaneous capture of $CD34^+$ cells and purging of breast cancer cells.[25]

Tumor Cell Detection: Standard Immunocytochemical Assay

Aliquots from the preselection leukapheresis product, $CD34^+$ and $CD34^-$ fractions, were used for the preparation of cytospins onto positively charged microscope slides. One million cells were deposited on each slide in a volume of 0.1 mL. Six slides were prepared per fraction, for a total of 6 million cells per specimen. Slides were immunostained using the cocktail of anti-cytokeratin monoclonal antibodies in an immunoalkaline phosphatase assay (Cytonex ImmunoCytoChemistry Kit; Nexell Therapeutics) according to manufacturer's instructions.

Tumor Cell Enrichment Procedure: Patient Specimens

Subsequent to the Isolex 300i cell selection process, the tumor cell enrichment procedure (Figure 2) was performed using 2.0×10^9 cells of the $CD34^-$ fraction and the entire $CD34^+$ fraction as described in the tumor cell enrichment procedure validation experiments described above. Twelve slides were prepared using tumor cell–bead rosettes obtained from each CD34 cell fraction. Slides were stained using the double-staining method described above and viewed with a standard light microscope. Tumor cells were manually enumerated to obtain a semiquantitative result. A mean of 7 slides (range, 2 to 11 slides) were stained and enumerated per fraction for all patient samples.

RESULTS

dsICC Validation Experiments

Results of the validation experiments in which CAMA-1 cells were seeded at varying ranges into MNCs indicated that recovery of the CAMA-1 cells ranged from a low of 11% (seeding level of 0.000002% in 2.0×10^9 MNCs) to a high of 60% (seeding level of 0.000002% in 1.0×10^8 MNCs). Percent tumor cell recovery was a function of the number of tumor cells seeded and the number of MNCs processed. No stained cells were detected in the unseeded MNC samples. In a series of 4 replicate experiments at all cell-seeding concentrations, the upper limit of detection sensitivity of the dsICC assay was 1 tumor cell in 5.0×10^7 MNCs. Thus, the dsICC assay increased tumor detection by 3 logs compared with standard ICC.

Table 1. Tumor Detection Results in Phase 2/3 Patient Specimens*

Patient ID (Selection) and Diagnosis	*PBSCs*	*Standard ICC Results*		*dsICC Results*	
		$CD34^-$ Fraction	*$CD34^+$ Fraction*	*$CD34^-$ Fraction*	*$CD34^+$ Fraction*
33 (+/–) Stage II A	Negative	Negative	Negative	Positive (1 in 1.7×10^8)	Negative
34 (+/–) Stage II B	Negative	Positive (1 in 5.5×10^6)*	Negative	Positive (1 in 4.6×10^8)	Negative
36 (+/–) Stage II B	Negative	Negative	Negative	Positive (1 in 9.2×10^8)	Negative
37 (+/–) Stage II A	Negative	Negative	Negative	Positive (1 in 9.2×10^8)	Negative
302 (+) Stage III	Negative	Positive (1 in 2.5×10^6)	Negative	Positive (1 in 2.5×10^8)	Positive (1 in 6.4×10^7)
304 (+) Stage II	Negative	Positive (1 in 2.5×10^6)	Negative	Positive (1 in 6.7×10^7)	Negative
306 (+/–) Stage IV	Positive (1 in 4.5×10^5)	Positive (1 in 2.5×10^6)	Negative	Positive (1 in 1.4×10^8)	Negative
402 (+) Stage IV	Negative	Positive (1 in 3.0×10^6)	Negative	Positive (1 in 5.0×10^8)	Negative
404 (+/–) Stage III	Negative	Negative	Negative	Negative	Negative
405 (+) Stage IV	Negative	Positive (1 in 5.0×10^6)	Negative	Positive (1 in 8.3×10^8)	Negative

**Tumor frequency in parentheses. dsICC, double-immunostaining immunocytochemical; ICC, immunocytochemical.*

Patient Specimens

Ten patient PBSC products from phase 2/3 studies were analyzed (Table 1). Four patient specimens were selected with the Isolex 300i using the CD34 positive procedure, and 6 were processed using the CD34 positive/negative procedure, using investigational software. Five patients in the study were diagnosed with stage II breast cancer, 2 with stage III, and 3 with stage IV. Using the standard ICC assay, 1 of 10 PBSC specimens showed immunostained tumor cells. Six of the 10 $CD34^-$ fractions were found to be positive for tumor cells as determined by standard ICC, compared with 9 of 10 when using the dsICC (tumor cell counts ranged from 1 in 1.67×10^7 to 1 in 9.2×10^8 hematopoietic cells). None of the $CD34^+$ fractions was positive for immunostained tumor cells using the standard ICC assay, whereas tumor was detected in 1 of 10 of the $CD34^+$ specimens when the dsICC was used for detection. This specimen was obtained from a patient with stage III disease whose PBSC product was processed with the CD34 positive procedure.

