Survival of Hematopoietic and Lymphoma Colony-forming Cells in Vivo following the Administration of a Variety of Alkylating Agents

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SUMMARY

Dose-survival curves for murine hematopoietic stem cells and transplanted syngeneic lymphoma cells were constructed for a variety of alkylating agents administered in vivo. A comparison of the slopes of the dose-survival curves, defined as the efficacy ratio, provided a quantitative measure of the differential sensitivity of these two cell populations to a given alkylating agent. Nitrogen mustard yielded no differential killing of the malignant cell population when compared with the normal, while cyclophosphamide, the most efficacious agent examined, was extremely selective for the lymphoma cells. The efficacy of the other agents tested fell between these two extremes. In increasing order of efficacy were nitrogen mustard, tris(1-aziridinyl)phosphine sulfide, phenylalanine mustard, triethylenemelamine, chlorambucil, NSC 82196, 1,3-bis(2-chloroethyl)-1-nitrosourea, and cyclophosphamide. While the mechanism for the difference in selectivity of cell lethality observed among the different alkylating agents is not known, a possible explanation is presented, together with its implications.

INTRODUCTION

Survival of both hematopoietic stem cells and lymphoma colony-forming cells decreases exponentially, with increasing dosage, for a wide variety of anticancer agents (4). The slopes of the dose-survival curves for both cell populations were shown to be similar for nitrogen mustard, while for cyclophosphamide the lymphoma cells were much more sensitive to the lethal action of this drug. Since the majority of the lymphoma cells were progressing through cell cycle at the time of treatment, while most of the hematopoietic stem cells were not in cell cycle, an explanation for the differential cell killing was advanced that was based on this difference in the proliferative state of the 2 cell populations (5). Further, on the basis of their selectivity of cell lethality, the agents were placed into 1 of 2 categories: nonspecific agents, such as nitrogen mustard, which killed cells either in or out of the cell cycle with equal efficiency; and cycle-specific agents, such as cyclophosphamide, which preferentially killed cells in the cell cycle (4).

While this classification system postulates 2 categories for those anticancer agents that yield exponential dose-survival curves, it is interesting to note that alkylating agents were found in both categories. It seems possible that the 2 alkylating agents mentioned above represent 2 extremes in the class of agents termed “alkylating agents” which harbor other members with differential sensitivities lying between these 2 extremes. We examined this possibility by comparing the effects of a number of alkylating agents on the survival of the 2 cell populations. A range of differential sensitivities for the alkylating agents was found, and an interpretation of this finding, as well as some of its implications are presented.

MATERIALS AND METHODS

Mice. Female AKR mice weighing about 22 g and approximately 7 weeks of age (from either The Jackson Laboratory, Bar Harbor, Maine, or National Laboratory Animal Co., Crève Coeur, Mo.) were used for the experiments.

Transplanted Cell Line. A transplantable, syngeneic lymphoma cell line was used. It was initially isolated as a spontaneous lymphoma and has been passaged weekly for the past 8 years. For this transfer, a cell suspension was prepared from the spleens of lymphoma-bearing mice late in the course of their disease, and 2 X 10⁴ cells were injected i.v. into each recipient mouse. The lymphoma-bearing mice used in the experimental studies received lymphoma cells at the same time as did the transfer mice but, in this case, 10⁷ cells were injected and the animals were given the anticancer agent 4 days later. Some of the growth characteristics of this line have been previously described (3).

Assay for Lymphoma Colony-forming Cells. Suspensions of lymphoma cells were prepared from the femoral marrow of tumor-bearing mice as previously described (3). The suspensions were then assayed for the number of cells that had sufficient proliferative capacity to produce colonies in the spleens of recipient mice upon transplantation (7). For the assay, the cells were diluted with tissue culture Medium

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Chart 1. Dose-survival curves for both normal hematopoietic and transplanted lymphoma CFU. Normal or tumor-bearing mice were given different doses of a given alkylating agent, and their femoral marrows were assayed 24 hr later for their content of CFU. The values shown are normalized to untreated controls so that the surviving fraction could be recorded. Errors shown are 1 S.E. Three separate experiments were carried out for each drug and cell population, and the method of least squares was used to fit the data. Two of the dose-survival curves, A and H, have been published previously (5). The $D_{50}$ value is the dose required to reduce the survival to 50% on the exponential portion of the curve. A, nitrogen mustard; B, thio-TEPA; C, sarcolysin; D, triethylenemelamine; E, chlorambucil; F, NSC 82196; G, BCNU; H, cyclophosphamide.

