ABSTRACT

Soybean agglutinin (SBA) was used as a differential reagent to achieve selective elimination of human breast cancer cells (T-47D cell line) from human marrow contaminated with tumor cells. Two successive cycles of direct agglutination by soluble SBA resulted in depletion of 3.5 logs of tumor cells as determined by radiolabeling, whereas removal of more than 4 logs of tumor cells was demonstrated by a clonogenic bioassay. A more convenient procedure for tumor purge involved the use of SBA bound to either polyglutaraldehyde magnetic beads or to commercial polystyrene magnetic beads. After one cycle of magnetic separation, 2 to 3.5 logs of tumor cells were removed. A second separation cycle using fresh magnetic beads improved depletion to more than 4 logs. Neither of these purging procedures affected the hematopoietic potential of granuloid-macrophage colony-forming unit cells. We suggest the use of SBA bound to magnetic beads as a convenient tool for effective ex vivo purging of marrow aspirates contaminated with metastatic breast cancer cells in patients with advanced disease. A similar procedure is applicable for all SBA-positive neoplasms.

INTRODUCTION

High-dose chemotherapy or chemoradiotherapy followed by rescue with cryopreserved autologous BM is a potentially curative therapeutic modality for an increasing number of patients with various neoplastic disease (1–4). A variety of immunological and pharmacological procedures have been investigated for ex vivo purging of marrow grafts contaminated with leukemia, lymphoma, neuroblastoma, or small cell lung carcinoma cells (5–10). Breast cancer, a most frequent malignant disorder in adult females, is one of the frequent malignant disorders in which the availability of cryopreserved autologous BM would allow escalation of cytotoxic therapeutic doses, thereby enabling more efficient eradication of metastatic tumor cells and prolongation of disease-free survival time. Unfortunately, at advanced stages of disease, BM involvement occurs in some 40 to 70% of patients (11).

We have recently reported a preliminary study in which SBA was utilized to remove cells of a breast cancer cell line from artificial mixtures of tumor and human BM cells (12). Our decision to attempt selective removal of breast cancer cells from human marrow aspirates using SBA was based on the following two observations: (a) binding of SBA to breast cancer cells was demonstrated in histological sections of breast carcinoma tissue (13, 14), and only 5% of 130 studied were totally negative; and (b) hematopoietic stem cells are SBA negative (15). SBA-treated marrow is used routinely in many centers as part of the T-cell depletion procedure in allogeneic BMT following marrow ablative doses of chemoradiotherapy (16–18).

In the present study we have evaluated the use of SBA for purging tumor cells using various types of magnetic beads as SBA carriers, as well as by direct agglutination with soluble SBA. Assessment of the degree of tumor cell elimination was accomplished by a clonogenic bioassay.

MATERIALS AND METHODS

Breast Cancer Cell Lines. Breast cancer cell lines T-47D (19) (a gift from Dr. I. Kedar, University of Tel Aviv, Israel), MDA-MB 231 (20), and MCF/7 (21) (a gift from Dr. D. Sulizeanu, Hebrew University, Jerusalem, Israel) were grown in RPMI medium, as detailed in our previous paper (12). Insulin (0.2 units/ml) was added only to the medium for culturing the T-47D cell line.

Flow cytometric analysis, labeling of T-47D, preparation of tumor/normal bone marrow cell mixtures, and depletion of tumor cells by agglutination were carried out, as previously described by us (12).

Binding of SBA to Magnetic Beads. PG magnetic beads were prepared by Dr. S. Margel (Weizmann Institute of Science, Israel) as previously described in detail (22). Binding of SBA was carried out by mixing 5 mg of magnetic beads with 0.5 mg of SBA in a total volume of 3 ml of distilled water for 3 h with continuous gentle agitation at room temperature. Unbound SBA was removed by six washes with water using a magnetic field. The SBA-PG magnetic beads were quenched with 3 ml of 0.5% human serum albumin in water and kept in this solution at 4°C until use. PS magnetic beads (Dynabeads M-450) were kindly supplied by Dynal, Norway. Binding of SBA was carried out by mixing 105-mg beads with 5 mg of SBA in a total volume of 6.6 ml of PBS for 16 h at 4°C by end-over-end rotation. Dynabeads were collected by placing the suspension in a cobalt samarium MFC kindly supplied by Dynal. Coated beads were washed twice immediately and then twice, 2 h each, by end-over-end rotation at 4°C. The fifth wash was done overnight in PBS containing 0.1% BSA. SBA-PS-magnetic beads were resuspended to a concentration of 30 mg/ml with the same buffer (PBS/BSA) and kept at 4°C until use. Conjugates of SBA-magnetic beads were found to be effective for at least 2 mo.

