Drug Therapy against a Transplantable Guinea Pig Leukemia

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SUMMARY

The effects of six clinically active drugs were tested against a transplantable leukemia in inbred strain 2 guinea pigs. Cytoxan and 6-mercaptopurine were found to elicit a therapeutic response against this leukemia based on complete tumor regression of the established tumor as well as a substantial increase in survival time. Animals dying in the untreated control and drug-treated groups revealed typical generalized lymphoblastic leukemia. However, only Cytoxan-treated animals that had relapsed exhibited central nervous system involvement originating from the arachnoid membrane.

A two-drug combination of Cytoxan and 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea was found not only to prevent meningeal leukemia development but also to result in "curing" all animals from their leukemia. This observation was based on a complete clinical, hematological, and histopathological "remission" period up to 176 days. The administration of 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea alone was observed not only to control the systemic leukemia but also to prevent central nervous system involvement. No relapses occurred after the first "remission" period was achieved in the groups of animals that received 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea.

INTRODUCTION

The L2C strain of guinea pig leukemia first described by Congdon and Lorenzy (4) exhibits a striking hematological and pathological similarity to acute lymphoblastic leukemia occurring in man (4, 10, 15, 16). Perk et al. (17) recently reported meningeal leukemia development in the L2C guinea pig leukemia model and described its similarity to meningeal leukemia observed in childhood leukemia. Of particular interest was that meningeal leukemia developed only after the life-span of the animals was extended as a result of a chemotherapeutically induced remission. Children with prolonged drug-induced bone marrow remissions frequently develop leukemic involvement of the central nervous system. In addition, once meningeal involvement occurs in humans with acute lymphoblastic leukemia, the disease is more difficult to control even with the present aggressive chemotherapeutic and radiotherapeutic regimens (1, 3, 7, 12). The occurrence of meningeal leukemia following chemotherapy in our previous study (17) prompted us to use the guinea pig leukemia system to evaluate other clinically active drugs in controlling both the systemic leukemia and the development of central nervous system leukemia.

MATERIALS AND METHODS

Tumor. The L2C strain of guinea pig leukemia (10) was kindly supplied by Dr. Ludwik Gross. The transplantable leukemia has been maintained in our laboratory for over 33 passages by s.c. inoculation into the inguinal area of a homogenous tumor cell suspension passed every 10 to 12 days in inbred strain 2 male guinea pigs.

Guinea Pigs. Young male strain 2-N-guinea pigs, approximately 4 weeks old, were obtained through the Frederick Cancer Research Center Animal Farm, Frederick, Md. The animals were housed in stainless steel cages and fed Wayne guinea pig chow and cabbage daily with water ad libitum. All animals were aged until they weighed 300 to 500 g before use in experimentation.

Drugs. All drugs utilized in this study were kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Md. Cytoxan (cyclophosphamide), vincristine, and prednisolone were dissolved in 0.9% NaCl solution (pH 7.0). MTX was dissolved in 0.2% NaHCO₃. 6MP was suspended in 2% NaHCO₃. 6MP was added dropwise until a solution was attained. HCl, 1 N, was then added dropwise until 1 drop caused precipitation. One or 2 drops of 1 N NaOH were then added to dissolve the precipitate, and then the drug solution was made up to a final volume of 10 ml with water (pH 9 to 9.5). All drugs except prednisolone were administered i.p. in a constant volume of 0.001 ml/g of body weight. Prednisolone was injected s.c. in the axillary area at 0.001 ml/g of body weight. MeCCNU was solubilized in an ethanol-Emulphor vehicle and then further diluted in 0.9% NaCl solution as previously described (6). MeCCNU was then added s.c. in the axillary area at 0.001 ml/g of body weight. MeCCNU was then added s.c. in the axillary area at 0.001 ml/g of body weight.

The abbreviations used are: MTX, methotrexate; 6MP, 6-mercaptopurine; MeCCNU, 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea.

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Pathology. At specified time intervals during the course of the disease, at time of death or at the termination of experiments, samples of organs and tumors were taken for histology. The excised tissues were fixed in neutral formalin and routinely stained with hematoxylin and eosin. In addition, WBC and peripheral blood smears were prepared at different times during the course of the disease. The blood smears were stained with Wright's stain.

