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Differential Effects of Melphalan on Mouse Myeloma (Adj. PC-5) and Hemopoietic Stem Cells[1]

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SUMMARY

The effect of melphalan on mouse myeloma and hemopoietic progenitor cells was studied with a primary cell culture assay for mouse myeloma stem cells developed by Park, Bergsagel, and McCulloch and bone marrow cell culture and spleen colony assays for hemopoietic progenitor cells. Myeloma and bone marrow cells were exposed *in vivo* to graded doses of melphalan and harvested 3 and 24 hr later for assays of the surviving fractions. The sensitivity of myeloma cells was more than 70 times greater than that of normal bone marrow cells. Exposure of the 2 cell classes to melphalan in culture also showed a similar differential effect of melphalan.

The sensitivity of normal and regenerating bone marrow to melphalan was almost identical when assayed by cell culture or the spleen colony method. These results indicate that the sensitivity of hemopoietic progenitor cells to melphalan is not influenced by the proliferative state of these cells. It would appear that the vast difference in the sensitivity of these 2 cell classes to melphalan is dependent on intrinsic differences between myeloma stem cells and hemopoietic progenitor cells.

INTRODUCTION

The chemotherapy of myeloma is limited by the toxicity of the available drugs to normal hemopoietic cells. The effects of drugs, therefore, should be assessed on both myeloma stem cells and normal hemopoietic progenitors. For such a comparison to be valid, it is necessary that the assessment of cell kill be comparable for both cell classes. Recently, it has become possible to meet this requirement in the mouse, since colony assays in cell culture have become available for normal marrow granulopoietic progenitors (4, 8, 10) and myeloma cells (9). In this paper, we describe experiments in which melphalan, a drug known to be effective against both human and mouse myeloma, was assessed with cell culture methods. The assessment was validated further by comparing the survival of normal marrow cells in culture and *in vivo* and under conditions of rest and active proliferation. We found that the colony-forming capacity of myeloma cells was much more sensitive to melphalan than that of normal marrow progenitor cells. The finding could not be attributed to different cell cycle states for the 2 populations and may provide evidence for the existence of variation in the intrinsic sensitivities of different cell classes to this alkylating agent.

MATERIALS AND METHODS

Mice. BALB/c female mice were obtained from The Jackson Laboratories, Bar Harbor, Maine. Eight- to 10-week-old mice were used for transfers of myeloma and studies of the sensitivity of bone marrow progenitor cells. F_1 hybrids between C57BL/6J0ci and C3H/He0ci (hereafter called $C3B6F_1$) were used in experiments requiring irradiation of the mice. These animals were obtained from the animal colony of The Ontario Cancer Institute. Data for normal cells from either mouse were indistinguishable, and the mice have not been designated separately in the charts.

Melphalan. Injectable melphalan was purchased from Burroughs Wellcome & Co., London, England. One hundred mg of melphalan were dissolved in 10 ml of sterile propylene glycol at temperatures between 60 and 70°, and for a 1-week period the solution was used as stock solution. Further dilutions were made in 0.9% NaCl solution.

Mouse Myeloma (Adj. PC-5). The myeloma line was obtained originally in ascitic form from Dr. Michael Potter, NIH, Bethesda, Md. This tumor was adapted for growth in the spleen and has been transplanted fortnightly by i.v. injection of 1 to 2×10^6 cells. Experiments were carried out with cells from i.v. transplant generations 85 to 110.

Culture Assay for Mouse Myeloma Stem Cells. Tumor cell suspensions were prepared from spleen with a fine wire mesh screen (3) and were cultured with the method described by Park *et al.* (9), modified only in that α medium (11) (Flow Laboratories, Rockville, Md.) was used instead of CMRL 1066. The method depends on repeated feeding of cultures with medium containing human serum and vitamin C (C. H. Park, D. E. Bergsagel, and E. A. McCulloch. Ascorbic Acid: A Culture Requirement for Colony Formation by Mouse Myeloma Cells. *Science*, in press). Because of these special requirements for colony formation by myeloma cells, they may be assayed independently in mixtures with normal marrow (9). Each group of cells was cultured in 6 dishes with 3 different concentrations. Colonies were counted 6 or 7 days after plating with an inverted microscope at 35-fold magnification.

