Investigation of the Activity of Cytoxan against Leukemia L1210 in Mice

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Two prime limiting factors in the treatment of neoplastic disease are (a) the toxicity of active anti-tumor agents for the host, and (b) the origin, during therapy, of resistance to the agent being employed. The origin of resistant variant sublines to therapy with folic acid antagonists, antipurines, glutamine antagonists, etc., has been amply demonstrated (5, 6, 22, 25-31, 36, 37).

For purposes of overcoming the limitation of drug toxicity in therapy, a primary objective of screening programs and of detailed structure-activity studies within active groups of compounds has been to uncover drugs with increased anti-tumor activity and reduced host toxicity (7, 17, 21, 35, 39, 41). With respect to the origin of resistance to treatment, the effort has been twofold: (a) To uncover more active congeners within the same structural series (2, 7, 17, 41) and (b) to obtain drugs which inhibit at sites in different metabolic pathways or at different loci within the same metabolic pathway (8, 38, 40). Such compounds have been employed in clinical studies of concomitant and sequential therapy (9). Combinations of drugs presumed to act at different sites have been employed in studies of anti-tumor synergism, empirically or in attempts at multiple biochemical blockade (12).

In this laboratory, assay procedures for the quantitative evaluation of chemotherapeutic agents have been developed with the use of early and advanced leukemia L1210 in mice (14, 15). With these procedures, various purine and pyrimidine antagonists, nitrogen mustard, and folic acid antagonists etc. (18), demonstrated definite therapeutic effectiveness. The folic acid antagonists were particularly effective in increasing the survival time of mice with this leukemia (17, 18). Although amethopterin was markedly effective in this system, it was surpassed in therapeutic activity by various halogenated derivatives of amethopterin (11, 17). Notably, two of the dihalogenated derivatives of amethopterin, 3'-bromo-5'-chloroamethopterin and 3'-bromo-5'-chloroamethopterin, were capable of eliciting extensive survival time and apparently cured some of the mice, even when treatment was initiated after the disease was frankly systemic (11). However, despite the marked effectiveness of the antifolics in this system, host toxicity and the origin of resistant variants, particularly following very extensive treatment, appeared to limit the usefulness of therapy (20).

In seeking additional anti-leukemic compounds with differing modes of action for use in conjunction with folic acid antagonists, purine and pyrimidine antagonists, etc., the nitrogen mustard derivative, N,N-bis(β-chloroethyl)-N',O-propylenephosphoric acid ester diamide (Cytoxan) (1) was selected for more extensive study. This cyclic nitrogen mustard phosphamide ester appeared to be of particular interest because of (a) its marked inhibitory action against a variety of experimental tumors, and its apparently greater specificity of anti-tumor action as compared with a number of other alkylating agents (1, 3, 4, 19, 23, 24), (b) the postulated mechanism for increased specificity, involving splitting off of the phosphamide group and release of mustard activity at the tumor site (9), and (c) the apparent dissimilarity of this action as compared with that of the folic acid antagonists, antipurines, antipyrimidines, etc.

The current study was conducted to characterize the effectiveness of Cytoxan against leukemia L1210 and to determine its effectiveness against an antifolic-resistant subline and an antipurine-resistant subline of leukemia L1210.

MATERIALS AND METHODS

The general procedures for the assay of drug effectiveness are similar to those used previously in this laboratory (14, 15, 18).

The experiments were conducted in 20-28-gm. BDF₁ (C57BL/6 × DBA/2)F₁ hybrid, CDBA (BALB/c An × DBA/2)F₁ hybrid, or D₂BC (ZBC × DBA/2) hybrid mice.

Cytoxan, Mead Johnson brand of cyclophosphamide, was formerly designated as Endoxan ("Summary of data on Endoxan," A. G. Astawerke). The Cytoxan used in these experiments was obtained from Mead Johnson through the Cancer Chemotherapy National Service Center.

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The hybrids employed are designated with the individual experiments.

