Removal of Carcinoma Cells from Contaminated Bone Marrow Using the Lipophilic Cation Rhodamine 123

James R. Wong, Carole Ho, Peter Mauch, Norman Coleman, Stuart Berman, and Lan Bo Chen

Stitch Radiation Center, Department of Radiology, New York Hospital, Cornell University Medical College, New York, New York 10021 [J. R. W., S. B.], and Division of Cellular and Molecular Biology, Dana-Farber Cancer Institute [J. R. W., C. H., L. B. C.] and Joint Center for Radiation Therapy [P. M., N. C.], Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

Autologous bone marrow transplants for solid tumor treatment are severely limited by the potential presence of residual cancer cells in the reinjured bone marrow and can lead to future tumor recurrence.

This article presents a novel method of removing carcinoma cells from bone marrow with contaminating cancer cells. This method is based on our previous studies demonstrating that carcinoma cells have a higher uptake of lipophilic cations such as rhodamine 123 than their normal epithelial counterparts. When the relative differences in rhodamine 123 uptake are quantified, carcinoma cell lines demonstrated a 7.4-21 times greater uptake of rhodamine 123 than normal mouse bone marrow cells. More important, when normal bone marrow cells and carcinoma cell lines are mixed to simulate carcinoma-contaminated bone marrow, individual cell populations continue to exhibit characteristic and identifiable relative differences (10-20 times) in rhodamine 123 uptake. Differential sorting of bone marrow/carcinoma cell mixtures with respect to rhodamine 123 fluorescence intensity resulted in the removal of 95-99% of the "contaminating carcinoma cells." The recovered bone marrow cells were fully viable as ascertained by their ability to form splenic colonies. In our preliminary experiments, sorted bone marrow cells transplanted into lethally irradiated C57BL6 mice allowed the mice to survive for more than 8 months. In light of these promising results, we propose that lipophilic cations may play a role in the purification of autologous bone marrow used in transplants for patients with advanced solid tumors.

INTRODUCTION

Rhodamine 123, an extensively studied delocalized lipophilic cation, has been widely used in the past decade in various cellular systems. Some such systems include the study of mitochondria using rhodamine 123 as a vital stain, the use of rhodamine 123 and other lipophilic cations as a novel class of anticarcinoma agents (1-8), and the use of rhodamine 123 in detecting and sorting of bone marrow stem cells (9-11). In this article, a new role for rhodamine 123 is presented.

Because of the relatively high mitochondrial accumulation of rhodamine 123 by living cells, driven by plasma membrane and mitochondrial membrane potentials (4-6), rhodamine 123 has been used extensively as a mitochondrial stain. The mechanism of rhodamine 123 uptake by the mitochondria exploits the two membrane potentials which create a proton gradient facilitating the uptake and retention of the positively charged lipophilic cation. A 100-1000-fold concentration difference of lipophilic compound accumulation in the mitochondria as compared to the extracellular environment is readily achievable (4).

After examining mitochondrial fluorescent staining in over 200 cell lines/strains, our laboratory demonstrated a consistent difference in rhodamine 123 uptake between cancer cells and normal cells. Carcinoma cell lines/strains derived from human kidney, ovary, pancreas, lung, adrenal cortex, skin, breast, prostate, cervix, vulva, colon, liver, and testis have a higher mitochondrial uptake of rhodamine 123 than the cell lines/strains derived from the normal cell counterparts of these tumors (1, 6-8). Furthermore, upon examining other lipophilic cations in addition to rhodamine 123, the differential uptake of lipophilic cations by normal cells as compared to their tumorigenic counterparts led us to propose previously that lipophilic cations comprise a novel class of anticarcinoma agents (1-8). This hypothesis is supported by experiments demonstrating that lipophilic cations are selectively toxic to carcinoma cells as compared to their normal epithelial counterparts (3-6). The in vitro toxicity of lipophilic cations for carcinoma cells over normal cells ranges from 10- to 100-fold (2-5), and it has been shown that three such agents, rhodamine 123, dequalinium chloride, and AA1, are selectively toxic to carcinoma cells in vitro and display anticarcinoma activity in vivo. Other laboratories, using different lipophilic cations such as EDKC (13), Victoria blue BO (14), and tetr phenylphosphonium (15), have also confirmed the anticancer abilities of these compounds.

