Peripheral blood stem cells (PBSC) are being used as one alternative to autologous marrow rescue for patients with neuroblastoma and other solid malignancies. Some physicians prefer use of PBSC because less risk of tumor contamination is believed to exist. This hypothesis was evaluated by immunocytologic analysis of blood samples and concurrently drawn bone marrow (BM) samples and of PBSC harvests obtained from 31 patients with disseminated neuroblastoma. We found circulating neoplastic cells in 75% of specimens analyzed at diagnosis, in 36% during therapy, and in 14% of PBSC harvests. Tumor cells in blood obtained during therapy did not appear until 3 months after the time of diagnosis. Clearance of circulating neuroblastoma cells was documented after two courses of induction chemotherapy. Six of 13 patients with minimal or no BM disease had positive blood specimens. We conclude that substantial risk of tumor contamination of PB harvests exists and recommend that induction chemotherapy be administered before hematopoietic progenitor cells are collected from blood.

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Contamination of Peripheral Blood Stem Cell Harvests by Circulating Neuroblastoma Cells

By Thomas J. Moss, David G. Sanders, Larry C. Lasky, and Bruce Bostrom

Peripheral blood stem cells (PBSC) are being used with increasing frequency for patients with disseminated solid malignancies. For certain diseases, such as neuroblastoma, the extracted marrow is mixed with monoclonal antibodies (MoAbs) and various cell-removal techniques are used to isolate and remove residual neoplastic cells. Despite improved long-term patient survival, 50% of neuroblastoma patients who undergo ABMT eventually die of tumor relapse, possibly owing to inadequate removal of contaminating neoplastic cells. Because approximately 50% of harvested autologous BM samples contain residual neoplastic cells, other methods of obtaining tumor-free hematopoietic cells have been investigated.

A major purpose of this study was to determine the potential for PBSC harvests to contain tumor cells. To accomplish this, blood specimens obtained at diagnosis, during therapy, and at relapse from 23 patients with disseminated neuroblastoma were evaluated with a highly specific and sensitive immunocytologic assay for circulating tumor cells. To determine the relationship between minimal marrow disease and circulating neuroblastoma cells, 16 of these children also had concurrently drawn BM samples analyzed. Immunocytologic screening was also performed on PBSC harvests from eight additional patients with disseminated neuroblastoma. We found that a substantial risk exists for PBSC harvests to contain contaminating tumor cells irrespective of marrow disease.

MATERIALS AND METHODS

Patient population and samples tested. The diagnostic criteria for neuroblastoma included standard histologic and electronmicroscopic verification of tumor type and/or demonstration of tumor clumps in the BM of patients with elevated urinary catecholamine metabolites. Seventy-four blood and PBSC harvest specimens were analyzed from 31 patients with disseminated neuroblastoma. Thirty-seven blood samples were obtained from 23 children. Twelve of these specimens were evaluated at diagnosis, 23 during therapy, and two at relapse. BM specimens drawn within 1 week of the blood sampling were analyzed from 16 of these children. Twenty paired marrow and blood samples were analyzed in all; 10 at diagnosis, 8 during therapy, and 2 at relapse. An additional eight patients with disseminated neuroblastoma were tested for tumor cells in PBSC harvests. Each child had four to eight harvests performed, and immunocytologic analysis was conducted on 37 specimens.

Preparation of PB and BM. Blood samples were obtained from a central venous catheter or from the antecubital fossa. Two to 5 mL blood was collected in heparinized tubes before processing. Bilateral posterior iliac crest BM aspirates were collected in heparin (100 U/mL) and pooled into a single collection tube. BM and blood samples from referring hospitals arrived within 24 hours, and all specimens were processed within 48 hours of collection.

Blood samples were diluted to 10 mL with Liebowitz L-15 medium (L-15) supplemented with 2% fetal calf serum (FCS, GIBCO, Grand Island, NY). Diluted samples were layered over 3 mL Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and then subjected to density-gradient centrifugation at 600g for 15 minutes. BM samples were similarly diluted with 20 mL Hank’s balanced salt solution (HBSS) with 2% FCS, layered over 15 mL Ficoll-Hypaque, and subjected to density-gradient centrifugation at 600g for 15 minutes. The light-density mononuclear cell fraction from both blood and BM samples was resuspended and washed twice in L-15/FCS at a dilution of nucleated cells 1 x 10^6/mL. Four MoAbs (459, 459, 459, 459) were added to 0.1 mL of resuspended PB and BM.

From the Ahmanson Pediatric Center, Cedars-Sinai Medical Center and UCLA School of Medicine, Los Angeles, CA; and Bone Marrow Transplantation Program, Departments of Pediatrics and Laboratory Medicine and Pathology, University of Minnesota, Minneapolis.

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Address reprint requests to Thomas J. Moss, MD, Cedars-Sinai Medical Center, Department of Pediatrics, Room 3410, 8700 Beverly Blvd, Los Angeles, CA 90048.

