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Selective Elimination of Breast Cancer Cells from Human Bone Marrow Using an Antibody-Pseudomonas Exotoxin A Conjugate

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ABSTRACT

A pancarcinoma monoclonal antibody (NR-LU-10), homogeneously reactive with human breast cancer cells, was conjugated to Pseudomonas exotoxin A. The immunotoxin was evaluated for its potential for purging breast cancer cells from human bone marrow. The immunotoxin NR-LU-10 antibody did not react with normal bone marrow preparations yet readily detected 1% contamination of bone marrow by MCF-7 breast cancer cells added to normal bone marrow without significantly inhibiting the colony-forming ability of bone marrow progenitor cells. NR-LU-10-Pseudomonas exotoxin A has potential for purging bone marrow of breast cancer cells without impairing the growth of bone marrow progenitor cells.

INTRODUCTION

Toxicity to bone marrow is the limiting factor in the use of many conventional cytotoxic drugs. Larger doses can be given if a portion of the patient’s bone marrow is withdrawn before chemotherapy and the cryopreserved marrow is reinfused into the patient after the systemic concentration of cytotoxic drug has decreased below the marrow-ablative level. Breast cancer is one of the malignancies in which the availability of autologous bone marrow would allow dose escalation of cytotoxic drugs. However, during advanced stages of disease, metastases to bone marrow occurs in more than half of all patients (1). Thus, methods of removing metastases from bone marrow prior to reinfusion into the patient are needed.

Bone marrow transplantation following ablative therapy has been hampered by a number of limitations. The first is the presence of extramedullary residual disease which may be unresponsive to therapy. In breast cancer, current treatment modalities have not been applied in a sufficiently aggressive manner to know whether they can be curative. Second, the agents developed for marrow purging, especially in breast cancer, may not be able to eliminate all residual tumor cells. Finally, as a result of marrow ablation, the time course of hematopoietic recovery can be prolonged, further increasing the risk of infection. These limitations are being addressed by the use of more aggressive therapies and by the advent of colony-stimulating factors.

In this report, we provide data concerning a method for more efficient elimination of tumor cells from bone marrow without compromising hematopoietic potential as assayed in vitro.

MATERIALS AND METHODS

Materials. Purified PE2 was purchased from Swiss Serum and Vaccine Institute (Berne, Switzerland).

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The abbreviations used are: PE, Pseudomonas exotoxin A; ID50 inhibitory dose 50%; BFU-E, erythroid burst-forming units; CFU-GM, granulocyte-macrophage colony-forming units; FITC, fluorescein isothiocyanate; SMCC, succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate; PBS, phosphate-buffered saline.

The antibodies used in this study were NR-LU-10 (2) and NR-ML-05 (3), both murine IgG2b monoclonal antibodies. NR-ML-05, directed to human melanoma proteoglycan, was used as an irrelevant control antibody. NR-LU-10 showed strong reactivity with five of five human breast cancer cell lines, as analyzed by flow cytometry. Immunoperoxidase staining of frozen thin sections from breast carcinomas demonstrated that NR-LU-10 reacted with virtually every tumor cell in 23 of 23 tumor samples examined (2). NR-LU-10 did not react with peripheral blood cells, spleen lymphocytes, or bone marrow mononuclear cells.

Cell Lines. The cell lines used in this study were MCF-7 and ALAB, both human breast carcinoma lines, and A375 and M14, human melanoma lines. Cultures were maintained as described previously (2).

Preparation of Immunotoxins. Preparation and characterization of stably bonded thioether conjugates are described in more detail elsewhere (4). Briefly, PE in 0.1 M sodium phosphate-0.15 M sodium chloride, pH 7.5 (reaction buffer), was adjusted to pH 8.5 by the addition of 0.5 M sodium borate, pH 8.5. One mg of SMCC (Pierce Chemical Co., Rockford, IL) was dissolved in 1 ml of 10% (v/v) dimethyl sulfoxide in ethanol. A 10-fold molar excess of SMCC was added and reacted with PE for 30 min at room temperature. Unreacted SMCC was removed by chromatography on a PD-10 column (Pharmacia Fine Chemicals, Piscatway, NJ) equilibrated in reaction buffer.

NR-LU-10 and NR-ML-05 monoclonal antibodies in reaction buffer (1 mg/ml) were adjusted to pH 8.5 with the addition of 0.5 M sodium borate, pH 8.5, and reacted with 25 mM diithiothreitol for 30 min at room temperature. Diithiothreitol was removed by passage of the mixture over a PD-10 column.

