Limiting-Dilution Analysis for the Determination of Leukemic Cell Frequencies After Bone Marrow Decontamination With Mafosfamide or Merocyanine 540

By A. Porcellini, N. Talevi, M.T. Marchetti-Rossi, M. Palazzi, A. Manna, G. Sparaventi, C. Delfini, and M. Valentini

To simulate a leukemia remission marrow, cell suspensions of normal human bone marrow were mixed with human acute lymphoblastic or myelogenous leukemic cells of the CCRF-SF, Nalm-6, and K-562 lines. The cell mixtures were incubated in vitro with mafosfamide (AZ) or with the photoreactive dye merocyanine 540 (MC-540). A quantity of $10^6$ cells of the treated suspensions was dispensed into microculture plates, and graded cell numbers of the line used to contaminate the normal marrow were added. Limiting-dilution analysis was used to estimate the frequency of leukemia cells persisting after treatment with the decontaminating agents. Treatment with AZ or MC-540 produced a total elimination (ie, 6 logs or 5.3 logs respectively) of B cell acute leukemia cells (CCRF-SB), whereas nearly 1.7 logs and 2 logs of K-562 acute myelogenous blasts were still present in the cell mixtures after treatment with MC-540 and AZ, respectively. Treatment of the Nalm-6–contaminated cell mixtures with AZ resulted in 100% elimination of clonogenic cells, whereas nearly 80% decontamination was obtained with MC-540. Our results suggest that treatment with AZ could be an effective method of eliminating clonogenic tumor cells from human bone marrow. MC-540, shown by previous studies to spare sufficient pluripotential stem cells to ensure hemopoietic reconstitution in the murine model and in clinical application, has comparable effects and merits trials for possible clinical use in autologous bone marrow transplantation.

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AUTOLOGOUS BONE MARROW transplantation (ABMT) is now being used as an alternative transplant approach for patients in remission of acute leukemia who lack an HLA-identical donor. The major stumbling block in ABMT still remains the high relapse rate as compared with allogeneic marrow transplantation,1 evidently due to occult leukemic cells that contaminate the remission marrow. Several methods for eliminating these cells from autologous marrow have been proposed, but the efficacy of the purging procedures has yet to be established. Although the advantage of marrow purging is uncertain in acute nonlymphocytic leukemia (ANLL) treated by autotransplantation in the first complete remission (CR), there are some indications that cleansing of the marrow is linked with improved survival in autograft recipients in the second CR.1 Despite the preliminary nature of these data and the need for more prolonged follow-up, in vitro treatment of the autologous marrow harvest to eliminate or at least reduce leukemic cells would seem a desirable goal.2

4-hydroperoxycyclophosphamide (4HC) and 4-hydroxazaphosphorine or mafosfamide (AZ), both congeners of cyclophosphamide (CY), are the most commonly used drugs for chemical purging in ABMT. Evidence from animal models that 4HC and AZ may purge marrow of clonogenic cells14 while sparing the pluripotential stem cells15 has been presented previously. Furthermore, 4HC- or AZ-treated human marrow cell suspensions are able to reconstitute the hematopoietic system after ablative therapy despite a marked reduction in detectable progenitor cells.2,3,9

The photoreactive dye merocyanine-540 (MC-540) binds to fluid like domains in the outer leaflet of the lipid bilayer of intact cell membranes.11-14 Electrically excitable cells and some immature hematopoietic precursor cells show exceptional permeability to MC-540.15

Photoexcitation of membrane-bound dye with light of suitable wavelength causes increased dye uptake, impairment of the normal permeability properties of the plasma membrane, and eventually, cell death.15,16 Sieber et al17-20 have studied the sensitivity to MC-540–mediated photolysis of hematopoietic precursors and pluripotential stem cells as compared with the lymphocytic leukemia cell line L1210.

Their findings of a much greater reduction in the L1210 cell population suggest that this dye might serve as a cleansing agent for ABMT.

