Clonogenicity of Circulating Neuroblastoma Cells: Implications Regarding Peripheral Blood Stem Cell Transplantation

By Thomas J. Moss, Mitchell Cairo, Victor M. Santana, Joel Weinthal, Carole Hurvitz, and Bruce Bostrom

Peripheral blood stem cells (PBSCs) are being used as an alternative to autologous marrow rescue for hematopoietic reconstitution after high-dose chemotherapy in patients with neuroblastoma and other solid malignancies. Use of PBSCs is preferred by some because of the belief that there is less risk of tumor contamination. Because tumor stem cell contamination is thought to be one contributing cause of relapse after myeloablative therapy and autologous reconstitution, we examined the potential risk of reinfecting circulating neuroblastoma cells by in vitro evaluation of their clonogenicity. Immunocytologic and tumor cell clonogenic analyses were performed on 74 blood specimens obtained from 56 children with advanced-stage neuroblastoma. Concurrently drawn bone marrow specimens were evaluated in 30 instances. Circulating neoplastic cells were detected in 19 of 74 (26%) for all specimens and by immunologic techniques.

PERIPHERAL BLOOD stem cells (PBSCs) are being used with increasing frequency to enhance hematopoietic reconstitution after myeloablative therapy for a number of disseminated malignancies.1-3 To rescue the patient from lethal chemotherapy, PBSCs are infused either alone or in conjunction with marrow.4,10 PBSCs are also used to limit the neutropenic phase of patients undergoing conventional chemotherapy. Conditions currently being treated in this way include lymphoma, leukemia, myeloma, neuroblastoma, breast carcinoma, and ovarian carcinoma, as well as a number of other solid malignancies.1-3,5 PBSC reinfusion is gaining popularity because (1) the cells are easy to collect; (2) rapid hematopoietic recovery is achievable; and (3) PBSC collections may circumvent tumor contamination likely to be present in the bone marrow and are supposedly free of tumor contamination.

It has recently been shown that circulating malignant cells can contaminate PBSC harvests from patients with neuroblastoma, breast carcinoma, Hodgkin's lymphoma, and non-Hodgkin's lymphoma.11-14 No studies have been reported regarding their clonogenicity; thus, the clinical significance of reinfecting neoplastic cells into the patient is unknown.

Because tumor stem cells are thought to be responsible for the relapse of disease,15 we examined the potential risk of reinfecting circulating neuroblastoma cells by evaluating their clonogenic growth in vitro. We analyzed mononuclear cells isolated from blood specimens obtained at diagnosis and during therapy from 46 children with disseminated neuroblastoma in a soft agar tumor colony assay. Blood specimens were also evaluated with a highly sensitive and specific immunocytologic assay to determine the concentration of circulating malignant cells.

MATERIALS AND METHODS

Patient population and samples tested. The study was conducted as a multicenter trial. The diagnosis of neuroblastoma was established by standard histologic and electron microscopic evaluation and/or demonstration of tumor cells in the bone marrow of patients with elevated urinary catecholamines. From October 1990 to April 1993, 74 blood specimens from 56 patients with disseminated neuroblastoma (48 with stage IV, 7 with stage III, and 1 with recurrent stage II disease) were analyzed. There were 19 females and 37 males analyzed with an age range of 5 to 161 months (median, 33 months). Nine blood specimens were obtained at the time of diagnosis, 27 samples were analyzed 1 to 7 months after diagnosis, and 38 samples were drawn and evaluated greater than 7 months after diagnosis. Concurrently drawn bone marrows were available for immunocytologic analysis in 30 cases (6 at diagnosis, 13 at 1 to 7 months, and 11 after 7 months).

Monoclonal antibodies (MoAbs). Neuroblastoma cells are known to express a number of surface antigens16-19 not expressed by normal hematopoietic cells. A panel of four antineuroblastoma MoAbs was used for tumor cell detection and colony verification by immunostaining target antigens. The specific antibodies and corresponding antigens are shown in Table 1.18,20

Cell lines. Human neuroblastoma cell lines LA-N-1 and LA-N-5 were cultured at 37°C in Liebowitz L-15 medium (L-15) supplemented with 15% heat-inactivated fetal bovine serum (FBS), glutamine (100 mmol/L), and penicillin (1,000,000 U/L). Cells were removed for immunostaining when the flasks were confluent.

Preparation of peripheral blood and bone marrow. Methods for cell preparation and immunostaining have been previously reported.21,22 Briefly, blood was obtained from a central venous catheter or from the antecubital fossa and bone marrow was drawn from the posterior iliac crests. Approximately 3 to 5 mL of blood was collected in heparinized tubes before processing. Samples from the referring hospitals arrived within 24 hours and all specimens were processed within 48 hours of collection.

Upon arrival in the laboratory, the blood was diluted to 10 mL with L-15 supplemented with 10% FBS (GIBCO, Grand Island, NY). The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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Submitted April 1, 1993; accepted January 20, 1994.

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Diluted samples were layered over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and then subjected to density gradient centrifugation. The mononuclear cell fraction was tested for viability (Trypan blue exclusion), counted by a hemacytometer, and resuspended in L-15/FBS. Cell lines were removed by trypsin EDTA and washed with L-15/FBS. The four MoAb mixture was then added to 1 mL of the cell suspension (blood or cell lines) and incubated at room temperature. After incubation, the cells were washed, cytokeratin-fuged onto coverslips (75,000 nucleated cells per coverslip), and stored at 4°C for future immunoperoxidase staining.

