Elimination of Drug-Resistant Myeloma Tumor Cell Lines by Monoclonal Anti-P-Glycoprotein Antibody and Rabbit Complement

By Sulabha S. Kulkarni, Zhimin Wang, Gary Spitzer, Mokhtar Taha, Hirofumi Hamada, Takashi Tsuruo, and Karel A. Dicke

The effectiveness of ex vivo chemotherapy with drugs, such as vincristine, etoposide, and Adriamycin (doxorubicin), can be undermined by the presence of multidrug-resistant tumor cells in the bone marrow. Therefore, to supplement chemoseparation, we investigated whether MRK-16, a monoclonal antibody (MoAb) to the surface moiety of multidrug resistance-associated P-glycoprotein antigen, can eliminate drug-resistant tumor cells in the presence of rabbit complement (RC). Two doxorubicin (DOX)-resistant human myeloma tumor cell lines, 8226/DOX₉, (resistant to 4 × 10⁻⁷ mol/L DOX) and 8226/DOX₅ (6 × 10⁻⁸ mol/L DOX) with high and low amounts of cell surface P-glycoprotein, respectively, and the drug-sensitive parent cell line 8226/S were used as tumor models in this study. Using the limiting dilution assay, we have shown that three cycles of treatment with 25 μg/mL of MRK-16 MoAb and a 1:4 final dilution of RC eliminated 2.90 ± 0.10 logs of 8226/DOX₉ cells and 1.94 ± 0.18 logs of 8226/DOX₅ cells. One and two cycles of treatment were less effective, eliminating 0.47 ± 0.40 and 1.94 ± 0.36 logs of 8226/DOX₉ and 0.12 ± 0.20 and 1.63 ± 0.58 logs of 8226/DOX₅ cells, respectively. The 8226/S cell growth was unaffected by one to three cycles of treatment. The cell kill was not impaired when the antibody plus complement treatment was carried out on a mixture of 8226/DOX₉ or 8226/DOX₅ cells with a ninefold excess of irradiated bone marrow mononuclear cells (MNCs). The three cycles of treatment with antibody plus complement did not adversely affect granulocyte-macrophage colony-forming unit (GM-CFU) survival in hematologically normal marrows (92.5% to 104% survival) or in myeloma patient marrows (85% to 100%). These results show that it is possible to eliminate drug-resistant myeloma tumor cells from the admixed human bone marrow by treatment with MRK-16 MoAb plus RC. This method could prove to be effective for elimination of other drug-resistant tumor cell lines including those of leukemia and solid tumors, and will be further useful for supplementing chemopurging, and immunopurging of bone marrow with other antitumor cell antibodies.

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TUMOR RESISTANCE TO DRUGS often occurs in cancer patients during chemotherapy. It is detected with greater frequency in patients treated for ovarian cancer, breast cancer, multiple myeloma, lymphoma, and leukemia. Often tumors refractory to treatment with one drug are cross-resistant to other drugs of various classes that include antitumor antibiotics, such as the anthracyclines, vinca alkaloids, and epipodophyllotoxins." This form of resistance is termed multidrug resistance (MDR). Multidrug-resistant cell lines have been generated using hamster, rodent, and human cells. A characteristic feature of cultured MDR cells is a drug accumulation decrease associated with overexpression of a membrane glycoprotein termed P170 or P-glycoprotein. Binding of a drug to this membrane glycoprotein facilitates the influx of drug by an energy-dependent process. A positive correlation is found to exist between the amount of this protein on the cell surface and the degree of drug resistance. Elevated levels of MDR1 RNA have been found in intrinsically drug-resistant cancers of the colon, kidney, and adrenal gland, as well as in some tumors that had acquired drug resistance during chemotherapy. Using monoclonal antibodies (MoAbs) C219, MRK-16, and JSB-1, specimens from a variety of patients have been screened for the presence of membrane P-glycoprotein; overexpression of P-glycoprotein has been detected in tumor samples from patients with advanced ovarian cancer and sarcoma by immunoblotting technique with C219 MoAb. P-glycoprotein was also detected in two patients with drug-resistant acute nonlymphoblastic leukemia by means of an immunocytochemical assay with C219 MoAb. Using MRK-16 MoAb in an immunocytochemical assay, a high level of P-glycoprotein expression has been reported in one of ten untreated lung cancer cases and in one of nine breast cancer cases. One normal bone marrow sample tested was negative for P-glycoprotein. Screening of multiple myeloma specimens has shown positive staining with JSB-1 MoAb in several patients.

