Effect of Alkylating Agents on Meningeal Leukemia L1210 Arising in Methotrexate-treated Mice

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Summary

The current studies show that by extending the survival time of leukemia L1210-inoculated mice by methotrexate (MTX) therapy, the animals develop and succumb with meningeal leukemia. Continuous therapy with MTX retarded the disease in blood and spleen but did not retard the infiltration and progressive growth occurring in the brain. Enhanced therapeutic response was achieved by combining MTX therapy with either of 2 alkylating agents, Cytoxan and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Combined MTX and Cytoxan therapy was capable of managing the leukemia of blood and spleen but was ineffective in eradicating or decreasing the leukemia that became established in the brain. Combined MTX and BCNU therapy suppressed the leukemia of blood, spleen, and brain, resulting in greater increases in survival time and a significant number of survivors. The better therapeutic response achieved with BCNU treatment is attributable to its capacity to penetrate the blood-brain barrier. The experimental results achieved with this model system provide evidence to explain the apparent increase during the last 15 years of the meningeal leukemia syndrome that has occurred in humans with acute leukemia and who have been treated with chemotherapeutic agents.

Introduction

We have previously reported that in mice inoculated s.c. with leukemia L1210, the disease rapidly spreads to many organs and tissues. Despite the fact that leukemic cells circulated through cerebral blood vessels for several days prior to the death of the animals, only a few focal leukemic cell deposits were found in the dura or arachnoid of brain. Thus, diffuse leukemic cell growth in the brain (meningeal leukemia) was not established at the time the s.c. inoculated mice died (10).

In subsequent studies we were able to develop meningeal leukemia in mice inoculated s.c. with leukemia L1210 by treatment with methotrexate (11). Both the tissue transplantation data and the pathologic findings showed that the advanced leukemia was not eradicated by the dose of methotrexate employed but in fact slowly progressed. The metastasis of the disease resulted in progressive infiltration and growth of leukemic cells in both the dura and the arachnoid of brain in every animal that was treated with methotrexate.

It was considered of interest to determine whether the diffuse meningeal leukemia developed in methotrexate-treated animals (11) would respond to 2 alkylating agents reported to be active against intracerebra1lly inoculated leukemia L1210.

Cytoxan was reported effective in mice inoculated intracerebrally with leukemia L1210 (1). The prolonged survival time achieved with Cytoxan treatment was related to the effect of the drug on leukemic cells in the systemic organs and tissues and in the dura (10). Schabel et al. (8) reported the beneficial effect achieved by treating mice inoculated intracerebrally with leukemia L1210 with 1,3-bis(2-chloroethyl)-1-nitrosourea (NSC-409962) (BCNU).

Materials and Methods

The experiments were conducted in CDBA males, 3-4 months of age, weighing 25-28 gm. Mice were inoculated s.c. in the right inguinal region with 0.1 ml of a 3% physiologic saline suspension of cells prepared from L1210-infiltrated spleens of leukemia DBA/2J stock mice. Treatment with methotrexate (MTX) was started 7 days after tumor inoculation. The tumor at the site of inoculation was estimated by palpation to be 9-11 mm in diameter on the day MTX therapy was initiated.

MTX was given in 2% sodium bicarbonate. Cytoxan and BCNU were given in 0.85% saline. A constant volume of 0.01 ml/gm body weight was injected s.c. in the scapular region. The treatment schedules are presented in the tables.

For transplant donor tissue bioassay, 0.1 ml of undiluted whole blood, 0.2 ml of a 50% concentration of spleen or brain brei prepared in physiologic saline was implanted s.c. in the right inguinal region of the recipient mice.

Results

The effect of MTX therapy on survival time and tissue transplantability is shown in Table 1. Animals inoculated with L1210 were distributed to 6 groups, each group containing no less than 15 animals. Groups 2-6 were treated with 0.75 mg of MTX/kg. Mice were sacrificed at different time intervals, always allowing 24 hr to elapse after treatment before sacrificing the animal for bioassay. Untreated controls had a median survival time (MST) of 10.0 days, while mice receiving continuous daily treatment had a MST of 26.5 days. Treatment thus produced a greater than 2-fold increase in survival time.

In the 3 different tissues examined (Table 1, Group 1) suffi-

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(10) (BALB/c × AN × DBA/2J) F1 hybrid male. The hybrid and DBA/2J mice were obtained from the NIH breeding colony.
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Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Treatment schedule (days)</th>
<th>No. of treatments</th>
<th>Median survival time (days)</th>
<th>Results of donor tissue bioassay in recipients</th>
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</thead>
<tbody>
<tr>
<td>Donors</td>
<td>Blood</td>
<td>Spleen</td>
<td>Brain</td>
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<td>11</td>
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<tr>
<td>3</td>
<td>7-14</td>
<td>8</td>
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<td>15</td>
<td>3/3 (12-13) 3/3 (7-8) 3/3 (9-10)</td>
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<td>7-18</td>
<td>12</td>
<td></td>
<td>19</td>
<td>3/3 (12-15) 3/3 (9) 3/3 (10)</td>
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<td>7-21</td>
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<td>26</td>
<td>3/3 (12-16) 3/3 (9-10) 3/3 (8-9)</td>
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</tbody>
</table>

* A sufficient number of animals was distributed to each group to allow for bioassay and holding 10 for survival time observation.

