Variability in 4-Hydroperoxycyclophosphamide Activity During Clinical Purging for Autologous Bone Marrow Transplantation


We examined the effects of varying incubation conditions on the in vitro activity of 4-hydroperoxycyclophosphamide (4HC). 4HC activity against CFU-GM and against the K562 tumor cell line decreased with increasing the RBC concentration of the incubation mixture. Increasing the concentration of nucleated bone marrow cells in the incubation mixture also decreased the 4HC activity. Evaluation of 53 consecutive patients undergoing autologous bone marrow transplantation (BMT) revealed that the incubation RBC concentration during clinical purging showed a similar effect on CFU-GM recovery. Aldehyde dehydrogenase content of RBCs and nucleated marrow cells appears to be the cause of the inhibition of 4HC activity. Although there was no difference in individual CFU-GM sensitivity to 4HC among normals, previously treated patients undergoing autologous BMT showed significant variability in CFU-GM sensitivity to 4HC. The combined effects of incubation RBC concentration and individual patient 4HC sensitivity appear to account for most of the variability in CFU-GM recovery and speed of hematologic recovery after clinical purging with 4HC.

A UTOLLOGOUS bone marrow transplantation (BMT) is a useful treatment for resistant acute leukemias, non-Hodgkin's lymphomas, and Hodgkin's disease. However, a potential obstacle to the optimal utilization of this procedure is the contamination of the autologous graft with tumor cells, even when the tumor is absent morphologically. Sharkis and coworkers showed that 4HC could eliminate or purge leukemic cells from suspensions of marrow and tumor and still permit hematologic reconstitution. Subsequently, purging with 4HC has undergone extensive preclinical and clinical study. At our institution autologous marrow grafts are purged with 100 μg/mL of 4HC at a concentration of 2 × 10⁶ nucleated marrow cells/mL for 30 minutes at 37°C. This dose of 4HC was the highest dose that permitted hematopoietic reconstitution in essentially all patients. However, the time to hematopoietic recovery in these patients varied greatly. Recently work from our institution showed that the CFU-GM content of 4HC-purged bone marrow grafts predicted the speed of hematopoietic recovery. However, the recovery of CFU-GM following clinical 4HC-purging ranged from 0.07% to 23%. The reasons for this 300-fold range in CFU-GM recovery are not all known but may represent differences in 4HC activity resulting from varied incubation conditions, differences in individual hematopoietic progenitor sensitivity, or a combination of both factors. Although serum concentration, nucleated cell concentration, time, and temperature of the 4HC incubation mixture were held constant during clinical purging, the RBC concentration of the incubation mixture varied considerably among patients. Preliminary clinical data from our institution suggested a relationship between the incubation RBC concentration and CFU-GM recovery following bone marrow purging with 4HC. A similar relationship for mafosfamide has also been suggested.

To examine the possible causes of the variable CFU-GM recovery following clinical 4HC purging of autologous marrow grafts, we examined the effects of graded concentrations of RBCs and nucleated marrow cells on the in vitro activity of 4HC against normal human bone marrow and against the K562 myeloid leukemia cell line. We also studied the effects of the incubation RBC concentration during clinical purging with 4HC in 53 patients undergoing autologous bone marrow transplantation (BMT) for the treatment of malignancy. Individual CFU-GM sensitivity to 4HC was also determined for 21 patients receiving autologous transplants.

MATERIALS AND METHODS

Bone marrow cells. Bone marrow cells from healthy volunteers were obtained by aspiration from their posterior iliac crests. Samples of the bone marrow grafts harvested from 53 consecutive patients undergoing autologous bone marrow transplantation for the treatment of malignancy were obtained prior to and after clinical purging with 4HC. All healthy volunteers and patients gave informed consent for study participation as approved by The Johns Hopkins Joint Committee on Clinical Investigation. Mononuclear cells (density <1.078 g/mL) for CFU-GM assays were recovered by Ficoll-Hypaque (FH) density centrifugation.