In recent years, in vivo chemotherapy for cancer patients is often combined with ex vivo chemotherapy of patients' own bone marrow before transplantation for more effective management of the disease. 4-Hydroperoxycyclophosphamide (4-HC) has been used for ex vivo treatment of bone marrow from patients with acute myeloid leukemia or acute lymphoid leukemia. We thought that the efficacy of this procedure could be improved further by ex vivo killing of drug-resistant tumor cells from the bone marrow before ex vivo chemotherapy. This two-step strategy could be useful for treatment of a broad range of tumor types, both hematologic and nonhematologic. We therefore investigated the feasibility of eliminating drug-resistant tumor cells from the marrow using a myeloma tumor cell model. We show here that several logs of
drug-resistant tumor cells of two established myeloma tumor cell lines can be eliminated from the admixed excess irradiated bone marrow MNCs by treating the cell mixture with RC and MRK-16 MoAb that binds to the surface epitope of P-glycoprotein.28

MATERIALS AND METHODS

Cell lines. RPMI 8226 myeloma cells from the American Type Culture Collection (Rockville, MD) and its derivative cell lines 8226/DOX, and 8226/DOX selected for resistance to two different concentrations of DOX (Adria Labs, Columbus, OH) in the laboratory of Dr W. Dalton, the University of Arizona, Tucson, AZ, were all obtained from Dr W. Dalton. The cell lines 8226/DOX, and 8226/DOX selected for resistance to DOX concentrations of 6 \times 10^{-4} mol/L and 4 \times 10^{-5} mol/L, respectively, which are 6 and 40 times higher than the initial concentration (1 \times 10^{-4} mol/L) of DOX used in the selection process. The cells were maintained by continuous suspension culture at 37°C in 5% CO2, 12% O2, and balanced N2 in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1% (vol/vol) penicillin (100 units/mL), streptomycin (100 \mu g/mL), and 1% (vol/vol) L-glutamine (all from GIBCO). The drug-resistant cell lines were maintained in the continuous presence of respective concentrations of DOX. The cells were subcultured every 4 to 6 days. Cells were grown in drug-free medium for 1 week before immunohistochemical or cell elimination studies.

Antibody and normal mouse IgG2a. MRK-16, a murine MoAb of IgG2a class directed against the surface portion of P-glycoprotein present on the plasma cell membrane of multidrug-resistant cell lines,28 was obtained from Drs H. Hamada and T. Tsuuo of the Japanese Foundation for Cancer Research, Tokyo, Japan. MRK-16 MoAb was stored at −20°C in lyophilized form. When required for use, the lyophilized antibody was reconstituted with distilled water and stored in 5 \mu L aliquots (25 mg/mL) at −70°C. Further dilutions were made as required in RPMI 1640 medium supplemented with FCS or in phosphate-buffered salt (PBS) solution, pH 7.5 (GIBCO, Grand Island, NY), supplemented with 1% bovine serum albumin (BSA) (Sigma Chemical Company, St Louis, MO). Normal mouse IgG2a (NM1gG2a) derived from NS-1 hybridoma clone #T4-1F5 (Coulter Immunology, Hialeah, FL) was used as a negative control.

Rabbit complement. Two sources of RC i.e., an adult RC [GIBCO] and a baby RC [Pel-Freez Clinical Systems, Brown Deer, WI] were used in these studies. Adult RC showed negligible levels of toxicity on 8226/DOX cells and was therefore used in initial studies. Baby RC was, however, used in subsequent studies because of its lack of toxic effects on both 8226/DOX cells and human bone marrow cells.