Depletion of Tumor Cells by SBA–Magnetic Beads. SBA-PG magnetic beads were mixed with the contaminated BM cells (5% and 10% T-47D cells) as follows: 1.3 mg of SBA-PG were incubated with 20 x 10⁶ cells in a total volume of 0.3 ml for 15 min at room temperature. Magnetic particles were removed over a MFC, and the unbound cells were collected and washed with 1% BSA in PBS. When 2 cycles of separation were carried out, the SBA-negative cell fraction was subjected to another interaction with new SBA-PG magnetic beads in the following ratio: 0.40 mg of SBA-bead/4 x 10⁷ cells.

SBA-PS magnetic beads were mixed with tumor BM cell mixtures as follows: 12.3 mg of SBA-PS were incubated with 20 x 10⁶ cells in a total volume of 0.8 ml for 15 min at room temperature. Magnetic beads were removed by MFC, and the cells collected and were washed and subjected to a second cycle of cell separation. In the second cycle, 0.9 mg of SBA-PS beads were mixed with 2 x 10⁶ cells for 15 min at room temperature, and the SBA-negative cells were collected.

Clonogenic Assay. Various amounts of T-47D cells (10 to 1000) were plated with 2 x 10⁶ irradiated (4000 rads) fresh human BM cells suspended in 1 ml of culture medium supplemented with 15% FBS and 0.5% agar (Difco, MI). Cell suspensions were each plated over a layer...
of 0.5% agar and 10% conditioned medium supernatant collected from
with 0.3% agar and 20% FBS were plated over 1 ml of a layer consisting
of Pike and Robinson (23) with a slight modification, as previously
described (12). Briefly, 2 x 10^5 BM cells suspended in 1 ml of McCoy's
Medium 5A (Gibco Laboratories, Grand Island, NY) supplemented
with 0.3% agar and 20% FBS were plated over 1 ml of a layer consisting
of 0.5% agar and 10% conditioned medium supernatant collected from
5637 bladder carcinoma cell line (kindly supplied by Dr. E. Fibach,
Department of Hematology, Hadassah University Hospital, Jerusalem,
Israel). Plates were kept at 37°C in a 5% CO2-humidified incubator for 12 to 14 days.
T-47D cells were subjected to agglutination (6000 rads) before mixing
with human BM cells in order to avoid colony growth of T-47D origin
which might affect the growth of GM-CFU.

RESULTS

Binding of Fluorescein Isothiocyanate-conjugated SBA to
Breast Cancer Cell Lines. As shown in Fig. 1, fluorescence-
activated cell sorter analysis of 2 x 10^6 T-47D cells showed
bright fluorescent SBA staining which could be abrogated in
the presence of the SBA-specific inhibitor d-galactose. A large
fraction of MDA-MB 231 and MCF/7 cells also showed a
strong fluorescent stain (data not shown).

Depletion of T-47D Cells from BM/Tumor Cell Mixtures after
One Cycle of Separation by Soluble SBA or SBA-PG. Artificial
mixtures consisting of normal human BM cells and 5 to 10%
51Cr-labeled T-47D tumor cells were subjected to agglutination
by soluble SBA or to interaction with SBA-PG magnetic beads.
After removal of SBA-positive cells (T-47D as well as those
present in human BM), radioactivity remaining in the SBA-
negative fraction was measured and compared to the total
radioactive recovery. In Table 1 we compared the efficacy of T-
47D depletion obtained by soluble SBA with depletion accom-
plished by SBA-PG magnetic beads. Depletion of the same
order of magnitude was achieved by both means: 98.1% of the
radioactivity was removed from the SBA-negative fraction by
soluble SBA and 97.8% by SBA-PG magnetic beads. Total cell
recovery in the SBA-negative fraction was between 4 and 10%
by direct agglutination and 5 and 10% by SBA-PG magnetic
beads. These results indicate that SBA-PG magnetic beads are
as effective as soluble SBA for tumor cell depletion. None of
these separation procedures affected the recovery of GM-CFU
hematopoietic cells (data not shown).