The transplanted tumors employed were the sensitive leukemia (LI10) (32) carried in DBA/2J mice, an amethopterin-resistant subline of LI10 (M46R) carried in DBA/2 mice, and an 8-azaguanine-resistant variant (33) carried in DBA/2 mice. For each experiment, the mice were divided into groups of ten for each dose level and twenty untreated controls. The experimental design, including dosages and schedules of treatment, is indicated in the individual experiments.

The transplantable tumors employed were the sensitive leukemia (LI10) and the amethopterin-resistant LI10 (M46R) inocula. The amethopterin-resistant LI10 inoculum was prepared from ascitic fluid. The leukemia (LI10) and amethopterin-resistant LI10 (M46R) were prepared from leukemic spleen tissue. The 8-azaguanine-resistant LI10 inoculum was prepared from ascitic fluid. The concentration of the experimental tumor inoculum ranged from 3 × 106 to 3 × 107 cells per mouse for the various experiments.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Chemical designation</th>
<th>Molecular formula</th>
<th>Vehicle</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoxan (Endoxan)</td>
<td>N,N-Bis-[4-chloroethyl]-N',O-propyl-ene phosphoric acid ester dimide</td>
<td>C6H5Cl2N4O2P·H2O</td>
<td>0.85% saline</td>
<td>Mead Johnson</td>
</tr>
<tr>
<td>Nitrogen mustard (HN8)</td>
<td></td>
<td>C6H5ClN·HCl</td>
<td>0.85% saline</td>
<td>Merck</td>
</tr>
<tr>
<td>Triethylene melamine</td>
<td>2,4,6-Tris(1-aziridinyl)-s-triazine</td>
<td>C4H8N8</td>
<td>0.85% saline</td>
<td>Lederle</td>
</tr>
<tr>
<td>Degranol</td>
<td>1,6-Bis[2-chloroethylamino]-1,6-dideoxy-D-mannitol dihydrochloride</td>
<td>C8H16Cl2N2O2·2HCl</td>
<td>0.85% saline</td>
<td>Medimpex</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>4-[p-[4-Bis[2-chloroethylamino]phenyl]butyric acid</td>
<td>C6H5ClN2O3</td>
<td>MC*</td>
<td>Burroughs-Wellcome</td>
</tr>
<tr>
<td>Hydroquinone mustard</td>
<td>3- [p-[2-Chloroethylamino]phenyl]-1,6-xylohydroquinone dihydrochloride</td>
<td>C6H5ClN2O3·2HCl</td>
<td>MC</td>
<td>C. Weatherbee, Milliken Univ.</td>
</tr>
<tr>
<td>DL-Sarcolysin</td>
<td>3-[p-[2-Chloroethylamino]phenyl]-DL-alanine, dihydrochloride</td>
<td>C6H5ClN2O3·HCl</td>
<td>H2O</td>
<td>J. H. Burchhalter, Univ. of Kansas</td>
</tr>
<tr>
<td>L-Sarcolysin</td>
<td>3-[p-[4-Bis[2-chloroethylamino]phenyl]-L-alanine, dihydrochloride</td>
<td>C6H5ClN2O3·HCl</td>
<td>H2O</td>
<td>Chester Beatty Research Inst. Takeda</td>
</tr>
<tr>
<td>Nitroimin</td>
<td>2,4-Dichloro-N-methyl diethylamine, N-oxide hydrochloride</td>
<td>C6H5ClN2O3·HCl</td>
<td>H2O</td>
<td>Takeda</td>
</tr>
<tr>
<td>Uracil mustard</td>
<td>5-[2-Chloroethylamino]urate</td>
<td>C6H5ClN2O3</td>
<td>5% EOH-saline</td>
<td>Upjohn</td>
</tr>
<tr>
<td>Amethopterin</td>
<td>4-amino-N,N'-methylpteroylglutamic acid</td>
<td>C6H5N3S</td>
<td>2% NaHCO3</td>
<td>Am. Cyanamid Co.</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>6-Furfurin</td>
<td>C6H5N3S</td>
<td>Dil. NaOH</td>
<td>Burroughs-Wellcome</td>
</tr>
</tbody>
</table>

* MC = 0.5 per cent methyl cellulose solution.