In addition to its use as a supravital mitochondrial stain and its potential anticarcinoma activity, rhodamine 123 has been used in separating subpopulations of hematopoietic cells as well as a probe for the resolution and hierarchical ordering of primitive hematopoietic progenitor cells (9, 16). Rhodamine 123 was used for the isolation of murine CFU-S using multiparameter flow cytometric cell sorting (17, 18). It was found that bone marrow fractions that form CFU-S at day 8 and day 12 after transplantation in lethally irradiated mice differ in rhodamine uptake. This method is based on our previous studies demonstrating that carcinoma cells have a higher uptake of lipophilic cations than their normal epithelial counterparts. When the relative differences in rhodamine 123 uptake are quantified, carcinoma cell lines demonstrated a 7.4-21 times greater uptake of rhodamine 123 than normal mouse bone marrow cells. More important, when normal bone marrow cells and carcinoma cell lines are mixed to simulate carcinoma-contaminated bone marrow, individual cell populations continue to exhibit characteristic and identifiable relative differences (10-20 times) in rhodamine 123 uptake. Differential sorting of bone marrow/carcinoma cell mixtures with respect to rhodamine 123 fluorescence intensity resulted in the removal of 95-99% of the "contaminating carcinoma cells." The recovered bone marrow cells were fully viable as ascertained by their ability to form splenic colonies. In our preliminary experiments, sorted bone marrow cells transplanted into lethally irradiated C57BL6 mice allowed the mice to survive for more than 8 months. In light of these promising results, we propose that lipophilic cations may play a role in the purification of autologous bone marrow used in transplants for patients with advanced solid tumors.

Received 8/24/94; accepted 3/2/95.

The abbreviations used are: CFU-S, spleen colony-forming units; CHO, Chinese hamster ovary; 4-HC, 4-hydroperoxycyclophosphamide.
Removal of Carcinoma Cells from Contaminated Bone Marrow

123 uptake (19). CFU-S at day 8 incorporated a relatively large amount of rhodamine 123, while day 12 CFU-S stain heterogeneously. Day 8 CFU-S were also found to stain more intensely with the supravital DNA stain Hoechst 33342 than day 12 CFU-S (20); day 13 CFU-S go through the S-phase at least 10 less frequently compared to day 7 CFU-S, indicating that the later CFU-S are relatively quiescent compared to earlier CFU-S (21). From such studies, it was proposed that because pluripotent hematopoietic stem cells are in G0, they have a lower mitochondrial activity and, thus, a lower uptake of rhodamine 123 (22).

Other studies also support the hypothesis that pluripotent hematopoietic stem cells have a lower uptake of rhodamine 123. Bone marrow cells with low uptake of rhodamine 123 were found to have a significantly increased ability to repopulate marrow (9, 23, 24) and give rise to clonogenic cells in long-term culture (25, 26), suggesting that the hematopoietic stem cells may be contained in such rhodamine dull fractions. In fact, weakly fluorescent or rhodamine dull fractions of day 12 CFU-S predominantly mediate survival in mice after lethal irradiation (19). The intensity of rhodamine 123 staining correlates with the degree of differentiation of the hematopoietic progenitor cells, with more differentiated bone marrow cells taking up more rhodamine 123 (24).

The lower uptake of rhodamine 123 by primitive hematopoietic progenitors in conjunction with the demonstrated higher uptake of carcinoma cells suggests yet another application for rhodamine 123 and forms the central concept underlying the model for bone marrow purging presented in this article.

Over the past decade, high-dose chemotherapy or chemotherapy plus radiotherapy in conjunction with bone marrow rescue has been accepted as a standard therapy for certain advanced leukemias and lymphomas (27-31). Continuous complete remission achieved in as many as 40% of some non-Hodgkin's lymphoma cases and 35% of advanced Hodgkin's disease cases reflects the success of these transplants (32-34). More recently, high-dose chemotherapy and autologous bone marrow rescue are also being used in clinical trials for the treatment of advanced solid tumors such as breast, lung, neuroblastoma, and colon cancer (35-40).

The use of autologous rather than allogeneic transplants eliminates the risk of graft-versus-host reactions and graft rejection, but its potential efficacy is limited if the autologous bone marrow is contaminated with metastatic cancer cells. Bone marrow metastasis has been estimated in 40-60% of advanced breast cancer patients (41, 42), and immunofluorescent staining uncovered cancer cells in 60% of bone marrow specimens from small cell lung cancer patients diagnosed by conventional microscopy to be tumor free (43). Thus, even in the absence of microscopically identifiable cancer cells in the autologous bone marrow, neoplastic cells may still be present. Purging the bone marrow ex vivo prior to reinfusion may therefore decrease incidences of future tumor recurrence (41). Unfortunately, few, if any, specific antibodies against solid carcinoma cells are known, and equally few chemotherapeutic agents can effectively purge carcinoma-contaminated bone marrow without adverse effects on hematological stem cell viability. The use of delocalized lipophilic cations may provide a potential solution to this problem.