Assays for CFU-C.[3] Normal bone marrow cells were

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[3] The abbreviations used are: CFU-C, colony-forming units in culture; CFU-S, colony-forming units in spleen; myeloma CFU, myeloma colony-forming units in culture; D37, dose required to reduce survival to 37%.

obtained from femurs of female BALB/c mice and female F_1 hybrids ($C3B6F_1$) as described previously (12). Marrow cells capable of forming colonies in culture were assayed with a modification of the technique of Worton *et al.* (13). α medium was used instead of CMRL 1066, and bovine serum albumin was omitted.

Assay for CFU-S. Cells capable of forming colonies in the spleens of heavily irradiated mice were measured in femoral marrow cell suspensions of $C3B6F_1$ hybrids with the technique of Till and McCulloch (12).

Cells from Regenerating Bone Marrow. Mice with regenerating bone marrow were prepared by the i.v. injection of 1 to 2×10^7 nucleated $C3B6F_1$ marrow cells into isologous animals that had received 950 rads of ^{137}Cs radiation. The femoral marrow of these mice was harvested 5 to 6 days later.

***In Vivo* Exposure to Melphalan.** Groups of mice were given 2×10^6 tumor-infiltrated spleen cells i.v. Twelve days later, graded doses of melphalan were administered i.p. After 3 or 24 hr, groups of mice were sacrificed, and cell suspensions were prepared from the spleens of each group. These suspensions were assayed for myeloma CFU. For the study of the sensitivity of bone marrow CFU-C and CFU-S, melphalan was injected i.p. into normal mice in varying doses. Cells from the femurs of each group were pooled and assayed for colony-forming units either *in vivo* or in culture.

Exposure to Melphalan in Culture. Single-cell suspensions of normal bone marrow and tumor-infiltrated spleens were prepared at a concentration of 1×10^6 cells/ml in α medium. The suspensions were incubated with melphalan in 15-ml plastic tubes (Falcon Plastics, Los Angeles, Calif.) at 37° in 5% CO_2 in air for 3 hr. Tubes were inverted every 0.5 hr. At the conclusion of incubation, cells were washed 3 times by centrifugation, and the suspensions were assayed for hemopoietic and myeloma CFU.

Statistical Analysis. Most calculations were carried out with General Electric computer time-sharing systems. Each group of cells was cultured in 3 or 4 concentrations, and the p values of the correlation coefficients were less than 0.01. The intercept of the line obtained by the least squares method did not differ significantly from 0 (within 95% confidence limits).

RESULTS

Sensitivity to Melphalan *in Vivo*. A comparison of the sensitivity of CFU-C from marrow and myeloma CFU from spleen is presented in Chart 1. Similar results were obtained when animals were taken 3 or 24 hr after injection of the drug, and the data have been combined in Chart 1. For both cell classes, survival decreased exponentially with increasing dosage of drug. However, the D37 was much less for myeloma CFU (0.002 mg/mouse) than for normal CFU-C (0.14 mg/mouse).

Sensitivity to Melphalan in Culture. The 70-fold difference in slope between the survival curves of CFU-C and myeloma CFU might be a consequence of the different site of drug exposure (marrow for CFU-C and spleen for myeloma CFU). This possibility was examined with 2 experimental designs. In the first, either myeloma-infiltrated spleen cell suspensions or suspensions of normal marrow were exposed to varying concentrations of melphalan for 3 hr in culture at 37°, washed