Although marrow cleansing offers an interesting approach because the aforementioned research and clinical trials have shown that this procedure is not harmful to pluripotential stem cells and not hazardous, the real efficacy of this procedure in terms of residual clonogenic cell removal still needs to be determined. Several assays have been developed in an attempt to solve this problem.21-26

This study was designed to test the ability of AZ or MC-540 to eliminate clonogenic leukemic cells from a mixture of leukemic and normal human bone marrow mononuclear cells (HBMMC). For this purpose, a highly sensitive in vitro clonogenic assay was developed by using established human leukemia cell lines. This assay, which enables enumeration of residual leukemia cells, was sufficiently sensitive to detect as much as a 6-log reduction in leukemia cells that contaminate human bone marrow.

MATERIALS AND METHODS

Preparation of bone marrow suspensions. Bone marrow specimens were aspirated into heparinized syringes from the posterior iliac crests of healthy volunteers after obtaining their informed consent. The mononuclear marrow cell line suspensions were prepared as described previously.27

From Divisione di Ematologia, Ospedale Civile, Pesaro, Italy. Submitted August 8, 1986; accepted July 10, 1987.

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Address reprint requests to Adolfo Porcellini, MD, Divisione di Ematologia, Ospedale Civile, Via Lombroso 61100 Pesaro, Italy.

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Leukemia cell lines. The following established cell lines were used: pre-B cell line Nalm-6; B cell line CCRF-SB, and the myeloid-monocytic line K-562. All cell lines were kindly supplied to us by Dr Hansen, Fred Hutchinson Cancer Research Center, Seattle. The cell lines were maintained in RPMI 1640 medium with 15% fetal calf serum (FCS), 1% penicillin-streptomycin antibiotics (10,000 U), 1% l-glutamine, and 1% minimal essential medium (MEM) sodium pyruvate solution and were kept at 37°C in a fully humidified incubator with 5% CO2. The cells were maintained in exponential growth by keeping the cell concentration in a range from 0.5 to 1.0 x 10^6 cells/mL. The viability of all cell lines was >90% as judged by trypan blue dye exclusion.

Purging agents. AZ (Asta Werke, Bielefeld, FRG) was diluted with TC 199 to a concentration of 10 μg/mL immediately before use.

MC-540 (Eastman Kodak Co., Rochester, NY) was diluted from a freshly prepared stock solution of 1 mg/mL in 50% (vol/vol) water-ethanol to a final concentration of 15 μg/mL.

Purification with AZ. To simulate remission bone marrows from leukemia patients, 10^6 leukemia cells of the CCRF-SB, Nalm-6, or K-562 cell lines were mixed with a 19-fold excess of HBMMC; the suspensions were adjusted to 20 x 10^6 cells/mL with TC 199 medium, placed in 12-mL plastic tubes, and incubated for 30 minutes at 37°C in a water bath with 50 or 100 μg/mL of AZ. After incubation, the cell suspensions were placed in ice for five minutes to stop the reaction and then washed twice in RPMI 1640.

Photosensitization with MC-540. A quantity of 2 x 10^6 leukemia cells of the CCRF-SB or K-562 lines were mixed with a 19-fold excess of HBMMC; the suspension was adjusted to 4 x 10^6 cells/mL with MEM α medium (GIBCO, Grand Island, NY), containing 15% FCS (Seromed, FRG) and placed in Petri dishes, and 15 μg of MC-540 dissolved in 50% ethanol was added. Clear polystyrene tissue culture dishes (60 x 15 mm) containing the cell suspension were placed in the center of a rectangular frame (58 x 40 cm) fitted with six daylight fluorescent tubes (Phillips, TL M 20W/133 RS, 56 cm long), three above the dishes and three below, and illuminated for 45 or 90 minutes. Illumination at each side of the sample (at 20 cm from the light source) was 0.0007 W/cm².

The dishes were shaken manually every five minutes to keep the cells in suspension. After light exposure, the cell suspensions were washed twice in MEM α medium containing 5% FCS.

Limiting-dilution Assay. After incubation with AZ or MC-540, 10^6 cells from those treated were suspended in 100 μL RPMI 1640 and plated in microculture plates (Nunc, Roskilde, Denmark).

Graded cell numbers of the same line used to contaminate the HBMMC were suspended in 100 μL RPMI 1640 containing 15% pooled human serum and added to the treated cell feeders and incubated for 13 days. Refeeding of the cultures with fresh medium was carried out every third day.

 Twelve hours before harvesting, the cells were pulse-labeled with 1 μCi; 3H-thymidine. The cells were then harvested by means of a Skatron apparatus (Lierbyen, Norway), and incorporated thymidine was quantitated by scintillation counting.

