Comparison of Two Antibody-based Methods for Elimination of Breast Cancer Cells from Human Bone Marrow

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ABSTRACT

Three monoclonal antibodies reactive with antigens abundantly expressed on human carcinoma cells were used to develop and compare the efficacy of immunotoxins (ITs) and immunobeads for purging breast cancer cells from bone marrow. ITs constructed as conjugates of the monoclonal antibodies and Pseudomonas exotoxin A showed high specific cytotoxicity against three breast cancer cell lines, inhibiting protein synthesis by 50% at concentrations of 4 × 10⁻¹³ m to 1 × 10⁻¹⁰ m. Tested in a reproducible clonogenic assay, two of the ITs used at a concentration of 0.1 μg/ml killed >5 log units of MCF7 cells, the maximal sensitivity for ascites carcinoma cells from bone marrow. ITs constructed as conjugates of the monoclonal antibodies and Pseudomonas exotoxin A showed high specific cytotoxicity against three breast cancer cell lines, inhibiting protein synthesis by 50% at concentrations of 4 × 10⁻¹³ m to 1 × 10⁻¹⁰ m. Tested in a reproducible clonogenic assay, two of the ITs used at a concentration of 0.1 μg/ml each of the three ITs eliminated >5 log of both cell lines. The immunobead procedure removed 2.0–4.1 log of tumor cells with one purging cycle and up to 6.0 log with two cycles. The mixture of three ITs or immunobeads was not clearly superior in efficacy, compared to the use of individual molecules, probably reflecting an overlap in expression of the respective antigens in these cell lines. For both methods, the purging efficacy was not reduced when the tumor cells were admixed with normal bone marrow cells at a ratio of 1:10. The survival of colony-forming units, granulocyte/macrophage, was 49–86% with the immunobeads and 44–75% even at high concentrations (up to 2.5 μg/ml x 3) of the ITs. The results indicate that each of the two immunological methods can be safely used for effective elimination of tumor cells from the graft of breast cancer patients undergoing autologous bone marrow transplantation.

INTRODUCTION

In several types of cancers, the frequency and degree of tumor response to chemotherapy correlate with the total drug dose given to the patient. For many drugs, the dose-limiting factor is toxicity to the bone marrow. In these cases, high dose chemotherapy may be administered as part of an ABMT3 approach with marrow harvested before treatment. ABMT has become the treatment of choice in selected groups of patients with T- or B-cell malignancies or with neuroblastoma (1) and is also increasingly being used in patients with breast cancer (2–9). In a review of published data (10), it was concluded that in women with metastatic breast cancer ABMT resulted in increased complete remission and response rates, but the overall survival rate remained unchanged. One of several possible explanations for the lack of effect on relapse rate could be that the harvested BM might have been infiltrated with malignant cells. Micrometastatic disease is known to be more common than previously anticipated (11), even when the BM is negative as assessed by morphological examination (12, 13). To avoid reinfusion of malignant cells, which subsequently could give rise to relapse of the disease, several methods for eliminating breast cancer cells from the marrow have been developed. These include mechanical (14), chemical (15, 16), and immunological procedures (17–23).

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3 The abbreviations used are: ABMT, autologous bone marrow transplantation; BM, bone marrow; IT, immunotoxin; MAb, monoclonal antibody; PE, Pseudomonas exotoxin A; 4HC, 4-hydroperoxycytophosphamide; IC50, concentration resulting in 50% reduction in protein synthesis; PBS, phosphate-buffered saline; FCS, fetal calf serum; SAM, sheep anti-mouse; CFU-GM, colony-forming units, granulocyte/macrophage.

We previously developed immunological methods in which MAbs can be used together with paramagnetic beads or conjugated to the bacterial toxin PE for bone marrow purging of non-Hodgkin lymphoma (24, 25) and small cell lung cancer cells (26, 27). In the present study with breast cancer cell lines, we tested and compared the purging efficacy of ITs and immunomagnetic beads, in both cases involving the same three anticarcinoma MAbs. With each of the two methods, >5 log units of T-47D and MCF7 breast cancer cells could be eliminated without significant reduction in the clonogenic capacity of CFU-GM normal progenitor cells.