Cytotoxicity assay by trypan blue exclusion method. The initial antibody titration and determination of optimal RC concentration was done using a standard microcytotoxicity assay. In this assay, 2.5 \times 10^6 cells in a 25 \mu L volume were plated per well in a microtitre plate (U-bottom 96-well tissue culture plate; Corning Glass Works, Corning, NY). For determination of antibody concentration for cell elimination studies, 1 \times 10^6 cells in 250 \mu L were plated per tube in polystyrene round-bottom tubes (12 \times 75 mm style tubes; Falcon 2054, Becton-Dickinson, Lincoln Park, NJ). The specific cytotoxicity was calculated using the formula:

\[
\% \text{c} = \frac{\% \text{Ab cytotoxicity} - \% \text{RC/NM1gG2a cytotoxicity}}{100 - \% \text{RC/NM1gG2a cytotoxicity}} \times 100
\]

Antibody treatment and limiting dilution analysis. The 8226/DOX and 8226/DOX cells were subcultured in RPMI 1640 medium with 10% FCS in the absence of added DOX prior to harvesting the cells for the test. Cells including those of cell lines 8226/S, were then washed and resuspended in RPMI 1640 with 5% FCS. Cell viability was determined by trypan blue exclusion, and only the cell suspensions showing >95% viability were used in the tests.

The antibody treatment was carried out as follows. Aliquots of 1 \times 10^6 pellet cells were resuspended in 0.25 mL of medium and incubated with 0.25 mL of MRK-16 MoAb, NM1gG2a control solution, or the medium at 4°C for 15 minutes. Rabbit complement, diluted 1:2 in medium, was added in a final dilution of 1:4, and the tubes were incubated at 37°C for 30 minutes. The cells were then pelleted by centrifugation, washed twice with medium, and resuspended in 1 mL of RPMI 1640 with 10% FCS. When more than one cycle of treatment was used, the cells were washed once after RC treatment and the whole cycle of treatment was repeated.

For limiting dilution analysis, 10 serial fivefold dilutions were made in the medium, and each dilution was plated in 0.1 mL volume in 6 wells of a U-bottom 96-well tissue culture plate (Corning Glass Works, Corning, NY) with 0.1 mL of medium. The cultures were incubated for 10 days at 37°C in 5% CO2, 12% O2, and balanced N2. The cultures were refed with medium every three to four days. At the end of the incubation period, the cultures were observed under an inverted scope, and the wells showing cell growth were scored as positive wells. This assay showed linear relationship between the number of clonogenic units per well and the logarithm of the proportion of negative wells both in the untreated and the antibody-treated samples (r^2 = .98), thus excluding significant effects of nonviable cells on the plating efficiency.

The log kill of cells by MRK-16 MoAb + RC was determined by using the calculations described earlier.28

Mixing experiments. Light-density MNCs in normal bone marrow were separated by centrifugation over Ficoll-Hypaque (density, 1.077 g/mL; Ficoll from Pharmacia Fine Chemicals, Piscataway, NJ; Hypaque from Winthrop Laboratories, New York) and were irradiated by exposure to 4,000 Gy. The irradiated cells were incubated overnight at 37°C in humidified air containing 5% CO2, 12% O2, and balanced N2.

Aliquots of mixtures of cells containing 1 \times 10^6 8226/DOX or 8226/DOX and 9 \times 10^6 irradiated bone marrow MNCs were incubated with MRK-16 MoAb, NM1gG2a or medium, and RC as described earlier in antibody treatment. Also, an additional control tube containing 10^7 irradiated MNC was incubated with medium alone. Cells were washed twice with medium, and the limiting dilution analysis was performed as described earlier. After incubating for 10 days, the results were scored in the same manner as described earlier in the limiting dilution analysis.

Antibody treatment and granulocyte-macrophage colony-forming unit assay. The light density MNCs from the bone marrow were separated by centrifugation over Ficoll-Hypaque. The cells were resuspended in RPMI 1640 medium supplemented with 5% FCS. For the antibody treatment, aliquots of 1 \times 10^6 pellet cells were resuspended in 0.25 mL of medium and the additional treatment was carried out as described previously for the limiting dilution analysis. For the granulocyte-macrophage colony-forming unit assay (GM-CFU) assay, after completion of the antibody treatment and the washings, the cells were resuspended and plated in a bilayer soft agar system28 at 100,000 cells/plate in 0.32% agar with a minimum essential medium (GIBCO) and 15% FCS over an underlayer of 0.5% agar containing α-minimum essential medium with 15% FCS and 20% human placental-conditioned medium.29 Corning 35-mm tissue culture dishes (Corning Glass Works) were used. After 10 days incubation at 37°C in a humidified atmosphere of 5% CO2, balanced N2, and 12% O2, the number of GM-CFUs on each plate was counted.