MATERIALS AND METHODS

Cell Cultures. CX-1 cells were grown in 50% RPMI 1640 and 50% DMEM (GIBCO Laboratories, Grand Island, NY) supplemented with 5% calf serum and 5% NU-serum (M. A. Bioproducts, Walkersville, MD). All other cell lines/types were grown in DMEM supplemented with 10% calf serum. All tissue cultures were incubated at 37°C with 5% CO2 and 100% humidity. The CHO cell line was a gift from Dr. L. Hlatky (Harvard Medical School). CV-1, an African Green monkey kidney epithelial cell line, and HeLa, a human cervical adenocarcinoma cell line, were obtained from American Type Culture Collection. CX-1, a human colon carcinoma cell line, was obtained from Dr. S. D. Bernal (Harvard Medical School). EJ, a human bladder transitional cell carcinoma cell line, was obtained from Dr. V. Summerhayes (Harvard Medical School). MCF-7, a human mammary carcinoma cell line, was provided by Dr. M. Rich (Michigan Cancer Foundation). OVCA-III, a human ovarian papillary adenocarcinoma cell line, and LOX, a human melanoma cell line, were provided by Dr. R. Salem (Harvard Medical School).

Animals and Irradiation. C57BL/6J mice (6-10 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Swiss nu/nu mice were obtained from Taconic Farm, Inc. (German Town, NY). Animals were irradiated in groups of 6-10 within a cylindrical lucite chamber between two opposing 137Cs sources as described previously (44). Each irradiated animal received a total body dose of 950 cGy.

Bone Marrow Preparation. Bone marrow cell suspensions from donor mice were prepared by flushing the medullary cavity of the humerus, tibia, and femur with cold HBSS or with cold DMEM. Nucleated cells were stained with crystal violet and counted using a hemocytometer. All bone marrow cell suspensions were kept in ice until ready to be used.

Fluorescence Microscopy. Cell suspensions (1 X 10⁶ cells/ml) were incubated with 4 μg/ml rhodamine 123 in DMEM for 60 min at 5% CO2/95% air and 100% humidity. The cell suspensions were then spun down and washed with cold DMEM. After two washings, cells were spun down and almost all media were removed to form a concentrated cellular suspension. The respective suspensions were placed on slide under coverslips and viewed under a Zeiss Axioshot microscope equipped with epifluorescence optics as described previously (1). All pictures were photographed at ASA 1600 on TMax Kodak film.

Rhodamine 123 Labeling for Flow Cytometry. Cell suspensions (1-2.5 X 10⁶ cells/ml) were incubated with rhodamine 123 (1-4 μg/ml) in 100- or 250-ml Erlenmeyer beakers to increase nutrient and oxygen contact, thus achieving enhanced separation by preventing hypoxic conditions. Cells were incubated for the time periods indicated and washed and spun down as described in "Fluorescence Microscopy." The cells were then resuspended to 2-3 X 10⁶ cells/ml and kept on ice until flow cytometry analysis.

Fluorescence-activated Cell Sorting and Flow Cytometry. Fluorescence-activated cell sorting and analysis were conducted with a Coulter Epics 750 dual laser flow cytometer. Rhodamine 123 was excited by 488 nm light from an argon laser. Selection of emission signal was achieved by the addition
of 525-nm band-pass filters. Prior to flow sorting and analysis, all cell samples were made to a final suspension of $3 \times 10^6$ cells/ml in DMEM supplemented with 20 µg/ml gentamicin. Cells were sorted at a rate of 1000–2000 cells/s. Cell sorting on the basis of rhodamine 123 fluorescence was performed after gating out dead cells based on high propidium iodide fluorescence.

### Clonogenic Assays of Carcinoma Cells for Determination of Degree of Bone Marrow Purging

Cells sorted on the basis of rhodamine 123 fluorescence were collected in 50% RPMI 1640 and 50% DMEM supplemented with 5% calf serum and 5% NU-serum and with 20 µg/ml gentamicin. Cells were seeded in 60-mm Petri dishes to yield samples with 1,000, 5,000, 10,000, and 50,000 cells/dish for both the unsorted and sorted bone marrow samples. Only carcinoma cells, not bone marrow cells, form colonies under such conditions. Control plates were seeded with an unsorted (rhodamine-treated) mixture of bone marrow and carcinoma cells. The ratio of colonies formed in the sorted versus the unsorted group determined the degree by which carcinoma cells were removed from the contaminated bone marrow. All plates were made in triplicate and incubated at 37°C with 5% CO₂ and 100% humidity. Media was changed after 24 h of seeding and again at day 5. Colonies were scored on day 8 after staining with 0.2% methylene blue.

