Eradication of Neuroblastoma Cells in Vitro by Monoclonal Antibody and Human Complement: Method for Purging Autologous Bone Marrow

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ABSTRACT

We describe an in vitro method which is useful for purging autologous bone marrow of neuroblastoma cells. The method utilizes a single murine monoclonal antibody 3G6 (an immunoglobulin Mκ) which we have previously developed against the ganglioside GD2, undiluted human complement; and unfractionated whole bone marrow at 1 x 10^7 nucleated cells/ml. Tumor cell clonogenic assays, Hoechst 33342 fluorescent nuclear stain, and trypan blue viability stain methods were used to assay cytotoxicity. This complement-mediated cytotoxicity technique killed 99.9-100% of neuroblastoma cell lines NMB-7, LAN-1, LAN-5, and IMR-6, while normal marrow precursor cells were not detectably damaged. The presence of normal bone marrow did not inhibit the human complement-mediated cytotoxicity. Applying the cytotoxicity method to whole unseparated bone marrow demonstrated killing of seeded neuroblastoma cells, with no gross hemolysis or cell clumping. The method did not require expensive special equipment, use of animal complement sera, or prior fractionation of the bone marrow. The average marrow nucleated cell recovery was 95%. These studies indicate that in vitro purging of autologous marrow infiltrated with neuroblastoma with monoclonal antibody 3G6 and human complement is both technically feasible and effective in eradicating residual tumor while preserving bone marrow stem cells.

INTRODUCTION

Over 65% of children with neuroblastoma present with disseminated disease at diagnosis and are virtually incurable with conventional therapy. Complete or partial remission periods can be achieved with chemotherapy together with irradiation and surgery (1, 2). Bone marrow transplantation coupled with intense chemotherapy is a new, encouraging treatment modality for these patients (3-8). Unfortunately more than half of the patients do not have a histocompatible sibling donor. In this setting, autologous bone marrow transplantation (9) has been utilized to circumvent marrow toxicity after the administration of otherwise lethal doses of chemotherapy and irradiation. However, in childhood NB, as in many other malignant diseases, the bone marrow is frequently infiltrated with tumor cells at the time of diagnosis, and occult tumor cells may be detectable in the marrow by new, more sensitive methods (10) even during apparent remission. This contamination of the bone marrow by tumor limits the usefulness of autologous bone marrow transplantation.

To overcome the difficulty arising in autologous marrow contaminated by malignant cells, techniques are being developed to remove tumor cells from the harvested bone marrow in vitro, before cryopreservation and/or reinfusion into the patient (11).

We now describe an effective method to purge bone marrow of microscopic NB in vitro by using one potent, specific Mab against NB and human C' serum. The method does not require expensive special equipment, use of animal C', or fractionation of the bone marrow.

MATERIALS AND METHODS

Neuroblastoma Cell Lines. The NB cell lines LAN-1 and LAN-5 were kindly provided by Dr. Robert Seeger, University of California at Los Angeles, Los Angeles, CA; IMR-6 and NMB-7 by Dr. Shuen-Kuei Liao, McMaster University, Hamilton, Ontario, Canada. IMR-32 cell line was purchased from American Type Culture Collection, Bethesda, MD. Cells were grown in monolayers in RPMI 1640 with 15% heat-inactivated fetal calf serum from Hyclone (Logan, UT), containing penicillin (100 units/ml), streptomycin (100 µg/ml), and 2 µM glutamine.

Normal Bone Marrow Cells. After obtaining informed consent, bone marrow was aspirated from the posterior iliac crest of healthy volunteer donors into preservative-free heparin. Fresh marrow was used for analysis.

Monoclonal Antibodies against Neuroblastoma. Murine Mabs 3A7, 3G6, and 3F8 were produced by the ascites method. The IgM antibodies 3A7 and 3G6 are IgM κ antibodies, and 3F8 is an IgG3 antibody. These Mabs are very potent and specific and do not bind to nonneuronal normal human tissues, including normal bone marrow (12).