Immunoperoxidase staining method. Immunoperoxidase staining was performed on acetone-fixed cytospin preparations (Cytospin
Fig 1. Immunoperoxidase staining of cytocentrifuge preparations of 8226/DOX\textsubscript{82} and 8226/DOX\textsubscript{4} drug-resistant cell lines, and 8226/S drug-sensitive parent cell line. (Left vertical column), 8226/DOX\textsubscript{82} stained with MRK-16 (A) and NM\textsubscript{lg}G2a (B); Giemsa staining (C). (Right vertical column), 8226/DOX\textsubscript{4} stained with MRK-16 (D), NM\textsubscript{lg}G2a (E); 8226/S stained with MRK-16 (F).
ELIMINATION OF DRUG-RESISTANT MYELOMA CELL LINES

2. Shandon Inc, Pittsburgh, PA) of cells using a Universal Immuno-peroxidase staining kit (Cambridge Research Laboratory, Cambridge, MA). All reagents in the kit are prediluted and ready for use. Each reagent is to be used in 2 to 3 drops at approx. measurements per slide. The staining was carried out as follows. The slides were first incubated with normal goat serum (blocking reagent) for 20 minutes to block any nonspecific reactions with primary or secondary antibodies. This was followed by successive incubations with (1) 50 µL of MRK-16 MoAb (primary Ab) in 1% BSA in PBS, pH 7.5, at a concentration of 20 µg/mL for 30 minutes, (2) goat antimouse IgG (linking reagent) for 20 minutes, (3) peroxidase-labeled mouse IgG (labeling reagent) for 20 minutes, and (4) substrate solution for 5 minutes. The substrate solution was freshly prepared before use by mixing one drop each of substrate chromogen (2% 3-amino-9-ethylcarbazole in sodium and substrate reagent (1% hydrogen peroxide in water) with 2 mL of 0.1 mol/L acetate buffer, pH 5.2 (all reagents from the kit). The slides were washed with 0.5% BSA in PBS between successive incubations. The final step consisted of counterstaining with hematoxylin. The slides were then cleaned and mounted with Aqua Mount, an aqueous mounting medium (Lerner Laboratories, New Haven, Connecticut). In negative control slides, the primary antibody MRK-16 was excluded and replaced by NMlgG2a.

RESULTS

MRK-16 MoAb binding to 8226/DOX40 and 8226/DOX4 cells. In immunocytochemical analysis of acetone-fixed cytopsin preparations of 8226/DOX40 cells by immunoperoxidase staining method, MRK-16 exhibited a strong positive reaction with 8226/DOX40 cells. Almost all the cells were brightly stained, and the staining was located primarily on the surface of cells. Control slides stained with NMlgG2a showed no positive reaction with 8226/DOX40 cells. A moderately positive reaction with MRK-16 MoAb was noted in 8226/DOX4 cells. The control slides stained with NMlgG2a were negative. MRK-16 MoAb did not react with 8226/S drug-sensitive parent cells, thereby showing the specificity of MRK-16 antibody reactivity with drug-resistant cells (Fig 1).

Cytotoxicity of MRK-16 MoAb + RC treatment on 8226/DOX40 and 8226/DOX4 cells. Trypan blue exclusion assay was used for preliminary evaluation of cytotoxic activity of MRK-16 MoAb + RC against the drug-resistant and the drug-sensitive myeloma tumor cell lines and for determination of cytotoxic concentrations of MRK-16 MoAb for use in cell elimination experiments employing the limiting dilution analysis. The latter method was selected for use in cell elimination experiments because of its greater accuracy and sensitivity for measuring cell kill which is up to 5.6 logs when an initial concentration of 10^5 cells/well is used. However, prior to plating the treated cells in limiting dilution assay, the trypan blue exclusion method was routinely used to check on cytotoxicity of treatments. The results of these tests are described later along with limiting dilution analyses.

