Model System for Removing Neuroblastoma Cells from Bone Marrow Using Monoclonal Antibodies and Magnetic Immunobeads

C. Patrick Reynolds, Robert C. Seeger, Dai Dang Vo, Alfred T. Black, John Wells, and John Ugelstad

ABSTRACT

Variables effecting removal of neuroblastoma cells from bone marrow using monoclonal antibodies and magnetic immunobeads were studied. Human neuroblastoma cell lines were labeled with the supravital DNA stain Hoechst 33342, seeded into normal bone marrow, incubated with monoclonal antibodies recognizing neuroblastoma cell surface antigens (HSAN 1.2, antibody 459, antibody 390, BA-1, and Leu-7), and then mixed with magnetic microspheres coated with goat anti-mouse immunoglobulin. Tumor cells that attached to the magnetic immunobeads were then removed from the marrow with magnets. The efficacy of tumor cell removal depended on the amount of monoclonal antibody bound to tumor cells and the immunobead/tumor cell ratio. In addition, two cycles of purging with both monoclonal antibodies and immunobeads was superior to one cycle. Using a cocktail of the five antibodies, 3 to 4 logs of tumor cells could be depleted from marrow with good recovery of viable hematopoietic cells.

INTRODUCTION

Disseminated neuroblastoma that occurs after 1 year of age has had an extremely poor prognosis with conventional therapy. Recent pilot studies suggest that improved survival may be achieved with intensive multidrug chemotherapy and total body irradiation followed by BMT3 (1-3). Because only 20 to 25% of patients have an HLA compatible sibling to donate marrow, autologous or HLA incompatible marrow must be used to reconstitute most patients. Autologous BMT avoids the risk of graft versus host disease, and of graft failure, that is associated with non-HLA identical BMT. However, there is a possibility of infusing tumor cells with the marrow since marrow metastases are present in 75% of patients who have disseminated tumor at diagnosis. Removal of contaminating tumor cells from autologous marrow will be essential in many cases to permit autologous BMT early in the course of treatment. In addition, a randomized trial comparing autologous BMT, allogeneic BMT, and conventional chemotherapy will require an effective means of removing tumor cells from contaminated marrow so that all patients randomized to autologous BMT can be studied.

A number of methods for selective removal of cells from marrow have been reported, including monoclonal antibodies and complement (4, 5), lectin agglutination (6), immunotoxins (7), cytotoxic drugs (8, 9), and physical separation by equilibrium centrifugation (10, 11). Recently, monoclonal antibodies have been used to attach magnetic microspheres or magnetic colloid to the target cells, which are then removed with magnets (12-14).

We established a model system to study removal of neuroblastoma cells from bone marrow. Cultured tumor cells were premarked with the supravital DNA-binding fluorochrome Hoechst 33342, which provides intense nuclear fluorescence; when seeded into normal bone marrow, as few as one viable tumor cell can be detected among 1 million normal marrow cells (15, 33). We have used this sensitive detection method to study variables effecting the removal of neuroblastoma cells from bone marrow with monoclonal antibodies and magnetic immunobeads.

MATERIALS AND METHODS

Cell Lines. Human neuroblastoma cell lines used in this study were established in the laboratories of the authors (16-18). Cell lines were maintained in RPMI 1640 supplemented with 15% fetal calf serum.

Characteristics of Monoclonal Antibodies. Monoclonal antibody 390 (19) was purified from tissue culture supernatant using staphylococcal protein A affinity chromatography, and antibody 459 (20) was purified from mouse ascites by precipitation with ammonium sulfate and then size fractionation through Sephacryl S-300. Antibody HSAN 1.2 was purified from mouse ascites by affinity chromatography with Affi-Gel protein A (Bio-Rad, Richmond, CA) (21). These antibody preparations were assessed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified antibodies BA-1 and BA-2 (22, 23) were provided by Hybritech, Inc., and purified Leu-7 (HNK-1) (24, 25) was provided by Becton Dickinson.

Antibodies 390, 459, and HSAN 1.2 bind to neuroblastoma but not normal bone marrow cells (19-21). Antibodies BA-1 and BA-2 are anti-B-cell antibodies that bind to human neuroblastoma cells (25, 26). BA-1 and BA-2 react with a small percentage of cells in normal marrow but not hematopoietic stem cells (5, 22). Analysis of the binding of all of these antibodies to neuroblastoma cells has been presented elsewhere (19, 21, 25), and their characteristics are summarized in Table 1.