We next describe an effective method to purify bone marrow of microscopic NB in vitro by using one potent, specific Mab against NB and human C' serum. The method does not require expensive special equipment, use of animal C', or fractionation of the bone marrow.
were then pelleted at high speed (2000 rpm) for 10 min at 4°C. The plasma was removed and allowed to clot at room temperature in the presence of glass. The serum was separated and frozen (within 1.5 h of blood drawing) and kept at −70°C until used. The heat inactivation of C’ serum (for control purposes) was performed by heating to 56°C for 30 min.

Complement-mediated Cytotoxicity. NB cells were washed in PBS with 0.5% of human serum albumin (Sigma). Fifty μl of target cell suspension, with 1–3 x 10^6 cells/ml, were incubated in round-bottomed microtiter wells of a 96-well plate with 50 μl of different dilutions of Mab at 4°C for 45 min. All samples were run in duplicate. The microtiter plate was then centrifuged at 1000 rpm for 5 min, the supernatant was removed, and 50 μl of C’ serum per well were added. Incubation was continued for 60 min at 37°C, after which the microtiter plate was put on ice, and the cytolysis in the wells was assessed.

C’-mediated cytotoxicity was also performed in sterile test tube sets using 1.0–1.5 ml of cell suspension and equal volumes of Mab (0.5 mg/ml) and C’ (undiluted human serum) each. The sterile procedure with these larger volumes was used for clonogenic tumor cell assays and bone marrow colony assays.

Cell Viability Test with Trypan Blue Dye Exclusion. At the end of the analysis, a sample of cell suspension was stained with an equal volume of 0.4% trypan blue, and the percentage of viable cells was enumerated in a hemocytometer (14). The mean viabilities of duplicate samples were calculated. The percentage of specific kill due to Mab was determined according to the following formula (15):

\[
\text{% of kill} = \frac{\% \text{ of viable cells in C’ control} - \% \text{ of viable cells in test sample}}{\% \text{ of viable cells in C’ control}} \times 100\%
\]

In the formula above, the control sample was run without Mab, but with the same source, dilution, and amount of active C’ serum as the test sample.

In Vitro Assay for Hematopoietic Colony Growth. After obtaining informed consent, bone marrow was aspirated from the posterior iliac crest of normal volunteers, as described above. Low density mononuclear bone marrow cells were isolated by Ficoll-Hypaque density centrifugation (16). The cells were incubated in the presence of Mab and human C’ in the cytotoxicity procedure as described above, washed to remove C’, and resuspended in RPMI 1640 culture medium with 15% fetal calf serum at 2 x 10^6 cells/ml. All incubations were performed in duplicate. Each cell suspension was assayed for erythroid, myeloid, and mixed hematopoietic progenitors using established methods (16-19). Briefly, the colony formation by these cells was measured in 0.3% agar as previously described (16, 20) in triplicate 1-ml aliquots in 35-mm Lux culture dishes using a final cell concentration of 1 x 10^4 cells/ml. The culture medium was modified to include 30% fetal calf serum, Iscove’s modified Dulbecco’s medium supplemented with 2 x 10^{-4} M α-thioglycerol, 10 mm L-asparagine, 2 mm glutamine, and 1% bovine serum albumin (17, 18). Erythroid colony formation was stimulated by the presence of erythropoietin (0.5 units/ml) (Step 3; Connaught Laboratories), and granulocyte/macrophage colonies were stimulated by 10% phytohemagglutinin-leukocyte-conditioned medium (16). Cultures were incubated at 37°C in 5% CO2 On Day 14, the colonies and bursts were counted (16-18) using an AO sterenoscope. Enumeration of colony types was performed following fixation of the agar culture on a glass slide, as previously described (16), and staining with benzidine (17), followed by a counter stain with Mayer’s hematoxylin.

This culture system was designed to optimize the growth of granulocyte/macrophage colonies and burst-forming units-erythroid. Large, individual colony-forming units-erythroid were scored separately. In preliminary experiments, the amount of erythropoietin and phytohemagglutinin-leukocyte-conditioned medium used produced erythroid and myeloid growth which was at the upper end of the linear portion of the dose-response curve for colony formation relative to stimulant added. We chose these conditions to maximize the chance of detecting damage to the hematopoietic stem cells. It is known, for instance, that the inhibition of colony formation by interferon can be overcome by excess amounts of colony-stimulating activity (20). By choosing adequate but not excessive amounts of stimulatory factors, we attempted to increase the sensitivity of detecting stem cell damage.