Only one cycle of treatment with Ab at RC was tested in all the following trypan blue exclusion assays. When 8226/DOX4 and the 8226/DOX40 cells were treated simultaneously in a microtiter assay (2.5 x 10^5 cells/well), identical levels of cytotoxic activity from 50% to 90% in increasing order of activity were observed against both cell lines with concentrations of MRK-16 MoAb ranging from 2 to 30 µg/mL and a 1:4 final dilution of RC. Cytotoxic activity was negligible against 8226/S cells. In macrotitration (1 x 10^5 cells/well) where 8226/DOX40 and 8226/DOX4 were tested at separate times, maximum specific cytotoxicity was 60% to 65% against 8226/DOX40 and 84% against 8226/DOX4 cells with 25 µg/mL of MRK-16 MoAb and 1:4 final dilution of RC. Lower level of cytotoxicity observed in macrortitration against 8226/DOX40 cells is probably due to elimination of cell proliferation without sufficient cell membrane damage to incorporate the dye.

Based on the above findings, concentrations of 25 µg/mL of MRK-16 MoAb and a 1:4 final dilution of RC were selected for use in cell elimination experiments. However, before embarking on these studies, the specificity of MRK-16 MoAb plus RC cytotoxicity against drug-resistant cell lines was confirmed by comparing the cytotoxic effects of MRK-16 MoAb plus RC on 8226/DOX40 with that on drug-sensitive parent 8226/S cells in a trypan blue exclusion assay using 1 x 10^5 cells per test. The results showed that the cytotoxicity of MRK-16 MoAb + RC was significantly higher on 8226/DOX40 cells (67%) than on 8226/S cells (only 10% similar to complement treatment alone) (Table 1). Antibody treatment alone showed 7% cytotoxicity on 8226/DOX40 cells.

Determination of toxicity of MRK-16 MoAb + RC on normal bone marrow. The toxic effects of antibody plus complement treatment on normal bone marrow (hematologically normal marrow from breast carcinoma patients) at serially increasing antibody concentrations ranging from 0.98 µg/mL up to 31.2 µg/mL were assessed using the GM-CFU cells as a target. The results showed that survival of GM-CFU cells was not significantly affected at these antibody concentrations as compared with GM-CFU survival using complement alone. Survival was 90% and 80% of control values with 15.6 µg/mL and 31.2 µg/mL of antibody, respectively. Additional evidence for minimal toxicity of MRK-16 MoAb + RC is provided in later experiments.

Limiting dilution analysis of cell kill of drug-resistant cell lines 8226/DOX40 and 8226/DOX4 and the drug-sensitive parent cell line 8226/S by MRK-16 MoAb plus RC. The results of three replicate experiments showed that one cycle of treatment (1 x 90 min) of cells with 25 µg/mL of MRK-16 MoAb + 1:4 final dilution of RC (the concentrations that produced the maximum specific cytotoxicity in trypan blue exclusion assay) produced less than 1 log kill of either 8226/DOX40 (0.47 ± 0.40 log kill) or 8226/DOX4 (0.12 ± 0.20 log kill). Two cycles (2 x 45 min) and three

Table 1. Comparison of Cytotoxic Activity of MRK-16 MoAb + RC Against 8226/DOX40 and 8226/S Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Ab + RC</th>
<th>NMlgG2a + RC</th>
<th>RC</th>
<th>Ab</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>8226/DOX40</td>
<td>67</td>
<td>6</td>
<td>6.5</td>
<td>6.5</td>
<td>7.5</td>
</tr>
<tr>
<td>8226/S</td>
<td>10</td>
<td>4</td>
<td>7.5</td>
<td>3.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* 1 x 10^5 8226/DOX40 or 8226/S cells were treated with 25 µg/mL of antibody + a 1:4 final dilution of RC. Controls consisted of cells treated with NMlgG2a + RC, RC, antibody, and medium. Cell viability was determined by trypan blue exclusion.
cycles (3 x 30 min) of treatment, however, increased the kill significantly from less than 1 log kill to 1.94 ± 0.36 (P < .001) and 2.90 ± 0.10 (P < .001) log kills, respectively, of 8226/DOX cells and 1.63 ± 0.18 (P < .001) and 1.94 ± 0.18 (P < .001), respectively, of 8226/DOX6 cells. The three cycle treatment was superior to the two cycle treatment for 8226/DOX cell line (P < .001) but not for 8226/DOX6 line. This difference is probably related to differences in P-glycoprotein densities between the two cell lines. Cell growth of 8226/S was unaffected by one to three cycles of treatment with MRK-16 MoAb + RC (Table 2). These results show that treatment with MRK-16 MoAb plus RC produced a significant kill of both drug-resistant cell lines as compared to the treatment with NMIgG2a + RC; 8226/DOX6 cell kill was, however, lower than that of 8226/DOX4 cells (P = .001 when three cycle treatments were compared).