Immunostaining. Cell lines and patient specimens were removed from storage and tested for immunoreactivity. Immunostaining was accomplished using the avidin-biotin-peroxidase technique (ABC method). Coverslips were incubated in a paraformaldehyde/methanol fixative, followed by normal goat serum, biotinylated goat antimouse Ig (Vector Laboratories, Burlingame, CA), phenylhydrazine (Sigma, St Louis, MO) to block endogenous peroxidase, and then avidin-biotin-peroxidase complexes (Vector Laboratories). Visualization of bound peroxidase was made possible by incubation with diaminobenzidine/H2O2 (diluted in citrate buffer, 0.3% H2O2) followed by Ehrlich’s hematoxylin counterstain. Coverslips were dehydrated with ethyl alcohol and Xylene and attached to slides with Cytoseal.

Analysis for circulating neuroblastoma cells. Neuroblastoma cells were identified both immunologically (positive immunostaining for the cell surface membrane) and morphologically (large cells alone or in clusters with high nuclear to cytoplasmic ratio, with no clumping of chromatin, and with prominent nuclei). 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%, 4 fields of 200 cells each were assessed on at least 2 slides; for specimens with fewer than 0.5% tumor cells, at least 4 slides, each containing 50,000 cells, were evaluated.

Neuroblastoma tumor colony assay. Ten million mononuclear cells from blood samples were added to 3 mL of double-strength Iscove’s modified Dulbecco’s medium (IMDM; Sigma) and combined with 2 mL of heat-inactivated FBS (GIBCO). The media cell mixture was added to 5 mL of 0.6% sea-plaque agar (Sigma) and 1×10^6 cells (1 mL) were placed in 35-mm petri dishes containing 10% growth factors (20 U recombinant human granulocyte-macrophage growth factor [Collaborative Research, Boston, MA]). Cells incubated without growth factors served as negative controls. All cultures were plated in triplicate. Plates were incubated for 10 days in a 100% humified chamber at 37°C with 5% CO2. Tumor colonies were defined as aggregates of 50 or more cells. They were identified and enumerated by phase microscopy.
and in 4 of 39 taken after 7 months. Tumor cell concentrations ranged from 1 to 160 per $10^5$ mononuclear cells with a median of 5 per $10^7$ (Table 2). An example of circulating neuroblastoma cells identified by immunoperoxidase staining is shown in Fig 1.

Clonogenic analysis of blood specimens. Three of the 74 samples could not be evaluable because of bacterial contamination. Tumor colonies grew from 13 specimens, from 5 samples at diagnosis, from 5 samples at 1 to 7 months, and from 3 samples after 7 months. The number of colonies ranged from 1 to 40 per plate, with a median of 3 (Table 2). Of the 19 samples positive by immunocytologic analysis, 10 produced neuroblastoma colonies; of the 53 specimens negative by immunocytology, 3 grew neoplastic colonies. Examples of tumor colonies are shown in Fig 2.

Tumor cell plating efficiency could be calculated for 10 specimens. Plating efficiencies ranged from 0.9% to 50%, with a median of 6% (Table 2). There was no significant difference in the efficiency of specimens obtained at diagnosis versus those obtained later (medians of both, 6.0%).

Immunocytologic evaluation of bone marrow. Concurrently drawn bone marrow specimens were evaluated for tumor cell contamination in 30 instances. Metastatic neuroblastoma cells were detected in 5 of 6 samples obtained at diagnosis, in 4 of 13 analyzed at 1 to 7 months, and in 3 of 11 drawn after 7 months (Table 3). The tumor concentration ranged from 2 to 80,000 per $10^7$ mononuclear cells, with a median of 43 per $10^7$. Of the 11 positive blood samples, 9 concurrent marrows contained neuroblastoma cells; of the 19 negative blood specimens, 3 concurrent marrows had metastatic disease (Table 3).

DISCUSSION

PBSC reinfusion is one way of using a 'supposedly' tumor-free marrow stem cell product for hematopoietic re-

![Image](Image)
study indicate that circulating neuroblastoma cells in patients may be particularly aggressive. The reported plating efficiency of primary and metastatic tumors in agar culture is substantially lower (<0.2%). In addition, previous reports on the clonogenicity of neuroblastoma cell lines, obtained from clinically virulent tumors, showed a plating efficiency ranging from 1.0% to 27%, with a median of only 2.4%. Thus, circulating neuroblastoma cells may have a greater growth potential than primary and metastatic neoplastic cells and may be similar to that observed in cell lines. However, because the number of samples and colonies per specimen are small, further evaluation is necessary before definitive conclusions can be made.

The use of PBSC collections is becoming a major therapeutic modality after conventional and myeloablative therapy. However, our data suggest that enthusiasm for this technique should be tempered with caution in patients with neuroblastoma. Currently, some clinical trials are being performed without adequate testing for tumor cell contamination. Our observations are in accord with previous reports that demonstrate the regularity with which neoplastic cells circulate and contaminate PBSC harvests. Moreover, we have shown these cells to be highly clonogenic and, if reinfused, to carry the potential to re-establish the malignant process.

Based on our observations, we recommend that future prospective clinical trials using PBSC collections address the issue of tumor cell contamination. If tumor contamination is detected, some form of purging procedure (MoAb or chemotherapeutic) of these specimens may be warranted.

**REFERENCES**


### Table 3. Comparison of Tumor Contamination in Blood and Bone Marrow Samples

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Time Sample Obtained (Mo after Diagnosis)</th>
<th>Blood Tumor Concentration*</th>
<th>Marrow Tumor Concentration*</th>
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<tbody>
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<tr>
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<td>15</td>
<td>0</td>
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</table>

Abbreviation: D, diagnosis.

* Neuroblastoma cells per 100,000 mononuclear cells.


Clonogenicity of circulating neuroblastoma cells: implications regarding peripheral blood stem cell transplantation

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