MATERIALS AND METHODS

Bone Marrow. BM aspirates were obtained from healthy volunteers or from patients at the Norwegian Radium Hospital with noncarcinoma malignancies who were free of tumor cells in their marrow. All samples were obtained with informed consent from the donors. Ten ml of BM were layered onto Lymphoprep (Nycomed, Oslo, Norway) and the mononuclear cell fraction was obtained by centrifugation at 1200 rpm for 30 min. The mononuclear cells were washed once in PBS before being used in the experiments.

Cell Lines. The T-47D, MCF7, and SK-BR-3 human breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). The small cell lung cancer line DMS-273 was acquired from Dr. George Sorensen, Dartmouth-Hitchcock Medical Center (Hanover, NH). The cell lines were grown in monolayer cultures in the following media (all from Gibco, Paisley, UK): T-47D and DMS-273 in RPMI 1640 medium plus 10% FCS, MCF7 in Dulbecco’s modified Eagle medium with 10% FCS (GIBCO), and SK-BR-3 in McCoy’s 5A medium with 15% FCS. All media contained 10 μg/ml insulin (Sigma Chemical Co., St. Louis, MO), 100 units/ml of penicillin, and 100 μl/ml streptomycin (GIBCO).

Antibodies and Toxin. The MOC-1 (28) and MOC-31 (29) MAbs were gifts from Dr. L. de Leij (University of Groningen, the Netherlands). MOC-1 (IgG1) binds to the neural cell adhesion molecule (CD56) (30), and MOC-31 (IgG2a) binds to an epithelial antigen (cluster 2). NRLa10, an IgG2b antibody recognizing a M, 39,000 antigen (31), and the 9.2.27 high molecular weight melanoma-associated IgG2a MAb (32) were supplied by NeoRx Corporation (Seattle, WA). MLuC1 (IgG1), provided by Dr. Sylvie Menard (National Cancer Institute, Milan, Italy), binds to a saccharide epitope carried by neutral glycolipids, glycoproteins, and mucins (33). PE was obtained from Swiss Serum and Vaccine Institute (Bern, Switzerland).

Measurement of Antigen Expression in Breast Cancer Cells. The expression of relevant antigens on the surface of the target cells was assessed by measuring the binding of radiolabeled MAbs to three different cell lines. The MAbs were labeled with 125I (Amersham, Buckinghamshire, UK) according to the method of Fraker and Speck (34) and were incubated with the cells for 1 h at 4°C. The cells were washed twice, the cell-bound radioactivity was counted in a standard gamma-counter, and the fraction of bound MAb was calculated and expressed as the percentage of the total labeled MAb added.

Preparation of ITs. Each of the antibodies was conjugated to PE via a thioether bond formed with sulfo-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL) as described previously (35), except that, after incubation of reduced MAb and maleimidomethylcyclohexane-1-carboxylate-PE at 4°C overnight, an equal volume of saturated ammoium sulfate was added and the preparation was further incubated for 1 h. The precipitate formed was collected by centrifugation at 10,000 × g for 10 min, dissolved in 1 ml of PBS, and subjected to gel filtration (36). Fractions containing purified conjugate as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (37) were pooled and used in the experiments. Protein concentration was determined by Bio-Rad protein assay, using bovine γ-globulin as the standard.
**METHODS FOR PURGING BM OF BREAST CANCER CELLS**

**IT Treatment Procedures.** The cytotoxic effect of the ITs was first assessed by measuring their ability to inhibit cellular protein synthesis. Breast cancer cells were seeded into Multidish 24-well plates (Nunc, Roskilde, Denmark), incubated for 3 h to let the cells adhere, and treated with four different concentrations of each of the ITs for 20 h at 37°C. Protein synthesis was measured using the [3H]leucine incorporation assay as described by Sandvig and Østnes (38), and the IC50 was calculated.

Clonogenic assays were used to examine the toxicity of the ITs on both tumor and normal cells. Mechanically dispersed tumor cells (2 x 10⁶), mononuclear BM cells (2 x 10⁶), or a mixture of both (total, 5 x 10⁶) were incubated for 2 h at 37°C with one or more ITs, in RPMI 1640 medium/FCS supplemented with antibiotics. The cells were washed twice in PBS with 1% FCS before being seeded for the appropriate clonogenic assays.

**Immunobeads and Immunomagnetic Treatment Procedure.** The immunomagnetic beads, Dynabeads SAM ST (Dynal, Oslo, Norway), are uniform, magnetic, polystyrene beads coated with covalently surface-bound sheep antimouse IgG. The SAM IgG antibodies bind all mouse IgG subclasses.