4HC incubations. The 4HC (prepared by one of the authors, O.M. Colvin) was freshly dissolved prior to use. All clinical purging was performed as previously described. Briefly, the nucleated cell buffy coat of the bone marrow graft was incubated with 100 μg/mL of 4HC in TC 199 medium containing 20% autologous plasma at a nucleated marrow cell concentration of 2 × 10⁶ cells/mL for 30 minutes at 37°C. The incubation mixture was rapidly cooled to 4°C and centrifuged at 2900 rpm for ten minutes. Bone marrow cells from normal donors or cells of the K562 myelogenous leukemia cell line were incubated at concentrations of 5 × 10⁶ cells/mL in enriched McCoy's 5A medium with graded concentrations of 4HC at graded concentrations of autologous RBCs for 30 minutes at 37°C. To remove background red cells from the plates, red cells were lysed by incubating the mixture with 0.87%...
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NH$_4$Cl for ten minutes after washing the cells. Parallel experiments revealed that the NH$_4$Cl incubation did not alter the CFU-GM recovery. 4HC incubations with various concentrations of mononuclear bone marrow cells, K562 cells, and fetal bovine serum (FBS), and individual patient serum replacing the FBS, were also performed.

Mononuclear bone marrow cells from 21 autologous bone marrow grafts, obtained prior to clinical purging with 4HC, and cells from 14 healthy volunteers were incubated with graded concentrations of 4HC at concentrations of 2 x 10$^4$ cells/mL for 30 minutes at 37°C to determine individual CFU-GM sensitivities to the drug in the absence of RBCs. All experimental 4HC incubations were arrested by the addition of chilled medium, and the cells were washed twice.

**Colony assays.** CFU-GM assays following the experimental incubations were performed using a modification of the technique described by Pike and Robinson.$^{14}$ 2 x 10$^4$ mononuclear bone marrow cells/mL were cultured in enriched McCoy's 5A medium containing 0.3% agar and 10% human placental-conditioned medium as a source of colony stimulating factor.$^{15}$ CFU-GM assays performed on the clinical autologous marrow grafts and those done on normal marrows to establish individual CFU-GM sensitivity to 4HC were performed as previously described.$^{16}$ Briefly, 5 x 10$^4$ to 1 x 10$^5$ mononuclear marrow cells/mL were cultured in 1.32% methylcellulose containing alpha medium, 5% phytohemagglutinin-stimulated leukocyte-conditioned medium, 1% bovine serum albumin (BSA), 30% fetal bovine serum (FBS), 10$^{-4}$ mol/L methylprednisolone, 10$^{-4}$ mol/L 2-mercaptoethanol, and 1 unit/mL erythropoietin.

Clonogenic assays of K562 cells were performed using 0.3% agar, enriched McCoy's 5A medium, and between 200 and 10$^4$ cells/mL. Parallel experiments have shown that K562 colony growth is linear over this range, with inhibition of growth occurring at K562 cell densities greater than 10$^4$ cells/mL. One milliliter of the bone marrow or K562 cell mixture was plated in 35-mm petri dishes. The plates were incubated for ten to 14 days in a humidified atmosphere containing 5% CO$_2$ at 37°C. The CFU-GM survival (log) was significantly linearly related to the incubation dose of 4HC at each concentration.

**Intracellular 4HC metabolism.** Determination of intracellular metabolites of activated cyclophosphamide used 4HC prepared from [H]$^3$-side chain] cyclophosphamide (Amersham) by the method of Hohorst et al.$^{21}$ Cells were exposed to the radiolabeled 4HC (20 µCi/µmole) at 37°C before being rapidly separated from the radioactive medium by centrifuging through oil to give a cell pellet that is extracted by freeze-thawing at neutral pH. A protein-free extract was applied to a reverse phase C$_{18}$ high-pressure liquid chromatography column and eluted with an ion-pairing solvent. Fractions of the eluate were collected and their radioactivity determined by scintillation counting. The intracellular concentration of individual components of the mixture of metabolites was determined from the specific activity of the [H]$^3$ 4HC and the intracellular volume of the cells being analyzed.

**Statistical evaluations.** All the graphed data points from the preclinical studies were the means (±SEM) of two to four separate experiments performed in quadruplicate. The logarithms of percent surviving CFU-GM after incubation with 5 x 10$^4$ mononuclear marrow cells/mL for 30 minutes at 37°C showed a significant linear relationship with the 4HC concentration. The logarithms of surviving CFU-GM from healthy donors was assessed. Graded concentrations of 4HC were incubated with 5 x 10$^4$ mononuclear marrow cells/mL for 30 minutes at 37°C at incubation-packed RBC volumes of 0%, 1%, 5%, 10%, and 20%. A significant linear relationship was found between the 4HC concentration and the logarithm of CFU-GM survival for each packed RBC volume tested (Fig 1). The CFU-GM activity against CFU-GM decreased with an increase in the RBC content of the incubation mixture. Similar effects of 4HC on RBC activity against K562 cells were observed (Fig 2). Again there was a significant linear relationship between 4HC concentration and the recovery of K562 clonogenic cells. Adding RBCs to the incubation mixture increased the survival of K562 cells.