Seeding Experiments and Clonogenic Assays of Neuroblastoma Line Cells. Normal bone marrow low density mononuclear cells were collected following Ficoll-Hypaque density centrifugation, washed, and resuspended at 2 x 10^6 cells/ml. The marrow cell suspension was seeded with 10%, 1%, and 0.1% of NB cells (NMB-7). The cytotoxicity procedure was performed with Mab 3G6 at 0.5 mg/ml and undiluted human C’ serum as described above, after which the cell counts and viabilities by trypan blue dye exclusion were assessed.

For clonogenic assay of viable NB cells, the test samples were suspended in culture medium of RPMI 1640 with 15% fetal calf serum and fresh bone marrow mononuclear cells, adjusted to a concentration of 3 x 10^5 cells/ml. The cell suspensions were plated in duplicate 10-ml aliquots on culture plates and incubated at 37°C. After 7 days of incubation, the culture medium was removed, and the plates were washed carefully with PBS, air-dried, and stained with Wright’s stain. The NB cell colonies were counted by a Biotran II automated colony counter (New Brunswick Scientific Co., Edison, NJ). Under conditions of this clonogenic assay, normal hematopoietic colony-forming cells were not expected to form colonies, because no colony-stimulating activity or erythropoietin was provided.

Seeding Experiments by Using the Hoechst 33342 Fluorescent Nuclear Stain. Another approach to examine the effectiveness of our cytotoxicity procedure on NB cells seeded in bone marrow was the use of Hoechst 33342 (H342) supravital DNA stain (21). Stain H342 does not affect cell viability. It gives bright nuclear fluorescence and does not leak to adjacent, nonstained bystander cells. The H342 fluorescence is quenched by trypan blue dye, permitting detection by fluorescence of only viable cells (21).

The NB cells (NMB-7) were prestained before the seeding experiment as follows. The NMB-7 cell suspension of 1 x 10^6 cells/ml was incubated with 1 μg of H342 per ml in RPMI 1640 and 15% fetal calf serum for 1 h at 37°C, washed once, reincubated in RPMI 1640 with 15% fetal calf serum for 2 h to leak out cytoplasmic dye, and finally again washed. The H342-stained NMB-7 cells were seeded in normal bone marrow at different concentrations (10, 5, 1, 0.1%). We used either low density mononuclear bone marrow cells separated by Ficoll-Hypaque density centrifugation, in concentrations of 2 x 10^5 cells/ml and 1 x 10^6 cells/ml, or whole, unfractonated bone marrow, adjusted to 1 x 10^6 nucleated cells/ml by adding Hanks’ solution.

The cytotoxicity procedure was performed with Mab 3G6 (0.5 mg/ml) and undiluted human C’ as described above. The low density mononuclear bone marrow cell and NMB-7 cell mixtures were incubated in duplicate 50-μl samples on a microtiter plate, while the unfractionated, whole bone marrow mixed with NMB-7 cells was incubated as 2-ml samples. The percentage of NMB-7 cells killed was compared to a pure NMB-7 suspension tested at a concentration of 2 x 10^6 cells/ml. The untreated control cell suspensions were kept at room temperature in the dark. The treated suspensions were exposed to Mab and human C’ in the cytotoxicity procedure described above. To facilitate detection of fluorescent cells, RBC were removed from the samples after the procedure by Ficoll-Hypaque centrifugation.

Finally the samples were counterstained with 0.4% trypan blue and examined in a hemocytometer using a Leitz fluorescence microscope. The microscope was equipped with a B-2 filter block with excitation within the violet range (350–410 nm), at which setting bright fluorescence of H342 was obtained. The fluorescent cells (viable tumor cells), trypan blue-positive cells (dead cells), and the total cell counts were enumerated.
RESULTS

Complement-mediated Cytotoxicity by Monoclonal Antibodies against Neuroblastoma. The three Mabs 3A7, 3G6, and 3F8 all fixed C’ and lysed target NB cells. The C’ fixation was not species specific; guinea pig, rabbit and human C’s were all cytolytic. Baby rabbit C’ caused a high nonspecific kill and was inferior to human and guinea pig C’s in specific kill (Chart 1). Human and guinea pig C’s were about equally effective in specific kill, and both had low nonspecific kill (Chart 1).