As mentioned earlier, prior to plating the treated cells in limiting dilution assay, a rapid measurement of cytotoxicity of treatments was made using trypan blue exclusion method. The results showed that specific cytotoxicity ± SD values (n = 3) with one, two, and three cycles of treatment were 64.94 ± 14.17, 91.43 ± 4.50, and 92.06 ± 2.60, respectively, for 8226/DOX4 cells and 59.64 ± 17.05, 91.76 ± 7.06, and 97.72 ± 1.44, respectively, for 8226/DOX6 cells. Similar tests failed to show any cytotoxicity against 8226/S cells.

These findings show the limitations of trypan blue exclusion method, which failed to measure the increased cell kill of 8226/DOX6 by three cycles compared with two cycles of treatment, demonstrated above by limiting dilution assay. These data support the use of the latter method for more accurate and sensitive determination of cell kill.

Table 2. Limiting Dilution Analysis of Cell Kills by MRK-16 MoAb + RC of Drug-Sensitive and Drug-Resistant Myeloma Cell Lines: Effect of Number of Treatment Cycles

<table>
<thead>
<tr>
<th>No. of Treatments x Time (min)</th>
<th>Treatment*</th>
<th>Mean Log Kill ± SD (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8226/S</td>
<td>8226/DOX4</td>
</tr>
<tr>
<td>1 x 90</td>
<td>MRK-16 + RC</td>
<td>0.12 ± 0.20</td>
</tr>
<tr>
<td>2 x 45</td>
<td>MRK-16 + RC</td>
<td>1.63 ± 0.58</td>
</tr>
<tr>
<td>3 x 30</td>
<td>MRK-16 + RC</td>
<td>1.94 ± 0.18</td>
</tr>
</tbody>
</table>

*1 x 10^6 cells of each of the cell lines (8226/S, 8226/DOX4, and 8226/DOX6) were treated individually with 25 µg/mL of MRK-16 MoAb + a 1:4 final dilution of adult RC in a total volume of 1 mL.

Table 3. Limiting Dilution Analysis of Cell Kills by MRK-16 MoAb + RC of Drug-Resistant Myeloma Cell Lines in the Presence of Excess Marrow MNCs

<table>
<thead>
<tr>
<th>No. of Treatments x Time (min)</th>
<th>Treatment*</th>
<th>Mean Log Kill ± SD (n = 2)</th>
<th>Mean Log Kill ± SD (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 x 30</td>
<td>MRK-16 + RC</td>
<td>2.39 ± 0.41</td>
<td>2.91 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>MRK-16 + RC</td>
<td>2.21 ± 0.16</td>
<td>2.91 ± 0.16</td>
</tr>
</tbody>
</table>

8226/DOX4 and 8226/DOX6 cell kills by MRK-16 MoAb + RC in the presence of excess marrow MNCs. The adult RC used in the previous experiments was replaced by baby RC in this series of experiments because unusually high marrow toxicity was detected in the next batch of adult RC tested. The efficacy of baby RC in killing 8226/DOX4 cells in the presence of MRK-16 MoAb was at first established in two replicate experiments. The results showed 1.17 ± 0.33 log kills with one cycle of treatment and 4.36 ± 0.25 with three cycles of treatment. In mixing experiments, 8226/DOX4 or 8226/DOX6 cells mixed with a ninefold excess of irradiated bone marrow MNCs were treated with 25 µg/mL of MRK-16 MoAb + a 1:4 final dilution of RC. The results showed that cell kills of 8226/DOX4 and 8226/DOX6 were not affected by the addition of normal marrow MNCs (Table 3), thereby indicating that the presence of marrow cells did not compromise cell kill efficiency of MRK-16 MoAb + RC.