DISCUSSION

Although the clinical relevance of infusion of breast cancer cells in the HDC/autoSCT setting remains to be elucidated, the fact remains that many autoSCGs contain contaminating tumor cells.[1–3,7–16,20–23] Prospective studies analyzing the clinical correlation of the infusion of tumor cells with outcome in the breast cancer HDC/autoSCT setting have provided conflicting results. Using standard ICC techniques, 2 studies showed no correlation of tumor contamination of autoSCG with posttransplant outcome in stage IV patients[8] or high-risk stage II/III and metastatic stage IV patients.[7] In contrast, Solano et al.[13] recently used a similar standard ICC assay to analyze PBSC collections from 52 high-risk stage II patients (>9 positive axillary nodes). At median posttransplant follow-up of 42 months, median disease-free survival was better in the patients who received a tumor-free graft than in those who received a tumor-contaminated graft (P=.002). Multivariate analysis concluded that tumor contamination of the PBSC product was the only prognostic predictor of posttransplant relapse (P<.01). Vannucchi et al.[12] used an RT-PCR assay with reported tumor detection sensitivity greater than standard ICC techniques to analyze PBSC grafts from 33 stage II/III breast cancer patients. They reported that there was a trend toward longer relapse-free survival (P=.053) posttransplant in patients who received a tumor-negative graft. In a subset of 4 patients with RT-PCR–positive PBSC specimens, the infused grafts became RT-PCR–negative after $CD34^+$ selection.

As illustrated above, highly sensitive assays that detect low numbers of tumor cells are crucial in investigating the role that the infusion of tumor cells may play in the HDC/autoSCT treatment setting. Several recent studies have employed tumor-enrichment technology to increase the detection sensitivity of tumor cells in breast cancer autoSCGs.[20,24,26,27] Collectively, these studies have demonstrated that tumor contamination of breast cancer PBSC collections is more prevalent than that reported by studies using standard tumor detection techniques. Umiel et al.[20] used a tumor enrichment assay to evaluate PBSC specimens and $CD34^+$-selected specimens from breast cancer patients with high-risk stage II/III and metastatic stage IV disease. Their results indicate that the tumor-enriched assay detected a greater level of tumor contamination of PBSC specimens (86%) than did the standard ICC assay (7%).

Taken together, these studies suggest that tumor contamination of autoSCGs is more prevalent than previously appreciated, and that $CD34^+$ cell selection procedures alone may not remove all tumor cells from the grafts. Laboratory experiments using tumor cell–seeded specimens have indicated that the inclusion of an additional tumor-purging step to the existing $CD34^+$ cell selection technology increases the depletion of contaminating tumor cells.[25,28] For these reasons, we are conducting studies directly comparing the tumor-purging capability of the Isolex

300i CD34 positive selection procedure versus the Isolex 300i positive/negative (investigational software) procedure.

To more accurately analyze the levels of tumor contamination in these PBSC specimens, we developed a novel double-staining ICC assay using tumor-enriched cell samples. This dsICC assay demonstrated increased sensitivity of tumor cell detection over our standard nonenriched ICC assay. In tumor cell–seeding experiments, we were able to document a 3-log increase in tumor cell detection, up to 1 tumor cell in 50 million PBSCs. Further, the double-immunostaining assay allowed for simultaneous microscopic evaluation of the binding of breast cancer purging antibodies on cytokeratin-positive cells. Thus, the combination of the bead-enrichment methods with the dsICC assay provided enhanced detection and microscopic visualization of extremely low numbers of seeded tumor cells in PBSC specimens (1 in 5.0×10^7 hematopoietic cells).

Our preliminary analyses of 10 patient specimens indicate that the bead-enriched dsICC assay provided enhanced detection of contaminating tumor cells. Eight of 10 PBSC patient specimens that were deemed to be tumor-negative using the standard ICC assay proved to be tumor-positive with the dsICC assay of the $CD34^-$ fraction. None of the 10 $CD34^+$ fractions analyzed by standard ICC analysis showed immunostained tumor cells; however, using the dsICC assay, 1 of the $CD34^+$ fractions (processed with the CD34 positive selection protocol) from a patient with stage III breast cancer showed double-immunostained tumor cells. These preliminary data suggest that the dsICC assay is more sensitive than the standard ICC assay in detecting low numbers of tumor cells in patient PBSC collections and, potentially, in $CD34^+$-selected fractions from patients with breast cancer.

In conclusion, there is concern that tumor contamination of breast cancer autoSCGs may contribute to poor posttransplant clinical outcome. As novel therapies evolve that result in additional in vivo tumoricidal effects (eg, Herceptin therapy), and as HDC/autoSCT protocols are refined to target those patients who might derive greater benefit,[29] it is possible that the infusion of tumor cells may take on added significance. Our preliminary studies indicate that tumor contamination of PBSCs can be more accurately detected using our dsICC enhanced assay, and that tumor cell removal of contaminated products is feasible using the approved Isolex 300i CD34 positive selection procedure and the investigational Isolex 300i CD34 positive/negative selection procedure. Clinical studies are ongoing to address this issue.

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