Removal of 51Cr-radiolabeled T-47D Cells from BM/Tumor
Cell Mixtures by Two Cycles of Agglutination with SBA. Fresh
human BM cells mixed with 10% 51Cr-radiolabeled T-47D cells
were subjected to agglutination with SBA. In order to improve
the degree of BM purging, the SBA-negative fraction collected
in the first step was subjected to a second cycle of SBA agglu-
tination. The degree of depletion was calculated by comparing
radioactivity left in the second SBA-negative fraction to the
total radioactivity recovered in both SBA-positive fractions of
the 2 cycles and the SBA-negative fraction collected in the
second cycle. After 2 cycles of agglutination, the residual radio-
activity left was 0.05% and 0.025% in the 2 experiments pre-
sented in Table 2. Depletions of tumor cells expressed in orders
of magnitude were 3.3 and 3.6, respectively. Log depletions of
tumor cells calculated in relation to radioactivity added to the
original mixtures were 3.5 and 3.8, respectively. The total
number of cells recovered in the SBA-negative fraction after 2
cycles of agglutination was between 3.5 and 5%.

T-47D Clonogenic Assay. In order to improve the evaluation
of tumor cell depletion, we developed a sensitive biological
assay which provides better biological information than the
radiolabeling assay. Various amounts of T-47D cells (10 to 10^3)
with or without 2 x 10^5 BM cells were plated in agar. Tumor
colonies consisting of more than 10 cells each were formed
between Days 12 and 14.

The number of T-47D colonies formed was not affected by
the presence of BM cells (data not shown). Irradiation of BM
cells before mixing with T-47D cells was carried out in order to
prevent colony growth from BM origin, thus enabling exact
titration of tumor cells for establishment of a standard clono-
genic assay in the experimental model. Double logarithmic
plots of the number of T-47D colonies formed as a function of
the number of cells plated gave a straight line with a slope of
0.91, indicating the clonal origin of the colonies. Clonogenic
efficiency ranged between 30 and 80% when small numbers of
tumor cells were plated and between 60 and 80% when large
numbers of cells were plated. Fig. 2 demonstrates a titration
curve which summarizes 3 different experiments. A titration
curve of this type was later performed for each experiment of
tumor cell depletion, and the number of tumor cells left after
purging was calculated by extrapolation on the titration curve.

Depletion of T-47D Cells by Two Cycles of Agglutination
with SBA as Assessed by Clonogenic Assay. Fresh human BM cells
were mixed with 10% T-47D cells and subjected to 2 cycles of
agglutination with SBA. A sample of 2 x 10^5 cells obtained
from the second cycle SBA-negative fraction was plated in a T-
47D clonogenic assay. From a standard titration curve done on
the same day, we extrapolated the number of tumor cells present

Table 1: Depletion of 51Cr-radiolabeled T-47D cells from 5 to 10% tumor/bone
marrow cell mixtures by direct agglutination with SBA or by SBA covalently
bound to polyglutaraldehyde magnetic beads (SBA-PG)

<table>
<thead>
<tr>
<th>% of radioactivity in SBA-negative fraction out of total radioactivity recovered</th>
<th>Median</th>
<th>Range</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBA agglutination</td>
<td>1.9</td>
<td>0.2-9.2</td>
<td>12</td>
</tr>
<tr>
<td>SBA-PG</td>
<td>2.2</td>
<td>1.3-3.3</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2: Depletion of 51Cr-radiolabeled T-47D cells by 2 cycles of agglutination
with SBA