The effect of varying the concentration of marrow mononuclear cells in the incubation mixture on 4HC activity was measured. Graded concentrations of 4HC were incubated with 5 x 10$^4$, 10 x 10$^4$, and 20 x 10$^4$ cells/mL for 30 minutes at 37°C. The CFU-GM survival (log) was significantly linearly related to the incubation dose of 4HC at each mononuclear cell concentration tested (Fig 3). The activity of 4HC also decreased with an increase in the concentration of mononuclear cells in the incubation mixture. Increasing

**RESULTS**

The effect of RBC concentration on the activity of 4HC against CFU-GM from healthy donors was assessed. Graded concentrations of 4HC were incubated with 5 x 10$^4$ mononuclear marrow cells/mL for 30 minutes at 37°C at incubation-packed RBC volumes of 0%, 1%, 5%, 10%, and 20%. A significant linear relationship was found between the 4HC concentration and the logarithm of CFU-GM survival for each packed RBC volume tested (Fig 1). The CFU-GM activity against CFU-GM decreased with an increase in the RBC content of the incubation mixture. Similar effects of 4HC on RBC activity against K562 cells were observed (Fig 2). Again there was a significant linear relationship between 4HC concentration and the recovery of K562 clonogenic cells. Adding RBCs to the incubation mixture increased the survival of K562 cells.

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**Fig 1.** CFU-GM dose response to 4HC as a function of incubation hematocrit: 0% - 0%; 1% - 0.057 x (n - 0.017 x (n - 0.97)); 5% - 0.027 x (n - 0.94). All correlation coefficients are significant at P < 10$^{-4}$.
the incubation concentration of K562 cells produced similar effects on the 4HC activity against these cells (data not shown).

Evaluation of the relationship between the RBC concentration of the incubation mixture during clinical purging with 4HC and CFU-GM recovery was performed in 53 consecutive patients undergoing autologous BMT. The incubation packed RBC volumes ranged from 4% to 23% with a mean of 8.7%. The CFU-GM recovery after 4HC treatment ranged from 0.01% to 23.8% (mean 1.4%). There was a statistically significant positive correlation between the incubation-packed RBC volume and CFU-GM recovery (Fig 4).

The individual CFU-GM sensitivity to 4HC was tested in 21 of the patients undergoing autologous BMT and 14 normal volunteers. Mononuclear bone marrow cells, obtained from autologous bone marrow grafts prior to clinical purging with 4HC, were incubated with graded concentrations of 4HC at cell concentrations of $2 \times 10^5$ cells/mL for 30 minutes at 37°C. The individual CFU-GM sensitivity to 4HC was expressed as the slope of the individual dose response obtained by plotting the concentration of 4HC ($\mu$g/mL) in the incubation mixture versus the logarithm of the percent CFU-GM survival. The mean ($\pm$SD) 4HC sensitivity for the 14 normals was 0.03 $\pm$ 0.008. CFU-GM sensitivity to 4HC was greater than 1 SD above the mean in only one of 14 normal donors, while ten of 21 patients showed CFU-GM sensitivity to 4HC that was greater than 1 SD above the mean ($P = .03$). In addition, the CFU-GM sensitivity to 4HC was over 4 SD above the mean in two of these ten patients. No patient or normal donor had CFU-GM sensitivity to 4HC that was less than 1 SD below the mean. Differing concentrations of FBS or incubations with serum from different patients did not alter the 4HC sensitivities (data not shown).

The metabolism of 4HC in RBCs was followed by analysis of the intracellular concentrations of activated cyclophosphamide (4-hydroxycyclophosphamide), carboxyphosphamide, and phosphoramid mustard, which together make up 90% of the cyclophosphamide metabolites. Figure 5 indicates the concurrent disappearance of 4-hydroxycyclophosphamide and the appearance of carboxyphosphamide for 10 RBCs/mL. This model predicts that 10 RBCs/mL (or approximately a 1% incubation-packed RBC volume) would
metabolize approximately 5 nmol (1.5 μg) of 4HC to inactive carboxyphosphamide during each minute of incubation.