Electron Microscopy. Representative samples of the local s.c. tumor, brain, lungs, thymus, lymph nodes, spleen, liver, kidneys, adrenal gland, and pancreas were fixed in 3% glutaraldehyde followed by 1% chrome osmium (5). The tissues were dehydrated in ethyl alcohol and embedded in a mixture of Epon 816 and Araldite (14). Sections were cut on an LKB ultratome and stained with uranyl acetate and then with lead citrate (9, 19). Electron micrographs were taken with an Elmiskop 101 microscope.

RESULTS

Table 1 shows the results of a series of experiments in which 5 drugs were tested to ascertain their therapeutic effects against the L2C guinea pig leukemia. Adult guinea pigs were inoculated s.c. in the inguinal area with approximately 1.0 x 10⁶ tumor cells on Day 0. Twelve days later, when tumor size ranged between 10 to 15 mm and WBC counts were approximately 20,000/cu mm with a blast cell population of approximately 28 to 42%, individual groups of animals were treated with different drugs to observe their effect against the disease. Cytoxan and 6MP treatment provided a significant increase of life-span when compared to the control group. Treatment with vincristine, MTX, or prednisolone did not alter the course of the disease. However, testing with these drugs was limited, and it is possible that more beneficial effects could have been achieved if different treatment regimens were used. Gross signs of central nervous system leukemia (i.e., hind leg paralysis, body tremors, and lethargy) occurred in only the Cytoxan-treated groups.

Data presented in Chart 1 shows the therapeutic response achieved following a single or 2 courses of treatment with Cytoxan. Tumors grew progressively in the untreated control group with all animals succumbing to the leukemia within 15 to 21 days. Treatment with 10- or 20-mg/kg doses of Cytoxan on Day 12 resulted in regression of the established tumor within 5 to 6 days following treatment. Histological examination of brains removed from the Cytoxan-treated animals 5 days after therapy showed the presence of small clusters of leukemic cells along the walls of the superficial arachnoid veins (Fig. 1), although all other internal organs were free of leukemia.

The hematological “remission” obtained following drug therapy (Chart 1) revealed an abrupt drop in the WBC count and percentage of blast cell population in the peripheral blood within 7 days following a single Cytoxan administration of either 10 or 20 mg/kg. The effectiveness of 2 courses of Cytoxan therapy is evidenced by the extension of the hematological “remission” period. Animals that received a single injection of Cytoxan, regardless of dose, on Day 12 began to exhibit elevated WBC counts as well as increased blast cells in the peripheral blood 17 days following treatment. In contrast, a hematological “relapse” did not occur until 27 days following 2 courses of Cytoxan treatment. At this time, all animals that had received either 10- or 20-mg/kg doses of the drug had already succumbed to their disease. Following the hematological “relapse” regardless of the regimen of Cytoxan therapy, all animals exhibited central nervous system involvement with death occurring within 12 to 24 hr after the initial signs of central nervous system leukemia. Central nervous system involvement was not observed in the untreated control group.

Table 1

Response of a transplantable guinea pig leukemia to chemotherapy

<table>
<thead>
<tr>
<th>Drug given on Day 12</th>
<th>Dose (mg/kg)</th>
<th>No. of toxic deaths</th>
<th>No. of animals dead with tumor/total inoculated</th>
<th>Central nervous system involvement</th>
<th>Median survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>43/43</td>
<td>+</td>
<td>15.0</td>
</tr>
<tr>
<td>Cytoxan</td>
<td>20</td>
<td>0</td>
<td>14/14</td>
<td>+</td>
<td>36.5</td>
</tr>
<tr>
<td>Cytoxan</td>
<td>10</td>
<td>0</td>
<td>9/9</td>
<td>+</td>
<td>31.0</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.1</td>
<td>0</td>
<td>5/5</td>
<td></td>
<td>16.0</td>
</tr>
<tr>
<td>MTX</td>
<td>60</td>
<td>0</td>
<td>16/16</td>
<td></td>
<td>16.0</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>100</td>
<td>0</td>
<td>16/16</td>
<td></td>
<td>15.0</td>
</tr>
<tr>
<td>6MP</td>
<td>25</td>
<td>0</td>
<td>8/8</td>
<td></td>
<td>30.0</td>
</tr>
<tr>
<td>(daily for 5 treatments)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoxan</td>
<td>10</td>
<td>0</td>
<td>8/8</td>
<td>+</td>
<td>46.0</td>
</tr>
</tbody>
</table>