Statistical analysis. Clonogenic cell frequency was determined by the maximum likelihood (ML) method from the plot of the Poisson distribution relationship between the cell number seeded per well and the percentage of negative wells.

The ML equation was obtained by modifying the Taswell method, ML regression analysis, x-intercept values, and confidence limits on the population regression line for prediction of y at a given value of x were obtained by using the MINUIT computer program (CERN Library, Geneva) in a VAX-750 computer.

RESULTS

For this study, a limiting-dilution (LD) analysis was adopted to evaluate the frequency of leukemic cells of the CCRF-SB, Nalm-6, and K-562 lines surviving in an HBMMC mixture after treatment with AZ or MC-540.

The mean 3H-thymidine uptake in the wells in which no leukemia cells were added (ie, containing only 10^6 cleansed HBMM cells) was determined (Fig 1). Only wells that exceeded the mean of the control wells by at least 3 SD were scored as positive (responders). The number of responders increased progressively with the number of blasts added (Fig

![Figure 1](https://www.bloodjournal.org/...)

Fig 1. Distribution of 3H-thymidine uptake in an LD assay for residual CCRF-SB lymphoblasts. Groups of 24 microwells containing suspensions of 10^6 mixed cells treated with AZ (left-hand panel) or MC-540 (right-hand panel) were seeded with graded numbers of CCRF-SB cells per well (see Materials and Methods) and incubated for 13 days. The cell proliferation rate was assayed by 3H-thymidine uptake. The dotted line represents 3 SD above the mean 3H-thymidine uptake of the nonresponding microcultures with no added CCRF-SB clonogenic cells.
leukemic cells added/well

1). Figures 2 and 3 represent the data obtained from LD assays of cleansed HBMM and leukemic cell mixtures in which the equations of the line of best fit were derived by ML methods.

The cloning efficiencies of CCRF-SB, K-562, and Nalm-6 cells in this experiment, calculated as the slope of the regression line when the ordinate is the natural log of the percent nonresponders (Fig 2), were 22%, 44%, and 26% respectively.

The estimate of the number of leukemic cells still present in the cell mixtures after cleansing is represented by the x-intercept of the regression line. In the experiment shown in Fig 2, complete decontamination of CCRF-SB and Nalm-6 cells (panels A and C) in each well containing 10^5 mafosfamide-purged mixture cells was obtained, and 0.08 K-562 leukemic cells (Panel B) survived after treatment.

The 95% confidence intervals (95% CI) for the value of the x-intercept of this experiment, calculated as described by Cox and Hinkley, were −0.71 to 0, −0.28 to 0, and −0.39 to 0 for the three leukemic lines respectively. Thus, there was <5% probability that the frequency of leukemic cells would be greater than 1/10^4 cleansed mixture cells. It should be noted that the threshold sensitivity of this assay was about one clonogenic cell per 10^4 HBMM cells (Figs 2 and 3).

Similar results were obtained in two replicate experiments in which a complete clearing of all leukemic cells was obtained, with the exception of 0.16 residual K-562 blasts in one single experiment. In all these replicate studies, the 95% CI indicated that there was always a 5% probability that the frequency of residual blasts would be >1/10^4 purged mixture cells.

Figure 3 shows the results obtained by purging the cell mixtures with MC-540. With this cleansing agent, the calculated residual clonogenic cells in each well were 0 CCRF-SB cells (95% CI, <0.38, panel A) and 0.11 K-562 cells (95% CI <0.53; panel B). Pre-B Nalm-6 cells proved less responsive to MC-540 in that 1.78 residual leukemic cells (95% CI, <4.74) were found after photosensitization.

The cloning efficiencies of leukemic cells in this experiment as judged by the ML regression line were 13.4% (CCRF-SB), 40.3% (K-562), and 8.7% (Nalm-6).
These results were confirmed in two replicate experiments. The B cell lymphoblasts were eliminated completely by the MC-540 treatment; however, 0.023 to 0.18 clonogenic cells were present after purging cell mixtures contaminated with the K-562 cells. Again, the 95% CI confirmed that there was a 5% probability of the residual blasts being present at a frequency greater than 1/10^6 purged mixture cells.

