A Combined Approach for Purging Multidrug-Resistant Leukemic Cell Lines in Bone Marrow Using a Monoclonal Antibody and Chemotherapy

By Michiko Aihara, Yukoh Aihara, Gabriele Schmidt-Wolf, Ingo Schmidt-Wolf, Branimir I. Sikic, Karl G. Blume, and Nelson J. Chao

Selective removal of malignant cells (purging) from bone marrow (BM) is a concern in autologous BM transplantation (ABMT). Use of vincristine, etoposide, or doxorubicin for purging could be rendered ineffective by the presence of multidrug-resistant (MDR) tumor cells. To circumvent this particular problem, we investigated whether 17F9, a monoclonal IgG2b antibody directed against the cell surface product of the MDR gene, P-glycoprotein, is effective in selective removal of MDR cells from BM when used with rabbit complement (C'). Using two different cell lines we have demonstrated that 17F9 + C' selectively lysed MDR-positive cells. Three rounds of antibody + C' resulted in 96.4% ± 3.6% kill of K562/DOX and 100% ± 0% of CEM/VLB cells. Mixtures of malignant cells and normal BM resulted in 99.85% removal of K562/DOX and 99.91% removal of CEM/VLB clonogenic cells. This treatment did not affect normal committed precursors compared with C' alone. The addition of the cytotoxic agent etoposide (VP-16) following antibody purging resulted in a 4.6 log reduction of malignant cells. Furthermore, this antibody was effective when used against patients' leukemic blasts. These results suggest the use of 17F9 + C' is effective and selective for removal of MDR cells from BM before ABMT and the addition of VP-16 enhances the purging efficacy.

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MATERIALS AND METHODS

Cells. CCRF-CEM (human leukemic lymphoblasts) selected for resistance to vinblastine (CEM/VLB) were obtained from Dr William T. Beck.9 K562 (human leukemic erythroblasts) selected for resistance to doxorubicin (K562/DOX) were obtained from Dr Kevin Ross.9 Both of these resistant cell lines express high levels of mdr-1 RNA. All cells were grown as standard suspension cultures in minimal essential media (MEM) containing 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT). BM and peripheral blood were obtained from healthy volunteers. Mononuclear cells were prepared by Ficoll-Hypaque density gradient. Mononuclear cells were radiated to 20 Gy at 39 Gy/min to prepare mixtures with leukemic cells for clonogenic assays.

Monoclonal antibodies (MoAbs) and complement. MoAb 17F9 (murine IgG2b) was kindly provided by Dr David Ring (Cetus Corporation). Briefly, 17F9 hybridoma was made by immunization of Balb/c mice with murine 3T3 cells transfected with the human mdr-1 gene. Reactivity of 17F9 with P-glycoprotein was established by differential staining of the transfected murine 3T3 cells (Ring D, manuscript in preparation). The 17F9 antibody was obtained from ascites fluid of Balb/c mice bearing the cells and purified by ion exchange chromatography. As a control, MoAb 17CS (IgG1), which reacts with P-glycoprotein but fixes complement poorly, was also obtained from Dr Ring. Newborn rabbit complement was obtained from Pel-Freez (Brown Deer, WI). This complement gave maximum killing at a final dilution of 1:8 in a 51Cr release assay.

Antibody and complement treatment. Leukemic cells (1 x 10^6)

AUTOLOGOUS BONE MARROW transplantation (ABMT) for malignant diseases is most often performed after extensive prior chemotherapy to the patient.12 Most patients have relapsed disease with potential BM involvement with resistant tumor cells. Reinfection of marrow containing malignant clonogenic cells could place the patient at risk for recurrence of the underlying disease. Selective removal of these malignant cells may be possible by methods that exploit differences between tumor cells and normal hematopoietic precursors. Such methods include antibody-complement-mediated cytotoxicity, immunophotophysical techniques, in vitro chemotherapy, photoactivation, and cellular cytotoxicity.16 All such methods have been described as effective in removing malignant cells. However, one concern has been the persistence of drug-resistant malignant cells following exposure to conventional chemotherapy.