Determination of toxic effects of three cycles of MRK-16 MoAb + RC on normal marrow GM-CFU. Toxicity of three cycles of treatment was assessed using bone marrow specimens from three myeloma and three breast cancer patients. The patients were in remission at the time of bone marrow aspiration. Marrow from breast cancer patients was used as a source of hematologically normal marrow. After treatment with MRK-16 MoAb + RC, GM-CFU cell survival was 85% to 100% in the myeloma patient marrow (Table 4) and 92.5% to 104% in the breast cancer patient marrow (Table 5), thereby showing that three cycles of treatment with MRK-16 MoAb + baby RC produced little or no adverse effect on marrow GM-CFU. GM-CFU sur-

Table 4. Determination of Toxicity of MRK-16 MoAb + RC Treatment on Human Myeloma Patient Bone Marrow GM-CFUs

<table>
<thead>
<tr>
<th>Treatment of Bone Marrow*</th>
<th>No. of GM-CFU/1 x 10^6 Bone Marrow Cells Mean ± SD (% of medium control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ex Experiment 1</td>
</tr>
<tr>
<td>Medium (unincubated)</td>
<td>114 ± 11</td>
</tr>
<tr>
<td>Medium</td>
<td>120 ± 1</td>
</tr>
<tr>
<td>MRK-16 + RC</td>
<td>102 ± 4 (85)</td>
</tr>
<tr>
<td>NMIgG2a + RC</td>
<td>106 ± 4 (88)</td>
</tr>
<tr>
<td>RC</td>
<td>102 ± 4 (85)</td>
</tr>
<tr>
<td>MRK-16</td>
<td>108 ± 9 (90)</td>
</tr>
</tbody>
</table>

*1 x 10^6 bone marrow MNCs were incubated with 25 µg/mL of MRK-16 MoAb + a 1:4 final dilution of RC. Control sets of cells consisted of cells left unincubated or incubated with medium, NMIgG2a + RC, RC alone, and MRK-16 MoAb alone.
vival was not significantly affected by treatment with MRK-16 MoAb, RC alone, or NMlG2a plus RC.

**DISCUSSION**

Intrinsic or acquired multidrug resistance is a common clinical problem in the treatment of cancer. It is likely that more than one mechanism contributes to the phenomenon of multidrug resistance. P-glycoprotein, the product of the MDR1 gene, has been shown to mediate one kind of multidrug resistance in cultured cells. Clinical studies have suggested that drug resistance in some tumors may be related to expression of the MDR1 gene. The purpose of this study was to investigate the ability of anti-P-glycoprotein antibody MRK-16 + RC to eliminate P-glycoprotein containing drug-resistant human myeloma cells from the bone marrow. Because P-glycoprotein is located in the plasma membrane, antibodies can be used to kill cells containing this protein. Several MoAbs against P-glycoprotein have been isolated. Some of these, such as MRK-16 and 32G7, were developed against the P-glycoprotein of human origin, and they react with the epitopes exposed on viable cells. The others, such as the C219 and JSB-1, developed against P-glycoproteins in different multidrug resistant mammalian cells, react with a cytoplasmic region of P-glycoprotein. Some degree of membrane permeabilization is required for binding of the latter antibodies to multidrug-resistant cells. Therefore, MRK-16 MoAb, which binds to viable cells, was selected for use in our study. Furthermore, MRK-16 is the only antibody shown to affect drug transport in resistant cells. Therefore, if used for treatment before ex vivo chemotherapy, MRK-16, with its dual action of complement-mediated cytotoxicity and its modulation of drug transport by simply binding to cells, might be more effective.

Recently, several studies have demonstrated a lack of correlation between net drug accumulation and degree of resistance in various drug-resistant cell lines. A lack of correlation was found between drug accumulation and membrane protein alterations in various drug-resistant murine tumors. However, the studies by Dalton et al have shown that the amount of P-glycoprotein detected in the plasma membranes of 8226/DOX6 cell line correlated well with the degree of drug resistance. Moreover, the level of P-glycoprotein was found to be inversely related to the net intracellular accumulation of doxorubicin, and the decrease in drug accumulation appeared to be a major cause of drug resistance in this cell line.