CMRL-1066 (Grand Island Biological Co., Grand Island, N. Y.), and 0.5 ml of this mixture was injected into the tail vein of the recipients. Eight days later, the mouse spleens were removed and fixed in Bouin's solution, and the macroscopic colonies were counted. Since lymphoma cells proliferate readily in the spleens of unirradiated recipients, an estimate of the lymphoma cells initially present in the femoral marrows of the donor mice is obtained.

Assay for Hematopoietic Colony-forming Cells. The assay for this population of cells was similar to that described above for the leukemic colony-forming cells and has been described in detail (15). Briefly, fractions of the cell suspension of femoral marrow to be assayed were injected i.v. into supralethally irradiated (800 R, whole body) recipients. The X-radiation was administered by a machine operated at 220 kV and with filtration of 0.5 mm Cu plus 1 mm Al. Ten days following marrow transplantation, the spleens of the animals were removed and placed in Bouin's fixative, and the number of macroscopic colonies were counted. This provided an estimate of the number of hematopoietic stem cells in the femoral marrow of the donor mice.

Chemotherapeutic Agents. Most of the alkylating agents were supplied by the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md. The drugs were suspended in ice-cold 0.5% carboxymethylcellulose with the aid of a Dounce homogenizer. This initial suspension was further diluted with ice-cold 0.9% NaCl solution, and 0.5-ml volumes were injected i.p. into recipient mice within 5 min of preparation.

RESULTS

The effect of a number of alkylating agents on the survival of both normal and lymphoma colony-forming cells was determined. Increasing doses of the agents were administered as single i.p. injections to groups of 5 mice, and 24 hr later the femoral marrows were assayed for their content of either normal or lymphoma colony-forming cells. Results obtained with each drug are shown in Chart 1, A to H, wherein the surviving fraction per femur of both normal and lymphoma CFU$^2$ is plotted as a function of dose of drug injected. For some of the curves, a shoulder region was observed, but all of the curves were exponential in shape.

As will be outlined in more detail below, for systemically administered chemotherapeutic agents, a pronounced selective killing of malignant cells over normal cells is necessary. Therefore, in order to objectively define a drug's differential killing activity, a parameter that dose required to kill 50% of the mice in 30 days; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.
sensitivity, we have defined a parameter termed the “efficacy ratio,” which is simply the ratio of the slopes of the 2 dose-survival curves. As a measure of the slope, we have obtained a $D_{1/2}$ value for each drug measured on the exponential portion of the curve. The $D_{1/2}$ value is that dose which reduces the survival of the cell population by a factor of one-half. For example, the results for NSC 82196 (Chart IF) yield a $D_{1/2}$ value of 2.07 mg/mouse for the hematopoietic CFU and a value of 0.27 mg/mouse for the lymphoma CFU. The efficacy ratio is then \( \frac{2.07 \text{ mg/mouse}}{0.27 \text{ mg/mouse}} = 7.7 \). This implies that, ignoring any shoulder region, when the survival fraction of the hematopoietic CFU is at $1/2$ or $2^{-1}$, that of the leukemic CFU is $2^{-7.7}$, or, alternatively, when the survival of the former is $10^{-1}$, that of the latter is $10^{-7.7}$. The implications of this ratio are discussed below.

A summary of all our results are shown in Table 1. The chemical name of the agent, together with a more common name by which it is known and its NSC number, is given in Column 1. The chemical structure is given in Column 2. The
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$D_{1/2}$ values for both cell populations are presented in Columns 3 and 4, and the calculated efficacy ratio is indicated in Column 5. Column 6 shows the LD$_{50/30}$ dose for these drugs injected into normal mice. To determine the LD$_{50/30}$, we gave groups of 5 mice injections of different doses of the agents, administered as single doses. Their survival was followed weekly, and the number of animals alive in each group was recorded at 30 days. At least 3 different determinations were carried out for each drug. We have also shown, in brackets in Column 6, the fraction of hematopoietic CFU that would survive at 24 hr following the administration of the LD$_{50/30}$ dose, a value which was obtained directly from Chart 1.