Among the many known biochemical mechanisms of drug resistance, multidrug resistance (MDR) is of particular interest.79 Models of MDR have been developed by exposure of cells to single drugs usually an antitumor antibiotic, vinca alkaloid, or podophyllotoxin. These cells become cross-resistant to many cytotoxic drugs with diverse structures and mechanisms of action. MDR is associated with amplification or overexpression of the mdr-1 gene and the phenotypic expression of a transmembrane glycoprotein of 170 Kd (P-glycoprotein).10,11 This protein has two adenosine triphosphate (ATP) binding sites and appears to act as an efflux pump, resulting in decreased intracellular drug concentration. P-glycoprotein is not expressed at high levels in normal BM cells, and its expression may be a cause of drug resistance following exposure to chemotherapy.12 This expression of P-glycoprotein allows for a differential target for in vitro purging of BM containing MDR malignant cells.10,16 Because the expression of P-glycoprotein is heterogenous in tumor cells, we reasoned that the addition of a cytotoxic agent that is effective yet spares normal hematopoietic precursors, VP-16, would enhance tumor cell removal ex vivo.17,18 In this report we present the use of an antibody against P-glycoprotein plus the chemotherapeutic agent VP-16 to purge contaminating tumor cells from BM before autologous transplantation.

From the Bone Marrow Transplantation Program, Hematology Division, Oncology Division, Department of Medicine, Department of Pathology, Stanford University School of Medicine, Stanford, CA. Submitted July 23, 1990; accepted January 8, 1991. Supported by National Institutes of Health Grants CA 49605/01-03, Leukemia Society Fellowship (N.J.C.), and American Cancer Society Grant CH-411 (B.I.S.). N.J.C. is a Fellow of the Leukemia Society of America.

Address reprint requests to Nelson J. Chao, MD, Stanford University Medical Center, Bone Marrow Transplantation Program, Room H1353, 300 Pasteur Dr, Stanford, CA 94305-5306.

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(5% of the total cell number) were mixed with $19 \times 10^6$ BM or peripheral mononuclear cells before antibody treatment. Deoxyribo- nuclease I (DNase-I from bovine pancreas; Sigma, St Louis, MO) was added to these mixtures at 50 U/mL to minimize cell clumping and incubated with 17F9 on ice for 30 minutes. After addition of complement at a final concentration of 1:8, the cells were incubated for 60 minutes at 37°C in a shaking water bath. The cells were washed and this procedure was repeated once or twice as indicated. Normal BM cells alone were also treated at a cell concentration of $2 \times 10^6$ mononuclear cells/mL in the same fashion. Tumor cells and normal BM were incubated with VP-16 at a concentration of 50 μmol/L at 37°C for 2 hours. All experiments were performed in triplicate.

**31Cr release assay.** Leukemic cells were labeled with 200 μCi of $^{31}$Cr (Dupont-NEN, Boston, MA) for 1 hour at 37°C. These cells were washed and added to the mononuclear cells to a final concentration of 5% leukemic cells. After treatment with antibody and complement, the cells were pelleted and the activity of $^{31}$Cr in the supernatant was measured and expressed as a percentage of maximum release corrected for complement control.

Maximum release was accomplished by lysing the $^{31}$Cr-labeled leukemic BM mixtures with 1% NP-40. To examine the relationship between antibody dose and cytotoxicity, $1 \times 10^6$ $^{31}$Cr-labeled cells were treated with various concentrations of antibody and complement in 96-well round bottom plates. After the first round of antibody and complement, the plates were centrifuged and the activity of $^{31}$Cr in the supernatant was measured. To assess nonspecific cell kill, control samples were treated with an irrelevant mouse IgG2b antibody and complement. All experiments were performed in triplicate.