### Splenic Colony Forming Assay

The viability of bone marrow samples was determined using the CFU-S assay (30). Unfractionated and sorted bone marrow cell suspensions were injected through the tail vein of lethally irradiated C57BL/6J mice. Ten days after transplantation of the bone marrow samples, the mice were sacrificed, and their spleens were obtained for colony count determination.

### RESULTS

#### Qualitative Analysis of Differential Uptake of Rhodamine 123: Bone Marrow Cells versus Carcinoma Cells

Fluorescent microscopy was used to qualitatively ascertain the difference in uptake of rhodamine 123 by single-cell samples of C57BL/6J mouse bone marrow and CX-1 (human colon carcinoma) cells. The difference in uptake between CX-1 cells and bone marrow cells incubated under identical conditions and exposed for the same length of time is illustrated in Fig. 1. Under low magnification ($\times 10$, Fig. 1, A and B), the CX-1 cells fluoresce brightly while minimal staining of the bone marrow cells is observed. At higher magnification ($\times 100$, Fig. 1, C and D), the mitochondrial staining by rhodamine 123 is again more intense for the CX-1 carcinoma cells. These results are similar to the previous observations that carcinoma cells have a higher...
uptake of rhodamine 123 than their epithelial counterparts (1–8). In addition to the brighter rhodamine 123 staining, the carcinoma cells are larger in size than the bone marrow cells.

**Cellular Uptake of Rhodamine 123 Analyzed Using Flow Cytometry: Bone Marrow Cells versus Carcinoma Cells in Single-Cell Samples.** The uptake of lipophilic cations such as rhodamine 123 by living cells has been quantified by: (a) direct butanol extraction followed by excitation and emission spectra measurement, (b) liquid scintillation measurement of radioactivity, (c) fluorescence microscopy, and (d) flow cytometry analysis (4, 6, 45). We selected flow cytometry analysis because, unlike the other methods, it is rapid, easy to perform, and can determine the amount of compound uptake within a natural cellular environment. In addition, cells analyzed using flow cytometry can be sorted by their differential uptake of rhodamine 123 and recovered for further study.

Separate cell samples of bone marrow cells (from C57BL/6J and Swiss nu/nu mice), carcinoma cells (LOX, CX-1, MCF-7, EJ, HeLa), and normal cells (CV-1, CHO), all at a concentration of 2.5 × 10⁶ cells/ml, were incubated for 30 min with 5 μg/ml rhodamine 123 and then processed as described in “Materials and Methods.”

Fig. 2 and Table 1 display and compare amounts of rhodamine 123 uptake in the various cell samples, respectively. CV-1, CHO, and bone marrow cells from both C57BL/6J mice and Swiss nu/nu mice are all normal cells that exhibit a characteristically low uptake of rhodamine 123, consistent with previous reports that normal cells have either a lower plasma membrane potential, a lower mitochondrial membrane potential, or fewer mitochondria (1–6, 33). Carcinoma cells were found to have a high uptake of rhodamine 123 when incubated in identical conditions as the control (C57BL/6J) mouse bone marrow cells. CX-1 (human colon carcinoma), EJ (human bladder carcinoma), HeLa (human cervical carcinoma), MCF-7 (human mammary carcinoma), MB49 (mouse bladder carcinoma), and LOX (human melanoma) cell lines took up 15.5 times, 10.3 times, 7.4 times, 21.0 times, 15.2 times, and 12.6 times more rhodamine 123 than control bone marrow cells, respectively (Table 1).

**Table 1** Uptake ratios of rhodamine 123 of various cell types or cell lines compared to C57BL/6J bone marrow cells

<table>
<thead>
<tr>
<th>Cell type or line</th>
<th>Comment</th>
<th>Relative uptake ratio of rhodamine 123</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV-1</td>
<td>Monkey epithelial; nontransformed</td>
<td>2.0</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary; nontransformed</td>
<td>1.0</td>
</tr>
<tr>
<td>Swiss nu/nu mice bone marrow</td>
<td>Nontransformed</td>
<td>0.9</td>
</tr>
<tr>
<td>CX-1</td>
<td>Human colon carcinoma</td>
<td>15.5</td>
</tr>
<tr>
<td>EJ</td>
<td>Human bladder carcinoma</td>
<td>10.3</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical carcinoma</td>
<td>7.4</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human mammary carcinoma</td>
<td>21.0</td>
</tr>
<tr>
<td>LOX</td>
<td>Human melanoma</td>
<td>12.6</td>
</tr>
</tbody>
</table>

**Differential Uptake of Rhodamine 123 in Mixed Cell Populations Mirrors Relative Uptake in Single-Cell Samples: Time Course Assay to Determine Optimal Separation.** After determining that bone marrow cells did take up significantly less rhodamine 123 than representative carcinoma cells, studies were done to determine whether the same uptake differ-
ences could be demonstrated in mixed cell samples simulating carcinoma-contaminated bone marrow.