Uracil mustard was kindly provided by Dr. John S. Evans, Research Division, Upjohn Co. Amethopterin was obtained from the Lederle Research Div., American Cyanamid Co. The remaining compounds were obtained from the Cancer Chemotherapy National Service Center, National Cancer Inst.

The results are summarized in Tables 2–4 and Charts 1–3.

Table 2 shows summary data of a series of experiments comparing the relative effectiveness of daily administration of Cytoxan, various other alkylating agents, and amethopterin in increasing the survival time of mice with systemic leukemia. At the optimal daily dose (62.5 mg/kg) (Table 2, Exp. 1), Cytoxan increased the median survival time of the mice from 11.0 days (untreated controls) to 26.5 days. The extension in survival time provided by Cytoxan, in this experiment, was 97 per cent as great as that observed with amethopterin. The antileukemic action of Cytoxan was also reflected in an inhibitory effect on the growth of the local tumor at the site of leukemic inoculation. At the optimal dose, the local tumor, approximately 7–9 mm. in diameter, was nonpalpable after about 1 week of treatment. Relative to amethopterin, employed as a standard, Cytoxan displayed greater antileukemic activity than did any of the other alkylating agents tested. Uracil mustard, in one of two experiments (Table 2, Exp. 2), appeared to be next in effectiveness among the alkylating agents, producing an increase in...
median survival time which was 63 per cent as great as that observed with amethopterin. In a second experiment, however, uracil mustard was only 25 per cent as effective as amethopterin (Table 2, Exp. 3). Triethylene melamine, DL-Sarcolysin, L-Sarcolysin, Degranol, nitrogen mustard (HN2), and hydroquinone mustard gave increases in median survival time ranging from 12 to 81 per cent as great as that of amethopterin. Chlorambucil and nitromin were essentially ineffective.

In a previous report from this laboratory (18), 38 compounds, including 23 employed in the Gellhorn-Hirschberg report (10), were compared with amethopterin with respect to their capacity to increase the survival time of mice with advanced leukemia. Of the compounds tested, reserpine, 8-azaguanine, 6-mercaptopurine, thio
guanine, 5-fluorouracil, 2-amino-1,3,4-thiadiazole, and aminopterin were relatively effective, producing increases in median survival time which were 42–62 per cent as great as those produced by amethopterin. Up to the present, the only compounds which have been shown to provide an antileukemic specificity as great as, or greater than, amethopterin have been other congeners of folic acid; notably, the halogenated derivatives of amethopterin and aminopterin, adenopterin and N\textsuperscript{16}-ethyl-aminopterin (11, 17). Cytoxan, then, is the only drug, other than other antifolies, shown to be as effective as amethopterin in increasing the survival time of mice with systemic leukemia. More detailed studies were therefore undertaken against the sensitive line of leukemia L1210.

An experiment was conducted (Chart 1) to determine the influence of the schedule of treatment