The C’ effect of human serum was abolished by heat inactivation. Dilution of human C’ in heat-inactivated serum, instead of complement buffer, diminished the nonspecific kill to negligible levels (Chart 1). Human C’ effect was dose dependent, diminishing drastically after a dilution higher than 1:4. We compared different human C’ donor sources and found no major differences in C’ activity among six healthy individuals studied (Chart 2).

Kill of Different Neuroblastoma Cell Lines by Complement-mediated Cytotoxicity. We studied 5 different NB lines (NMB-7, LAN-1, LAN-5, IMR-6, IMR-32) by using the three Mabs (3A7, 3G6, 3F8) and human or guinea pig C’. The percentage of specific kill is illustrated in Chart 3. The best percentage of specific kill was achieved with Mab 3G6 and undiluted human C’; the percentage of specific kill was 99.9-100% in four cell lines and 98% in one (IMR-32) (Chart 3).

Effect of Complement-mediated Cytotoxicity Procedure on Normal Bone Marrow Colony-forming Cells. Normal bone marrow low density mononuclear cells were incubated with Mab 3A7 or 3G6 and human C’, and the colony growth of bone marrow cells in culture was subsequently assayed. The human C’ serum was selected to be ABO compatible with the bone marrow cells used in the analysis. Table 1 demonstrates that bone marrow colony formation was unaffected by incubation in the presence of Mab and human C’. Specifically, the number of total colonies and the numbers of erythroid, myeloid, and mixed colonies present in the bone marrow samples after incubation in the cytotoxicity procedure were not reduced.

Seeding Experiments and Clonogenic Assays. The cytotoxicity procedure was performed on samples of normal bone marrow seeded with NB cells (NMB-7) at different concentrations, and the clonogenic assay was used subsequently to estimate the number of residual viable tumor cells. In the untreated cell mixtures, the number of tumor colonies formed increased with the number of tumor cells plated, regardless of the seeding percentage. When more than $3 \times 10^6$ tumor cells were plated, confluence of tumor colonies and a tendency to overgrowth exceeded the counting capacity of the automated colony counter. No growth of normal hematopoietic colony-forming cells was observed, because no source of colony-stimulating activity was provided. In contrast to the untreated control samples which showed tumor colony growth (Table 2), when the cell mixture were treated with Mab 3G6 and human C’ in the cytotoxicity procedure, no tumor colony growth was observed (Table 2). Kill of tumor cells which were seeded in bone marrow...
PURGING BONE MARROW OF NEUROBLASTOMA

could be reproducibly demonstrated in four subsequent experiments. The total percentage of cells killed in the seeding series, evaluated by trypan blue dye exclusion, closely agreed with the percentage of NB cells initially seeded in the marrow.

Seeding Experiments and Use of Hoechst Nuclear Stain (H342). The percentage of fluorescent cells enumerated by fluorescence microscopy corresponded to the initial seeding percentages in the control samples (Table 3), even with seeding into whole bone marrow followed by FicolHypaque density centrifugation (Table 3). The minor differences found probably were due to difficulties of NB cells to remain in single cell suspensions. In contrast to control samples, only rare viable tumor cells, if any, were detected in samples treated by the cytotoxicity procedure (Table 3). Occasional resistant tumor cells were present in the 5% and 10% seedings, but not in the 1% or 0.1%. The percentage of dead cells in the test samples was essentially the same as the percentage of fluorescent cells in the control samples (Chart 4).

The accuracy of detecting viable tumor cells by the nuclear fluorescence method depends on the total number of cells examined. In our experiments, the detection level was 1 in 3,000-10,000 at the initial setting of 1 x 10^7 cells/ml and 1 in 500-800 at initial 2 x 10^6 cells/ml. Fluorescent cells were extremely bright and easily recognized.

When the cytotoxicity procedure was applied to whole unseparated bone marrow seeded with NB cells, we observed no gross hemolysis and neither macroscopic nor microscopic cell clumping. The marrow nucleated cell recovery after the cytotoxicity procedure, without FicolHypaque density centrifugation, was 94.7 ± 8.8% (SD) in 16 samples studied.