Flow cytometry analysis was performed on a treated 1:1 mixture of C57BL/6J mouse bone marrow cells and human colon carcinoma, CX-1 cells, which simulated carcinoma-contaminated bone marrow. The cell mixtures were incubated with rhodamine 123 at concentrations of 1, 2, and 4 μg/ml, with 1 × 10^6 cells/ml in each sample. Samples were incubated for 1, 2, and 4 h at each respective concentration.

Optimal separation was determined at 4 μg/ml for 1 h and as can be seen in Fig. 3A, the difference in uptake of bone marrow fractions over carcinoma cell fractions in the histogram is remarkable, as is readily apparent by the two distinct peaks in the histogram in Fig. 3A.

In subsequent experiments comparing the differential uptake of rhodamine 123 between coincubating murine bone marrow and carcinoma cells, carcinoma cells exhibited a 10–21-fold greater uptake of rhodamine 123 as compared to coincubating bone marrow cells, mirroring differences found in Table 1. As shown in Fig. 2, C and D, CX-1 and LOX cells were observed as distinct peaks further to the right of the normal bone marrow cells in Fig. 2, A and B, respectively, indicating a substantially higher uptake of rhodamine 123. Fig. 3A reflects these results and demonstrates that uptake and retention properties of rhodamine 123 by bone marrow cells and CX-1 carcinoma cells are not effected by coincubation. A two-dimensional scatterplot of the same cell populations in Fig. 3B further emphasizes the low rhodamine 123 uptake of hematological cells and the higher uptake characteristic of cancer cells. Any doubts concerning which peaks in the histogram corresponded to which cell populations are resolved by the distinction of cell size in these two-dimensional scatterplots with the carcinoma cells being the larger cells (also shown in Fig. 1, C and D). The smaller bone marrow cells take up substantially less rhodamine 123 than larger carcinoma cells. The CX-1 cells have a wide range of cell diameters (10–25 μm) and yet even in CX-1 cells with diameters comparable to that of the mouse bone marrow cells, the uptake of rhodamine 123 is still much higher than that of bone marrow cells. Moreover, as shown in Table 1, CV-1 cells and CHO cells, two normal epithelial cell lines with cell diameters similar to that of CX-1 (i.e., much larger than that of bone marrow cells), display rhodamine 123 uptake comparable to that of bone marrow cells. These results taken together indicate that the difference in uptake of rhodamine 123 between bone marrow cells and carcinoma cells are not secondary to the volume or diameter of the cells, but secondary to an intrinsic difference of mitochondrial uptake of rhodamine 123. In subsequent experiments, tissue cultures of recovered cells in the ‘‘bone marrow peak’’ will demonstrate that the peak contains very few carcinoma cells.

Removal of Carcinoma Cells from Experimentally "Contaminated Bone Marrow" Using Rhodamine 123 and Purification Achieved. The above results demonstrate that human carcinoma cells such as CX-1 take up 20 times more rhodamine 123 than normal bone marrow cells when incubated either with the bone marrow samples or separately. This difference in rhodamine 123 uptake may be exploited for removal of carcinoma cells from contaminated bone marrow.

A contaminated bone marrow model was designed by mixing carcinoma cells and bone marrow cells. CX-1 cells were mixed with bone marrow cells in a 1:9 ratio and incubated with rhodamine 123 at 4 μg/ml for 1 h. This 1:9 ratio was selected to demonstrate the difference in uptake of carcinoma cells versus the bone marrow cells in the following fluorescence-activated cell sorter generated plots (Figs. 4 and 5). If a lower fraction of carcinoma cells is used (e.g., 1:10^2–10^3), then the carcinoma cell peaks would be barely visible, making the demonstration here difficult. Nevertheless, from individual cell staining as shown in Fig. 1, it would imply that even in situations simulating lower tumor burden (1:10^2–10^3), the difference in rhoda-
Removal of Carcinoma Cells from Contaminated Bone Marrow

The fraction of bone marrow cells sorted out is shown between the two vertical bars in each respective diagram. A, 25% of the bone marrow peak; B, 50% of the bone marrow peak; and C, 75% of the bone marrow peak.