Reduced antibodies and SMCC-derived PE were mixed at a 1:1 molar ratio and allowed to react at room temperature for 1 h. Conjugation was monitored by analysis of aliquots of the reaction mixture on size exclusion chromatography using a Superose 12 column (Pharmacia). Unconjugated PE was removed on the Superose 12 column equilibrated in 10 mM sodium phosphate-0.5 mM sodium chloride, pH 7.5, at a flow rate of 0.5 ml/min. Fractions corresponding to conjugate and unconjugated antibody were pooled. Unconjugated antibody was removed by anion exchange chromatography on a Mono Q column (Pharmacia). Conjugate enriched in the 1:1 (monoclonal antibody:PE) species was eluted with a linear gradient of 0–0.5 mM sodium chloride in 5 mM sodium phosphate, pH 7.6.

[3H]Leucine Cytotoxicity Assay. MCF-7 and ALAB human breast carcinoma cells (antigen positive) and A375 melanoma cells (antigen negative) were harvested with a trypsin/EDTA solution (0.5/0.2%; Sigma Chemical Company, St. Louis, MO) and then seeded at 105 cells/well in 96-well plates (Costar, Cambridge, MA). Cultures were incubated for 24 h and then NR-LU-10, NR-ML-05, or PE was added in serial dilutions at 100 μl/well in growth medium (Dulbecco’s modified Eagle’s medium) containing 5% heat-inactivated fetal calf serum (MA Bioproducts, Walkersville, MD) and 7% Serum Plus (Hazelton Research Products, Inc., Lenexa, KS). After 24 h, cells were pulsed with 0.5 μCi of tritiated leucine/well (Amersham, Arlington Heights, IL; 186 Ci/mM) for 4 h. Cells were then collected onto glass fiber filters using a PhD cell harvester (Cambridge Technology, Inc., Cambridge, MA). The filters were washed and counted in a scintillation counter (LS-2800, Beckman Instruments, Inc., Palo Alto, CA). The ID50 of [3H]leucine incorporation was extrapolated from the titration curve.

Normal Human Bone Marrow. Bone marrow samples were obtained from normal volunteer donors after informed consent, according to
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guidelines of the Human Subjects Review Committee at the Fred Hutchinson Cancer Research Center. Bone marrow was aspirated from the posterior iliac crest, and the mononuclear fraction was isolated either by density centrifugation over Ficoll-Hypaque or after centrifugation and collection of buffy coat cells.

Long-Term Marrow Cultures. Buffy coat marrow cells were subjected to hypotonic lysis in ammonium chloride and were used to establish long-term marrow cultures according to a method modified from Garner and Kaplan (5). Marrow cells were plated in 25-cm² flasks (Corning, Corning, NY) in 10 ml of complete α-medium (Gibco, Grand Island, NY) with 12.5% horse serum (Flow, McLean, VA), 12.5% fetal bovine serum (Hyclone, Logan, UT), and 1 μmol hydrocortisone sodium succinate (Sigma). Cultures were incubated at 37°C in 5% CO₂ for 1 week and 33°C thereafter. They were fed once a week by demidepletion of the culture medium.

Hematopoietic Progenitor Assays. Erythroid bursts (BFU-E) and granulocytic/monocytic colonies (CFU-GM) were assayed in methylcellulose culture systems containing 0.9% methylcellulose with 20% pooled human serum and 2% bovine serum albumin in Iscove’s medium. Four % phytohemagglutinin-stimulated leukocyte-conditioned medium and 4% human placenta-conditioned medium were added as a source of burst-promoting activity and colony-stimulating activity, respectively. Recombinant erythropoietin was added at 3 units/ml. Cultures were established in triplicate. Plates were incubated at 37°C in a fully humidified incubator with 5% CO₂ in air. BFU-E containing >200 cells were identified on the basis of their orange-red color. Colonies composed of >50 non-erythroid cells were scored as CFU-GM. Both types of colonies were scored with an inverted microscope at 12-14 days of culture.

Immunofluorescence Microscopy. To detect antigenic determinants on the external cell surface, immunofluorescence was performed on viable cells before fixation for viewing. Cells were grown at least 2 days on 12-mm diameter glass coverslips to allow adhesion. Coverslips were washed briefly in Dulbecco’s PBS at room temperature and then placed on a glass plate on ice. The coverslips were overlaid with 20 μl of the first antibody or conjugate (NR-LU-10-PE, NR-LU-10, or NR-ML-05) diluted in PBS (antibody concentration, 250 ng/ml). After 30 min, the coverslips were washed briefly with ice-cold PBS, returned to the cold glass plate, and overlaid with 20 μl of affinity-purified rhodamine-labeled goat anti-mouse immunoglobulin (TAGO, Burlingame, CA) for an additional 30 min. The coverslips were then washed in PBS and mounted in Elvanol (Monsanto, St. Louis, MO) on glass slides. Mounted cells were viewed with a Zeiss Axiovert microscope equipped with epillumination. Photographs of fluorescent images were taken on Kodak T-MAX 400 film.