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cpm recovered in 2 cycles</td>
<td>2.8 x 10^6</td>
</tr>
<tr>
<td>Radioactivity left in SBA-negative fraction out of total radioactivity recovered</td>
<td>0.050%</td>
</tr>
<tr>
<td>Log depletion</td>
<td>3.3</td>
</tr>
<tr>
<td>Radioactivity left in SBA-negative fraction out of radioactivity added</td>
<td>0.030%</td>
</tr>
<tr>
<td>Log depletion</td>
<td>3.5</td>
</tr>
</tbody>
</table>
**Removal of Breast Cancer Cell by Soybean Agglutinin**

Fig. 2. Titration curve of T-47D clonogenic assay. Points, mean of 3 different experiments (each performed in triplicate); bars, SE.

**Table 3 Depletion of tumor cells by 2 cycles of agglutination with SBA as assessed by the T-47D clonogenic assay**

Artificial mixtures consisting of 10% T-47D cells and fresh bone marrow cells were subjected to 2 cycles of agglutination with soluble SBA. The number of T-47D cells left in the second SBA-negative fraction was determined in a clonogenic assay.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of T-47D colonies formed in 2 x 10^6 cells of SBA-negative fraction</th>
<th>No. of T-47D present in 2 x 10^6 cells of SBA-negative fraction</th>
<th>Total no. of T-47D cells left in SBA-negative fraction</th>
<th>Total no. of T-47D cells added to original BM/tumor cell mixture</th>
<th>Log depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20 ± 0.73^a</td>
<td>34</td>
<td>425</td>
<td>8.0 x 10^4</td>
<td>4.3</td>
</tr>
<tr>
<td>2</td>
<td>20 ± 1.47</td>
<td>29</td>
<td>188</td>
<td>4.0 x 10^6</td>
<td>4.3</td>
</tr>
<tr>
<td>3</td>
<td>281 ± 15.8</td>
<td>400</td>
<td>3260</td>
<td>3.8 x 10^6</td>
<td>3.0</td>
</tr>
</tbody>
</table>

^a Mean ± SE of triplicates.

Experiment 1 was done with SBA-PG magnetic beads, while Experiments 2 and 3 were done with SBA-PS magnetic beads.

DISCUSSION

The present report describes the use of the lectin SBA to remove tumor cells from artificial mixtures of breast carcinoma.

**Table 4 Depletion of tumor cells by two cycles of separation with SBA-magnetic beads, as assessed by T-47D clonogenic assay**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of T-47D colonies present in 2 x 10^6 cells of first SBA-negative fraction</th>
<th>Total no. of T-47D cells in SBA-negative fraction</th>
<th>Log depletion</th>
<th>No. of T-47D colonies present in 2 x 10^6 cells of second SBA-negative fraction</th>
<th>Total no. of T-47D cells in second SBA-negative fraction</th>
<th>Yield of total cells in second SBA-negative fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ND^b</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>7.5%</td>
</tr>
<tr>
<td>2</td>
<td>64 ± 4.0^c</td>
<td>800</td>
<td>3.22</td>
<td>2 ± 0.8</td>
<td>9 ± 2.5</td>
<td>11.5%</td>
</tr>
<tr>
<td>3</td>
<td>46 ± 0.5</td>
<td>575</td>
<td>3.55</td>
<td>9 ± 2.5</td>
<td>8 ± 2.5</td>
<td>8.7%</td>
</tr>
</tbody>
</table>

^b ND, not determined.

^a Mean ± SE.

Removal of T-47D Cells and GM-CFU Recovery from the BM/Tumor Cell Mixture Subjected to Two Cycles of Interaction with SBA-Magnetic Beads. Artificial mixtures consisting of 10% T-47D tumor cells and fresh human BM cells were subjected to 2 cycles of interaction with SBA bound to either PG or PS magnetic beads. Efficacy of tumor cell depletion was assessed by T-47D clonogenic assay. Results of 3 experiments are summarized in Table 4. In Experiment 1, PG magnetic beads were used, and of 2 x 10^6 T-47D cells added to the original mixture, 127 were left after 2 cycles of cell separation. In Experiments 2 and 3, PS magnetic beads were used, and samples of both steps of cell separation were taken. In Experiment 2, of 1.3 x 10^6 T-47D cells added to original mixture, 800 tumor cells were left after the first cycle. The second step further improved removal of tumor cells; only 15 T-47D cells were left in the SBA-negative fraction. In Experiment 3, of 2 x 10^6 T-47D cells in the original mixture, 575 tumor cells were left in the SBA-negative fraction after the first step of separation and 78 tumor cells after the second cycle.