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Day of initial treatment</th>
<th>Compound name</th>
<th>Dose range, daily (mg/kg)</th>
<th>Optimal dose level, daily</th>
<th>Median survival time at optimal dose (days)</th>
<th>Per cent increase in median survival time over controls</th>
<th>Relative increase in median survival time (Amethopterin increase=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 8</td>
<td>Amethopterin</td>
<td>0.39–3.02</td>
<td>1.09</td>
<td>27.0</td>
<td>145</td>
<td>145</td>
<td>100</td>
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<tr>
<td></td>
<td>Cytoxan</td>
<td>31.3–1000</td>
<td>62.5</td>
<td>26.5</td>
<td>141</td>
<td>141</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Hydroquinone mustard</td>
<td>2.00–63.0</td>
<td>63.0*</td>
<td>13.0*</td>
<td>18</td>
<td>18</td>
<td>12</td>
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<tr>
<td></td>
<td>Chlorambucil</td>
<td>5.04–180</td>
<td>14.0</td>
<td>11.5</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td>11.0</td>
<td>11.0</td>
<td>11.0</td>
</tr>
<tr>
<td>2 8</td>
<td>Amethopterin</td>
<td>0.47–2.16</td>
<td>1.30</td>
<td>20.5</td>
<td>86</td>
<td>86</td>
<td>100</td>
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<tr>
<td></td>
<td>Uracil mustard</td>
<td>0.78–6.00</td>
<td>9.16</td>
<td>17.0</td>
<td>55</td>
<td>55</td>
<td>63</td>
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<td>11.0</td>
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<td>3 7</td>
<td>Amethopterin</td>
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<td>1.30</td>
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<td>100</td>
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<td>Uracil mustard</td>
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<td>9.16</td>
<td>14.5</td>
<td>32</td>
<td>32</td>
<td>22</td>
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<td>11.0</td>
<td>11.0</td>
</tr>
<tr>
<td>4 7</td>
<td>Amethopterin</td>
<td>0.39–3.02</td>
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<td>25.5</td>
<td>182</td>
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<td>100</td>
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<td></td>
<td>DL-Sarcolysin</td>
<td>1.09–38.9</td>
<td>28.3</td>
<td>15.5</td>
<td>41</td>
<td>41</td>
<td>31</td>
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<tr>
<td></td>
<td>Degranol</td>
<td>1.81–108</td>
<td>64.8</td>
<td>14.0</td>
<td>27</td>
<td>27</td>
<td>21</td>
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<tr>
<td></td>
<td>L-Sarcolysin</td>
<td>0.65–38.9</td>
<td>38.9*</td>
<td>15.0*</td>
<td>18</td>
<td>18</td>
<td>14</td>
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<tr>
<td></td>
<td>Nitromin</td>
<td>3.02–108</td>
<td>38.9</td>
<td>11.5</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td>11.0</td>
<td>11.0</td>
<td>11.0</td>
</tr>
<tr>
<td>5 7</td>
<td>Amethopterin</td>
<td>0.39–3.02</td>
<td>1.09</td>
<td>25.5</td>
<td>155</td>
<td>155</td>
<td>100</td>
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<tr>
<td></td>
<td>Triethylene melamine (TEM)</td>
<td>0.33–3.02</td>
<td>1.09</td>
<td>14.0</td>
<td>40</td>
<td>40</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Nitrogen mustard (HN2)</td>
<td>0.23–1.81</td>
<td>0.65</td>
<td>12.0</td>
<td>20</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>6 11</td>
<td>Amethopterin</td>
<td>0.39–3.02</td>
<td>0.65</td>
<td>27.0</td>
<td>108</td>
<td>108</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>L-Sarcolysin</td>
<td>23.8–108</td>
<td>38.9</td>
<td>16.5</td>
<td>27</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Chlorambucil</td>
<td>14.0–108</td>
<td>None</td>
<td>13.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td>13.0</td>
<td>13.0</td>
<td>13.0</td>
</tr>
</tbody>
</table>

* The highest daily dose employed was most effective. The mice succumbed, however, with considerable weight loss, which suggests that higher dose levels would not have produced appreciably greater increases in median survival time.

Mice employed in the various experiments: BDF\textsubscript{1} male (Exps. 1, 3, 4); D\textsubscript{2}BC, male (Exp. 5); D\textsubscript{2}BC, female (Exps. 2, 6). Ten mice per treated group; twenty mice per control group.
TABLE 3
EFFECT OF CYTOXAN, ALONE AND COMBINED WITH NICOTINAMIDE, ON EARLY LEUKEMIA L1210