**Clonogenic assays.** After treatment, leukemic cells and irradiated mononuclear cells mixtures were washed twice and cultured in 1% methylcellulose and Iscove's Modified Dulbecco's Medium (IMDM) containing 20% FCS. The cell mixtures were plated and the number of tumor clonogenic units was determined as described. All cell lines used for the experiments had a colony forming efficacy of 20% to 40% in the presence or absence of excess BM or peripheral blood cells. When nonirradiated BM cells ($10^7$ cells/mL) were cultured after treatment, placenta-conditioned media containing 0.01% endotoxin, recombinant erythropoietin, or Mo-conditioned media were added as stimulants for colony-forming unit granulocyte-macrophage (CFU-GM), burst-forming unit erythroid (BFU-E), or CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM), respectively. Plates were incubated at 37°C and 5% CO₂ in a humidified incubator and colonies on methylcellulose were scored on day 7 (leukemic clonogenic units), day 10 (CFU-GM), or day 14 (BFU-E, CFU-GEMM). The effects of treatments on colony formation are expressed as percent of untreated control. Analysis of significance was performed using the Student's t-test.

**Immunofluorescence analysis.** Cells were indirectly stained with unconjugated 17F9 followed by fluorescein isothiocyanate (FITC)-conjugated goat antimouse Ig. Cells ($1 \times 10^7$) were analyzed on a FACSTAR (Becton Dickinson, Mountain View, CA), modified as

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**Fig 1.** Flow cytometry analysis of MDR (K562/DOX and CEM/VLB) and drug-sensitive (K562/CEM) leukemic cell lines stained with 17F9 antibody and goat antimouse Ig (dark curve). Cells stained only with FITC-conjugated goat antimouse Ig were used as background staining (light curve).
PURGING MDR+ CELLS WITH MoAb + C' AND VP-16

Fig 2. Lysis of 51Cr-labeled leukemic cells with complement and increasing concentration of 17F9 and 17C5. The results shown are the mean of three experiments (SD < ±5%). Complement alone causes 3% to 6% 51Cr release. Note the lack of activity of 17F9 on CEM and K562, the drug-sensitive parental cell lines.

previously described. Dead cells were excluded from analysis by propidium iodine (PI) staining. Cells stained only with FITC-conjugated goat antimouse IgG were used as background control.

RESULTS

Fluorescence-activated cell sorter (FACS) analysis of MDR cells. To evaluate the expression of P-glycoprotein antigen recognized by 17F9 on drug-resistant and drug-sensitive cell lines, immunofluorescence analysis was performed. The results shown in Fig 1 indicate that 98.7% of K562/DOX and 98.9% CEM/CLB express the antigen with a single staining pattern. The parental cells K562 and CEM express 1.3% and 1.6% P-glycoprotein, respectively. These results support the data that K562/DOX and CEM/CLB express high levels of mdr-1 mRNA. Normal BM cells showed less than 1% staining with 17F9 (data not shown).

Optimal concentration of antibody. To determine the optimal concentration of MoAb, leukemic cells were incubated with different concentrations of 17F9 (3 ng/mL to 10 μg/mL) before incubation with rabbit complement. The data presented in Fig 2 are based on analysis of cytotoxicity by 51Cr release assay. The MoAb 17F9 mediated specific lysis of K562/DOX and CEM/CLB at a concentration greater than 30 ng/mL. There was no specific lysis of K562 and CEM, the parental cell lines, even at 10 μg/mL. The addition of 20-fold excess of normal BM cells showed no effect on the lysis of K562/DOX and CEM/CLB over this range of antibody concentration (data not shown). Because both cell lines showed greater than 60% release at a concentration greater than 1 μg/mL, 3 μg/mL of 17F9 was chosen for subsequent experiments. At this concentration, 17C5 (the IgG1 control antibody) and complement treatment showed less than 10% of 51Cr release using K562/DOX and CEM/CLB cells. Treatment with the same range of a nonspecific IgG2b control antibody and complement showed less than 5% of 51Cr release with all cell lines used here (data not shown). Treatment with complement alone resulted in 3% to 6% of 51Cr release compared with phosphate-buffered saline (PBS) control.

