The Effectiveness of Cyclophosphamide (Cytoxan) against Well Established Transplanted Rodent Tumors*

MONTAGUE LANE

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SUMMARY

The survival of mice and rats bearing well established transplanted carcinoma, leukemia, and lymphosarcoma was markedly extended by weekly doses of the cyclic phosphamide alkylating agent, Cytoxan. Complete regressions were obtained with four types of tumors.

The activity of Cytoxan against these tumors was compared with the effects previously obtained with two other alkylating agents, uracil mustard and nitrogen mustard. Under the conditions of these experiments Cytoxan was the most effective of these antitumor agents, and it had substantial activity over a wide dose range.

The synthesis of N,N-bis(β-chloroethyl)-N',O-propylene phosphoric acid ester diamide (cyclophosphamide) by Arnold and Bourseaux (1) has provided an alkylating agent of unusual interest. In earlier work (7, 8) it was reported that Cytoxan markedly extended the survival of mice bearing advanced L1210 leukemia and that the effectiveness of this drug was significantly influenced by treatment schedule. Daily doses of Cytoxan prolonged the survival of leukemic mice to the same extent as the folic acid antagonist, Methotrexate. On the other hand, weekly doses of the alkylating agent were at least twice as effective. Some of these studies were subsequently confirmed by Venditti et al. (11).

The present paper deals with the effectiveness of weekly doses of Cytoxan on a spectrum of well established transplanted tumors in mice and rats. The antitumor activity of Cytoxan in these systems is shown to be considerably greater than that of two other alkylating agents, uracil mustard and nitrogen mustard.

MATERIALS AND METHODS

Preparations of drug.—Cytoxan was dissolved in 0.9 per cent saline and injected within 1 hour of preparation on a dose level basis of 0.01 ml/gm of rodent body weight. Injections were given intraperitoneally except in the oral toxicity studies, in which solutions were administered by gavage.

Tumors.—The tumors, implantation sites, and host strains used are shown in Table 1. Animals were 2–3 months old at the start of each experiment. Transplantation procedures and randomization technics were the same as described previously (9). Treatment was begun when the tumors were well established, in some instances just prior to the first deaths in untreated groups. Complete regression of tumor was defined as no palpable recurrence of tumor for 10 weeks after cessation of therapy. The median survival of treated compared with untreated animals was used to evaluate antitumor activity, except in one of the studies with Carcinoma 241-6, in which survivors were killed 10 days after treatment and tumors and carcasses were weighed separately.

Statistical methods.—The LD₅₀ values were determined by a modification of the method of Karber (4). In survival studies with tumor-bearing animals a direct comparison was made of the survival of treated groups with untreated controls given implants concomitantly. The effect of treatment was analyzed statistically by the nonparametric method of Festinger (5). A probability (P) of...
less than 0.05 was considered a significant difference due to treatment.

RESULTS

LD₅₀ values for single doses of Cytoxan administered to normal mice and rats are shown in Table 2. Cytoxan was more toxic for rats than for mice. The drug was less toxic on oral than on intraperitoneal administrations for the mouse, but toxicity was the same by either route for the rat, in agreement with the data of Rall. The details of Cytoxan toxicity in the mouse have been described elsewhere.

Table 3 illustrates the effects of Cytoxan on survival of mice bearing intramuscular implant of the L₂ lymphosarcoma. Treatment was begun 7 days after tumor implantation, and the first deaths in untreated animals occurred on the 10th day. Injections of drug were discontinued after day 42 (a total of six weekly injections). At this time no tumors were palpable in the surviving mice (250 and 180 mg/kg groups). A maximum increase in survival beyond controls was obtained at a dose of 180 mg/kg, and there was complete regression of tumors in 30 per cent of this group. The broad range of efficacy of Cytoxan against this tumor is illustrated by the significant increases in median survival of all groups in the dose range from 90–350 mg/kg.

A study of the inhibitory effect of Cytoxan on the growth of mammary carcinoma 241-6 is shown in Table 4. Tumors were at least 1 cm in diameter at the start of treatment. In many instances no tumor was detected on post-mortem examination of treated mice. All dose levels from 125 to 310 mg/kg were approximately equally effective in producing tumor regression (tumor weights 1–3 per cent of untreated controls), and there was little change in carcass weight. A study of the influence of Cytoxan on the survival of mice bearing this tumor was carried out concomitantly in mice given implants of the same tumor mash. The results are indicated in Table 5. The range of survival of untreated animals was 11–29 days, and 80 per cent of deaths occurred between the 11th and 16th days. The median survival of this group was 15 days. Four weekly injections of Cytoxan were given beginning 6 days after tumor implantation. Treatment was discontinued because no tumors were palpable in any group, and it was apparent that animals at the high dose levels were dying from drug toxicity. A maximum effect on survival (990 per cent increase) was