DISCUSSION

We have developed a method for the in vitro purging of autologous bone marrow of NB cells, which utilizes a single Mab, human C' serum, and unfractionated whole bone marrow. Our data indicate that this method can produce effective cytolysis of NB cells; usually 99.9-100% tumor cell kill was achieved with different NB cell lines.

In Table 4 we compare our method with techniques reported...
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Table 1

Bone marrow hematopoietic colony growth after C'-mediated cytotoxicity with Mabs 3G6 or 3A7 and undiluted human C'.

Data from one of five representative experiments are shown.

<table>
<thead>
<tr>
<th>Test samples with monoclonal antibody</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>3G6 + human C'</td>
<td>3A7 + human C'</td>
</tr>
<tr>
<td>Viability by trypan blue (%)</td>
<td>98.5 ± 0.1 ± 0.1a</td>
</tr>
<tr>
<td>Total no. of colonies/10⁵ marrow cells</td>
<td>196 ± 8</td>
</tr>
<tr>
<td>BFU-E (%)</td>
<td>6</td>
</tr>
<tr>
<td>CFU-E (%)</td>
<td>19</td>
</tr>
<tr>
<td>CFU-G (%)</td>
<td>43</td>
</tr>
<tr>
<td>CFU-M (%)</td>
<td>27</td>
</tr>
<tr>
<td>CFU-GEM (%)</td>
<td>5</td>
</tr>
</tbody>
</table>

* Mean ± SE of six plates.

Table 2

Tumor cell growth in clonogenic assay after cytotoxicity procedure on mixtures of NB and bone marrow cells.

NMB-7 cells were seeded into normal bone marrow mononuclear cells separated by Ficoll-Hypaque density centrifugation. C'-mediated cytotoxicity was performed with Mab 3G6 (250 ng/10⁶ cells) and undiluted human C' at a cell suspension of 2 x 10⁷ total cells/ml.

<table>
<thead>
<tr>
<th>NMB-7 seeded in bone marrow only</th>
<th>Bone marrow only</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of tumor cells plated</td>
<td></td>
</tr>
<tr>
<td>Treated + nontreated</td>
<td>3 x 10⁶</td>
</tr>
<tr>
<td>3 x 10⁷</td>
<td>3 x 10⁷</td>
</tr>
<tr>
<td>3 x 10⁸</td>
<td>3 x 10⁸</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No. of tumor colonies on Day 7</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>635</td>
</tr>
<tr>
<td>612</td>
<td>169</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>NMB-7 seeded in normal bone marrow</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>10%</td>
</tr>
<tr>
<td>5%</td>
<td>1%</td>
</tr>
<tr>
<td>0.1%</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

Table 3

Complement-mediated cytotoxicity of neuroblastoma cells mixed with bone marrow.

NMB-7 cells, prestained with Hoechst fluorescent nuclear stain, were seeded in normal bone marrow and treated with Mab 3G6 and human C'. The percentages of fluorescent cells (percentages of viable tumor cells) after the procedure are given.

<table>
<thead>
<tr>
<th>NMB-7 seeded in normal bone marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
</tr>
<tr>
<td>10%</td>
</tr>
<tr>
<td>5%</td>
</tr>
<tr>
<td>1%</td>
</tr>
<tr>
<td>0.1%</td>
</tr>
</tbody>
</table>

Seeding in marrow mononuclear cells separated by Ficoll-Hypaque density centrifugation.

Cells (2 x 10⁶/ml) Mab + C' Untreated 100 13.6 6.2 2.0 0.5

Cells (1 x 10⁶/ml) Mab + C' Untreated 100 0.16 0.02 0 0

Seeding in whole, unseparated bone marrow with 1 x 10⁷ nucleated cells/ml Mab + C' Untreated 0.3 0.01 0 0 0