Determination of Antibody Binding to Neuroblastoma. Human neuroblastoma cell lines in log phase were removed from the substrate with Puck’s Saline A containing 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid and 1 mM EDTA (8), pipeted into a cell suspension, counted, and dispensed in 105-cell aliquots into 12- x 75-mm conical tubes. The cells were washed once with PBS without divalent cations that contained 5% GS and 0.2% AZ. After the cells were pelleted by centrifugation, the wash solution was aspirated so as to leave 50 µ1 in the tube, and the cells were resuspended by vigorous agitation. The monoclonal antibody (or antibodies) to be tested was then added to the cells in a volume of 50 µl (final volume, 100 µl, 10 million cells/ml). Tubes were then incubated on wet ice for 30 min, washed twice in 4 ml of PBS/GS/AZ, and resuspended in 50 µl of PBS/GS/AZ, and then 50 µl of a saturating amount of fluorescein isothiocyanate-sheep anti-mouse immunoglobulin (Capp Laboratories, Malvern, PA) diluted in goat serum were added. After 30 min on wet ice, the cells were washed once in 4 ml of PBS/GS/AZ and once in 4 ml of PBS/AZ and finally resuspended in PBS/AZ for analysis with an Ortho Diagnostics 50 H/H cyttofluorograph. Electronic gating of cells using 90-degree versus forward angle scatter was used to exclude debris and nonviable cells.

Received 4/17/86; accepted 7/15/86.

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1 This investigation was supported by Naval Medical Research and Development Command Work Unit MF58.527.004.0004; Grant CA12800 awarded by the National Cancer Institute, Department of Health and Human Services; and the Concern Foundation, Inc.

2 Supported in part by Cancer Center Support Grant CA16042 from the National Cancer Institute, Department of Health and Human Services.

3 The abbreviations used are: BMT, bone marrow transplantation; PBS, phosphate buffered saline; heat inactivated GS, goat serum; A2, sodium azide; GAM, goat anti-mouse immunoglobulin; CFU-GM, colony forming units-granulocyte-macrophage (myeloid stem cells); CFU-C, colony forming units-cell; Ab, antibody; H342, Hoechst 33342.
Green fluorescence was quantitated for 5000–8000 cells. Gates were set so that 2% or less of controls (omission of primary antibody or use of nonbinding monoclonal antibody of the same subclass) fell into the positive channels. The mean fluorescence and percentage of cells positive were then calculated.

**Immunoaffinity Purging Method.** The method of immunoaffinity purification is outlined in Fig. 1. Monoclonal antibodies that bind to the tumor cells but not to marrow stem cells are used to coat the tumor cells with mouse immunoglobulin. Magnetic microspheres absorbed with GAM will then selectively attach to the tumor cells. Tumor cells with microspheres attached can be removed from the marrow using high energy magnets.

**Immunomagnetic Beads.** Polystyrene porous magnetic beads (prepared from styrene-divinyl benzene polymer, 3 μm in diameter, magnetite content corresponding to 27.4% by weight of iron) were prepared as described previously (14, 27). Affinity purified GAM (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a concentration of 200 μg/mg of beads, was incubated with the beads (5 mg/ml) in 0.1 M phosphate buffer, pH 7.4, on a rotating wheel at 4°C for 18 h. Unbound GAM was removed by washing four times in 12 ml of RPMI 1640 containing 15% fetal calf serum.

**Preparation of Neuroblastoma-Normal Marrow Mixture.** Normal human bone marrow was obtained from cadaveric vertebral bodies (28) and viable mononuclear cells were separated by density centrifugation with Ficoll-Hypaque (29). Cultured neuroblastoma cells, which were marked with the supravital DNA stain H342 (15, 30) (Calbiochem, La Jolla, CA), were then seeded into the marrow as single cells and small to moderate sized clumps, thus simulating metastatic neuroblastoma in the marrow of patients. The bright nuclear fluorescence of the H342 labeled cells allows detection of one marked tumor cell per million marrow cells. Counterstaining the tumor/marrow mixture with the vital dye trypan blue limits the detection of tumor cells to only viable cells, because trypan blue quenches the nuclear H342 fluorescence when it penetrates into nonviable cells (15, 33).