The effectiveness of both AZ and MC-540 proved to depend directly on the dose level (AZ) and the light exposure time (MC-540) (Fig 4). Incubation of the cell mixture containing CCRF-SB clonogenic cells with 50 µg/mL of AZ caused decontamination of 3.2 logs; hence about 620 clonogenic cells persisted after purging. The cloning efficiency in this experiment was 8.6%. By increasing the dose of AZ to the usual level of 100 µg/mL, complete clearing of CCRF-SB lymphoblasts was obtained, and the cloning efficiency was increased to 16.5% (Fig 4, left-hand panel).

With an exposure time of only 45 minutes, MC-540 was able to decrease the CCRF-SB lymphoblast frequency in the cell mixture by 3.7 logs. About 40 clonogenic cells were present after purging, and the ML regression line showed a cloning efficiency of 2.3%. By contrast, an exposure time of 90 minutes not only caused complete clearing of the lymphoblasts but also increased the cloning efficiency to 7.3%. By lengthening the exposure time to 120 minutes, we could not detect a higher degree of decontamination; however, the cloning efficiency obtained in this case was 26.1% (Fig 4, right-hand panel).

**DISCUSSION**

Removal of occult malignant cells may be of critical importance for improving the efficacy of ABMT in acute leukemia.1 A major complication in this procedure is the impossibility of evaluating the effects of in vitro treatment both on cells responsible for engraftment and on clonogenic cell removal.

Evidence obtained in experimental models that 4-HC, AZ, or MC-540 does not impair the ability of marrow cell suspensions to restore hematopoiesis has already been presented.3,5,18

A test for pluripotential stem cell survival in humans is not currently available. However, a body of evidence from both experimental and clinical studies indicates that a sufficient number of cells responsible for hematopoietic reconstitution survive exposure to either CY derivatives or MC-540. Indirect confirmation thereof was obtained in two in vitro studies. Normal human marrow cell suspensions treated with 4-HC and then seeded in a long-term marrow culture system, although completely devoid of detectable progenitor cells, showed recovery of CFU-GM.40 A similar study in which marrow from leukemic patients in CR was incubated with AZ obtained comparable results.4 Both studies tested a variety of drug dose levels including those commonly used in clinical ABMT and clearly indicated that purged marrow suspensions retain their self-regenerating ability.

The effect of both 4-HC and AZ on normal hematopoiesis was evaluated by Gordon et al31 and Degliantoni et al32 by means of a new colony assay33 designed to detect very immature blast progenitor cells. Both these studies showed that in vitro treatment with either drug did not impair the formation of blast colonies containing very primitive cells capable of self-renewal and differentiation.

An indirect but persuasive demonstration that ex vivo purging with CY derivatives is not harmful to pluripotential stem cells may be inferred from the prompt restoration of hematopoiesis after reinfusion of purged marrow suspensions following myeloablative treatment.2,7,10 In these reports the
median time of hematopoietic recovery was comparable to that obtained in ABMT with untreated marrow.

In the first clinical application of ABMT purged with MC-540, the photoreactive dye showed that it did not compromise the graft’s ability to reconstitute the hematopoietic system.44

The other major concern in ex vivo marrow cleansing is to quantitate the number of clonogenic cells present in the remission marrow and to estimate those removed by the cleansing procedures.

Unfortunately there is no means available for detecting minimal residual disease in remission marrow. However, by using leukemia cell lines it is possible to develop clonogenic assays that measure the elimination of malignant cells from an excess of normal HBMMC. Although these model systems may differ somewhat from the clinical situation, such in vitro simulation experiments permit evaluation of the efficiency of the drug used in terms of leukemic cell removal.

The LD assay has been used extensively to study the response of antigen-reactive cells both in animal models35,36 and in humans.37-39 To meet our requirements, we have adapted an LD assay originally described by Moretta et al.40 and modified by Martin and Hansen.41

In all the experiments performed, the ML regression lines were consistent with the single-hit model (Figs 2 and 3). Assuming that the test mixture cell suspensions are homogeneous, the leukemic cells sampled into each microwell will occur randomly according to the Poisson probability distribution. In a single-hit model, it is not possible to determine whether a positive response is due to one or more cells. However, no response occurs in the absence of cells.