DISCUSSION

The results of these studies indicate that the classification scheme of Bruce et al. (4) for alkylating agents must be modified. There do not seem to be 2 separate classes for those agents that yield exponential survival curves but, rather, agents exist in a spectrum of differential activities from the least selective agent (nitrogen mustard, efficacy ratio = 1) to the most selective agent (cyclophosphamide, efficacy ratio = 15.3).

The efficacy ratio defined here for a given chemotherapeutic agent is a quantitative measure indicating the usefulness of the agent. For example, the transplantable lymphoma becomes "clinically diagnosable" when the animal harbors about $10^9$ lymphoma cells. Therefore, to cure such an animal, it is necessary to destroy every lymphoma cell, that is, reduce the survival of the malignant cell population to $10^{-9}$ or less. At the same time, however, survival levels of the hematopoietic stem cells must not be reduced to less than about $10^{-3}$ or the animal will succumb to drug-induced toxicity. Those agents with efficacy ratios of the order of 3 or greater should be most useful in the treatment of this lymphoma. Our results indicate that any of the following agents, chlorambucil, NSC 82196, BCNU, or cyclophosphamide, meet this criteria and should, in theory, be curative when administered as a single, large, nonlethal dose. That this is not confirmed experimentally (16) seems to result from a change in sensitivity of the lymphoma cells at low (less than $10^{-6}$) cell survivals. As a first approximation, however, the results do conform to the predictions of the efficacy ratio (6).

Previous investigators have attempted to classify chemotherapeutic agents as to their efficacy. The compilation of the efficacy of a large number of alkylating agents by Goldin and Wood (9) shows BCNU to be the most effective agent, followed by 1-(2-chlorethyl)-3-cyclohexyl-1-nitrosourea, cyclophosphamide and, at the end of the list, nitrogen mustard, which is the least efficacious. Their classification system based on the percentage increase in life-span of mice bearing leukemia L1210 compares favorably with that found by us with AKR-transplanted lymphoma. Similarly, in the study by Schmidt et al. (13), which involved an exhaustive analysis of a number of alkylating agents on a variety of tumor systems, the most efficacious agents found were cyclophosphamide and phenylalanine mustard, with cyclophosphamide being the most active and the remainder of the alkylating agents being somewhat similar but with a very low efficacy. While the effectiveness of these agents is based on the results from a few transplanted tumor systems, it is heartening to realize that those agents that are the most effective in these
Table 1

Summary of cellular characteristics of the alkylating agents investigated

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Chemical structure</th>
<th>Normal CFU</th>
<th>Lymphoma CFU</th>
<th>Efficacy ratio</th>
<th>LD&lt;sub&gt;50/30&lt;/sub&gt; (mg/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2'-Dichloro-&lt;i&gt;N&lt;/i&gt;-methyl-diethylamine (nitrogen mustard), NSC 762</td>
<td><img src="image1.png" alt="Chemical structure" /></td>
<td>0.041</td>
<td>0.041</td>
<td>1</td>
<td>0.15 (10^{-2})&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tris(1-aziridinyl) phosphine sulfide (thio-TEPA), NSC 6396</td>
<td><img src="image2.png" alt="Chemical structure" /></td>
<td>0.048</td>
<td>0.025</td>
<td>1.9</td>
<td>0.70 (10^{-4})</td>
</tr>
<tr>
<td>3-&lt;i&gt;p-&lt;/i&gt;[Bis(2-chloroethyl)amino] phenylalanine mustard, NSC 14210</td>
<td><img src="image3.png" alt="Chemical structure" /></td>
<td>0.064</td>
<td>0.025</td>
<td>2.6</td>
<td>0.50 (10^{-2})</td>
</tr>
<tr>
<td>2,4,6-Tris(1-aziridinyl)s-triazine (TEM), NSC 9706</td>
<td><img src="image4.png" alt="Chemical structure" /></td>
<td>0.0055</td>
<td>0.0020</td>
<td>2.8</td>
<td>0.10 (10^{-4})</td>
</tr>
<tr>
<td>4-&lt;i&gt;p-&lt;/i&gt;[Bis(2-chloroethyl)amino] phenyl)-butyric acid (chlorambucil),</td>
<td><img src="image5.png" alt="Chemical structure" /></td>
<td>0.33</td>
<td>0.072</td>
<td>4.6</td>
<td>1.0 (10^{-1})</td>
</tr>
<tr>
<td>5-[3,3-Bis(2-chloroethyl)-1-triazeno] imidazole-4-carboxamide, NSC 82196</td>
<td><img src="image6.png" alt="Chemical structure" /></td>
<td>2.07</td>
<td>0.27</td>
<td>8</td>
<td>15 (10^{-3})</td>
</tr>
<tr>
<td>1,3-Bis(2-chloroethyl)-1-nitrosoare (BCNU), NSC 40962</td>
<td><img src="image7.png" alt="Chemical structure" /></td>
<td>0.28</td>
<td>0.023</td>
<td>12.2</td>
<td>1.0 (10^{-1})</td>
</tr>
<tr>
<td>2-[Bis(2-chloroethyl)amino] tetrahydro-1,3,2-oxazaphosphorine-2-oxide</td>
<td><img src="image8.png" alt="Chemical structure" /></td>
<td>1.0</td>
<td>0.068</td>
<td>15.3</td>
<td>8 (10^{-3})</td>
</tr>
<tr>
<td>(cyclophosphamide), NSC 26271</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup> No. in parentheses, normal CFU survival.
test systems also appear to be the most effective ones presently in clinical use.