* +, clinical signs of central nervous system leukemia.
Chart 1. Drug therapy against guinea pig leukemia. Adult guinea pigs inoculated s.c. on Day 0 with approximately $1.5 \times 10^6$ tumor cells. On Day 12, a time of generalized leukemia, animals given injections of a single Cytoxan dose of either 10 or 20 mg/kg. One group of animals (20 mg/kg) received an additional treatment (10 mg/kg) of drug on Day 19. Bars, total white cell count; numbers within each bar, percentage of blast cell population on day of observation; horizontal lines, normal white cell count range (6,000 to 10,000/cu mm); number above each bar, total number of surviving animals within each group on days of observation. CNS, central nervous system; MST, median survival time.

Interestingly, in the drug-treated animals no tumor recurrence occurred at the original inoculation site. Postmortem examination revealed a typical generalized leukemia with a remarkable infiltration of tumor cells into the superficial and deep arachnoid areas of the brain and in some cases into the brain parenchyma.

Even though Cytoxan therapy resulted in (a) prolongation of survival time associated with disappearance of the tumor mass at the site of inoculation, (b) a decrease in the degree of organ involvement, and (c) a normal peripheral blood picture, it was established from histological examinations that leukemic involvement of the central nervous system occurred within 5 days after the initial course of Cytoxan treatment. Therefore, it was of interest to determine what effect MeCCNU, the high lipid solubility of which facilitates its rapid transport into cells and across the blood-brain barrier, would have on the central nervous system involvement. Adult guinea pigs were inoculated s.c. in the inguinal area with approximately $1.3 \times 10^6$ tumor cells on Day 0 followed by treatment of the leukemic animals on Day 12 with Cytoxan, 20 mg/kg. Seven days later, a time when brain involvement has already occurred, specific groups of Cytoxan-treated animals were given injections of varying doses of MeCCNU. The results of this study are presented in Chart 2. Complete tumor regression and hematological “remission” were obtained by Day 19 as evidenced by the rapid decrease in the WBC and the lack of blast cells in the peripheral blood. In contrast, 9 untreated control animals that were still alive on Day 19 exhibited an average WBC count of 90,000/cu mm and between 86 and 96% blast cells in the peripheral blood. All animals died within 12 to 21 days with a median survival time of 16.0 days. The administration of varying doses of MeCCNU on Day 19 to the animals previously treated with Cytoxan resulted in nearly 100% long-term survivors free of central nervous system involvement and generalized leukemia when the study was terminated at 176 days. The effectiveness of this 2-drug combination against this L2C guinea pig leukemia was further confirmed by the results of the histological examination. All internal
organs, bone marrow, as well as brains removed from all long-term survivors, when the study was terminated, were free of leukemic cells. On the basis of gross as well as histological examination, all animals that received the drug combination were considered "cured" of their leukemia at the time the study was terminated.

It was apparent that supportive MeCCNU treatment was effective in prolonging the period of Cytoxan-induced "remission" against this transplantable guinea pig leukemia (Chart 2). It became obvious that, if supportive MeCCNU treatment was effective in retarding or eliminating the relapse and central nervous system involvement that occurs with Cytoxan treatment, MeCCNU should also be tested alone. Adult male guinea pigs were inoculated s.c. in the inguinal area with approximately 1.0 x 10^6 tumor cells on Day 0. On Day 12 the leukemic animals were given MeCCNU, 20 mg/kg, for the primary induction of "remission." One week later, another group received a 2nd course of MeCCNU (10 mg/kg). The results of this experiment are shown in Chart 3.

Treatment with MeCCNU, 20 mg/kg, resulted in a complete hematological "remission" by Day 19 as evidenced by a rapid drop in the WBC count from approximately 20,000/cu mm (Day 12) to approximately 5,000/cu mm. Similarly, the peripheral blood was free of blast cells. In contrast, a WBC count of 90,000/cu mm and a 86 to 96% blast cell population were observed in the peripheral blood of the untreated control group. Complete tumor regression occurred in the drug-treated animals by Day 19. However, following the 2nd course of MeCCNU therapy (10 mg/kg) on Day 19, drug toxicity occurred as 4 of 7 animals died within 7 days following treatment. The toxicity was characterized by severe body weight loss and anorexia. Although drug toxicity was apparent especially in the group of animals that received 2 treatments, a dramatic therapeutic response was elicited by MeCCNU when given alone. All untreated control animals died within 16 to 21 days with a median survival time of 17.0 days. In contrast, both a clinical and hematological "remission" was maintained for a period of 176 days as a result of MeCCNU.
treatment. The survivors were considered "cures" since all the internal organs and brains were histologically examined at autopsy and found to be free of leukemic cells.