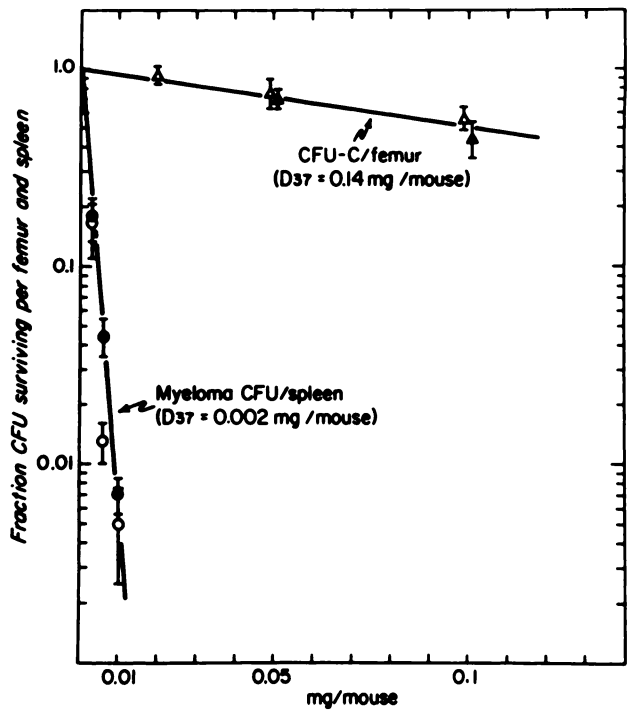

Chart 1. *In vivo* exposure to melphalan. Femoral marrow cells and myeloma-infiltrated spleen cells were harvested for culture assays 3 hr (*open symbols*) and 24 hr (*closed symbols*) after drug administration. The surviving fractions of CFU-C per femur and myeloma CFU per spleen were plotted on a semilogarithmic scale. *Bars*, 1 S.D.

free of the drug, and tested for colony formation. A 2nd experimental design was based on the special cultural requirements of myeloma stem cells (9) which permits them to be assayed independently of CFU-C in mixtures. Mixtures of myeloma-infiltrated spleen and normal femoral marrow cell suspensions were exposed to melphalan in culture and, after washing, plated under appropriate conditions for each cell class. The results of experiments of both designs are similar and are presented together in Chart 2. Exponential survival curves were obtained, and myeloma CFU were again found to be approximately 70-fold more sensitive to melphalan than normal CFU-C.

The Sensitivity of Normal and Regenerating Marrow to Melphalan. The data obtained from drug exposure in culture made it unlikely that drug distribution *in vivo*, or influences extrinsic to the cells, could explain the difference in sensitivity between myeloma CFU and normal CFU-C. Two biological explanations were considered. First, the dissimilarity in cell cycle states between normal CFU-C and myeloma CFU might account for the difference in the survival curves, since this mechanism is known to be the basis of differential killing of lymphoma CFU compared to normal CFU-S (5, 6). Second, since CFU-C is considered a class of early committed descendents of pluripotent CFU-S, differentiation might confer upon CFU-C a lessened sensitivity to melphalan. Both these possibilities were tested by obtaining marrow during the exponential phase of growth following transplantation (see "Materials and Methods"), when both CFU-C and CFU-S are known to be in active cell cycle (2, 7), and by testing for

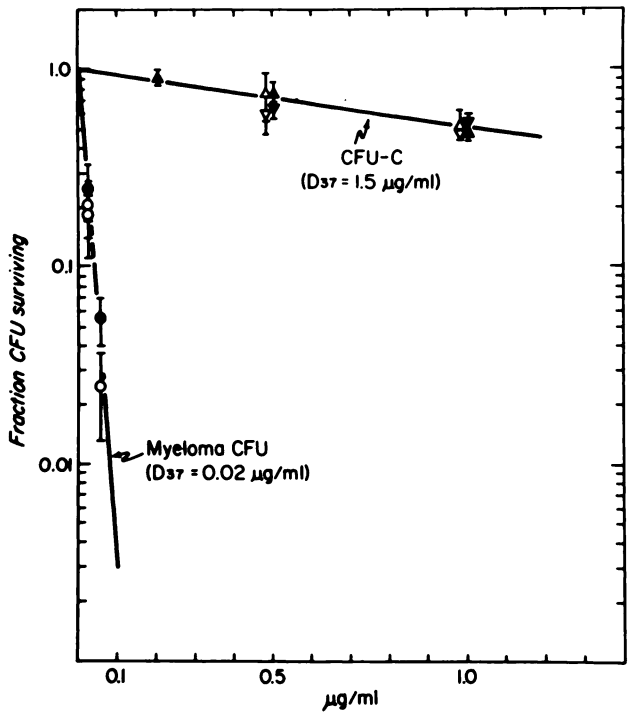

Chart 2. Exposure to melphalan in culture. Normal marrow cells and myeloma-infiltrated spleen cells were incubated with melphalan for 3 hr separately (*closed symbols*) and together as 1:1 mixture (*open symbols*) and were assayed for surviving fractions.