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Table 1. Relationship Between Appearance of Circulating Neuroblastoma Cells and Time From Diagnosis

<table>
<thead>
<tr>
<th>Months From Diagnosis</th>
<th>Negative Blood Specimens</th>
<th>Positive Blood</th>
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<tr>
<td>0</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>1–2</td>
<td>4</td>
<td>0</td>
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<td>3–5</td>
<td>6</td>
<td>4</td>
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<td>&gt;5</td>
<td>5</td>
<td>4</td>
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390, HSAN 1, 2, and 126-4) were then added to 1 mL cell suspension at a concentration of 10 μg per antibody/10⁶ cells and incubated at room temperature for 45 minutes. After incubation, the cells were washed twice with L-15/FCS at 300g for 10 minutes, cytocentrifuged onto coverslips (75,000 nucleated cells per coverslip) at 600g for 1 minute, and stored at 4°C for future immunoperoxidase staining.

Collection and preparation of PBSC harvests. PBSC transplantation was performed at the University of Minnesota. PBSC were collected using the Fenwal CS3000. This procedure has been previously described. After collection, 10⁷ WBCs were placed in

Fig 1. Photomicrographs of membrane-labeled neuroblastoma cells identified by immunocytology. (A) A single circulating neuroblastoma cell in a patient during therapy (arrow) (original magnification × 400). (B) Arrow shows a single tumor cell contaminating a PBSC harvest. (Original magnification × 600.)
L-15 and shipped overnight to Cedars-Sinai Medical Center. On arrival, samples were processed according to the methods described for PB and BM.

MoAbs. MoAbs 459, 390 (anti-Thy 1), HSAN 1.2, and 126-4 (anti-GD3) were used in the staining procedure. They were established using the following immunogens: LA-N-1 human neuroblastoma cells (MoAbs 459 and 126-4), SMS-SAN human neuroblastoma cells (MoAbs HSAN 1.2), and human fetal brain (MoAb 330). The immunoglobulin subtype was established by standard methods and identified as IgM for MoAbs 390 and HSAN 1.2 and as IgG for MoAbs 459 and HSAN 1.2. The antibodies were strongly reactive for neuroblastoma, but were unreactive for normal hematopoietic cells. All antibodies were purified using protein A-Sepharose (IgGs) or Sephacryl S-300 (IgMs).

**Immunoperoxidase staining.** Staining was performed using the avidin biotin-peroxidase technique (ABC method) and has been completely described elsewhere. Coverslips were removed from cold storage and sequentially incubated with paraformaldehyde/methanol, 10% goat serum, biotinylated goat anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA), phenylhydrazine (Sigma, St Louis, MO) and avidin-biotin-peroxidase complexes (Vector). Bound peroxidase was visualized with diaminobenzidine (Sigma) followed by Ehrlich’s hematoxylin counterstain. Coverslips were dehydrated with graded ethyl alcohols and xylene and mounted to slides with Cytoseal (Cornwell, Oak Ridge, NJ). Fifteen-minute washes between incubations were performed with complete Dulbecco’s phosphate-buffered saline (PBS).

Positive controls included cultured neuroblastoma cell lines (LA-N-1 and LA-N-5) prepared and stained in the same manner as described above. Cytopreparations of these same cell lines not incubated with MoAbs served as negative controls. Nonreactive normal hematopoietic cells on the patient cytopreparations served as additional internal negative controls for immunoperoxidase staining. Other negative controls included the previously published nonreactive specimens from 12 normal individuals and seven children with other malignancies.

**Analysis for circulating neuroblastoma cells.** Neuroblastoma cells were identified both immunologically (positive immunostaining on at least 50% of the surface membrane) and morphologically (large cells alone or in clusters, high ratio of nuclear to cytoplasmic cells, no clumping of chromatin, and obvious nucleoli). To minimize the possibility of false-positive analysis, detection of at least two tumor cells was required before a blood specimen was recorded as positive. Quantitation of neuroblastoma cells was performed for each specimen by the following criteria: for samples containing tumor cell concentrations greater than 0.5%, four fields of 200 cells each were assessed on at least two slides; for specimens with fewer than 0.5% tumor cells, two–four slides, each containing 20,000 to 50,000 cells, were evaluated.

**RESULTS**

**Detection of circulating neuroblastoma cells.** Circulating tumor cells were detected by immunoperoxidase staining in 9 of 12 blood specimens drawn from patients at the time of diagnosis (Table 1). In three subjects, repeat samples drawn after the second cycle of chemotherapy had converted to negative in all. Eight of 23 specimens obtained during therapy (Table 1) were positive for neuroblastoma cells. Both patients evaluated at relapse had detectable circulating neuroblastoma cells. The concentration of tumor cells ranged from 2 to 1,000/100,000 mononuclear cells at diagnosis (median 35 cells), and 2 to 500/100,000 mononuclear cells during therapy (median 23 cells). Figure 1 shows an example of immunoperoxidase-stained tumor cells from a positive blood specimen.