Two drug-resistant cell lines, namely 8226/DOX6 and 8226/DOX6 with high and low levels of P-glycoprotein expression, respectively, were used in this study to cover a range of antigenic densities that might be encountered in the clinical specimens. Comparison of levels of P-glycoprotein expression as determined by a computerized cell analysis system, in 8226/DOX6 and 8226/DOX6 cell lines, with that in a multiple myeloma patient with a drug refractory disease showed that the level of P-glycoprotein in the myeloma patient cells was slightly higher than that in the 8226/DOX6 cell line. The 8226/DOX6 contained slightly more than twice the amount of P-glycoprotein in 8226/DOX6 cell line. These findings support the use of 8226/DOX6 and 8226/DOX6 cell lines in our study.

More than one cycle of treatment with MRK-16 + RC was required to produce a significant cell kill of either 8226/DOX6 or 8226/DOX6 cells. These results agree with the findings of other laboratories that have employed MoAbs to tumor antigens + RC for depleting tumor cells from bone marrow. It was important to ensure that the reagents used in our study were not toxic to marrow stem cells. Our results showed that treatment with MRK-16 + RC did not affect GM-CFU cell survival adversely.

Our approach to purge drug-resistant tumor cells from the bone marrow could serve as a valuable supplementation to existing methods of purging, such as chemopurging and immunopurging in a number of tumor types, for example, acute leukemia and myeloma. Because single antibody treatments are shown to be inferior to treatments with combinations of two or three antibodies, addition of MRK-16 MoAb to PCA-1, an antibody directed to a protein on the surface of myeloma cells or myeloid antibodies in leukemia, could significantly increase the efficacy of purging. More importantly, the presence of MRK-16 MoAb could ensure the killing of any drug-resistant tumor cells that might escape treatment with other antibodies. It is possible that drug-resistant cells might have a survival advantage over drug-sensitive cells once they are infused into the patient.

Purging of marrow prior to autologous transplantation has not yet been unequivocally proven to be beneficial in terms of disease-free survival or overall survival of patients. Therefore, the search for new and improved methods of purging continues. Our results have shown that it is possible to obtain

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**Table 5. Determination of Toxicity of MRK-16 MoAb + RC Treatment on Human Breast Carcinoma Patient Bone Marrow GM-CFU**

<table>
<thead>
<tr>
<th>Treatment of Bone Marrow*</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium (unincubated)</td>
<td>76 ± 4.5</td>
<td>93 ± 6</td>
<td>84 ± 8</td>
</tr>
<tr>
<td>Medium</td>
<td>67 ± 7</td>
<td>68 ± 4</td>
<td>83 ± 6</td>
</tr>
<tr>
<td>MRK-16 + RC</td>
<td>62 ± 5 (92.5)</td>
<td>70 ± 10 (103)</td>
<td>79 ± 8 (95)</td>
</tr>
<tr>
<td>NMlG2a + RC</td>
<td>65 ± 2 (97)</td>
<td>68 ± 9 (100)</td>
<td>78 ± 4 (95)</td>
</tr>
<tr>
<td>RC</td>
<td>62 ± 4 (92.5)</td>
<td>66 ± 5 (97)</td>
<td>79 ± 4 (95)</td>
</tr>
<tr>
<td>MRK-16</td>
<td>68 ± 3 (101)</td>
<td>66 ± 2.5 (97)</td>
<td>86 ± 8 (104)</td>
</tr>
</tbody>
</table>

* 1 x 10⁸ bone marrow MNCs were treated with 25 µg/mL of MRK-16 MoAb + a 1:4 final dilution of RC. Control sets of cells consisted of cells left unincubated or incubated with medium, NMlG2a + RC, RC alone, and MRK-16 MoAb alone.

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a satisfactory cell kill of 8226/DOX and 8226/DOX<sub>40</sub> cells by treating the cells with MRK-16 MoAb + RC and that this form of treatment does not have an adverse effect on marrow stem cells. This method has potential as a modality to use in combination with other methods for marrow purging in multiple myeloma and in other forms of cancer.

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