Chemotherapy of Mouse Myeloma: Quantitative Cell Cultures Predictive of Response In Vivo

By M. Ogawa, D. E. Bergsagel, and E. A. McCulloch

The effects of chemotherapeutic agents on mouse myeloma and bone marrow precursor cells were studied using cell culture and spleen colony assay techniques, and the results were assessed for their values in predicting therapeutic effectiveness. For cyclophosphamide, nitrogen mustard, and 1,3 bis (2 chloroethyl)-1-nitrosourea (BCNU), selective toxic effects on mouse myeloma (Adj. PC-5) cells were seen while no such differential effect was observed for 5-fluorouracil. The differential effects of these agents could not be explained by the cell cycle effects but appeared to be dependent on the intrinsic properties of the two cell classes. Marked differences were noted in the sensitivity of three mouse myelomas to melphalan. The ratio of the melphalan dose required to reduce marrow CFU to 37% (D37) to the dose required to reduce myeloma CFU equally was 75 for Adj. PC-5, 18 for MOPC 46B, and 7 for MOPC 460D. These results predict that melphalan would be more effective than 5-fluorouracil for Adj. PC-5 and that melphalan would be relatively ineffective for MOPC 460D. These predictions were confirmed by survival tests.

SUPPRESSION OF THE HEMOPOIETIC SYSTEM often limits the clinical use of chemotherapeutic agents. Hence, the effectiveness of such agents should be assessed in terms of their differential effects on tumor and hemopoietic cells. Recently we described experiments in which melphalan was tested for such differential effects utilizing cell culture assays for mouse myeloma (Adj. PC-5) and hemopoietic progenitors. The sensitivity of myeloma cells to melphalan was much greater than that of normal bone marrow, both in vivo and in culture. Furthermore, the differential effect of melphalan appeared to be dependent upon intrinsic properties of these cell classes rather than on differences in their proliferative states.

These studies have been extended to consider four additional chemothera-
peutic agents and two more mouse myelomas. These tumor lines yielded different survival curves for each of the drugs tested. Where differential drug effects between tumor and normal cells were found in culture, drug therapy was effective against that tumor line in vivo. These findings support the view that cell culture methods may be appropriate for assessing the response of individual tumors to therapy.

MATERIALS AND METHODS

Mice

BALB/c female mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. For studies requiring irradiation of mice, (C57Bl/6)Oci × C3H/HeOci F1 (C3B6F1) were obtained from the animal colony of The Ontario Cancer Institute. Data for normal cells from BALB/c and C3B6F1 mice were similar and have not been designated separately.

Drugs

Injectable cyclophosphamide was obtained from Frank W. Horner, Ltd., Montreal, Canada, and was dissolved in sterile distilled water. Nitrogen mustard was purchased from the Boots Co., Nottingham, England. 1,3 bis (2 chloroethyl)-1-nitrosourea (BCNU) was obtained through the courtesy of Cancer Chemotherapy, National Cancer Institute, Bethesda, Maryland, and kept at -40°C. When used it was dissolved first in ethyl alcohol, then in distilled water. 5-fluorouracil was purchased from Hoffmann-La Roche Ltd., Montreal, Canada. Injectable melphalan was purchased from Burroughs Wellcome & Co., London, England. Melphalan, 100 mg, was dissolved in 10 ml sterile propylene glycol at temperatures between 60°C and 70°C, and for a 1-wk period was used as stock solution. Subsequent dilutions of all agents were made in 0.9% NaCl solution. BCNU was added to cell suspensions within 10 min after being dissolved.

Mouse Myeloma

All the lines were obtained from Dr. Michael Potter, National Institutes of Health, Bethesda, Md. Adj. PC-5 was originally obtained in ascitic form and subsequently adapted for both subcutaneous growth and growth in the spleen. Transplantation of spleen line Adj. PC-5 was carried out every 2 wk by intravenous injection of 5 × 10^5–10^6 cells and experiments were carried out using cells from transplant generations 105–128. The subcutaneous Adj. PC-5 was transplanted every 2 wk using a 15 gauge needle. Experiments were carried out using transplant generations 118–130. Both MOPC 46B and MOPC 460D were obtained in the form of subcutaneous solid tumor and were transplanted similarly. Experiments were carried out using cells from transplant generations 115–167 and 49–57, respectively.