**Purging of Marrow.** For purging experiments 50 to 100 million bone marrow cells were seeded with 10 to 20% H342 stained neuroblastoma cells. The cell mixture was incubated with monoclonal antibodies at saturating concentrations in RPMI 1640 containing 10% fetal calf serum, and DNase (50 units/ml; Sigma Chemical Co., St. Louis, MO) for 1 h at 4°C, 10 million cells/ml, and mixed with GAM coated beads for 1 h at 4°C on a rotating wheel. The sample was then diluted 3-fold and passed over two 15- x 48-mm samarium-cobalt magnets (Edmund Scientific, Barrington, NJ) to retain free beads and bead coated cells (31). For the second cycle of depletion, the same amount of beads determined by the starting tumor concentration was used, but the total amount of beads was reduced in proportion to the total number of cells remaining, i.e.,

\[
\text{Initial amount of beads} \times \% \text{of nucleated cells recovered} = \text{Amount of beads used in second cycle}
\]

The amount of tumor removed was quantitated by counting the H342 stained cells before and after depletion. Cells stained with H342 were counted by examining drops of marrow/tumor mixture under a coverslip in a Leitz Orthoplan fluorescent microscope for H342 stained cell concentrations down to 0.1%. For H342 stained cell concentrations less than 0.1%, 100,000 or 1,000,000 cells of the marrow/tumor mixture were examined in microwells of a 96-well multiplate and examined with a Leitz inverted fluorescent microscope. Microscopes were equipped with 100-W mercury lamps and UV excitation (350 nm)/blue fluorescence (460 nm) emission "D" cubes. The total number of tumor cells before and after purging was derived from the concentration of H342 stained cells counted, and the log of tumor cells removed was calculated.

**Quantitation of myeloid stem cells (CFU-GM) was done after 10 days of growth in agar with leukocyte conditioned medium (32).**

### RESULTS

Coating of Neuroblastoma Cells with Monoclonal Antibodies. The first step in defining the optimal monoclonal antibody coating of the tumor cell surface was titration of the antibodies by flow cytometry. Representative titrations for three of the antibodies used in this study are shown in Fig. 2. The amount of antibody bound to the SMS-KCNR neuroblastoma cell line for each individual antibody at saturating concentration is shown in Fig. 3. The binding profiles for SMS-KCNR are typical of several human neuroblastoma lines, with Ab459 consistently giving the brightest fluorescence. The additive effect of a mixture of multiple antibodies is also shown in Fig. 3.

### Table 1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Subclass</th>
<th>Reactivity</th>
<th>Ref.</th>
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</thead>
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<tr>
<td>Ab390</td>
<td>IgG3</td>
<td>Anti-human Thy-1</td>
<td>19</td>
</tr>
<tr>
<td>Ab459</td>
<td>IgM</td>
<td>Anti-human fetal brain</td>
<td>20</td>
</tr>
<tr>
<td>HSAN 1.2</td>
<td>IgG1</td>
<td>Anti-human neuroblastoma</td>
<td>21</td>
</tr>
<tr>
<td>BA-1</td>
<td>IgM</td>
<td>Anti-B cell lineage</td>
<td>22, 23</td>
</tr>
<tr>
<td>BA-2</td>
<td>IgG3</td>
<td>Anti-B cell lineage</td>
<td>23</td>
</tr>
<tr>
<td>Leu-7</td>
<td>IgM</td>
<td>Anti-natural killer cell</td>
<td>24, 34</td>
</tr>
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**Fig. 1.** Indirect method of immunoaffinity cell depletion. Monoclonal antibodies selective for target cells are used to coat the target cells with mouse immunoglobulin. Magnetic microspheres coated with GAM will then attach to the antibody coated cells. Cells with microspheres attached can be removed with high energy magnets.

**Fig. 2.** Titration of antibodies HSAN 1.2 (Δ), Ab459 (○), and BA-1 (□) on the LA-N-5 human neuroblastoma cell line. Quantitation of antibody binding was by flow cytometry. Neat antibody concentrations: Ab459, 200 μg/ml; HSAN 1.2, 100 μg/ml; BA-1, 200 μg/ml.