Mechanically dispersed tumor cells (1 x 10⁷), mononuclear BM cells (2 x 10⁶), or mixtures of both (total, 5 x 10⁶) were incubated in plastic tubes for 30 min at 4°C with one or more MAbs (10 μg of each), in 1 ml of RPMI 1640 medium with supplements. The cells were washed twice in PBS with 1% FCS before addition of the immunomagnetic beads suspended in 0.5 ml of medium and were then incubated at 4°C for 30 min. In the experiments with the cells, the ratio of beads to tumor cells was 50:1, and in the control experiments to test the effect on BM progenitor cells the ratio of beads to nucleated BM cells was also 50:1. To ensure proper contact between cells and MAbs/beads during the incubations, the tubes were rolled on a mixer (Coulter Electronics, Luton, UK). Tumor cells were removed by the use of a flat cobalt samarium magnet as described earlier (24).

Remaining clonogenic tumor cells and BM cells in the suspension were assessed in colony-forming assays as described below. In some experiments, a repeat cycle of immunomagnetic separation was performed.

** Colony-forming Assays for Tumor and BM Progenitor Cells.** The number of colony-forming tumor cells was assessed in a soft agar assay (39), as described previously (40). Briefly, soft agar cultures were set up in triplicate in 10 ml tubes by adding 0.2 ml August rat blood cells diluted 1:8, 0.2 ml of appropriately diluted BM/tumor cell suspensions, and 0.6 ml of 0.5% agar (Difco Laboratories, Detroit, MI). The tubes were incubated at 37°C in 5% O2/5% CO2/90% N2 and, after 21 days of incubation, colonies of >50 cells were counted using a Zeiss stereo microscope.

The viable clonogenic progenitor cells in the BM after cytotoxic or immunomagnetic treatment were assessed in a modified version (24) of the method described by Burgess et al. (41). Mononuclear BM cells were suspended to a concentration of 2 x 10⁶ cells/ml in McCoy's 5A medium containing 0.3% agar, 15% FCS, 20 ng/ml recombinant human granulocyte/macrophage colony-stimulating factor (provided by Schering Plough, Kenilworth, NJ), 100 units/ml penicillin, and 100 ml of streptomycin/ml of medium. The granulocyte/macrophage colony-stimulating factor concentration used was chosen on the basis of titration experiments. Triplicate 1-ml aliquots were cultured in 35-mm plastic dishes at 37°C and 5% CO2 in air and incubated for 14 days, and colonies of >40 cells were counted.

**RESULTS**

**IT Inhibition of Protein Synthesis and Relationship to Antigen Expression.** The MOC-1 antibody was not conjugated to PE, because it binds to the neural cell adhesion molecule antigen, which is not internalized from the cell surface, making it unsuitable as an immunotoxin. PE conjugates of the other three antibodies were highly cytotoxic to the three breast cancer cell lines examined (Table 1). In most cases, the IC50 values for the different conjugates in MCF7 and T-47D cells ranged between 9 x 10⁻¹² M and 4 x 10⁻¹³ M. Lower activity was seen for MLuC1 and NrLu10 ITs tested against the SK-BR-3 cell line, with IC50 values of 1 x 10⁻¹⁰ M and 5 x 10⁻¹¹ M, respectively.

The most sensitive cell line, MCF7, showed the highest expression of the three antigen epitopes, as measured by binding of radiolabeled free antibody. The fractions of the added amounts of MOC-31, NrLu10, and MLuC1 that bound to MCF7 cells were 29, 21, and 19%, respectively, whereas the corresponding values in the least sensitive cell line, SK-BR-3, were 7, 4, and 5% (Table 1).

The IC50 value for the nonbinding control IT (9.2.27-PE) in MCF7 cells was 1 x 10⁻⁹ M (data not shown). The DMS-273 small cell lung cancer line, which did not express detectable antigen levels, showed low sensitivity to the MOC-31 and NrLu10 conjugates (IC50 values of 2 x 10⁻⁹ M and 1 x 10⁻⁹ M, respectively). Based on these results, the three anticarcinoma ITs were further tested in clonogenic assays for T-47D and MCF7 cells.