Dose response. Previous studies with MoAbs that react with leukemic cells suggest that multiple treatments with antibody and complement are more effective than a single treatment for elimination of these leukemic cells from normal BM. K562/DOX and CEM/CLB mixed with normal mononuclear cells were incubated with 3 μg/mL of 17F9 and complement for 1 hour for each round to compare the effects of one to three rounds of treatment. Figure 3 shows the data of 51Cr release assay from three individual experiments with each cell line. Two rounds of treatment were more effective than a single treatment. There was slight additional cytotoxicity between rounds 2 and 3. After two

Fig 3. Lysis of 10^9 51Cr-labeled MDR leukemic cells in the presence of 2 × 10^6 mononuclear cells (simulating a remission marrow) after one to three rounds of treatment with 17F9 antibody and rabbit complement. Two and three rounds of treatment resulted in significant lysis (P < .01) for CEM/CLB. Compared with a single treatment for K562/DOX, two rounds were significantly different from a single round (P < .01). A third cycle did not result in a significant difference. The results show the mean of three experiments (SD < ±5%). ( ), K562/DOX; ( ), CEM/CLB.
rounds of treatment, there was 92% ± 2.3% of $^{51}$Cr release for K562/DOX and 97% ± 0.5% for CEM/VLB cells (both $P < .01$). After three rounds there was 96.4% ± 3.6% ($P = N S$) and 100% ± 0% ($P < .01$) for K562/DOX and CEM/VLB, respectively.

Selective purging experiments. We next investigated the efficacy of 17F9 and complement treatment using a clonogenic cell assay. K562/DOX and CEM/VLB were mixed with a 20-fold excess of irradiated normal mononuclear cells and treated with 3 μg/mL of 17F9 and complement for two rounds. After treatment, cells were cultured in methylcellulose. The results from four experiments are shown in Table 1. Two rounds of treatment with 17F9 and complement were quite effective in eliminating leukemic cells in the presence of normal mononuclear cells. The percentage of suppression of clonogenic units after 17F9 and complement treatment was approximately 99.9%, or a 3-log reduction. Three rounds of treatment did not show any additional effect as compared with two rounds of treatment (data not shown). These comparisons were made in a single experiment.

We examined whether the treatment with 17F9 and complement was toxic to normal hematopoietic progenitors. As shown in Table 2, two rounds of treatment with 3 μg/mL of 17F9 and complement did not affect any of the hematopoietic precursors as assayed by CFU-GM, CFU-GEMM, and BFU-E. Although there was a slight decrease in the number of CFU-GM and BFU-E after 17F9 and complement, this was also observed after treatment with complement alone.

Combination purging with antibody and chemotherapy. Following the 3-log kill achieved with antibody and complement, VP-16 was added to the mixture at a concentration of 50 μM/L and the cells incubated for 2 hours at 37°C. The results in Table 3 show a marked reduction in the number of clonogenic units with a decrease of 4.6 logs in tumor cells compared with the drug-sensitive cell line.

However, the addition of VP-16 results in toxicity to hematopoietic precursors as seen in Table 4 with CFU-GM decreasing to approximately 2%. This decrease was not different from that observed with 17F9 alone.

Use of 17F9 + C’ in patients’ leukemic blasts. Fresh patient samples were assayed for the expression of mdr phenotype by FACS or in situ hybridization. All patients carried the diagnosis of leukemia and were heavily pretreated or had suffered at least one relapse. Samples that expressed P-glycoprotein were incubated with 17F9 + C’ or 17C5 + C’ or saline at concentrations described above. Cytotoxicity was assayed by vital dye exclusion and chromium release assay. As shown in Fig 4, patients samples demonstrated similar percent cell kill as the leukemic blast cell line K562/DOX. Another patient’s sample that did not express the mdr phenotype showed no lysis (data not shown).

**DISCUSSION**

The ability of malignant cells to survive exposure to cytotoxic agents is a major obstacle to cure in cancer patients. MDR and the expression of P-glycoprotein is emerging as a cause of chemotherapy failure. Several investigators have recently correlated poor clinical response with the expression of P-glycoprotein in untreated as well as treated leukemias. The presence of P-glycoprotein allows the cell to actively decrease intracellular chemotherapy drug levels in the presence of relatively high extracellular drug concentrations.