[Diagram of monoclonal antibody binding to target cells with magnetic beads, showing binding index graph for different antibody dilutions.]
REMOVAL OF NEUROBLASTOMA CELLS FROM BONE MARROW

Fig. 3. Comparison of the binding to human neuroblastoma SMS-KCNR of individual antibodies and Cocktail 1 (HSAN 1.2, BA-1, BA-2, Ab390, Ab459) or Cocktail 2 (HSAN 1.2, BA-1, BA-2, Ab390, Ab459, and Leu-7).

Fig. 4. Effect of adding Leu-7 to cocktail of HSAN 1.2, BA-1, BA-2, Ab390, and Ab459 on removal of neuroblastoma cells from marrow. SMS-KCNR cells marked with H342 were seeded into bone marrow and the mixture was treated twice with either Cocktail 1 (without Leu-7) or Cocktail 2 (with Leu-7) and with GAM coated beads (10 ng/target cell). The log of the number of tumor cells removed was determined from the total H342 cells in the marrow before and after 2 cycles of depletion. Columns, mean of 3 determinations; bars, SD.

An increase in the amount of antibody bound per cell (shown by an increase in binding index) can be seen when the cells were stained with a mixture of five antibodies. Addition of a sixth antibody, Leu-7, further increases the binding index by 90 channels.

Comparison of Antibody Binding and Depletion of Tumor. To determine if an increase in the amount of antibody on the cell surface results in more efficient purging with magnetic immunobeads, we compared the depletion of SMS-KCNR neuroblastoma cells from bone marrow using a cocktail of antibodies (HSAN 1.2, BA-1, BA-2, Ab390, Ab459) with or without Leu-7. In agreement with the increased fluorescence observed with addition of Leu-7 to the cocktail, the amount of tumor cells removed from the marrow was also increased (Fig. 4).

Effect of Bead/Target Cell Ratio on Tumor Removal. Depletion of neuroblastoma cells from marrow was found to depend on the number of beads per target cell used. Increasing the amount of beads per target cell up to 10 ng of beads per target cell increased the efficacy of depletion (Fig. 5).

One Cycle versus Two Cycles of Treatment. We compared a single treatment with the antibody cocktail and magnetic immunobeads to two sequential treatments. As shown in Fig. 6, an additional 1 to 2 logs of tumor were removed with the second treatment for each of the cell lines tested. A second treatment with both monoclonal antibodies and beads was required to achieve this effect. Treatment with monoclonal antibodies once, even if the antibody concentration was doubled, and then treating with magnetic immunobeads twice was not as effective as two treatments with both antibodies and beads (data not shown).

Recovery of Hematopoietic Cells. Some of the antibodies bind to cells found in normal marrow (BA-1, BA-2, and Leu-7). Thus, it was not surprising that normal marrow cells were also depleted in the process of purging tumor from the marrow. The yield of total nucleated cells varied among experiments from 10 to 60% (mean, 30%). However, viability of the recovered marrow always exceeded 90% by dye exclusion. As a measure of the depletion of hematopoietic stem cells that occurs with this procedure, CFU-GM were performed on marrows after 2 cycles of purging with a 5-antibody cocktail (HSAN 1.2, Ab459, Ab390, BA-1, and Leu-7). As shown in Table 2, an average of 76% of the total CFU-C is recovered after immunomagnetic purging, and the CFU-C per nucleated cell increases.

DISCUSSION

We have demonstrated that a 1,000- to 10,000-fold reduction in the number of neuroblastoma cells in bone marrow can be
achieved using monoclonal antibodies and magnetic immunobeads. To reach this level of removal, it was necessary to carefully evaluate the procedure with a sensitive and quantitative assay for tumor cells in the marrow. By premarking neuroblastoma cells with H342, a supravital DNA binding fluorochrome, detection of one tumor cell seeded among 1 million roblastoma cells with H342, a supravital DNA binding fluorochrome, detection of one tumor cell seeded among 1 million normal marrow cells was possible (15, 33).