Table 2 Purification of carcinoma-contaminated bone marrow by fluorescence-activated cell sorting

<table>
<thead>
<tr>
<th>No. of cells seeded</th>
<th>Sorted sample</th>
<th>Unsorted sample</th>
<th>% Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>2.3</td>
<td>49</td>
<td>95.3</td>
</tr>
<tr>
<td>5,000</td>
<td>4.3</td>
<td>268</td>
<td>98.4</td>
</tr>
<tr>
<td>10,000</td>
<td>6.7</td>
<td>677</td>
<td>99.1</td>
</tr>
<tr>
<td>50,000</td>
<td>51</td>
<td>1776</td>
<td>97.1</td>
</tr>
</tbody>
</table>

Fig. 5 The fraction of bone marrow cells sorted out is shown between the two vertical bars in each respective diagram. A, 25% of the bone marrow peak; B, 50% of the bone marrow peak; and C, 75% of the bone marrow peak.

mine 123 uptake between carcinoma cells and bone marrow cells should be similar to the 1:9 ratio of cells as used here. Using the fluorescence-activated cell sorter, this mixture of cells was sorted according to the amount of rhodamine 123 uptake. The fraction of bone marrow cells sorted out is shown between the two vertical bars in Figs. 4 and 5. In the initial experiment, only the fraction of bone marrow cells with the lowest 20% uptake was collected (Fig. 4) to ensure less contamination by any carcinoma cells exhibiting a slightly lower uptake of rhodamine 123. Since it has been shown that more primitive hematopoietic stem cells are rhodamine dull (8, 9, 23), selecting the lower rhodamine 123 fraction further concentrates the bone marrow stem cells (22, 24). Of these sorted cells, 1,000–50,000 were then plated onto Petri culture dishes and checked for colony formation 8–10 days later. The ratio of colonies formed in the sorted versus the unsorted group reflects the degree to which carcinoma cells were removed from the contaminated bone marrow. From the results of this assay, we concluded that this simple purging procedure removed 95–99% of the contaminating carcinoma cells (Table 2).

In a subsequent experiment using MB49 cells, the degree of flow cytometry selectivity of the bone marrow correlated with the degree of purification. Here, MB49 cells mixed with bone marrow cells in a final ratio of 1:9 were incubated with rhodamine 123 at 4 μg/ml for 1 h. A clonogenic assay of purification was done as before; data on colony counts can be found in Table 3 and Fig. 5. In Fig. 5, the larger peak represents the bone marrow cells while the smaller peak to the right represents MB49 cells. The fluorescence-activated cell sorter was used to sort fractions including 25% of the bone marrow peak, 50% of the bone marrow peak, and 75% of the bone marrow peak. As can be seen in Table 3, the degree of hematopoietic purification directly correlates with the stringency imposed on the fractions of bone marrow selected. When 75% of the bone marrow peak is selected (Fig. 5C), 76–92% purification is obtained (average, 85% purification). When the fraction of the bone marrow cells with the lowest 50% uptake was collected (Fig. 5B), the purification improved to 86–96% (average, 93%). When even a lower uptake fraction (25%) of the bone marrow peak is selected (Fig. 5A), a 98–100% purification was achieved (average, 99.3%).

Clonogenic Ability of Sorted Bone Marrow Cells. The clonogenic ability of the sorted bone marrow cells is examined by reinfecting these cells into lethally irradiated mice. Cells recovered from the “sorted bone marrow group” were injected i.v. via tail vein to mice receiving total-body irradiation of 950 cGy, while similarly irradiated control group mice received injections with untreated bone marrow cells. Ten days after these injections, the treated mice were sacrificed, and their spleens were harvested for colony counting. Spleens from the sorted bone marrow group contained at least as many, if not more, colonies than the “unsorted bone marrow” group (Table 4). As evidenced by the above spleen colony assay, subjecting the cells to differential sorting does not affect the viability of the bone marrow cells.

In a similar sorting experiment, mice receiving 300,000–400,000 sorted bone marrow cells all continue to survive for more than 8 months (mice were sacrificed after 8 months) while control group mice receiving irradiation alone all died by day 8, thus demonstrating that the long-term viability of the transplanted bone marrow was not adversely altered.
carcinoma cells take up 10-20 times more rhodamine 123 than mAbs for such purposes (43, 53-55). However, considerable purge autologous bone marrows. In this article, we show that tumor samples obtained from different metastatic sites of the instance, large quantitative antigenic variations were expressed small cell lung cancer, melanoma, and neuroblastoma precludes antigenic heterogeneities present in breast cancer, colon cancer, and thus would include not only pure pluripotent stem cells, but probably committed progenitor cells as well. Nevertheless, it has been shown that primitive hematopoietic progenitor cells, the most important cellular population in a successful bone marrow graft, may have an even lower uptake of rhodamine 123 than other populations in the bone marrow (25, 26, 46) and therefore would not be left out in the flow cytometry separation method. This infusion of committed progenitor cells in conjunction with the pluripotent stem cells would be an advantage during bone marrow transplantation, as the time to recovery would be shorter if committed progenitor cells are infused. As demonstrated here, by differential fluorescence-activated cell sorting in the manner of the proposed model, 95-99% of carcinoma cells can be removed from the contaminated bone marrow (in one round of cell sorting) without adversely affecting the viability of the hematopoietic cells (Tables 2 and 3). In addition, in an in vivo experiment similar to the one described, cells recovered from differential fluorescence-activated cell sorting are able to rescue mice that were otherwise lethally irradiated.