Culture Assay for Mouse Myeloma Stem Cells (Myeloma CFU)

Tumor cell suspensions were prepared from spleen using a fine wire mesh screen and were cultured using the method described by Park et al., modified only in that alpha medium (Flow Laboratories, Rockville, Md.) was used instead of CMRL (Connaught Medical Research Laboratory) 1066. Each group of cells was cultured in six dishes using three or more cell concentrations. Colonies were counted 6 or 7 days after plating, with an inverted microscope at 35-fold magnification.

Assays for Bone Marrow Cells Forming Colonies in Culture (CFU-C)

Normal bone marrow cells were obtained from femurs of female BALB/c mice or F1 hybrids (C3B6F1) as described previously. Marrow cells capable of forming colonies in culture (CFU-C) were assayed, using a modification of the technique of Worton et al. Alpha medium and normal horse serum were used instead of CMRL 1066 and fetal calf serum. Bovine serum albumin was omitted. Each group of cells was cultured in four dishes with three cell concentrations.
Spleen Colony Assay (CFU-S)

Cells capable of forming colonies in the spleens of heavily irradiated mice (CFU-S) were measured in femoral marrow cell suspensions of C3B6F1 hybrids using the technique of Till and McCulloch.6

Cells from Regenerating Bone Marrow

Mice with regenerating bone marrow were prepared by the intravenous injection of $10^7 - 2 \times 10^7$ nucleated C3B6F1 marrow cells into isologous animals that had received 950 rads of $^{137}$Cs radiation. The femoral marrow of these mice was harvested 6 days later.

Exposure to Cyclophosphamide in vivo

Groups of mice were given $2 \times 10^6$ myeloma cells intravenously. Twelve days later, graded doses of cyclophosphamide were administered intraperitoneally. After 3 hr, mice were sacrificed, and cell suspensions were prepared from the spleens of each group for myeloma CFU assay. For the study of the sensitivity of bone marrow CFU-C and CFU-S, cyclophosphamide was injected intraperitoneally into normal mice in varying doses. Cells from the femurs of each group were pooled and assayed for CFU-C and CFU-S.

Exposure to Drugs in Culture (Nitrogen Mustard, BCNU, 5-Fluorouracil, and Melphalan)

Details have been described in a previous report.1 Single cell suspensions of bone marrow and myeloma cells were prepared at a concentration of $1 \times 10^8$ nucleated cells/ml and were incubated with melphalan for 3 hr and with nitrogen mustard, BCNU, and 5-fluorouracil for 1 hr, in 15 ml plastic tubes at 37°C. At the conclusion of incubation, the cells were washed twice by centrifugation and the suspensions were assayed for surviving hemopoietic and myeloma CFU. Exposure to 5-fluorouracil was carried out in alpha medium devoid of nucleosides. Prior to exposure to 5-fluorouracil both bone marrow and myeloma cells were washed once with alpha medium without nucleosides. Subsequent washings and plating were done using complete alpha medium. The concentration of nucleosides in the exposure medium did not influence the killing effect of nitrogen mustard, BCNU, and melphalan; therefore, for these three agents the entire procedure was carried out using complete alpha medium.

Survival Studies

For spleen line Adj. PC-S, $2 \times 10^8$ cells were transplanted intravenously. In studies with Adj. PC-S in subcutaneous form and MOPC 460D, small tumor pieces of similar size were transplanted with a 15 gauge needle subcutaneously. Seven days later, groups of mice received varying doses of melphalan and cyclophosphamide and the survival dates were recorded. Autopsy was done on all of the mice.

Statistical Analysis

Values obtained from plates seeded with three and four different cell numbers did not deviate significantly from linearity ($p<0.05$), and the intercept of the line, obtained by the method of least squares, did not differ significantly from zero (within 95% confidence limits).