The major concern in ABMT is the potential contamination of tumor cells at the time of BM harvest and subsequent reinfusion of these cells, especially in leukemias and
PURGING MDR+ CELLS WITH MoAb + C’ AND VP-16

variable expression markers, where we achieve an average results clearly show that a 3-log reduction of malignant observation to different cell lines (chronic myelogenous leukemia 

is more than a theoretical concern as prior chemotherapy tumor cells would significantly decrease this concern. This able to achieve a less than 2-log reduction of malignant clonogenic ceIls.2'

directed against other determinants such as T- or B-cell observed in using VP-16 together with MDR-modulating directed against P-glycoprotein, and rabbit complement obtained in our other experimental models using MoAbs leukemic cells can be achieved by using the MoAb 17F9, malignant cells using 17F9 MoAb is equivalent to that tion in the clonogenic malignant cells. The addition of VP-16 does result in enhanced cytotoxicity to normal hematopoietic precursors. However, our data and others demonstrate sufficient survival of progenitor cells for successful marrow reconstitution.l*~" We have also performed dose escalation studies of VP-16 purging in long-term marrow cultures.25 In such cultures, there were large numbers of CFU-GM remaining following incubation of BM with VP-16 (50 μmol/L) several weeks after the exposure. A combined approach of MoAb against P-glycoprotein with complement and chemotherapeutic agents (cyclosporine and verapamil) where we have been able to achieve a less than 2-log reduction of malignant clonogenic cells.29

Recently, various investigators have published their results using different MoAbs directed against P-glycoprotein, showing that it is effective with myeloma cell lines.20,29 Our results confirm their experience, but extend this observation to different cell lines (chronic myelogenous leukemia [CML]/acute lymphoid leukemia [ALL]). Moreover, we have demonstrated that this same combination of MoAb and complement is useful when directed against fresh leukemic blasts that express P-glycoprotein. These data suggest that the use of this MoAb may be effective for removal of MDR cells in leukemias and potentially other solid tumors.

One concern with using MoAbs against P-glycoprotein alone to purge drug-resistant cells in a patient is the variable expression of P-glycoprotein in malignant cells. This heterogeneity may allow for escape of cells that do not express P-glycoprotein on the cell surface, if purging with antibodies against P-glycoprotein alone. We have performed cytospins of these MDR+ cells and stained with fluoresceinated MoAb against P-glycoprotein. We observe variable expression of P-glycoprotein per cell. Most cells stain brightly, but other cells stain weakly, and a few cells do not stain at all. As shown in Fig 1, approximately 1% to 2% of drug-resistant cells do not express detectable amounts of P-glycoprotein. If 17F9 and complement was effective only in cells expressing high levels of MDR, we would expect at best removal of less than 2 logs of tumor cells. The data demonstrate a nearly 3-log tumor cell removal. Moreover, remaining tumor cells expressing less or no P-glycoprotein following MoAb treatment were sensitive to VP-16 because the addition of VP-16 resulted in an additional 1.7-log tumor cell kill. Addition of MDR modulators (cyclosporine [4 μmol/L] and verapamil [40 μmol/L]) did not increase the cytotoxicity of VP-16 against the MoAb-purged drug-resistant tumor cells (data not shown).

Therefore, 17F9 + C’ can be used sequentially with a chemotherapy drug such as VP-16 to ensure the removal of the tumor cells, resulting in a more effective purging process. Cells that express P-glycoprotein will be removed by 17F9 + C’ and other cells that do not express the mdr phenotype will be removed by the drug VP-16. This combined approach yields an impressive nearly 5-log reduction in the clonogenic malignant cells. The addition of VP-16 does result in enhanced cytotoxicity to normal hematopoietic precursors. Therefore, selective removal of these malignant cells is possible. An advantage of this approach is that it would cross specific cell types, ie, an MoAb against P-glycoprotein could be used for leukemias, lymphomas, or other solid tumors if such tumors express this antigen. We are prospectively gathering the incidence of expression of P-glycoprotein in tumor cells of our autologous transplant patients. Our experience in relapsed leukemia patients is that there is a significant increase in the expression of P-glycoprotein in malignant cells.3 Because ABMT is most frequently performed in patients following exposure to chemotherapeutic agents that can lead to higher levels of MDR expression, use of this purging technique may be more widely applicable.

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M Aihara, Y Aihara, G Schmidt-Wolf, I Schmidt-Wolf, BI Sikic, KG Blume and NJ Chao