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median survival time of total group (days)</th>
<th>No. toxic deaths</th>
<th>No. tumor deaths</th>
<th>Av. survival time of mice dying of tumor (days)</th>
<th>No. of tumor-free survivors (day 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 Nicotinamide+1000 Cytoxan</td>
<td>5.5</td>
<td>3</td>
<td>0</td>
<td>17.9</td>
<td>0</td>
</tr>
<tr>
<td>250 Nicotinamide+1000 Cytoxan</td>
<td>8.5</td>
<td>9</td>
<td>0</td>
<td>17.4</td>
<td>0</td>
</tr>
<tr>
<td>1000 Cytoxan</td>
<td>3.0</td>
<td>10</td>
<td>0</td>
<td>17.4</td>
<td>0</td>
</tr>
<tr>
<td>360 Amethopterin</td>
<td>5.5</td>
<td>3</td>
<td>0</td>
<td>17.4</td>
<td>0</td>
</tr>
<tr>
<td>CDBA female mice. Nicotinamide was administered intraperitoneally 15 minutes prior to the subcutaneous injection of Cytoxan (Exp. 7). Ten mice per group.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 4
RELATIVE EFFECTIVENESS OF CYTOXAN, AMETHOPTERIN, AND 6-MERCaptopurine AGAINST AN 8- AZAGUANINE-RESISTANT SUBLINE OF L1210

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose range, daily (mg/kg)</th>
<th>Optimal dose level, daily (mg/kg)</th>
<th>Median survival time at optimal dose (days)</th>
<th>Per cent increase in median survival time over controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoxan</td>
<td>14-0-180</td>
<td>64.8</td>
<td>25.5</td>
<td>118</td>
</tr>
<tr>
<td>Amethopterin</td>
<td>0.14-3.00</td>
<td>1.08</td>
<td>&gt;317</td>
<td>8</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>14-0-300</td>
<td>38.9, 108</td>
<td>28.5</td>
<td>27.0</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td>22.5</td>
<td>17.1</td>
</tr>
<tr>
<td>CDBA female mice. Ten per treated group, twenty untreated controls. Exp. 8. Daily treatment was begun 8 days after tumor inoculation.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
on the effectiveness of Cytoxan in increasing the survival time of mice with systemic leukemia. Cytoxan was given as a single dose, daily, every 2d-day, and every 4th day, starting at 7 days following leukemic inoculation. Comparison is made with daily treatment with amethopterin as the standard. The median survival time of the control mice was 10 days. In this experiment, on daily treatment, Cytoxan was slightly more effective than amethopterin. The alkylating agent gave a maximum median survival time of 27 days, as contrasted with 24.5 days for amethopterin.

On two other schedules of treatment, namely, every 2d day and every 4th day, Cytoxan was still more effective, giving maximum median survival times of 31 days and 30.5 days. A single treatment with Cytoxan given at 7 days following leukemic inoculation was also effective, increasing the median survival time to 23 days.

Thus, Cytoxan appears to be quite effective in increasing the survival time of mice with systemic leukemia over a wide range of schedules of treatment. This is in agreement with previous reports (23, 24). It is of interest to note that amethopterin has been shown to be relatively ineffective against advanced leukemia L1210 when given as a single treatment or when treatments are spaced 4 days apart (15).

With amethopterin daily, the last animal succumbed on the 30th day following leukemic inoculation, while on the four schedules of treatment with Cytoxan, 21 mice survived beyond this point, succumbing between the 30th day and the 48th day. There were two such mice on single treatment with Cytoxan and three on daily treatment. The remaining sixteen long-term survivors were in the groups treated with Cytoxan every 2 and every 4 days.

The results of treatment of early leukemia L1210 with Cytoxan are summarized in Table 3. Two schedules of treatment were employed, namely, a single treatment given 2 days following tumor inoculation and daily treatment beginning on the same day. Mice receiving a single treatment could be separated into three categories; those succumbing to drug toxicity, those succumb-
Chart 2.—Comparison of Cytoxan and amethopterin with respect to the limitation on anti-leukemic effectiveness imposed by the cumulative toxicity on daily treatment. Drug effects in normal and leukemia BDF1 male and female mice are compared. The numbers above the vertical arrows indicate the number of survivors out of ten mice in each normal (no tumor) group treated daily from day 7 through day 55 (Exp. 10).