RESULTS

Sensitivity of Myeloma CFU and Marrow CFU to Cyclophosphamide in vivo

Sensitivity of cells to cyclophosphamide was determined by exposing them in vivo by injecting the drug into animals to permit activation (see Materials and Methods). The results are presented as survival curves in Fig. 1. The
curves are simple negative exponentials. The most striking finding is that myeloma CFU are more sensitive than CFU-S or CFU-C obtained from normal marrow. Although CFU from regenerating marrow were somewhat more sensitive than CFU from intact mice, their sensitivity did not approach that of a myeloma CFU. Thus, as in previous experiments with melphalan, Adj. PC-5 was found to be more sensitive to cyclophosphamide than normal CFU and this sensitivity could not be attributed entirely to the proliferative state of the cells.

Sensitivity of Myeloma CFU and Normal Marrow CFU to Nitrogen Mustard, BCNU, and 5-Fluorouracil in Culture

Suspensions of myeloma Adj. PC-5, marrow from intact mice and regenerating marrow cells were exposed to nitrogen mustard, BCNU, or 5-fluorouracil in culture as described in Materials and Methods. The results are presented as survival curves in Fig. 2, 3, and 4. Both the nitrogen mustard (Fig. 2) and BCNU (Fig. 3) survival curves showed a marked difference in sensitivity between normal CFU and myeloma CFU. For nitrogen mustard, no difference was found between “resting” marrow and proliferating marrow, while for BCNU a small difference was observed at the highest drug level, where the survival curve deviated from the simple negative exponential form.

In contrast, the 5-fluorouracil survival curves, shown in Fig. 4, demonstrate that myeloma CFU are only slightly more sensitive than normal CFU-C from resting marrow and are less sensitive than the CFU-C obtained from regenerating marrow. In this instance, measurements of CFU-S were not made since variations in thymidine content in the culture medium for CFU-C and the animals used for the CFU-S assay might make the results misleading.

Sensitivity of MOPC 46B and MOPC 460D to Melphalan in Culture

The experiments presented in Figs. 1–4 and summarized in Table 1 provide evidence that the four agents, cyclophosphamide, nitrogen mustard, BCNU, and 5-fluorouracil have different lethal effects on the colony-forming capacity

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Fig. 1. Sensitivity of myeloma and bone marrow CFU to cyclophosphamide in vivo. Myeloma-infiltrated spleen cells and femoral marrow cells were harvested for culture 3 hr after drug administration. Filled square, myeloma CFU; filled circle, normal CFU-C; open circle, regenerating CFU-C; filled triangle, normal CFU-S; open triangle, regenerating CFU-S. Bars, 1 SD.
**Fig. 2.** Sensitivity of myeloma and bone marrow CFU to nitrogen mustard in culture. Cells were exposed to nitrogen mustard in culture for 1 hr and then tested for surviving CFU.

**Fig. 3.** Sensitivity of myeloma and bone marrow CFU to BCNU in culture. Cells were exposed to BCNU for 1 hr and then tested for surviving CFU.

**Fig. 4.** Sensitivity of myeloma and bone marrow CFU to 5-fluorouracil in culture. Cells were washed once and exposed to 5-fluorouracil in culture for 1 hr using alpha medium without nucleosides. An assay for the surviving CFU was done using complete alpha medium.
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Table 1. D37° of Myeloma and Bone Marrow CFU Following Exposure to Various Chemotherapeutic Agents