**Blood specimen contamination relative to degree of marrow disease.** The tumor cell concentration in BM ranged from 0 to 95,000/100,000 mononuclear cells. Seven marrow specimens contained more than 35% neuroblastoma cells (five were obtained at diagnosis, and two were obtained during therapy). The blood specimens from these same patients also contained circulating neuroblasts. Six additional BM samples contained less than 0.5% neuroblastoma cells (four were obtained at diagnosis, one was obtained during therapy, and one was obtained at relapse). Four of the six corresponding blood specimens from these patients were positive for tumor cells (Table 2). Seven BM samples showed no metastatic disease (one obtained at diagnosis and six obtained during therapy). Two blood specimens both obtained during therapy from different patients were positive for circulating neuroblasts (Table 2).

**Detection of neuroblastoma cells in PBSC harvests.** Eight patients had immunocytologic analysis of 37 PBSC harvests. Six had minimal or no BM disease by immunocytology (less than 0.5% total tumor cells; Table 3). Two children, both with minimal marrow disease, had neuroblastoma cells contaminating five harvests (Fig 1 and Table 3). One other patient had a suspicious specimen that contained one cell with malignant morphology and positive membrane staining. The harvests from the remaining five were free of tumor cells.

**DISCUSSION**

Autologous PBSC transplantation is a viable alternative to BM rescue for patients with disseminated cancer. Recently, several reports documented the success of this therapy in patients with small cell lung carcinoma, breast cancer, and neuroblastoma. Current interest may reflect a number of advantages that PBSC rescue offers as compared with use of ABMT. These include ability to collect PBSC without general anesthesia, less discomfort for the patient, rapid

| Table 2. Relationship Between Minimal or No BM Disease and Circulating Neuroblastoma Cells |
|---|---|---|---|---|
| Case | Time Sample Obtained | BM* | Blood* |
| 1 | D | 0 | 0 |
| 2 | DT | 0 | 0 |
| 3 | DT | 0 | 0 |
| 4 | DT | 0 | 0 |
| 5 | DT | 0 | 2 |
| 6 | DT | 0 | 2 |
| 7 | DT | 0 | 30 |
| 8 | D | 27 | 0 |
| 9 | D | 80 | 0 |
| 10 | D | 17 | 2 |
| 11 | D | 80 | 5 |
| 12 | DT | 76 | 45 |
| 13 | R | 500 | 2 |

*Neuroblastoma cells per 100,000 mononuclear cells.
hematopoietic recovery after marrow-ablative chemoradiotherapy, and potential for reduced risk of tumor cell contamination. Moreover, PBSC transplantation can be performed when autologous BM rescue is not feasible owing to residual marrow disease or owing to the inability to harvest adequate hematopoietic cells because of fibrosis from pelvic irradiation. The precise risk of tumor contamination in autologous PB harvests is not currently known. Circulating neoplastic cells have been shown to be detectable at diagnosis and at relapse, however, and two preliminary reports demonstrated the presence of circulating neuroblastoma cells in blood samples obtained during therapy and in PBSC harvests. These studies did not determine whether the appearance of such cells is common, however, or whether the quantity of cells is significant. In addition, correlation of circulating neoplastic cells to the degree of marrow involvement has not been determined.

In this study, we used sensitive immunocytologic techniques to detect circulating neuroblastoma cells in patients with disseminated disease at diagnosis, during therapy, at relapse, and in PBSC harvests. The highest frequency of positive blood specimens occurred at diagnosis. Circulating neuroblastoma cells were also commonly present in specimens obtained during therapy and in PBSC harvests. Although only 5 of 37 harvests contained neoplastic cells, this represents tumor contamination for two of eight patients (25%). The data indicate that the risk for contamination in autologous PBSC transplantation is significant, and further immunocytologic studies should be performed for patients with other solid malignancies who undergo this treatment.

Because tumor contamination of PBSC harvests is substantial, determining the optimal time for PBSC collection would be beneficial. Our data regarding blood samples analyzed during therapy indicate that the risk of circulating neuroblastoma cells appears to increase markedly beginning 3 months after diagnosis. Of the patients with specimens analyzed at diagnosis and during therapy, three children who were negative after one or two courses of chemotherapy had positive initial samples. These preliminary data suggest that PBSC harvests may be tumor-free after the second cycle of induction chemotherapy. Further studies may determine if increased delays in collecting stem cells lead to a higher incidence of tumor contamination.

Our observations show that neuroblastoma cells can circulate even when there is scant marrow disease. Three of five patients with minimal marrow involvement and two patients with no marrow involvement had positive blood specimens. Moreover, the two patients with tumor cell contamination of PBSC harvests also had minimal marrow disease. We conclude that the level of marrow disease cannot be used as a reliable index for circulating tumor cells and that rigorous testing of PBSC harvests for contamination should be performed routinely.

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REFERENCES


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TJ Moss, DG Sanders, LC Lasky and B Bostrom