An important parameter governing the efficacy of purging is the amount of antibody on the surface of the target cell. Titration of monoclonal antibodies on viable cells using flow cytometry determined the concentration of antibodies necessary to saturate cell surface antigens. Furthermore, use of a cocktail of antibodies resulted in more immunoglobulin per cell and helped overcome heterogeneity in cell surface antigen expression in tumor cell populations. The amount of antibody on the target cells, as determined by flow cytometry, correlated with the efficacy of target cell depletion. For example, addition of a single antibody to a cocktail of five other antibodies increased the total amount of antibody bound to target cells and also increased the efficacy of purging.

A critical new observation made in this study is the increased removal of tumor cells provided by two cycles of treatment compared to one cycle. A significant increase in the number of tumor cells removed in the second cycle was consistently seen with three different neuroblastoma cell lines, all established from bone marrow metastases (16, 17). In these experiments, cells were seeded into normal marrow in clumps and single cells, mimicking the appearance of metastatic tumor in the marrow of patients. Thus, the model closely resembled the actual clinical situation, suggesting that a second cycle of treatment will contribute significantly to effective removal of tumor cells from the marrow of patients. Other investigators have reported that greater removal of leukemia from marrow was achieved with three cycles than with one cycle when monoclonal antibodies and complement are used (4).

The reason for the increased removal of tumor cells with the second cycle of treatment is not clear. It is likely that the heterogeneity inherent in most malignant cell populations results in cells with low levels of cell surface antigens. Indeed, flow cytometry histograms of neuroblastoma cell lines clearly demonstrate a wide range in the binding of individual antibodies (25). The improved efficacy seen when treating the marrow with both monoclonal antibodies and magnetic immunobeads twice, compared to antibodies once and beads twice, supports the possibility that the second cycle removes tumor cells that bind antibody less efficiently than those removed in the first cycle.

The marrow recovered after purging of tumor cells was highly viable as assessed by dye exclusion and by growth of CFU-GM. Although the latter data do not prove the viability of pluripotent hematopoietic stem cells, it suggests that the purged marrow retains proliferative capability which is necessary for hematological and immunological reconstitution. The percentage of normal cells and CFU-GM recovered indicates the feasibility of applying this method to large scale marrow specimens obtained from patients for autologous transplantation.

The proportion of tumor cells that is clonogenic in humans is unknown and may vary for different tumors as it does for cell lines in vitro (16). Because of this, the number of viable tumor cells remaining in marrow to be reinfused into the patient should be decreased to the lowest possible level. Marrow with 1% tumor cells harvested for autologous transplant from a 20-kg patient would still contain 1000 tumor cells, even after 4 logs of depletion (10^4 total cells containing 0.0001% tumor cells infused). Our data suggest that increasing the number of anti-cell surface monoclonal antibodies in the cocktail could provide more effective purging, but other methods may also be required. Treatment of the marrow with 6-hydroxydopamine may selectively destroy neuroblastoma cells without damage to marrow stem cells (8). Physical separation of tumor clumps by centrifugal elutriation may also be an effective method of reducing the tumor burden (10, 11). These nonimmunological methods should be effective against tumor cells that are not removed by antibodies. Removal of 5 to 6 logs of tumor cells would make it unlikely that tumor cells in the marrow will reestablish disease in the patient.

The model system described has provided a rapid and accurate means of studying variables effecting separation of neuroblastoma cells from bone marrow with monoclonal antibodies and magnetic immunobeads. Using different monoclonal antibodies, a similar approach can be developed for other malignancies in which tumor cells need to be removed from autologous marrow. This system is also applicable to removing T-lymphocytes from marrow harvested for allogeneic transplantation.

ACKNOWLEDGMENTS

The authors thank Doug Feagans, Donna-Maria Jones, F. Hoover, and S. Rayner for excellent technical assistance; Dr. R. Bartholomew of Hybritech, Inc., for purified BA-1 and BA-2; Drs. N. Warner and K. Snow of Becton Dickinson Monoclonal Antibody Center for providing the Leu-7 antibody; SINTEF Applied Chemistry Division, Trondheim, Norway, for providing the magnetic microspheres; Kirkegaard and Perry Laboratories, Bethesda, MD, for providing goat anti-mouse immunoglobulin; Debra A. Reynolds for technical illustrations; and Carole Portis for preparation of the manuscript.

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