<table>
<thead>
<tr>
<th></th>
<th>Nitrogen Mustard</th>
<th>BCNU</th>
<th>5-Fluorouracil</th>
<th>Melphalan</th>
<th>Cyclophosphamide</th>
<th>Melphalan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adj. PC-5</td>
<td>0.0063</td>
<td>0.94</td>
<td>58.0</td>
<td>0.066</td>
<td>0.016</td>
<td>0.002</td>
</tr>
<tr>
<td>MOPC 46B</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.28</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MOPC 460D</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.72</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Normal CFU-C</td>
<td>0.15</td>
<td>31.3</td>
<td>104.0</td>
<td>4.93</td>
<td>2.90</td>
<td>0.14</td>
</tr>
<tr>
<td>Normal CFU-S</td>
<td>0.15</td>
<td>31.3</td>
<td>—</td>
<td>4.93</td>
<td>2.90</td>
<td>—</td>
</tr>
<tr>
<td>Regenerating CFU-C</td>
<td>0.15</td>
<td>26.2</td>
<td>25.4</td>
<td>4.93</td>
<td>0.70</td>
<td>—</td>
</tr>
<tr>
<td>Regenerating CFU-S</td>
<td>0.15</td>
<td>26.2</td>
<td>—</td>
<td>4.93</td>
<td>1.45</td>
<td>—</td>
</tr>
</tbody>
</table>

*The dose of drug reducing survival to 37° of initial population.
†Cells were exposed to 5-fluorouracil in alpha medium without nucleosides.
‡Taken from previous experiments (Ref. 1).

of Adj. PC-5 and that for each agent, a comparison between Adj. PC-5 and normal CFU yields a different result. Accordingly, we tested two additional lines of myeloma cells, MOPC 46B and MOPC 460D, for sensitivity to melphalan in order to find out whether or not the quantitative response to this agent was a characteristic of mouse myelomas. The survival curves for these two tumors are presented in Fig. 5 and compared with the previous data for Adj. PC-5 and normal marrow CFU. It is apparent that each of the myelomas yielded a different survival curve, indicating a specific individual intrinsic sensitivity to this agent. While all three myelomas were more sensitive to melphalan than adult normal bone marrow CFU, the sensitivity of MOPC 460D was closer to that of normal cells than the other two lines of myeloma.

Effect of Melphalan and 5-Fluorouracil on Survival of Tumor-Bearing Mice

The cell culture data presented above form the basis of two predictions: first, melphalan should be more effective than 5-fluorouracil in the treatment of mice bearing Adj. PC-5, and, second, melphalan should be more effective against Adj. PC-5 than against MOPC 460D. Both of these predictions were borne out by experiments in which animals were treated with drugs 7 days after tumor transplantation. Figure 6 contains the results of an experiment in which groups of ten mice received varying doses of melphalan or 5-fluorouracil 7 days after the transplantation of 2 × 10⁶ Adj. PC-5 cells. Doses of melphalan between 0.05 and 0.2 mg/mouse yielded survival in excess of 50 days, while 5-fluorouracil had little effect over the range of 1–4 mg/mouse. No evidence of splenic enlargement was found in animals surviving after treatment with melphalan, while those that received 5-fluorouracil had grossly enlarged spleens at autopsy.

The effects of melphalan on Adj. PC-5 (sensitive in cell culture) and MOPC 460D (resistant in cell culture) are shown in Table 2. In these experiments,
Fig. 5. Sensitivity of various myeloma lines to melphalan in culture. Cells were exposed to melphalan in culture for 3 hr and then assayed for surviving fractions of CFU.

Fig. 6. Median survival time of tumor (Adj. PC-5) bearing mice treated with melphalan and 5-fluorouracil. Groups of ten mice received varying doses of melphalan and 5-fluorouracil 7 days after tumor transplantation. None of the autopsied mice treated with melphalan showed evidence of splenic enlargement while all of the autopsied mice in the 5-fluorouracil group had grossly enlarged spleens.

Adj. PC-5 and MOPC 460D tumor cells were transplanted subcutaneously. It is evident that, as anticipated from the culture results, melphalan was much more effective against Adj. PC-5 than against MOPC 460D. Table 2 also contains survival data for mice bearing MOPC 460D treated with cyclophosphamide. These animals all appeared to be cured by a cyclophosphamide dose that might be anticipated to reduce CFU-S only slightly (see Fig. 1). Thus, insensitivity to one alkylating agent does not imply insensitivity to another.