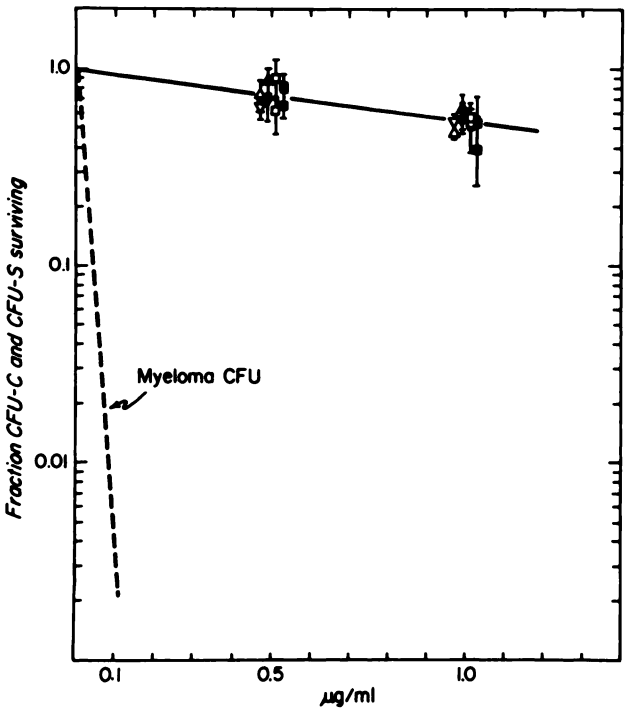

Chart 3. Melphalan sensitivity of normal and regenerating marrow cells. Cells from normal and regenerating marrows were exposed to melphalan in culture for 3 hr and were tested for surviving CFU-S and CFU-C. □, normal CFU-S; ■, regenerating CFU-S; △, normal CFU-C; ▲, regenerating CFU-C; *dashed line*, sensitivity curve for myeloma CFU taken from Chart 2.

survival of both CFU-S and CFU-C after exposure to melphalan in culture. The results of several experiments of this design are presented in Chart 3. The dotted line in the chart is the survival curve for myeloma CFU taken from Chart 2. Normal CFU-S and CFU-C were found to have equivalent melphalan sensitivities, and the values for proliferating CFU-S and CFU-C also were similar to those obtained with normal marrow. Thus, both pluripotent CFU-S and committed CFU-C are less sensitive to melphalan than myeloma, and the difference cannot readily be attributed to the proliferative state of the cells.

DISCUSSION

The chemotherapy of human myeloma might be improved if it were possible to assess drugs in culture rather than by time-consuming clinical trials. Accordingly, we have investigated a model system in the mouse where it is possible to compare survival curves for myeloma stem cells and granulopoietic progenitors with comparable culture techniques.

We chose to study the effects of melphalan, since this drug is known to prolong the survival of myeloma patients (1) and to cure transplantable mouse myeloma (Adj. PC-5) after a single injection (D. E. Bergsagel, personal observation). The cell culture results were compatible with the known therapeutic efficacy of melphalan since myeloma stem cells were found to be much more sensitive than granulopoietic progenitors. The extrapolation of culture results to the *in vivo* condition was further strengthened when the sensitivity of pluripotent stem cells, assayed by the spleen colony technique *in vivo*, yielded similar survival curves to granulopoietic progenitors assayed in cell culture.

Our experiments do not provide a ready explanation for the difference in sensitivity between myeloma stem cells and normal marrow. Explanations based on the distribution of the drug in the animal are made improbable by the results of exposure to the drug in both classes in culture, and factors local to either marrow or spleen, but extrinsic to the cells themselves, were not uncovered by mixing experiments. In the absence of evidence for an extrinsic mechanism explaining the difference in sensitivity, we considered an explanation based on cell physiology. Mouse lymphoma cells have been shown to be more sensitive than normal CFU-S to a number of chemotherapeutic agents, because the latter are in a state of rest while the former proliferate actively (5, 6). This mechanism cannot account for our results, since marrow cells taken during the exponential phase of growth were not more sensitive to melphalan than cells from resting marrow.

Regardless of the mechanism, it is reasonable to assume that the differential sensitivity observed between myeloma stem cells and normal marrow may contribute to the therapeutic efficacy of melphalan. If this assumption is valid, model systems in culture, such as that used in our experiments, may prove useful in the evaluation of chemotherapeutic agents. Further, the elucidation of the mechanism responsible for the observed difference in sensitivity may contribute to our understanding of the cellular basis of chemotherapy.

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