Chart 3.—Relative effectiveness of Cytoxan, amethopterin, and 6-mercaptopurine in increasing the survival time of mice with early or advanced amethopterin-resistant L1210 (M46R). Ten CDBA male mice per group. (Exp. 11).
ing to tumor growth, and those showing recovery from the leukemic implant (13). Animals succumbing to drug toxicity, in general, lost considerable body weight, displayed no local tumors at the time of death, and died prior to untreated controls. All the mice receiving daily treatment succumbed, and no clear separation of toxic deaths from tumor deaths could be made.

A single treatment with Cytoxan administered early in the course of the disease was particularly effective (Table 3). Extensive median survival times (beyond 60 days) were obtained, accompanied by a relatively high percentage of total recovery from the leukemic implant. Out of 60 mice treated with Cytoxan, over a series of dose levels, there were eighteen 60-day survivors showing no evidence of tumor.

On single treatment Cytoxan was considerably more effective than amethopterin. The maximum median survival time achieved with a single treatment with amethopterin was 21.0 days, and the last mouse succumbed on the 28th day.

On daily treatment of early leukemia L1210 the maximum median survival time achieved with Cytoxan was 29 days, which was considerably less than that observed with amethopterin on the same schedule (median survival time 39.0 days) (Table 3). With daily treatment beginning early, neither Cytoxan nor amethopterin produced any instances of total recovery from the leukemic implant. However, at doses effective in increasing survival time, both Cytoxan and amethopterin retarded the growth of the local tumor at the site of leukemic inoculation.

Because of a previous report (19) that Cytoxan behaves as a nicotinamide antagonist, an attempt was made to block the antileukemic action of Cytoxan with that vitamin, both on the single and multiple treatment schedules (Table 3). Doses of 250 mg/kg and 500 mg/kg of nicotinamide were administered 15 minutes prior to Cytoxan. No evidence was obtained that nicotinamide could block the antileukemic action of Cytoxan. Nicotinamide did not reverse the increase in survival time elicited by single or daily administration of Cytoxan, nor did it overcome the inhibition of local tumor growth at the site of leukemic inoculation. No reduction was observed in the number of "no takes" obtained on single-dose administration of Cytoxan. Nicotinamide also failed to diminish the toxicity of the alkylating agent for the host.

It was considered desirable to obtain further information on the extent to which the cumulative toxicity of Cytoxan may serve to limit the therapeutic efficacy of the drug. The importance of the limitation of toxicity was indicated in several experiments: (a) Following the achievement of a maximum median survival time with increasing dose on daily treatment (Chart 1) the median survival time diminished precipitously with further increase in dosage. (b) At the optimal dose on daily treatment for mice with advanced leukemia, there was considerable evidence of weight loss. (c) Single treatment appeared to be more effective than multiple treatment against early leukemia, suggesting that the effectiveness of multiple treatment was limited by the cumulative toxicity of the drug (Table 3).

The results of an experiment designed to test the limitation of drug toxicity for the host are shown in Chart 2. Comparison was made of the survival time of normal mice and mice with advanced leukemia L1210, employing daily treatment with Cytoxan and with amethopterin. This was done in both male and female mice. The data show that, in both the male and female mice, the toxicity of Cytoxan provided a greater limitation on this schedule of treatment to extended therapy than did the toxicity of amethopterin. At the daily dose yielding the maximum increase in survival time in leukemic mice, Cytoxan toxicity for normal mice was fairly extensive, although not as severe as had been observed with 5-fluorouracil (34). As had been observed in earlier studies (34), amethopterin, at its optimal daily dose in leukemic mice, produced little indication of toxicity in normal mice. Thus, the data suggest that, although at optimal dose levels on daily treatment the two compounds displayed nearly equal effectiveness in prolonging the life of mice with advanced leukemia, drug toxicity played a more prominent role in the death of animals receiving Cytoxan than in animals receiving amethopterin. This emphasizes the importance of investigations of means of reducing the toxicity for the host of drugs such as Cytoxan.