Therefore, the zero term of the Poisson equation will indicate that the proportion of nonresponders is a negative logarithmic function of the number of leukemic cells added to each well; hence in our study, it was possible to establish an equation for the line of best fit to calculate the leukemic cell frequency in each sample. The efficacy of this assay hinges on its ability to provide quantal discrimination between wells with cell growth (responders) and wells with no cell growth (nonresponders).42 Definition of the latter is somewhat arbitrary because when total decontamination (100% purging) was not attained some leukemic cells were present in the wells after treatment even when none were added for the LD assay. However, in all our experiments, the outliers in 3H-thymidine uptake were quite obvious (Fig 1B) and were disregarded when calculating the cutoff between negative and positive wells. In some cases, when there is a high number of residual leukemic cells or when micrometastases are seeded with an excessive amount of leukemic cells, the increased proliferation rate of the leukemic cells, as measured by 3H-thymidine incorporation, can give rise to false-negative results and hence an unexpectedly high proportion of negative wells. In our microtray assay system, although cultures are refed periodically, medium supply may become a limiting factor in the case of an excessive cell proliferation rate. It is possible to adjust the LD assay for different degrees of blast cell purging by changing the number of clonogenic cells seeded per well.

LD assay techniques usually involve the use of irradiated feeders to which increasing numbers of cells containing an unknown proportion of precursors are added. Hence, it follows that when no precursors are added to the irradiated feeder-containing wells 100% nonresponders will be obtained, and the regression line will necessarily include the origin. In our experiments, the feeders (ie, 10^6 purified HBMM) cells) contained an unknown proportion of residual leukemia cells to which further successive known doses of blasts were added. Thus, one would not expect semilog plots of blast cells added and the proportion of nonresponders to necessarily include the origin, except in the case in which no residual blasts remained in the wells.

Should residual blasts persist after purging, the x-intercept that represents an estimate of the number of blast cells that would have to be removed from the feeders to obtain 100% nonresponders can be calculated from the ML regression line.

In our model system, after AZ or MC-540 treatment at the highest dose level tested, no acute leukemia CCRF-SB lymphoblasts were detectable in the cell mixtures. In the Nalm-6--contaminated mixtures, AZ obtained 100% decontamination, whereas the figure for MC-540 was 80%. In the mixtures containing K-562 cells, both agents yielded a result of 99.97% decontamination. Our data do not indicate whether it would be possible to obtain a higher degree of decontamination, either by increasing the dose level of AZ or by increasing both the dose level and the light exposure time of MC-540. Clinical experience has shown that 100 μg/mL of AZ is the maximum safe dose level for this drug, and further increases might be critical.3 As far as MC-540 is concerned, further studies are needed to determine potential harmful effects on pluripotential stem cells at doses higher than those used in our model.

It is possible that occult leukemic cells contaminating a remission marrow are more difficult to eliminate than exponentially growing leukemic cells such as those used in our model system. It may be more difficult to obtain 99.97% decontamination in the clinical situation if the clonogenic leukemic cells are quiescent or slowly cycling. Thus the figure of 99.97% may represent an overestimate of the cleansing efficiency of both AZ and MC-540. Moreover, because the threshold limit for detection with this assay is about one per 500 clonogenic cells, it is evident that when complete decontamination (as calculated by our LD assay) is obtained as many as 3 to 4 logs of clonogenic cells could persist after purging, given the supposed initial leukemic cell population in the autologous harvest of between 10^6 and 2 x 10^7 cells.

It has been suggested that the susceptibility of tumor cells to alkylating agents may be due to a decrease in their aldehyde dehydrogenase content (compared with normal cells) rather than to their proliferative state.32-34 However, actively proliferating cells are likely to prove more susceptible to alkylators than are dormant cells. Moreover, if ABMT is performed in the second or higher CR, residual malignant cells may have developed a relative resistance to alkylating agents. One potential advantage of using MC-540 is that this
agent causes impairment of the normal permeability properties of the plasma membrane regardless of the proliferation rate of target cells. This suggests that this approach could effectively eliminate noncycling malignant cells as well.18 The viability of marrow treated with MC-540 is yet to be evaluated in a phase I clinical trial. However, our in vitro data suggest that chemical treatment of marrow with this photoreactive dye might provide a new approach for removing malignant cells, regardless of their cycling rate, without unduly damaging the pluripotential stem cells.

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