Flow Cytometry. NR-LU-10 and NR-ML-05 were conjugated with FITC using the method described by Rinderknecht (6). Breast carcinoma cells, MCF-7, were detected in bone marrow by direct immunofluorescence and flow cytometric analysis. Briefly, 10⁵ bone marrow cells (with or without breast carcinoma cells) in 100 μl were incubated for 30 min at 4°C with 5 μg of FITC-labeled antibody. Cells were washed three times and then analyzed.

RESULTS

Immunofluorescence Detection of NR-LU-10-PE and NR-LU-10 on Appropriate Target Cells. Viable cell fluorescence techniques were utilized, and the reactivity of NR-LU-10-PE was contrasted and compared with both NR-LU-10 and NR-ML-05 on target (MCF-7) and nontarget (M14 melanoma) cells. Both NR-LU-10 and NR-LU-10-PE reacted strongly with the surface of MCF-7 cells (Fig. 1), with virtually no difference in staining pattern, whereas NR-ML-05 produced only minimal staining. Neither NR-LU-10 nor NR-LU-10-PE produced detectable levels of staining on M14 antigen-negative cells (data not shown).

Preliminary Cytotoxicity Testing. NR-LU-10-PE conjugates were found previously to be highly cytotoxic to human colon cancer and ovarian cancer cell lines (2). Immunotoxins produced by the same methodology were tested for cytotoxicity against human breast cancer cell lines. Protein synthesis was assessed, and the immunotoxin was highly cytotoxic to the MCF-7 and ALAB human breast cancer cell lines, both of which express the antigen recognized by NR-LU-10 (Table 1). The ID₅₀ for these lines was 0.02–0.03 ng/ml. In contrast, the irrelevant control immunotoxin NR-ML-05-PE had an ID₅₀ of 100 ng/ml. The poor cytotoxicity of the appropriately matched control NR-ML-05-PE and other controls has been described (2, 4, 7, 8). When tested against the A375 human melanoma cell line, which lacks the antigen recognized by NR-LU-10, the ID₅₀ of NR-LU-10-PE was about 1 μg/ml, making it about...
Table 1  Cytotoxicity of NR-LU-10 for human cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>NR-LU-10 antigen</th>
<th>ID₅₀° (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Breast cancer +</td>
<td>0.03</td>
<td>5</td>
</tr>
<tr>
<td>ALAB</td>
<td>Breast cancer +</td>
<td>0.02</td>
<td>5</td>
</tr>
<tr>
<td>A375</td>
<td>Melanoma -</td>
<td>1000</td>
<td>40</td>
</tr>
</tbody>
</table>

* Mean of at least 3 separate determinations. The variability in ID₅₀° values ranged from 3- to 5-fold.

Bone Marrow Purging. We next determined whether NR-LU-10-PE conjugates are as potent and selective in bone marrow preparations and cultures. As shown in Fig. 2, the NR-LU-10-FITC antibody conjugate did not react with normal bone marrow mononuclear cells yet readily detected 1% contamination of bone marrow with MCF-7 breast carcinoma cells.

In a series of six experiments, normal bone marrow cells were mixed with either 10 or 50% MCF-7 cells prior to incubation with NR-LU-10-PE. In the initial three experiments, 5 x 10⁶ cells in a total of 1 ml was incubated for 24 h in the presence of various concentrations of NR-LU-10-PE. Two control groups were included: an untreated control and cells incubated in the presence of the irrelevant antibody-toxin conjugate (NRML-05-PE). After incubation, the cells were washed once, resuspended in α-medium, and then plated for progenitor assays as described. A proportion of the treated cells were resuspended in 10% fetal calf serum and RPMI and incubated at 37°C in Petri dishes. These latter conditions do not support the growth of normal hematopoietic cells but do support the growth of the MCF-7 cell line. The results shown in Fig. 3 indicate that at 10 ng/ml the NR-LU-10-PE had no effect on the growth of normal

![Figure 2. Flow cytofluorometric analysis of the binding of FITC-NR-LU-10 to breast cancer cells added to normal bone marrow cells (BMC). Varying amounts of MCF-7 cells were added to normal bone marrow cells as indicated. Numbers in parentheses, % of NR-LU-10-positive cells detected by flow cytometry. Control, cells stained with FITC-NR-ML-05.](image1)

![Figure 3. Cytotoxicity of NR-LU-10-PE for bone marrow progenitor cells (a and b) and the human breast cancer cell line MCF-7 (c). Cells were exposed to NR-LU-10-PE for 24 h, washed, and then plated as described in "Materials and Methods." The three separate curves in a and b represent triplicate determinations. Multiple determinations were also done for c, with results similar to those shown.](image2)
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hematopoietic progenitor cells, whereas >90% of the MCF-7 cells were killed. At 100 ng/ml, there was minimal inhibition of normal progenitor growth, whereas 100% of the MCF-7 cells were killed (Figs. 3 and 4). A second set of experiments was performed using a 4-h incubation instead of a 24-h incubation. In these experiments, 100% inhibition of MCF-7 occurred at 1000 ng/ml. As might be expected with a shorter incubation time, there was no inhibitory effect on progenitor growth at this dose concentration (data not shown).