In view of the marked activity observed in the treatment of early or advanced leukemia L1210 with Cytoxan, the studies were extended to an amethopterin-resistant subline and an antipurine-resistant subline of leukemia L1210.

A comparison was made of the relative effectiveness of Cytoxan, amethopterin, and 6-mercaptopurine in increasing the survival time of mice with the M46R resistant variant of leukemia L1210 when treatment was started at 3 days and also at 8 days following tumor inoculation (Chart 3). The M46R variant of L1210, in previous studies, had displayed marked resistance to amethopterin, and considerable resistance to 3'5'-dichloroamethopterin (20). In the current experiment the median survival time of the control mice was 11 days.
When treatment was started on the 3rd day or on the 8th day following leukemic inoculation, amethopterin was relatively ineffective in increasing the survival time of the mice. The most effective daily dose employed (3 mg/kg) increased the median survival time by 3.5 days when treatment was begun on the 3rd day and by 2 days when treatment was begun on the 8th day.

6-Mercaptopurine elicited a 6-day increase in survival time beyond that of the controls, when the treatment was started early or late following leukemic inoculation (Chart 3). This is in agreement with previous observations with this antifolic-resistant subline of L1210 (20). 6-Mercaptopurine also gave similar survival times with the sensitive L1210 (18).

Cytoxan produced rather extensive increases in survival time when treatment was started on either the 3rd or the 8th day after tumor inoculation (Chart 3). The median survival time was increased by 15 days when treatment was started early, and by 18.5 days when treatment was begun at 8 days following leukemic inoculation. The optimal daily dose starting treatment late (65 mg/kg) was higher than that observed when treatment was initiated early (39 mg/kg). This, and the lesser increase in survival time on early treatment, may, as with the sensitive leukemia, reflect the earlier appearance of cumulative toxicity when treatment was initiated early. These increases in survival time are similar to those observed with the sensitive L1210. Thus, the amethopterin-resistant subline of L1210 (M46R) was not resistant to Cytoxan.

Similarly, the 8-azaguanine-resistant subline of leukemia L1210 retained its sensitivity to Cytoxan (Table 4). The median survival time of the controls was 12 days. 6-Mercaptopurine increased the median survival time by only 1 day, giving an 8 per cent increase in survival time. Cytoxan, on the other hand, increased the median survival time by 13.5 days, giving a 113 per cent increase in survival time. Amethopterin, as had been reported previously (33), was particularly effective against this subline of L1210 and in this experiment gave more than a 317 per cent increase in survival time. Thus, with the 8-azaguanine-resistant variant of leukemia L1210 there was no loss in antileukemic effectiveness for Cytoxan.

Although definite maxima were not obtained, there was evidence of toxicity at the highest daily dose employed (8 mg/kg), indicating that higher doses would have been less effective in increasing survival time. In other experiments in which a clearly optimal daily dose of amethopterin was obtained for the treatment of early L1210 (M46R), extensions in median survival time greater than 3–4 days over controls were not realized.

DISCUSSION

The current studies show that Cytoxan, a cyclic derivative of nitrogen mustard, has pronounced antileukemic action against the sensitive line of leukemia L1210 in mice, and against an antifolic-resistant and an antipurine-resistant variant of this leukemia. Against advanced stages of the sensitive line of leukemia L1210, Cytoxan was approximately as effective as amethopterin, though not as effective as the halogenated derivatives of amethopterin (11) when daily treatments were given. Unlike the results with amethopterin (15) however, the effectiveness of Cytoxan against advanced L1210 increased as the interval between treatments was increased. Against an early stage of leukemia L1210, it was not quite as effective as amethopterin on daily treatment but considerably more effective than amethopterin when administered as a single dose.