**Sensitivity and Reproducibility of the Clonogenic Tumor Cell Assay.** When different numbers of T-47D or MCF7 cells were seeded in soft agar, the number of colonies formed was proportional to the number of plated cells (Fig. 1). For both cell lines the relationship was close to linear down to about 10 cells. The plating efficiency, calculated from the curves, was approximately 40% for the T-47D cells and 35% for the MCF7 cells. Similar curves were established in each therapy experiment and used to calculate the logarithm of the number of tumor cells eliminated by the treatment. The assay permitted reproducible detection of up to 5 log units of cell kill when using ITs and up to 6 log units of cell removal with immunobeads.

**Efficacy of IT Treatment.** The activity of the PE conjugates assessed in the soft agar assay is shown in Table 2. It can be seen that concentrations of the MOC-31 and NrLu10 ITs as low as 0.01 μg/ml eliminated 0.1–3.8 log units of MCF7 cells, with significant interexperiment variations. When the concentration was increased to 0.1 μg/ml, both ITs killed >5.0 log units of MCF7 cells and 1.5–1.7 log units of the more resistant T-47D cells. The MLuC1 conjugate showed only marginal effects in both cell lines at 0.1 μg/ml. At the highest concentration tested (1.0 μg/ml), however, all three ITs eradicated the tumor cells (>5.0 log units of cell kill), with the exception that only 2.2 log units of kill of T-47D cells were obtained with MLuC1-PE. It should be noted that when assessing the effect of cytotoxic treatment the sensitivity of the assay is limited to 5 log units of cell kill.

With the mixture of the three ITs >5.0 log units of MCF7 cells were eliminated at both 1.0 μg/ml and 0.1 μg/ml, and at 0.01 μg/ml 2.5 log units of cells were killed (Table 2). The results obtained with the mixture were slightly better than those seen with the individual ITs, but it should be noted that at each concentration level the total amount of IT added was 3 times higher. Also with the T-47D cells the mixture was only marginally superior to the most active individual IT. This may reflect the fact that the antigens recognized by both MOC-31 and NrLu10 are expressed on all tumor cells, and the efficacy of the treatment is therefore directly related to the total concentration of cell-bound IT.

![Table 1 Efficacy of PE conjugates of MOC-31, MLuC1, and NrLu10 to inhibit protein synthesis of human breast cancer cells](image-url)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Binding of MAb* (%)</th>
<th>IC50 of ITb (M)</th>
<th>Binding of MAb* (%)</th>
<th>IC50 of ITb (M)</th>
<th>Binding of MAb* (%)</th>
<th>IC50 of ITb (M)</th>
</tr>
</thead>
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<tr>
<td>MCF7</td>
<td>29</td>
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<td>21</td>
<td>8 x 10⁻¹³</td>
<td>19</td>
<td>4 x 10⁻¹³</td>
</tr>
<tr>
<td>T-47D</td>
<td>22</td>
<td>1 x 10⁻¹²</td>
<td>13</td>
<td>5 x 10⁻¹²</td>
<td>12</td>
<td>8 x 10⁻¹²</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>7</td>
<td>9 x 10⁻¹²</td>
<td>4</td>
<td>5 x 10⁻¹¹</td>
<td>5</td>
<td>1 x 10⁻¹⁰</td>
</tr>
</tbody>
</table>

* Binding of radiolabeled free antibody, expressed as percentage bound/total added.

b Activity of conjugates of each MAb and PE, expressed as IC50 (see "Materials and Methods").
Efficacy of Immunobead Purging. The same MAbs, as well as MOC-1, were also used together with SAM antibody immunobeads to assess the efficacy in removing T-47D and MCF7 cells in a magnetic field. MOC-1 did not show detectable binding to these cell lines. In concordance with this, MOC-1 showed only marginal effects with the immunobeads in one of the cell lines (T-47D), results that were not significantly different from those obtained in control experiments with SAM antibody beads alone (Table 3). With the other MAbs, the best results for both cell lines were obtained with MOC-31 and NrLu10, giving cell removals in the range of 3.7–4.1 log units with one purging cycle. MLuC1 was less effective, removing about 2.0 log units of tumor cells. With two purging cycles, the effect increased for all MAbs. Thus, MOC-31 and NrLu10 with immunobeads eliminated approximately 0.3 µg/ml. The immunomagnetic method resulted in a moderate reduction of tumor cells. With T-47D cells the effect of 0.1 µg/ml concentrations of the ITs increased from 2.3 log units to >5.0 log units. The inconsistencies in the results obtained at the two lower IT concentrations probably reflect inter-experiment variation and seem to suggest that the critical concentration for obtaining effective cell kill is approximately 0.3 µg/ml.