MTX (0.75 mg/kg) therapy was initiated 7 days postinoculation of L1210 and continued daily.

Animals were held for survival time in Groups 1 and 6 only.

Deaths from tumor per total number of animals inoculated (90-day observation).

Range of death in days.

Toxic leukemic cells were present in 2 of the 3 individual donor brains to transmit the disease to 2 of 3 recipient mice. Blood and spleen transplants produced tumors in all the recipient mice. Thus the leukemia was well disseminated to these 3 tissues by the 7th day after tumor inoculation. Since survival time depends upon the leukemic cell concentration of the inoculum (3, 5), the range of death of recipient mice inoculated with donor tissue is a useful parameter to judge whether leukemic cell concentration of donor tissue is increasing or decreasing. The continuous therapy with MTX did not appear to eradicate the leukemia, as evidenced by 100% transmission of the disease from the 3 tissues to recipient mice. Judged by the range of deaths, treatment appeared to retard the disease in blood and spleen but did not retard the infiltration and progressive growth occurring in the brain. The progressive growth in brain is evidenced by the shorter survival times of recipient mice receiving donor brain tissue inoculum. Thus the meningeval leukemia syndrome was developed in leukemic mice during the extended life-span resulting from MTX therapy.

Table 2 shows the results obtained in animals similarly treated with MTX; however, on the day of sacrifice for bioassay, they received a single injection of Cytoxan and were sacrificed 4 hr later. We have demonstrated that the biologic half-life of Cytoxan (1) and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) is 0.5 hr or less (2) and that of MTX is less than 24 hr (unpublished observations). Therefore, when mice were sacrificed 4 hr after treatment no significant residual drug was present in the bioassay tissue.

A single treatment with Cytoxan was sufficient to produce a 2-fold increase in MST. Continuous treatment with MTX followed by a single injection of Cytoxan resulted in greater increases in the MST of the leukemic animals. An explanation for this extension in survival time may be seen in the results obtained from donor tissue bioassay in the recipients. Cytoxan was quite effective in eradicating the leukemia in blood. The therapy was also effective in retarding the leukemic infiltration in spleen up to 26 days. At this time 100% tumor takes were obtained from spleen. Recipients inoculated with spleen from MTX-treated donors, sacrificed on Day 26, had a range of death between 9 and 10 days (Table 1, Group 6); in contrast, the recipient mice inoculated with spleen from donors treated with MTX and Cytoxan and sacrificed on Day 26 survived for a longer period, 23-31 days. This difference in MST of recipient mice inoculated with equivalent spleen concentrations indicates a resultant reduction of leukemic cells in spleen due to Cytoxan treatment. The results obtained with brain tissue bioassay were informative. Although the treatment was capable of managing the leukemia of blood and spleen, it was ineffective in eradicating or decreasing the leukemia that became established in the brain.

The results obtained in animals treated with MTX followed by a single injection of BCNU are shown in Table 3. BCNU therapy alone was quite effective in increasing survival time. Forty % of the animals in Group 2, which received only BCNU, survived for greater than 90 days and were free of local tumor. Forty % died within 25-45 days after treatment. Necropsy of the expired animals showed no evidence of local tumor; however, enlarged spleens indicative of leukemic metastasis were observed. Similar results were observed in Group 3.

It is of interest to point out that as a result of the therapy the leukemia was not transmissible to recipients (Groups 2-7). Four hr after treatment with BCNU, at which time the tissues were taken for bioassay, it appeared that as a result of the treatment the number of leukemic cells was decreased below the concentration detectable by bioassay technic. Eighty-two % of the animals in Groups 4-7 subsequently died. At necropsy the animals in Groups 4-6 were still free of local tumor but dis-


### TABLE 2

**Effect of Methotrexate (MTX) and Cytoxan Treatment on Tissue Disposition of Leukemia in s.c. Inoculated Mice**

<table>
<thead>
<tr>
<th>GROUP</th>
<th>No.</th>
<th>DONORS</th>
<th>Treatment schedule&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DAY OF DONOR BIOASSAY</th>
<th>RESULTS OF DONOR TISSUE BIOASSAY IN RECIPIENTS</th>
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<td>Cytoxan</td>
<td>Blood</td>
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</table>

<sup>a</sup> A sufficient number of animals was distributed to each group to allow for bioassay and holding 10 for survival time observation.

<sup>b</sup> MTX (0.75 mg/kg) therapy initiated 7 days postinoculation of L1210 and continued daily. Cytoxan (200 mg/kg) administered on day indicated.

<sup>c</sup> Deaths from tumor per total number of animals inoculated (90-day observation).