The observation that, for the sensitive line of leukemia L1210, over a wide dose range, on early treatment, Cytoxan was capable of total inhibition of growth of the leukemic implant indicates that the drug is capable of cytotoxic action on the tumor cells. This is supported by the preliminary observation that, in some cases, transplant of spleen failed to transmit the disease following treatment of systemic leukemia with Cytoxan. In contrast, single or multiple daily treatment of early leukemia with amethopterin failed to produce tumor-free survivors. However, in other experiments (16) it was demonstrated that two treatments with high doses of amethopterin spaced 4 days apart did yield a high percentage of "no takes" in mice with early leukemia.

The observation that Cytoxan retained its antileukemic effectiveness against an amethopterin-resistant variant of leukemia L1210 and against an 8-azaguanine-resistant variant of L1210 also resistant to 6-mercaptopurine indicates that Cytoxan may be useful in the exploration of a means to overcome leukemic cell resistance to antifolics and antipurines in experimental and clinical leukemia. Failure of cross-resistance with these variant tumors to Cytoxan also indicates that the drug exerts its action at sites other than those inhibited by metabolic antagonists in folic acid and purine metabolism. This would suggest that Cytoxan be employed in studies of synergism in conjunction with folic acid antagonists and purine antagonists in attempts to improve antitumor inhibitory action and therapeutic effectiveness.

The current demonstration of the use of studies of cross-resistance to detect differences in the
mode of antileukemic action of drugs also re-empha-
sizes the potential usefulness of the employment of
resistant variants in cancer screening and
development programs (25-31, 36, 37, 39).

The previous demonstration in other tumor systems that Cytoxan acts as a nicotinamide
antagonist could not be duplicated with leukemia L1210. However, it should be pointed out that, in
the previous studies, a series of treatments with
nicotinamide was given before and after a single
dose of Cytoxan (19).

The limitation of host toxicity in therapy with
Cytoxan, as evidenced by the toxicity for non-
leukemic mice of therapeutically active dosages,
appears to be greater than that observed for
amethopterin. However, methods may be sought
to reduce host toxicity with the retention of anti-
tumor activity. If nicotinamide or other metabo-
lites were to be found to overcome host toxicity,
employment of the metabolite with Cytoxan on
appropriate schedules could result in differential
protection for the host and resultant increased
antileukemic action.

Cytoxan has been demonstrated to be effective
gainst a wide variety of experimental neoplasms.
In the current studies, in the manner employed, it
was more effective than nitrogen mustard (HN2)
and various alkylating agents. This is in agreement
with previous studies of other tumors (1, 3, 4, 19,
23, 24). It has been suggested that Cytoxan itself
is essentially an inactive and nontoxic transport
form, and that it is converted to active form with
the enzymatic splitting off of the inert phospha-
mide and the release of active nitrogen mustard
(1). This type of transport and release of activity
at the tumor site may provide a basis for a high
degree of antitumor specificity of action. Further
clarification of the mechanism of action of Cy-
toxan and the testing of similar types of congeners
of nitrogen mustard may provide additional tools
for investigation of the enhancement of the ther-
apeutic usefulness of antineoplastic chemothera-
peutic agents.

SUMMARY

Cytoxan, a cyclic derivative of nitrogen mus-

tard, was found to be more effective than various
other alkylating agents and at least as effective as
amethopterin in increasing the survival time of
mice with advanced leukemia L1210. The anti-
leukemic effectiveness of Cytoxan was demon-
strable over a wide range of treatment schedules.
On all schedules, the therapeutic effectiveness of
Cytoxan was limited by the toxicity of the drug
for the host. The extent of the limitation of host
toxicity was determined by comparison of treat-
ment of normal and leukemic mice. Studies with
early leukemia suggest that the action of this
alkylating agent may result in destruction of im-
planted tumor cells. The anti-tumor effectiveness
of Cytoxan was not diminished in L1210 sublines
which were resistant to antifolic or antipurine
treatment. The significance of these results rela-
tive to problems such as the toxicity of known
anti-tumor agents for the host and the origin of
resistance during therapy is discussed.

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