Efficacy of tumor cell removal after one cycle of separation with PS magnetic beads was calculated to be 3.2 and 3.5 orders of magnitude in Experiments 2 and 3, respectively. After 2 cycles of separation, depletion of tumor cells achieved with PG magnetic beads was 4.2 orders of magnitude and 4.95 or 4.4 with PS magnetic beads. Total cell yield in the SBA-negative fraction after 2 cycles of separation was between 7.5 and 11.5%. Using SBA-PS magnetic beads for tumor cell depletion did not affect the recovery of GM-CFU after 2 cycles of cell separation. As can be seen in Table 5, the number of GM-CFU postseparation was approximately the same as found in the original untreated fresh human BM cells (58 versus 48 in Experiment 1 and 212 versus 233 in Experiment 2).

**Table 5 Recovery of GM-CFU in T-47D/bone marrow mixtures after 2 cycles of separation with SBA-PS beads**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>GM-CFU/2 x 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Untreated BM cells</td>
</tr>
<tr>
<td></td>
<td>SBA-negative fraction after 2 cycles of magnetic beads</td>
</tr>
<tr>
<td>2</td>
<td>Untreated BM cells</td>
</tr>
<tr>
<td></td>
<td>SBA-negative fraction after 2 cycles of magnetic beads</td>
</tr>
</tbody>
</table>

^a Mean ± SE.
cell line-derived cells (T-47D) and fresh normal human BM cells. This experimental system was developed to simulate the BM aspirate of metastatic breast cancer infiltrating the marrow in patients with advanced disease, bearing in mind autologous BMT as a rational form of therapy. Nevertheless, because of the ubiquitous distribution of SBA in a variety of cancer cells, our method represents a model system for other SBA-positive tumors as well. Our studies demonstrate that one cycle of separation, carried out either by agglutination with soluble SBA or by SBA covalently bound to PG magnetic beads, results in a depletion of radiolabeled tumor cells of almost 2 logs. Hema-topoietic cells of the GM-CFU lineage were not affected by these procedures (data not shown). Moreover, tumor cell elimination of 3.5 orders of magnitude could be achieved by subjecting the mixtures to 2 successive cycles of agglutination with soluble SBA (Table 2) or, as we have already shown, by combining one cycle of agglutination with one cycle of polyacrolein magnetic bead separation (12). These results were obtained from a radiolabeling assay using $^{51}$Cr-labeled tumor cells, which provide an immediate evaluation of the efficacy of tumor cell depletion.

In order to evaluate the effect of our purging techniques on residual SBA-negative clonogenic tumor cells, we established the conditions for a tumor colony growth assay in agar. Clonogenic efficiency was mostly above 65%, with the exception of a few cases of low efficiency (<50%) for low doses of T-47D cells (10 cells) on the titration curve. Contamination as low as 0.005% of tumor cells seeded in $2 \times 10^5$ BM cells could be detected by our clonogenic assay. This provided us with a more precise, biologically meaningful evaluation of residual tumor cells than could be obtained from the radiolabeling assay. The degree of tumor cell depletion after 2 cycles of SBA agglutination was 3.5 logs, as determined by immediate radiolabeling, whereas the clonogenic assay indicated a depletion of 4.3 logs in 2 experiments and 3 logs in one experiment. The clonogenic assay is of practical relevance, since it can provide indication of the growth potential of residual tumor cells following marrow purging in clinical autologous transplantation.