A difficulty in extrapolating these results to the treatment of other experimental or human tumors is that the differential sensitivity that we observed for this tumor system may not apply to other tumor systems. In fact, the differential sensitivity between a transplanted murine myeloid leukemia and normal hematopoietic stem cells to both cyclophosphamide and chlorambucil yielded an efficacy ratio greater than 1 but less than 2 (14). That is, these alkylating agents are much less selective in their killing of myeloid leukemic cells when compared with the transplanted lymphoma used in the studies reported here. A quantitative cellular assay for human malignant cell populations seems a necessary step in evaluating the efficacy of different chemotherapeutic agents and their protocols on different tumors. With this available, no extrapolation would be necessary, and chemotherapy could be tailored to the sensitivity of the individual tumor.

A question remaining then is why different alkylating agents have different efficacies for the 2 cell populations used in this study. While some investigators feel that "it can be accepted as a generalization that the modes of action of the direct descendents of nitrogen mustard are essentially indistinguishable from each other" (10), this in fact may not be true. Agents may exert their lethal effects through different modes of interaction with the target cells. While it is generally thought that the major biochemical mechanism by which alkylating agents destroy the proliferative capacity of mammalian cells is the alkylation of the DNA in the nucleus of these cells (11), investigators have presented evidence implicating other biological macromolecules (2, 8). In fact, while native or denatured DNA can be quite sensitive to inhibition of template function following exposure to alkylating agents such as nitrogen mustard in vitro, it is likely that DNA in the form of nucleoprotein is well protected from those agents when exposure is carried out in vivo (12). For example, compounds like nitrogen mustard may alkylate the macromolecules in the cytoplasm very effectively and thereby produce "cytoplasmic" death of the target cells (14), irrespective of the proliferative state of the cell population. In contrast, following activation, cyclophosphamide may alkylate nuclear macromolecules and thereby cause "nuclear" cell death. One could then explain the differential sensitivity of cyclophosphamide by presuming that the sites of alkylating in the nucleus would be most critical in proliferating cells. For cells out of cell cycle, possibly fewer critical sites are available for reaction as a consequence of those macromolecules that are acting to inhibit transcription of these sites. A second, less complicated explanation of the differential sensitivity of the agents could be based on different efficiencies for the repair mechanisms in the 2 cell populations for different alkylating species. It is known that alkylation of DNA can be repaired in mammalian cells (1), and it is possible that the malignant cell population has a less efficient repair mechanism than does the normal cell population.

It does seem obvious, however, that the determination of the true mode of action of these compounds and, more specifically, of whether there is in fact any difference in the mechanism of cell lethality between these compounds, would be an extremely profitable line of investigation, since information concerning the reason for this differential sensitivity would be most useful not only in predicting which agents should be used with which tumor but also in constructing compounds that are more active than those presently available.

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REFERENCES


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