### TABLE 1

<table>
<thead>
<tr>
<th>TUMORS AND TRANSPLANTATION TECHNICS</th>
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<tbody>
<tr>
<td><strong>Mouse tumors:</strong></td>
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<tr>
<td>Carcinoma 241-6</td>
</tr>
<tr>
<td>Leukemia L1210</td>
</tr>
<tr>
<td>Lymphosarcoma L₂</td>
</tr>
<tr>
<td><strong>Rat tumors:</strong></td>
</tr>
<tr>
<td>Leukemia IRC 741</td>
</tr>
<tr>
<td>Lymphosarcoma R-2788</td>
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<table>
<thead>
<tr>
<th>Implantation site</th>
<th>Inoculum</th>
<th>Host</th>
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</thead>
<tbody>
<tr>
<td>S.C.</td>
<td>S.C. tumor</td>
<td>C57BL</td>
</tr>
<tr>
<td>S.C.</td>
<td>Ascitic fluid</td>
<td>DBA*</td>
</tr>
<tr>
<td>I.M.</td>
<td>Ascitic fluid</td>
<td>CAF₁</td>
</tr>
<tr>
<td>S.C.</td>
<td>Venous blood</td>
<td>Fischer 344/N</td>
</tr>
<tr>
<td>S.C.</td>
<td>S.C. tumor</td>
<td>(M580/AxC)F₁</td>
</tr>
</tbody>
</table>

* (BALB/cAnN × DBA/2 JN)F₁.
Abbreviations: S.C. = subcutaneous; I.M. = intramuscular.

### TABLE 2

<table>
<thead>
<tr>
<th>SINGLE DOSE TOXICITY OF CYTOXAN</th>
</tr>
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<tbody>
<tr>
<td><strong>Animal strain</strong></td>
</tr>
<tr>
<td>DBA/p mouse</td>
</tr>
<tr>
<td>Fischer 344/N rat</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sex</th>
<th>Route</th>
<th>LD₅₀ (mg/kg)</th>
<th>95 Per cent confidence limit (mg/kg)</th>
<th>Slope (probit-log dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>male</td>
<td>I.P.</td>
<td>300</td>
<td>277–325</td>
<td>13.7</td>
</tr>
<tr>
<td>male</td>
<td>P.O.</td>
<td>429</td>
<td>375–401</td>
<td>6.3</td>
</tr>
<tr>
<td>female</td>
<td>I.P.</td>
<td>142</td>
<td>133–178</td>
<td>8.2</td>
</tr>
<tr>
<td>female</td>
<td>P.O.</td>
<td>150</td>
<td>105–210</td>
<td>4.7</td>
</tr>
</tbody>
</table>
observed at the lowest dose (125 mg/kg), and three of seven tumors completely regressed. It is possible that an optimal therapeutic dose might be even lower. These results substantiate those in the prior experiment (Table 4) in demonstrating the marked effectiveness of Cytoxan against this tumor at all dose levels employed.

The response to Cytoxan of leukemia L1210 in CBDA mice is shown in Table 6. Treatment was begun on day 7, just 2 days before the median death of untreated mice, and was given once weekly. The wide range of doses producing increased survival is apparent, with a maximum effect at 259 mg/kg (230 per cent increase in median survival). Earlier studies from this laboratory utilized DBA mice as hosts, and similar results were obtained with Cytospan treatment (8).

Cytoxan was effective in the present studies against the Dunning lymphosarcoma R-2788 in female rats. Treatment was begun 17 days after tumor implantation, and five weekly injections of 15, 30, and 45 mg/kg, respectively, were administered to groups of five rats each. The first death in a group of ten untreated rats occurred on the 24th day, and the median survival was 33 days. In the 15- and 30-mg/kg groups there were 100 per cent complete regressions, and in the 45-mg/kg group 80 per cent of the tumors regressed completely.

Cytoxan was also effective against the Dunning leukemia IRC741, as illustrated by the two experiments in Table 7. Treatments were begun 7 days after tumor implantation and were discontinued on day 42, after six weekly injections. In experiment 1, increases in median survival were obtained at both levels of drug administra-
tion. At 30 mg/kg the rats appeared to die primarily as a result of continued tumor growth, and at 90 mg/kg as a result of drug toxicity. Insufficient dose levels were used to determine whether a maximum effect had been obtained with 30 mg/kg. In experiment 2, considerable increases in survival were obtained with all four levels of drug administration. The rats given 15 mg/kg appeared to die primarily from continued tumor growth and those at 60 mg/kg from somewhat delayed drug toxicity. No evidence of tumor was observed in six of eight rats at both intermediate dose levels, a remarkable effect in view of the fact that untreated controls died earlier in this experiment than in the first.