The use of lipophilic cations may offer another solution to do the normal bone marrow cells. In the experiments reported in this article, when the fraction of bone marrow cells with the lower 25-50% rhodamine 123 uptake are selected for separation, this portion of the bone marrow cells is a large number and thus would include not only pure pluripotent stem cells, but probably committed progenitor cells as well. Nevertheless, it has been shown that primitive hematopoietic progenitor cells, the most important cellular population in a successful bone marrow graft, may have an even lower uptake of rhodamine 123 than other populations in the bone marrow (25, 26, 46) and therefore would not be left out in the flow cytometry separation method. This infusion of committed progenitor cells in conjunction with the pluripotent stem cells would be an advantage during bone marrow transplantation, as the time to recovery would be shorter if committed progenitor cells are infused. As demonstrated here, by differential fluorescence-activated cell sorting in the manner of the proposed model, 95-99% of carcinoma cells can be removed from the contaminated bone marrow (in one round of cell sorting) without adversely affecting the viability of the hematopoietic cells (Tables 2 and 3). In addition, in an in vivo experiment similar to the one described, cells recovered from differential fluorescence-activated cell sorting are able to rescue mice that were otherwise lethally irradiated.

The results described suggest that lipophilic cations such as rhodamine 123 have great potential for enhancing the treatment of patients with advanced solid tumors undergoing autologous bone marrow transplants. However, to be clinically applicable, several issues need to be addressed. First, all of the experiments that were performed in this study utilized mice bone marrow cells instead of human bone marrow cells. Thus, further experiments are needed to establish that human bone marrow cells also exhibit similar low uptake patterns of rhodamine 123. Second, the carcinoma cells used here are all established cell

**DISCUSSION**

The bone marrow is a dose-limiting cell renewal tissue for chemotherapy and wide-field irradiation. In cases of high-dose chemotherapy, hematological toxicity can be reversed by autologous bone marrow transplants. Such transplants have been used with increasing frequency over the past decade, despite the potential presence of carcinoma cells in the bone marrow graft that could reinfect the disease along with the hematopoietic stem cells. Because the marrow graft used for autologous support in many patients presumably contains tumor cells, in vivo purging should be done prior to transplantation (41, 47, 48, 50).

The idea of purging carcinoma-contaminated bone marrow has been pursued by various laboratories. 4-HC, a cyclophosphamide derivative that does not require hepatic microsomal activation, has been used ex vivo in the treatment of bone marrow from patients with acute leukemia and breast cancer (46, 49). However, 4-HC purging can result in less than 1% of granulocyte-monocyte colony-forming units recovery in in vitro cultures. Moreover, in cells that have a high content of the enzyme aldehyde hydrogenase, 4-HC is rapidly oxidized to an inactive form, resulting in inefficient purging (46, 49, 50). Another agent, VP-16, has been used for purging of carcinoma-contaminated bone marrow cells. Like 4-HC, VP-16 is toxic to the bone marrow (51) and may not display a selectivity between carcinoma cells and bone marrow cells. Photosensitization with Merocyanine 540 has been clinically used for both the purging of leukemia, lymphoma, and neuroblastoma cells prior to autologous bone marrow transplants and in in vitro systems of small cell lung cancers and breast cancers (52). Nevertheless, the effect of Merocyanine 540 is both light exposure and time dependent. At exposures required to produce complete or almost complete elimination of cancer cells, significant toxicity to the bone marrow stem cells is observed (52).

Theoretically, mAbs can selectively eliminate carcinoma cells from the bone marrow without affecting the viability of the hematopoietic cells. Several laboratories have reported the use of mAbs for such purposes (43, 53-55). However, considerable antigenic heterogeneities present in breast cancer, colon cancer, small cell lung cancer, melanoma, and neuroblastoma precludes their clinical efficacy (56-58). With small cell lung cancers, for instance, large quantitative antigenic variations were expressed not only in tumor samples from different patients but even in tumor samples obtained from different metastatic sites of the same patients (57).