Various types of SBA-bound magnetic beads were tested in an attempt to identify the optimal purging technique. In a previous study (12) we compared direct agglutination by soluble SBA and SBA covalently bound to polyacrylamide magnetic beads of various sizes (0.7 to 5 $\mu$m). No difference was found in the degree of tumor cell depletion achieved by these 2 techniques. In the present investigation we used PG magnetic beads (0.2 $\mu$m) covalently bound to SBA, as well as the commercial PS magnetic beads (4.5 $\mu$m) bound to SBA. As with the case with direct agglutination by soluble SBA, 2 cycles of interaction with SBA-magnetic beads improved elimination of tumor cells when compared with one cycle of separation. A comparable degree of depletion was obtained by SBA-PS beads and SBA-PG beads. However, PS beads displayed somewhat more efficient tumor cell depletion (4.95 and 4.4 logs as compared to 4.2 logs) and better total recovery of nucleated cells. Both types of beads spare the GM-CFU hematopoietic cells.

The use of SBA magnetic beads has several advantages over direct agglutination by soluble SBA. (a) Separation of the SBA-negative fraction is technically simple and faster. This aspect is of particular importance when large volumes are involved, as is the case in clinical situations in preparation for autologous BMT. (b) The yield of total nucleated cells was better than with soluble SBA. It is likely that nonspecific cell loss in large-scale separation necessary for clinical BMT would be proportionately even less than that observed in small-scale experiments. Previous studies have indicated that, although 90% of the nucleated cells are lost following purging with SBA (and rosetting with sheep RBC) in allogeneic BMT as part of the T-cell depletion procedure, most GM-CFU are left in the final fraction, and fast hematopoietic reconstitution of lethally conditioned recipients is the rule (17). Therefore, most of the cell loss following fractionation by marrow with SBA is clinically irrelevant, since human pluripotent stem cells are clearly SBA negative (15-18). SBA agglutination or SBA bound to Sepharose columns was previously tested in 2 experimental models, including neuroblastoma and T-cell leukemia line cells. Only 1 log of depletion was achieved for neuroblastoma cells, while a 3-log depletion was accomplished for a T-cell leukemia line (8). The difference in depletion was ascribed to the total number of SBA receptors present on the tumor cells tested. Evidence for variable degree of SBA binding to breast carcinoma cells was previously tested in paraffin-embedded breast carcinoma sections (13, 14) and in a number of breast cancer cell lines examined in our laboratory (Ref. 12 and the present study). Based on the positive binding of SBA to breast cancer cells as well as other tumor cells and in view of the fact that hemopoietic stem cells are SBA negative, SBA can serve as a tool for selective elimination of breast cancer cells as well as other tumor cells and in view of the fact that hemopoietic stem cells are SBA negative, SBA can serve as a tool for selective elimination of breast cancer cells as well as other tumor cells without compromising hematopoietic marrow reconstitution following autologous BMT. In view of the heterogeneity of breast cancer cells, low affinity of SBA binding to certain cancer cell subsets in comparison with normal marrow cells might interfere with effective depletion of a low concentration of tumor cells in tumor/BM mixtures. Nevertheless, the use of 2 subsequent cycles of SBA separation is expected to give the cancer cell with low-affinity binding a better chance of interaction with SBA in the second separation cycle after removal of more avidly binding cells.

Previous reports on the use of magnetic beads for marrow purging in experimental models as well as in clinical BMT (6, 24) suggested the use of beads bound to monoclonal antibodies directed against the target cells or against a primary antibody bound to the target cells. In contrast to the need for tumor-specific antibodies required for each disease (which can be extremely variable in itself), SBA, a lectin which is commercially available, can be bound to magnetic beads and might serve as a general reagent to remove many types of tumor cells which bear the receptor for SBA, since SBA receptors are ubiquitous on a variety of tumor cells. Nevertheless, due to the heterogeneity of tumor cell populations (in some cases investigated, in tissue sections only a small proportion of breast cancer cells react with SBA; 14), a combination of various approaches will probably provide the most promising strategy for the most effective removal of tumor cells from heavily contaminated BM aspirates in conjunction with autologous BMT.

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Removal of Breast Cancer Cells by Soybean Agglutinin in an Experimental Model for Purging Human Marrow

Shoshana Morecki, Shlomo Margel and Shimon Slavin


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