Survival of Human BM Progenitor Cells after Treatment with Immunobeads or ITs. The effect of the two treatment procedures on the survival of normal BM progenitor cells is summarized in Table 5. The immunomagnetic method resulted in a moderate reduction of CFU-GM. Thus, with the individual MAbs 49–86% of CFU-GM survived, and when a mixture of all three MAbs was used the survival was 53%.

Toxicity of the ITs to the progenitor cells was also low to moderate. Treatment with each of the ITs tested individually at concentrations from 1.0 to 5.0 µg/ml resulted in a reduction of CFU-GM to about 75–45% of that in the control cultures. Even at concentrations as high as 10 µg/ml, ≥25% of the clonogenic cells survived the treatment. The mixture of three ITs also showed a moderate toxicity, with reduction of the surviving CFU-GM to 44% at the highest dose tested (2.5 µg/ml).

DISCUSSION

Two highly effective immunological methods for eliminating breast cancer cells from human BM were developed. The use of three MAbs and immunomagnetic beads removed up to 6.0 log units of tumor cells as assessed in a highly reproducible clonogenic soft agar assay. The other approach, involving conjugates of the same MAbs and PE, also eradicated all malignant cells, but when using ITs the assay does not permit detection of >5.0 log units of tumor cell kill. Both purging procedures reduced the survival of normal BM progenitor cells to only a limited extent, indicating that they can be safely used in a clinical setting in conjunction with ABMT.

The effect of high dose chemotherapy combined with ABMT in patients with breast cancer has been investigated in several studies. In a recent review (10) it was concluded that, although this approach can result in higher response rates, it is still unclear whether response duration and survival rates may also be improved. Clearly, careful selection of patients who could possibly benefit from the treatment is important. Peters et al. (42) have recently obtained promising results in patients with stage II/III breast cancer with 10 or more cancer-positive axillary lymph nodes. Thus, among 85 evaluable patients with a mean follow-up of 2 years, 72% had disease-free survival, results which are far better than those obtained in historic control trials in comparable patient groups. In none of the studies was purging of tumor cells from the harvested BM performed. There is, however, evidence that a high percentage of breast cancer patients have immunologically detectable micrometastases in the BM, even in cytologically negative cases (12, 13, 43–45). As previously concluded by others (15, 17, 23), it is therefore logical to include an effective purging procedure for patients receiving BM transplants.

Several methods for removing breast cancer cells from human BM have been reported (14, 16–23). The highest purging efficiencies, up to 4.5–5.4 log units of tumor cell depletion, were obtained with magnetic separation procedures involving MAbs or soybean agglutinin, with or without the addition of 4-HC (19, 20, 22, 23). Our immunomagnetic method was even more effective, with >6-log unit removal of breast cancer cells without harmful effects on normal hematopoietic cells. The recovery of BM progenitor cells was also good in some of the previous studies, but methods involving 4-HC reduced the survival of CFU-GMs to about 20% of the control values (19, 23). In agreement with these results, Vredenburgh et al. (46) recently demonstrated in a clinical study that the engraftment was
METHODS FOR PURGING BM OF BREAST CANCER CELLS

Table 2  Effect of three immunotoxins involving Pseudomonas exotoxin A in killing T-47D and MCF7 breast cancer cells

Tumor cells were incubated with immunotoxins for 2 h at 37°C and seeded in soft agar, and colony formation was assessed as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>PE ITs with MAbs</th>
<th>Cell line</th>
<th>Log cell killa at IT concentrations of</th>
<th>0.01 μg/ml</th>
<th>0.1 μg/ml</th>
<th>1.0 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Meanb</td>
<td>Range</td>
<td>Meanb</td>
<td>Range</td>
</tr>
<tr>
<td>MOC-31</td>
<td>T-47D</td>
<td>NTc</td>
<td>0.1-2.9</td>
<td>1.5</td>
<td>1.1-2.3</td>
</tr>
<tr>
<td></td>
<td>MCF7</td>
<td>1.5</td>
<td></td>
<td>&gt;5.0</td>
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</tr>
<tr>
<td>NrLu10</td>
<td>T-47D</td>
<td>NTc</td>
<td>0.1-3.8</td>
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<td>1.1-2.4</td>
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<tr>
<td></td>
<td>MCF7</td>
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<td></td>
<td>&gt;5.0</td>
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<tr>
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<td>T-47D</td>
<td>NTc</td>
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<tr>
<td></td>
<td>MCF7</td>
<td>NTc</td>
<td></td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>MOC-31 + NrLu10 + MLuC1d</td>
<td>T-47D</td>
<td>NTc</td>
<td>0.0-5.0</td>
<td>2.3</td>
<td>0.6-2.6</td>
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<tr>
<td></td>
<td>MCF7</td>
<td>2.5</td>
<td></td>
<td>&gt;5.0</td>
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</tr>
</tbody>
</table>

a Calculated as in Table 1.
b Mean of 2-4 independent soft agar experiments, each in triplicate.
c NT, not tested.
d Each immunotoxin was used at the concentration indicated.