The use of lipophilic cations may offer another solution to purge autologous bone marrows. In this article, we show that carcinoma cells take up 10-20 times more rhodamine 123 than

<table>
<thead>
<tr>
<th>No. of</th>
<th>25% sample</th>
<th>50% sample</th>
<th>% Purified for</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells seeded</td>
<td>50% sample</td>
<td>75% sample</td>
<td>25% sample</td>
</tr>
<tr>
<td>1,000</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>5,000</td>
<td>0</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>10,000</td>
<td>1</td>
<td>18</td>
<td>49</td>
</tr>
<tr>
<td>50,000</td>
<td>12</td>
<td>93</td>
<td>161</td>
</tr>
</tbody>
</table>

**Table 3** Degree of purification versus degree of selectivity

Percentages listed refer to a percentage of the bone marrow peak, not the sample as a whole. Note the direct correlation between degree of purging/purification and degree of selectivity.

<table>
<thead>
<tr>
<th>No. of bone marrow cells</th>
<th>No. of mice</th>
<th>No. of colonies/10^5 cells</th>
<th>Average no. of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted 100,000</td>
<td>3</td>
<td>12</td>
<td>11.8</td>
</tr>
<tr>
<td>Unsorted 200,000</td>
<td>4</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>Unsorted 300,000</td>
<td>5</td>
<td>13.3</td>
<td></td>
</tr>
<tr>
<td>Sorted 50,000</td>
<td>5</td>
<td>15.6</td>
<td>22.6</td>
</tr>
<tr>
<td>Sorted 100,000</td>
<td>5</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td>Sorted 200,000</td>
<td>5</td>
<td>22.3</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4** Viability of sorted bone marrow cells

The "sorted" and "unsorted" samples as indicated in Fig. 4 were injected into lethally irradiated mice (950 cGy) to determine the number of splenic colonies. The "Average No. of Colonies" were normalized to each 10^5 cells injected.
lines which have a rather homogeneous high uptake of rhodamine 123. Although previous experiments from the Chen Laboratory comparing many native human carcinoma cell strains have shown a high uptake of rhodamine 123 as compared to their epithelial counterparts (1, 6–8), it remains to be determined that metastatic human carcinoma cells to the bone marrow will show a similar high rhodamine 123 uptake pattern. Such experiments are possible and are currently under way in our laboratory.

A major shortcoming that would limit the present clinical feasibility of purging the autologous bone marrow is the speed of cell sorting using the flow cytometer. Current flow cytometers usually can sort between 1000 and 3000 cells/s. A typical bone marrow buffy coat graft contains about 10 × 10^9 cells, and, thus, the clinical practicality of this technique would await development of more advanced cell sorters. Nevertheless, it has previously been shown that a number of lipophilic cations display selective cytotoxic effects toward carcinoma cells as opposed to their epithelial counterparts at higher concentrations (2, 3, 6, 8). Finding a suitable lipophilic cation where selectivity for carcinoma cells is high and the toxicity of the drug to the bone marrow is low could provide a means for in vitro purging. For use of cytotoxic lipophilic compounds in the model presented in this article, a compound must be found that is not toxic to the bone marrow in the amounts taken up by these cells, yet has cytotoxic effects on coincubating carcinoma cells.

Future research may not only fine tune the model as presented but may also add other parameters to the purging process, enhancing the 95–99% purification achieved in the experiments described. For example, one may utilize specific hematopoietic growth factors such as various interleukins or stem cell factor to selectively induce proliferation of the recovered bone marrow cells without affecting the carcinoma cells, thus resulting in the amplification of splenic colonies upon reinfusion (44) and thus a lower amount of bone marrow buffy coat may be needed.

Another possibility for eliminating the residual 1–5% of carcinoma cells in the bone marrow after purging by the method described is by applying more stringent criteria for differential cell sorting. Carcinoma cells are much larger than bone marrow cells, thus size could be included in the parameters for differential cell sorting (Fig. 2B). Also, enhancing the difference in mitochondrial uptake between carcinoma cells and bone marrow cells may be attempted using drugs or agents which positively or negatively influence the uptake and retention of lipophilic cations by the mitochondria (4).

The model proposed, utilizing the lipophilic cation rhodamine 123, has potential in the clinic for use in purging autologous bone marrow prior to transplantation. The procedure is simple and from preliminary studies appears not to adversely affect the bone marrow, while eliminating over 95% of the carcinoma cells. Most important, the model proposed presents the novel concept of using differential cell sorting with lipophilic cations for bone marrow purging; future models for bone marrow purging may be developed from this model or used in conjunction with the proposed procedure.

ACKNOWLEDGMENTS

We thank Dr. Steven Neben for his advice and Dr. Margaret Wong for editorial assistance. We also thank Peter Lopez for technical help with the flow cytometer.

REFERENCES


Removal of carcinoma cells from contaminated bone marrow using the lipophilic cation rhodamine 123.

J R Wong, C Ho, P Mauch, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/1/6/621

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.