% TUMOR CELLS SEED IN MARROW

Chart 4. Cytotoxicity of NMB-7 cells by Mab 3G6 and human C' when seeded in bone marrow mononuclear cell suspension of 1 x 10⁷ cells/ml in concentrations of 0.1%, 1%, 5%, and 10%. The NMB-7 cells were prestained with Hoechst 33342 fluorescent nuclear stain, and the fluorescence of residual viable tumor cells after the procedure was assessed after counterstaining with trypan blue. Left columns, untreated samples; right columns, samples treated by the cytotoxicity procedure. Additional control samples consisting of bone marrow cells alone had 2.5% of dead cells at the end of the procedure. This "background level" of bone marrow cell death was subtracted from the dead cell percentages of the treated samples (right columns). Accordingly, the percentage of dead cells in the chart indicates dead tumor cells. Bars, SD.

by others (22–28). Our method of tumor cell removal is very effective, and our percentage of tumor cell kill is comparable to that reported by Treleaven et al. (26) using a 12-Mab panel and magnetic beads, a method which appears to be one of the best approaches yet reported. In soybean lectin agglutination (22–23) or physical cell separation (24) methods, the tumor cell removal is less complete. The effectiveness of our method probably is due to the properties of our monoclonal antibody; Mab 3G6 recognizes the tumor-specific glycolipid antigen G02 which is present in over 98% of NB cells (12). We do not know how many tumor cells can be reinfused safely, or whether the few residual viable cells by trypan blue and Hoechst stain methods are clonogenic. Our present data suggest that the 0.1% of tumor cells that occasionally remain viable after the cytotoxicity procedure might not be truly viable, since no growth was observed in the clonogenic tumor cell assays. If it is desired to improve our usual 99.9–100% tumor cell kill, one might consider adding Mabs that bind to antigenic determinants other than G02 on tumor cells, to be used either concurrently as a panel, or sequentially, to treat bone marrow.

Compared to other reported systems (Table 4), our method also seems to result in superior recovery of nucleated marrow...
C'-mediated T-cell depletion of allogeneic marrow in attempts to prevent graft-versus-host disease (29, 30) or for purging leukemic bone marrow cells (average, 95%). This is of clinical importance, when autologous bone marrow is harvested from small children, as most NB patients are. Most investigators treat marrow nucleated cells separated by Ficoll-Hypaque density centrifugation, which involves a substantial loss of marrow nucleated cells. The advantage of our method is that it can be applied on unfractionated, freshly harvested whole marrow. We have not encountered gross hemolysis or cell clumping. We feel that our high marrow nucleated cell recovery, and particularly the absence of cell clumping in unfractionated marrow, is due to the high specificity of our Mab (12). Mabs cross-reacting with other marrow elements might reduce the nucleated cell recovery, as might the use of rabbit C’ due to nonspecific toxicity.

We conclude that our in vitro cytotoxicity method can be used for purging autologous marrow. Our bone marrow colony assay data indicate that normal hematopoietic precursor cells are not detectably damaged by the procedure (Table 1). We specifically chose an assay system in which erythropoietin and colony-stimulating activity were present in amounts which would stimulate in vitro colony formation, and serve as indicators of cell kill and had high nonspecific background kill (Chart 1). One problem in the C’-mediated cytotoxicity systems has been the anticomplementary effect noticed in the presence of normal bone marrow (33). This anticomplementary effect has been recently attributed to heterophile antibodies (34). The use of human C’ and human bone marrow in our studies obviated this problem, as indicated by the data from the seeding experiments with the clonogenic assay (Table 2) and Hoechst nuclear stain (Table 3).

The effectiveness of human C’ was dose dependent, was independent of donor serum source, and was abolished by heat inactivation. We could demonstrate inhibition of the C’-mediated cytolyis by ethyleneglycol tetraacetic acid, suggesting involvement of the classic pathway of C’ activation (35). Human C’ is effectively activated by our GD2-specific murine IgM Mabs, is not inhibited by the presence of bone marrow, has negligible background toxicity, can be easily obtained, and is inexpensive. Human C’ has also been the subject of recent interest in purging bone marrow of leukemia (15) or T-cells (36).

Our method of purging autologous bone marrow of NB cells with a single Mab 3G6 and human C’ is simple to perform, effective in removing NB cells, and results in optimal recovery of hematopoietic precursor cells. Clinical trials are being designed to utilize this method to treat autologous bone marrow of patients with Stage III or IV neuroblastoma. These studies will help clarify issues of engraftment and achievement of long-term remissions and survivals.

### REFERENCES

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