Table 3  Efficacy of SAM antibody-coated paramagnetic particles (immunobeads) in removing T-47D and MCF7 breast cancer cells incubated with different primary antibodies, individually and in combination

Tumor cells were incubated for 30 min at 4°C with the MAbs (10 µg/ml of each) and then for 30 min with SAM Dynabeads at a ratio of beads to tumor cells of 50:1. After magnetic separation, the remaining cells were seeded in soft agar and colony formation was assessed as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>MAb</th>
<th>T-47D</th>
<th>MCF7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of purging cycles</td>
<td>Log cell depletiona</td>
</tr>
<tr>
<td>None</td>
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<tr>
<td>MOC-1</td>
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<td>MOC-31</td>
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<tr>
<td>MLuC1</td>
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<tr>
<td>MOC-31 + NrLu10 + MLuC1d</td>
<td>3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

a Calculated from the observed number of colonies, taking the plating efficiency into account, determined as the logarithm of the number of tumor cells depleted by the treatment.
b Mean of results obtained in 2-4 independent soft agar experiments, each performed in triplicate.

Table 4  Comparison of the efficacy of immunotoxins and immunobeads, involving the same MAbs, in eliminating T-47D and MCF7 cells from fresh human BM

Tumor cells were mixed with nucleated BM cells at a ratio of tumor cells to BM cells of 1:10, treated, seeded in soft agar, and assayed as in Tables 2 and 3. Tumor/BM cell mixtures were incubated either with Pseudomonas exotoxin conjugates of the MAbs for 2 h at 37°C or with MAbs and then with SAM Dynabeads at a ratio of beads to tumor cells of 50:1, in both cases for 30 min at 4°C. Two purging cycles were used.

<table>
<thead>
<tr>
<th>MAb</th>
<th>T-47D</th>
<th>MCF7</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No. of experiments</td>
<td>Log tumor cell eliminationb</td>
</tr>
<tr>
<td>MOC-31 + NrLu10 + MLuC1d</td>
<td>2</td>
<td>&gt;5.0</td>
</tr>
<tr>
<td>MCF7</td>
<td>2</td>
<td>&gt;5.0</td>
</tr>
</tbody>
</table>

a Concentration of each IT.
b Calculated as in Table 1.
c Mean of the results obtained in independent soft agar experiments, each performed in triplicate.
d Each immunotoxin was used at the concentration indicated. Ten µg/ml concentrations of each MAb were used for the immunobeads.

faster in patients receiving immunomagnetically purged BM, compared to patients for whom 4-HC was used alone or in combination with immunobeads.

A number of ITs have been used, with varying degrees of success, as means to purge BM of breast cancer cells. Studies have included the toxin ricin (17) or recombinant ricin A chain (19), whereas abrin A chain conjugates were used clinically to assess the toxicity of the IT treatment on the recovery of normal BM function (47). Bjorn et al. (18) used a conjugate of PE and NrLu10, i.e., a conjugate similar to one of the ITs studied in the present work. Among the previous studies, the highest tumor cell kill was reported by O'Briant et al. (19), who used a combination of five conjugates containing ricin A chain, but in their work the depletion effect varied considerably in different experiments. The specific activity of ITs to tumor cells is highly dependent on a favorable matching of MAbs and toxins (35). We found in pilot experiments that the most effective ITs with our MAbs were obtained by conjugation to PE (data not shown). The high efficacy and specificity of these conjugates were demonstrated in the protein synthesis inhibition assay (Table 1), in which the IC50 values in most cases were in the range of 9 × 10^{-12} M to 4 × 10^{-13} M.
Nucleated BM cells were treated with immunotoxins or immunobeads and seeded in culture dishes (2 × 10^6 cells/dish), and the number of CFU-GM was assessed as described in "Materials and Methods." The nucleated cells were incubated either with a ratio of beads to cells of 50:1, in both cases for 30 min at 4°C. Two purging cycles were used.