The ultrastructural appearance of the leukemic cells, whether they were obtained from the primary site of tumor inoculation or from a metastatic lesion, and the intracisternal virus-like particles contained in these cells were consistent as previously described for the L2C leukemia (8, 10, 11). Of interest in the present study was determination of the association between the intracisternal virus-like particles and the leukemic cells in the L2C leukemia at different periods of the disease, i.e., during (a) the peak period of the disease; (b) the time of drug-induced remission; and (c) the "cured" stage.

Intracisternal virus-like particles were evident in abundance in leukemic cells obtained from animals during the peak period of the disease and during the relapse period. These particles were evident in leukemic cells obtained from peripheral blood or any involved organ. However, electron microscopic examination of peripheral blood or other organs from animals in remission, or considered "cured," revealed the lack of characteristic leukemic cells and associated intracisternal virus-like particles. These observations indicate the intimate relationship of the intracisternal virus-like particles to the L2C leukemic cell.

**DISCUSSION**

Cytoxan treatment induced a primary clinical and hematological remission of this highly fatal lymphoblastic leukemia; however, characteristically, all animals relapsed with central nervous system symptoms prior to death, a response not observed in the untreated controls. It is quite probable that the central nervous system involvement was not observed in the untreated controls due to the very rapid death of the tumor-bearing animals from generalized leukemia; thus there was inadequate time for progression to the brain to occur.

Cytoxan therapy resulted in prolongation of survival time associated with disappearance of the tumor mass at the size of inoculation, a decrease in the degree of organ involvement, and a normal peripheral blood picture. Histologically, as early as 5 days after Cytoxan treatment, no intact leukemia cells could be found in the tumor or in other visceral or thoracic organs. However, in this "remission" period, central nervous system leukemia developed and followed a predictable anatomic pattern (17). Walls of the superficial arachnoid veins revealed neoplastic involvement followed by arachnoid trabecule destruction and the subsequent invasion of the cerebrospinal fluid channels by the infiltrating cells. In the advanced or terminal stages of the disease when the pia-glial membranes were disrupted infiltration of the brain parenchyma occurred. It is quite probable that the "relapse" and subsequent development of generalized leukemia evolved secondarily from the arachnoid leukemic cell clusters.

The effect of MeCCNU used in combination with Cytoxan or alone was dramatic in that all treated animals remained in "remission" for longer than 176 days without any overt signs of generalized leukemia or central nervous system involvement. MeCCNU has been evaluated in clinical trials and has also been shown to elicit a broad spectrum of antitumor activity (2, 13, 20, 21). In addition, MeCCNU has been observed to have activity against intracranial L1210 leukemia (2). In the present study, MeCCNU was administered following a primary Cytoxan-induced "remission" and more importantly at a time when tumor cells were present in the brain. Due to the total absence of central nervous system involvement in the MeCCNU-treated groups, it was apparent that this drug was capable of reaching the leukemic clusters in the arachnoids and eradicating the tumor cells. The effectiveness of this drug against the leukemia was further borne out by the number of long-term survivors obtained and apparently "cured" following a single administration of MeCCNU. It was apparent that the drug not only controlled the generalized leukemia but also prevented infiltration and/or eradicated tumor cells present in the brain.

The "barriers" for the effectiveness of chemotherapy are primarily not the "blood-brain barrier" located at the capillary-neural interface since, from a previous report (17) as well as this study, only at the terminal stages of the disease does the neural parenchyma become infiltrated. Similar observations have been reported in human cases (18). Therefore, additional "barriers" in treating arachnoid leukemia exist that are "transversed" by MeCCNU and apparently not by Cytoxan as exemplified in this study. Several possible "obstacles" may be considered, such as choroid plexus, endothelium, or cerebrospinal channels and the endothelium of meningeal vessels. The L2C leukemia model lends itself to studies concerned with role of the "blood-brain barrier" and other barriers to drug penetration and the effect on meningeal leukemia.

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