Since short-term assays of hematopoietic colony formation are not an adequate measure of the cells needed to attain successful engraftment, we next tested NR-LU-10-PE conjugate in long-term marrow cultures. In the first experiment in this series, long-term marrow cultures were inoculated with bone marrow buffy coat cells, and after successful establishment of a normal-appearing stromal cell layer, the NR-LU-10-PE conjugate was added directly to marrow cultures. The number of CFU-GM, BFU-E, and nonadherent cells produced by these cultures was assessed during a 4-week period. As shown in Fig. 5, the addition of NR-LU-10-PE at 1, 100, and 1000 ng/ml did not significantly affect the long-term production of hematopoietic cells in these cultures as compared to concurrent controls.

In a second set of experiments, bone marrow buffy coat cells were exposed to immunotoxin prior to the establishment of long-term cultures. Bone marrow buffy coat cells were incubated for 24 h at 37°C in the presence of 1, 10, 100, or 1000 ng/ml of NR-LU-10-PE. Cells were then washed and resuspended in appropriate long-term culture media as described. The number of nonadherent cells, CFU-GM, and BFU-E were enumerated at weekly intervals for 4 weeks. The data (not shown) were similar to those observed in Fig. 5, insofar as there was no significant effect of treatment on the production of hematopoietic cells compared to cultures that had been incubated without NR-LU-10-PE.

DISCUSSION

Considerable effort has been directed toward developing methods for the detection and purging of tumor cells from bone

Fig. 4. Inhibition of MCF-7 colony formation by NR-LU-10-PE. MCF-7 cells were exposed to NR-LU-10-PE for 24 h, washed, and then incubated for 2 weeks before colonies were stained and counted. The input number of cells was 5 x 10⁴/dish and the plating efficiency was approximately 5%. The experiment was done three times with comparable results each time. Multiple preparations of NR-LU-10-PE have been made with similar activity. The concentration of NR-LU-10-PE in each dish was: a, 1000 ng/ml; b, 100 ng/ml; c, 10 ng/ml; d, 1 ng/ml; e, 0.1 ng/ml; f, control (no NR-LU-10-PE was added).

Fig. 5. Effect of NR-LU-10-PE on the production of nonadherent, CFU-GM, and BFU-E cells in long-term marrow cultures. NR-LU-10-PE concentrations used were 1000 ng/ml (♦), 100 ng/ml (●), and 1 ng/ml (□). Shaded area, range of values obtained in untreated concurrent controls, which represents the range of values obtained in 6 concurrent controls done for each of the 3 different experiments.
marrow. Antibody with complement, drugs, lectin separation, and immunomagnetic separation are a few of the methods that have been described. Historically, antibody with complement has been the most widely used, but it suffers from irreproducible depletion and low potency (9).

For purging of breast cancer from bone marrow, Coombes et al. (10) have used an antibody-abrin A chain conjugate. The antibody, LICR-LON-Fib75, recognized the majority of cells in human breast cancer cell lines. Five of seven human breast cancer cell lines were killed by a 2-h exposure to 10^{-4} M conjugate. The ex vivo treatment of breast marrow with the abrin A chain immunotoxin did not significantly impair bone marrow recovery when cells were reinfused back into patients. However, this conjugate was of limited usefulness because of insufficient potency and because many target cells failed to express the antigen. In contrast, one of the advantages of the NR-LU-10 monoclonal antibody is that it reacts with all human breast tumor specimens thus far tested (23 of 23). Moreover, NR-LU-10 reacts with most or all identified tumor cells within each of the patient specimens. No binding of NR-LU-10 to blood cells, spleen cells, or bone marrow cells has been detected.

We have shown that an immunotoxin, NR-LU-10-PE, is effective in depleting breast cancer cells from human bone marrow without damaging the proliferative capacity of bone marrow progenitor cells. We chose PE as the toxin component of NR-LU-10, since immunotoxins made with it are generally more potent than those made with A chains (8, 11) and do not require soluble inhibitors (as with whole ricin and abrin). The immunotoxin described here was constructed using native PE. Of importance for immunotoxin specificity is the fact that following PE derivation with SMCC and conjugation to antibody, the immunotoxin becomes less toxic to antigen-negative cells. NR-LU-10-PE was about 10,000 times less toxic to human cell lines which lack the NR-LU-10 antigen, as compared to antigen-positive cell lines. This specificity allows one to use a dose of conjugate which can eliminate all detectable malignant cells from bone marrow by use of antibody and complement: critical appraisal. J. Natl. Cancer Inst., 80: 154–159, 1988.