### TABLE 3

**Effect of MTX* and BCNU Treatment on Tissue Disposition of Leukemia in s.c. Inoculated Mice**

<table>
<thead>
<tr>
<th>GROUP No.</th>
<th>DONORS</th>
<th>Treatment schedule&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MEDIAN SURVIVAL TIME (days)</th>
<th>DAY OF DONOR BIOASSAY</th>
<th>RESULTS OF DONOR TISSUE BIOASSAY IN RECIPIENTS</th>
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<sup>a</sup> MTX, methotrexate; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.

<sup>b</sup> A sufficient number of animals was distributed to each group to allow for bioassay and holding 10 for survival time observation.

<sup>c</sup> MTX (0.75 mg/kg) therapy initiated 7 days postinoculation and continued daily. BCNU (50 mg/kg) was administered as 1 injection on the day indicated.

<sup>d</sup> Number of survivors per total number of animals at 90 days' observation.

* MTX, methotrexate; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.
played enlarged spleens. Donor animals in Group 7 had enlarged spleens and local tumors of a size ranging between 9 and 13 mm.

Discussion

As a result of extending the survival time of mice inoculated s.c. with leukemia L1210 by MTX therapy, meningeal leukemia developed in all the animals. These results confirm and extend studies previously reported (11).

Based on the results obtained from tissue bioassay, combined MTX and Cytoxan therapy apparently destroyed all the leukemic cells in blood and, to some extent, in spleen. Leukemic cell growth was retarded in brain for a period of time; however, growth progressed rapidly, indicating that the drug did not penetrate in sufficient quantity to completely inhibit cell replication. Progressive growth of leukemic cells in the subarachnoidal and perivascular spaces was reported to occur in the brain of animals receiving daily s.c. doses of Cytoxan (10). The increased therapeutic effectiveness of this drug combination in the present study is considered to be largely attributable to Cytoxan. Eckhardt et al. (4), from their studies on the transplantability of L1210 from blood of Cytoxan-treated mice, reported that 1 hr after Cytoxan treatment, a significant decrease in the number of malignant cells in the peripheral blood occurred which was followed by the complete or partial disappearance of such cells for a period of 96 hr. Similar changes were observed in the cell content of bone marrow and spleen. We (1) previously demonstrated that the leukemia could not be transmitted from donor blood or brain as late as 7 days after a single treatment with Cytoxan.

The results attained with the combined MTX and BCNU therapy are of particular interest. This was the only drug combination which resulted in survival times of greater than 90 days and which apparently eradicated the leukemia. At 106 days the survivors were again challenged s.c. with L1210. Tumor growth and subsequent death ensued as a result of the challenge. Based on the bioassay results, it would appear that the tissues examined in the animals in Groups 4–7 (Table 3) were free of the disease at the time of tissue bioassay; however, 82% of the animals eventually succumbed with leukemia. A logical explanation is that a few residual cells in tissues, other than those tested, may have been unaffected by the drug and served as a reservoir for reinfection. We have previously reported that leukemic cell infiltration occurs in retroorbital tissues, olfactory bulb tissue, and mucous membranes of the nasal cavity (3).

The combination of MTX and BCNU appears to offer a better therapeutic advantage than employing either drug alone. Venditti et al. (12) reported the relative effectiveness of 1 injection of BCNU and daily MTX treatment, separately and combined, against advanced leukemia L1210.

The results of these experiments furnish evidence to explain the failure of the chemotherapeutic control of leukemia once it becomes established in the brain. A number of authors (7, 9, 13) have speculated that the increase in meningeal leukemia was due to a combination of 2 factors: (a) the increase in survival time of acute leukemia patients as a result of drug therapy, thereby allowing leukemic cell infiltration into the brain; and (b) the inability of most systemically administered chemotherapeutic agents to penetrate the "blood-brain" barrier to control leukemic metastasis in the brain.

The experimental animal model system employed in these studies fulfilled these 2 conditions. MTX therapy initiated after hematogenous dissemination of L1210 to the brain caused some abatement of the disease and prolonged the survival time of the animals. The bioassay data showed that the leukemia was not eradicated by the dose of MTX employed but in fact slowly progressed. Also progressive infiltration and growth of leukemic cells in brain occurred in every animal that was treated with MTX. Results of previous studies (11) showed that the progressive infiltration and growth of leukemic cells in the brain occurred in the dura and arachnoid. The clinical syndrome of meningeal leukemia has been shown to be closely correlated with the amount of leukemic cell infiltration in the arachnoid and subarachnoid spaces (6).

Of the 2 alkylating agents tested Cytoxan was of limited therapeuetic advantage in controlling meningeal leukemia. It was found to be capable of penetrating only to limited anatomic sites of the brain (10). BCNU was the most effective in penetrating the blood-brain barrier and suppressing the leukemia cells which infiltrated to sites of the brain unattainable by Cytoxan therapy.

References

10. Thomas, L. B., Chirigos, M. A., Humphreys, S. R., and Goldin, A. Pathology of the Spread of L1210 Leukemia in the Central
Effect of Alkylating Agents on Meningeal Leukemia L1210 Arising in Methotrexate-treated Mice

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