**DISCUSSION**

We found that variations in the concentration of RBCs or nucleated bone marrow cells in the incubation mixture during purging with 4HC will significantly alter the 4HC activity against CFU-GM. It appears that the actual 4HC effect associated with 100 μg/mL of 4HC incubated with 2 x 10^6 cells/mL and variable RBC concentrations ranges from that associated with 20 μg/mL to 80 μg/mL of 4HC in the absence of RBCs. This inhibitory effect of the RBCs on 4HC activity also exists toward tumor cells. Therefore a high incubation RBC concentration during clinical purging with 4HC would be expected to shorten hematologic recovery following autologous marrow transplantation but would also likely be associated with decreased tumor kill. Alternatively, a low incubation concentration of red cells should increase tumor kill but would significantly lengthen hematologic recovery. In addition, there is variation in CFU-GM sensitivity to 4HC among individual patients, as also occurs during purging with mafosfamide for autologous BMT. Therefore the variable CFU-GM recoveries, and hence rates of hematologic recovery, seen after marrow purging with 4HC result from variable concentrations of RBCs in the incubation mixture and individual patient differences in 4HC sensitivities.

It appears that the decreased 4HC activity seen with increasing the number of RBCs in the incubation mixture is due to the aldehyde dehydrogenase content of these cells. RBCs have been previously shown to contain measurable aldehyde dehydrogenase activity, and the metabolism of 4HC in RBCs, as shown in Fig 5, is characteristic of a cell whose cyclophosphamide metabolism is dominated by aldehyde dehydrogenase. The very rapid consumption of activated cyclophosphamide is consistent with the reduced in vitro activity of 4HC that occurs with increasing the RBC concentration. It is likely that the decreased activity of 4HC seen with increasing the number of mononuclear marrow cells or K562 cells in the incubation mixture is also due to aldehyde dehydrogenase activity in these cells. Bone marrow precursor cells and K562 cells have been shown to contain substantial amounts of this enzyme activity. It also has been shown that L1210 cells can develop resistance to cyclophosphamide through increased aldehyde dehydrogenase activity.

Bone marrow progenitors from patients previously treated with chemotherapeutic agents are frequently more sensitive to 4HC than progenitors from normal donors. This effect of previous therapy on drug sensitivity is the opposite of what occurs with malignant tumors. The reasons for the development of increased CFU-GM sensitivity to 4HC in previously treated patients undergoing autologous BMT are unknown. Radiation recall of normal tissues is another example of prior therapy causing normal tissues to become more sensitive to later chemotherapy.

Two different culture conditions for CFU-GM assays were used in this study. The specimens from the studies shown in Figs 1 and 3 were assayed using standard conditions as described by Pike and Robinson. The specimens from the autologous marrow grafts, and from normals used to determine individual patient CFU-GM sensitivity to 4HC, were assayed using an enriched methylcellulose culture system. We found that the CFU-GM sensitivity to 4HC was not altered by the different culture conditions. The slope of dose response for 2 x 10^6 mononuclear bone marrow cells from normals incubated with 4HC for 30 minutes at 37°C was 0.03 using either the standard culture conditions (Fig 3) or the enriched methylcellulose culture system. We did find, however, that the enriched culture conditions allowed nearly a tenfold increase in CFU-GM cloning efficiency (data not shown).

It is important to standardize incubation conditions during clinical marrow purging. As we have shown, RBCs contaminating the incubation mixture for purging with 4HC are not inert. The mononuclear cell concentration, duration, and temperature of the incubation all affect the progenitor survival following treatment with 4HC. We have recently begun to adjust all incubation-packed red cell volumes to 7% during clinical purging with 4HC. This incubation RBC concentration was the value predicted by the regression line in Fig 4 for a CFU-GM recovery of 1.4%, the mean recovery seen in our study. Because of differences in CFU-GM sensitivity to mafosfamide between patients, Gorin et al have suggested that the purging dose of this agent be individualized to ensure consistent engraftment. We have chosen, however, not to individualize the dosage of 4HC used in purging. We have found that just standardizing the RBC concentration of the incubation mixture prevents markedly prolonged granulocytopenia following autologous marrow transplantation. In addition, individualizing the dosage of a purging agent based on patient CFU-GM sensitivity does not take into account the potentially more important differences in individual tumor sensitivity to the purging agent.
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