DISCUSSION

In this paper we have reported survival curves for three transplantable mouse myeloma tumor lines exposed to a number of chemotherapeutic agents either in cell culture or in vivo. Results were compared with sensitivity of two classes of normal murine hemopoietic progenitor cells, CFU-S, the pluripotent
Table 2. Effect of Melphalan and Cyclophosphamide on the Survival Time of Mice Bearing Adj. PC-S and MOPC 460D

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Drug</th>
<th>Dosage (mg/mouse)</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adj. PC-S (spleen)</td>
<td>None</td>
<td>None</td>
<td>11</td>
</tr>
<tr>
<td>Adj. PC-S (spleen)</td>
<td>Melphalan</td>
<td>0.05</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Adj. PC-S (subc.)</td>
<td>None</td>
<td>29</td>
<td>18-36</td>
</tr>
<tr>
<td>Adj. PC-S (subc.)</td>
<td>Melphalan</td>
<td>0.05</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Adj. PC-S (subc.)</td>
<td>Melphalan</td>
<td>0.1</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Adj. PC-5 (subc.)</td>
<td>Melphalan</td>
<td>0.2</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Adj. PC-5 (subc.)</td>
<td>Cyclophosphamide</td>
<td>1.0</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Adj. PC-5 (subc.)</td>
<td>Cyclophosphamide</td>
<td>2.0</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Adj. PC-5 (subc.)</td>
<td>Cyclophosphamide</td>
<td>4.0</td>
<td>&gt;60</td>
</tr>
<tr>
<td>MOPC 460D</td>
<td>None</td>
<td>19</td>
<td>17-30</td>
</tr>
<tr>
<td>MOPC 460D</td>
<td>Melphalan</td>
<td>0.05</td>
<td>22</td>
</tr>
<tr>
<td>MOPC 460D</td>
<td>Melphalan</td>
<td>0.1</td>
<td>22</td>
</tr>
<tr>
<td>MOPC 460D</td>
<td>Melphalan</td>
<td>0.2</td>
<td>29</td>
</tr>
<tr>
<td>MOPC 460D</td>
<td>Cyclophosphamide</td>
<td>1.0</td>
<td>51</td>
</tr>
<tr>
<td>MOPC 460D</td>
<td>Cyclophosphamide</td>
<td>2.0</td>
<td>52</td>
</tr>
<tr>
<td>MOPC 460D</td>
<td>Cyclophosphamide</td>
<td>4.0</td>
<td>&gt;60</td>
</tr>
</tbody>
</table>

stem cell, and CFU-C, the committed progenitor of granulopoiesis. The results are summarized in Table 1; it may be seen from the table that each cell line had a characteristic and different sensitivity to each chemotherapeutic agent tested; further, varying degrees of differential sensitivity were found between normal and tumor cells. The findings provide a clear indication that different myeloma lines have different intrinsic sensitivities to chemotherapeutic agents. This conclusion supports the need for individualization of treatment with a cellular basis. The use of cell culture assays for this purpose has been tested by treating animals with the various drug regimens; in each instance, the response to therapy was consistent with that predicted by the cell culture data.

Two additional pieces of information emerged during these studies. First, we found that response of a tumor line to one alkylating agent was not predictive of response to other alkylating agents (Table 2). The absence of cross-resistance between melphalan and cyclophosphamide has also been demonstrated clinically in the treatment of myeloma patients. Second, as in previous experiments, the differential effect of some chemotherapeutic agents on tumor cells as compared to normal cells could not be explained on the basis of cell cycle differences. The demonstration that melphalan is equally toxic for resting and regenerating hemopoietic cells is consistent with the clinical observation that the therapeutic response to this drug is not dependent on schedule of administration. Cyclophosphamide, on the other hand, is somewhat more toxic for regenerating than resting marrow CFU, and this suggests that the optimal dosage schedule for this drug may be schedule-dependent.

These animal experiments provide a precedent for chemotherapy in man. If relevant assays for human myeloma cells were available, drug survival
PREDICTIVE TESTS FOR MYELOMA CHEMOTHERAPY

Curves obtained with such assays and compared with survival curves for human CFU-C might be anticipated to provide a useful guide to the choice of a chemotherapeutic regimen.

ACKNOWLEDGMENT

We thank Mrs. Annemarie Kaufman for her skillful technical assistance.

REFERENCES

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