Table 5 Survival of human BM progenitor cells after treatment with immunotoxins or immunobeads

<table>
<thead>
<tr>
<th>MAbs</th>
<th>ITs Concentration (μg/ml)</th>
<th>Fraction of colonies controlb mean range</th>
<th>Antibody concentration (μg/ml)</th>
<th>No. of colonies controlb mean range</th>
<th>Fraction of colonies controlb mean range</th>
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<tr>
<td></td>
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<tr>
<td>MOC-31</td>
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<tr>
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<td>66</td>
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<td>71-100</td>
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<tr>
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<td>MOC-31 + NrLa10 + MLuc1</td>
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<td>126</td>
<td>79</td>
<td>60-88</td>
<td>10 × 3</td>
</tr>
<tr>
<td></td>
<td>2.5 × 3</td>
<td>44</td>
<td>39</td>
<td>23-55</td>
<td>10 × 3</td>
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</tbody>
</table>

a Mean of the results obtained in 2-4 independent experiments, each performed in triplicate.
b Fraction (%) of remaining colonies calculated relative to the number of colonies in untreated control cultures.

(∼0.1–1.8 ng/ml), compared to about 1 × 10^-9 M in the control experiments. The specific activity of these ITs was apparent also in the colony-forming assays for breast cancer cells (>5-log unit cell kill) and normal BM cells (44–75% surviving CFU-GMs). It should be mentioned that the combination of ITs used here showed a similar colony-forming assay for breast cancer cells (>5-log unit cell kill) experiments. The specific activity of these ITs was apparent also in the (-0.1–1.8 ng/ml), compared to about 1 × 10^-9 M in the control experiments.

Our experimental procedures yielded the highest purging efficacies so far reported with breast cancer cells, as evaluated in an assay that permits a reliable and sensitive estimation of tumor cell kill, calculated and expressed as log units of tumor cell elimination. The validity of such calculations is dependent on a close to linear relationship between the number of plated cells and colonies formed, as was seen for the MOC-31 and MLuc1 cell lines. Each of the two purging methods may be used in a clinical setting, and no conclusive argument in favor of one or the other is available. The immunobead methodology is technically feasible and has successfully been used clinically with several tumor types (24, 48, 49), but because it is necessary to use two purging cycles the method is time-consuming and the beads are relatively expensive. The IT approach, on the other hand, is simple and can be completed 2-3 h faster than the immunobead technique. Although the use of ITs might be regarded as potentially harmful, it was shown here that the survival of BM progenitor cells was only modestly affected at IT doses of at least 2.5 μg/ml for each of the three conjugates, concentrations which were 2.5–25-fold more than needed to eliminate all tumor cells in the model experiments. Moreover, we have calculated that in a clinical situation the maximum amount of IT in the treated marrow that could possibly be reinfused into the patient is at least 100-fold lower than what might be expected to give any systemic toxicity (27).

To be clinically useful the antigens recognized by the MAbs should be expressed on all tumor cells in all cases of breast cancer. The epitopes of the glycoprotein antigen that bind MOC-31 and NrLa10 are present on the surface of most breast cancer cells, as revealed in studies on cell lines and fresh tumor specimens (18, 50). The third antibody, MLuc1, binds to an antigen closely related to the Lewis Y (LeY) antigen, which is abundantly expressed on human carcinoma lines (33, 51). Together, the three MAbs should be complementary in ensuring that the heterogeneity of antigen expression in clinical breast cancer is satisfactorily covered.

An alternative to conventional ABMT is to transplant stem cells positively selected from peripheral blood or BM. In theory, such procedures could eliminate the need for purging. It should be noted, however, that of patients with operable breast cancer and >10 positive nodes 62% had tumor cells in peripheral blood detected by fluorescein-activated cell sorting, compared to 2% revealed by conventional cytology (43). Moreover, it has recently been reported that the risk for tumor cell contamination of peripheral blood stem cell harvests is present (52–54). In such cases, CD34+ selection might effectively reduce the number of, but not necessarily eliminate, contaminating tumor cells (54). It seems, therefore, that it may be necessary to use effective purging procedures, like those developed in the present work, also with patients receiving BM or peripheral blood stem cell support.

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Comparison of Two Antibody-based Methods for Elimination of Breast Cancer Cells from Human Bone Marrow

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