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Model System for Removing Neuroblastoma Cells from Bone Marrow Using Monoclonal Antibodies and Magnetic Immunobeads[1]

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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 BMT[3] (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 filtration 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, 34) 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 10^6-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 μl 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 (Cappel 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 cytofluorograph. Electronic gating of cells using 90-degree *versus* forward angle scatter was used to exclude debris and nonviable cells.

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[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.

$$\text{Binding index} = \frac{\text{Mean fluorescence} \times \% \text{ of cells positive}}{100}$$

Immunomagnetic Purging Method. The method of immunomagnetic purging 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.*,

Initial amount of beads × % of nucleated cells recovered

= 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 microtiter plate 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).

Table 1 *Monoclonal antibodies used for depleting neuroblastoma from bone marrow, their immunoreactivity, and immunoglobulin subclass*

Antibody	Subclass	Reactivity	Ref.
Ab390	IgG3	Anti-human Thy-1	19
Ab459	IgM	Anti-human fetal brain	20
HSAN 1.2	IgG1	Anti-human neuroblastoma	21
BA-1	IgM	Anti-B cell lineage	22, 23
BA-2	IgG3	Anti-B cell lineage	23
Leu-7	IgM	Anti-natural killer cell	24, 34

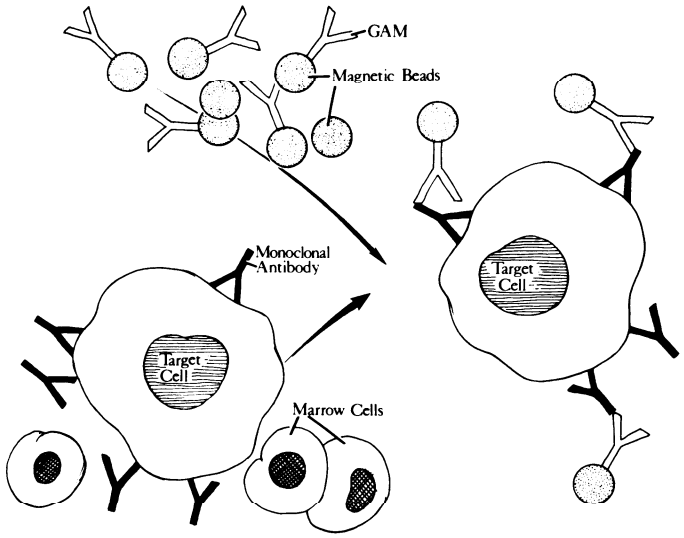

Fig. 1. Indirect method of immunomagnetic cell depletion. Monoclonal antibodies selective for target cells are used to coat the target cells with mouse immunoglobulin. Magnetic microspheres coated with GAM will then attach to the antibody coated cells. Cells with microspheres attached can be removed with high energy magnets.

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.

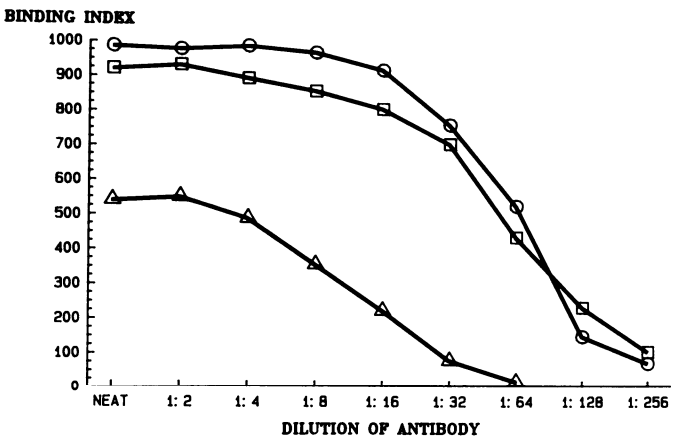

Fig. 2. Titration of antibodies HSAN 1.2 (△), Ab459 (○), and BA-1 (□) on the LA-N-5 human neuroblastoma cell line. Quantitation of antibody binding was by flow cytometry. Neat antibody concentrations: Ab459, 200 μg/ml; HSAN 1.2, 100 μg/ml; BA-1, 200 μg/ml.