In Table 8 the maximum increases in median survival obtained with Cytoxan treatment in the present studies are compared with the maximal effects of uracil mustard and nitrogen mustard in previous experiments (9) under conditions of optimal dose and treatment schedule. It is apparent that Cytoxan had a broad spectrum of activity. Marked increases in survival have been demonstrated for treated animals bearing each of the tumors studied. This is especially significant, since the tumors were well established at the onset of therapy, and supports previous observations by others on earlier treatment of rat (3) and mouse tumors, which indicated considerable activity for this agent.

In Table 8 the maximum increases in median survival obtained with Cytoxan treatment in the present studies are compared with the maximal effects of uracil mustard and nitrogen mustard in previous experiments (9) under conditions of optimal dose and treatment schedule. It is apparent that Cytoxan and uracil mustard were much more effective than nitrogen mustard in every case. Cytoxan was unquestionably the most effective agent against leukemias L1210 and IRC 741. Survival of mice with Carcinoma 241-6, markedly prolonged by Cytoxan (99 per cent), was not studied with uracil mustard and nitrogen mustard. However, the inhibition of growth of this tumor was measured. The extent of inhibition achieved with a single dose of each agent, unaccompanied by loss of carcass weight, was as follows: Cytoxan, 99 per cent; uracil mustard, 81 per cent; nitrogen mustard, 0 per cent.

**DISCUSSION**

These studies were designed to examine the antitumor activity of Cytoxan as measured by prolongation of the survival of tumor-bearing animals. A number of transplantable tumors were used to evaluate the activity of this alkylating agent. Tumors were allowed to become well established prior to treatment.

Previous experiments demonstrated that Cytoxan was more effective than either nitrogen mustard or Methotrexate in prolonging the survival of mice with advanced L1210 leukemia (7, 8). The therapeutic results achieved with a once-weekly dose schedule were superior to those obtained with less frequent schedules. The responsiveness of established L1210 leukemia implants to weekly doses of Cytoxan has been confirmed here and shown not to depend upon the host strain (Table 6).

<table>
<thead>
<tr>
<th>TUMOR</th>
<th>TREATMENT BEGUN (DAY)</th>
<th>PER CENT INCREASE MEDIAN SURVIVAL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemia L1210</td>
<td>7</td>
<td>233 (0)</td>
</tr>
<tr>
<td>Lymphosarcoma L4</td>
<td>7</td>
<td>291 (30)</td>
</tr>
<tr>
<td>Carcinoma 241-6</td>
<td>6</td>
<td>992 (45)</td>
</tr>
<tr>
<td>Leukemia IRC 741</td>
<td>7</td>
<td>(75)</td>
</tr>
<tr>
<td>Lymphosarcoma R-2788</td>
<td>17</td>
<td>(100)</td>
</tr>
<tr>
<td>* Numbers in parentheses represent per cent complete regressions.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It is apparent that Cytoxan had a broad spectrum of activity. Marked increases in survival have been demonstrated for treated animals bearing each of the tumors studied. This is especially significant, since the tumors were well established at the onset of therapy, and supports previous observations by others on earlier treatment of rat (3) and mouse tumors, which indicated considerable activity for this agent.

In addition to prolonging survival and produc-
ing marked decreases in tumor size, Cytoxan can produce complete regression of lymphosarcoma L2, mammary adenocarcinoma 241-6, Dunning lymphosarcoma R-2788, and Dunning leukemia IRC741.

Comparison of the maximum increases in median survival produced with Cytoxan, uracil mustard, and nitrogen mustard (Table 8) indicates that nitrogen mustard was the least effective agent against each of the tumors studied.Cytoxan was much more active against leukemias L1210 and IRC741 and carcinoma 241-6 than either uracil mustard or nitrogen mustard. Uracil mustard and Cytoxan were approximately equally capable of increasing the median survival of animals bearing the lymphosarcomas L2 and R-2788. Cytoxan more frequently produced complete regression of tumor. Both drugs were effective over a wide dose range.

In view of the considerable antitumor activity of Cytoxan, its suggested requirement for some in vivo "activation mechanism" (2), and its ability to "spare" megakaryocytes and induce their hyperplasia (8), further study of this agent and of any congeners which may be developed is indicated.

Screening data become particularly significant if they accurately predict the order of activity of chemotherapeutic compounds for the treatment of neoplasms of man. Clinical studies, therefore, must assess antitumor activity reliably. The techniques of evaluating drugs in quantitative controlled clinical trials have been applied only recently to the cancer problem (6, 10, 12, 13). In several Cooperative Chemotherapy Groups, clinical studies are now in progress to compare, qualitatively and quantitatively, the antitumor activities of Cytoxan with those of other alkylating agents. The results of these studies should determine, retrospectively, whether the greater effectiveness of Cytoxan than of nitrogen mustard observed in animal experiments accurately predicted the activity of this new agent against human tumors. The meaning of data obtained from various screens may be clarified as reliable clinical information becomes available.

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