The Effect of the Sequence of Administration of Cytoxan and Methotrexate on the Life-span of L1210 Leukemic Mice

Marc J. Straus, Nathan Mantel, and Abraham Goldin

Drug Research and Development, Chemotherapy [M. J. S., A. G.], and Biometry Branch, National Cancer Institute [N. M.], Bethesda, Maryland 20014

SUMMARY

The purpose of this study was to determine whether the sequence of administration of two chemotherapeutic agents, cytoxan (Cyt) and methotrexate (MTX), would alter antileukemic effectiveness. CDF1 mice were inoculated i.p. with 10^5 L1210 ascites tumor cells. In one experiment, mice were treated with one dose of MTX 2 days after tumor inoculation (Day 2) and one dose of Cyt on Day 4 or 6.

For each group of mice in this study there was a corresponding group that received the same doses of the drugs but for which the order of administration was reversed; i.e., Cyt was given on Day 2 and MTX was given on Day 4 or 6. When the doses of Cyt and MTX were 80 and 30 mg/kg, respectively, significantly longer survivals were noted when Cyt was administered prior to MTX.

In other experiments, MTX was administered in a series of doses at 2- or 4-day intervals to L1210 mice. When a high dose of Cyt (120 mg/kg) was substituted for a regularly scheduled dose of MTX, the earlier the substitution, the longer was the survival. No effect of replacement was noted when Cyt (40 mg/kg) was substituted for MTX. When a higher dose of MTX (36 mg/kg) replaced a dose of MTX (4 mg/kg), earlier substitution appeared to increase survival.

The advantage to therapy of a high priming dose may reflect a prior observation that the percentage kill of tumor cells of cell cycle stage-specific agents such as MTX is greater for smaller populations of tumor cells. Early administration of a high priming dose of Cyt or MTX apparently lowers the tumor cell population so that scheduled doses of MTX can kill tumor cells more effectively.

INTRODUCTION

The trend of protocol designs of cooperative cancer chemotherapy groups has been in the direction of utilization of greater numbers of drugs in combination and in the use of more complex schedules. In acute lymphocytic leukemia there has been a higher frequency of remission induction with combinations of drugs than with single agents (11, 14). Similarly, in Class III and IV Hodgkin's disease, combinations of agents have increased the frequency of remission (2). Many studies have included the use of 4 or more agents (5, 11, 16). Clinically, it has not been established whether combinations of drugs are best given together or sequentially (14). Sequential studies have generally involved either cyclic chemotherapy, in which the drugs are rotated, or the utilization of a drug until there is clinical deterioration, followed by administration of the next agent. Neither system has provided a definitive advantage in therapy (1, 3-5, 14).

Experimental models for the study of synergistic relationships of combinations of 2 drugs have been used in a number of laboratories (8-10, 19, 23, 24). More recently, sophisticated designs for seeking enhanced chemotherapeutic effect with combinations of 3 drugs (13) and for sequential chemotherapy (15, 21-23) have been devised. There have also been studies to determine whether it is possible to identify combinations of drugs that will be synergistic and which combinations may be effective in remission induction or remission maintenance (11). However, there have been few systematic studies designed to determine the optimal sequence in sequential chemotherapy.

On the basis of experimental results obtained by Laster et al. (17), it was suggested (17, 18) that the utilization of an alkylating agent prior to an antimetabolite would result in increased chemotherapeutic effect. Goldin et al. (11) have suggested the use of a reciprocal design (Drug A -> B, Drug B -> A), as one means for determining the influence of drug scheduling on the effectiveness of drugs in sequential therapy. Straus et al. (20) showed that the use of priming doses of MTX resulted in increased survival of mice with leukemia L1210.

The study reported here was designed to determine whether the sequence in which effective chemotherapeutic agents are given may alter the life-span of L1210 leukemic mice and whether the specific dose levels of the drugs play an important role in determining an optimal sequence. Specifically, we proposed to determine whether the time of administration and dose of the alkylating agent Cyt relative to the time of administration and dose of the antimetabolite MTX would alter therapeutic effectiveness. It was considered that the results of such a laboratory study might provide insight into the kinetics of drug action which in turn might enable clinicians to plan more effective sequences when administering more than 1 drug.

MATERIALS AND METHODS

Stock tumor of lymphoid leukemia L1210 was carried i.p. in DBA/2 male mice. Ascites tumor cells were counted in a

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1 The abbreviations used are: Cyt, cytoxan; MTX, methotrexate; MST, mean survival time; CCSN, cell cycle stage nonspecific; CCSS, cell cycle stage specific.
hemocytometer and diluted in Hanks' balanced salt solution to 1 X 10^6 cells/ml, and the suspension was kept in an ice bath. Tumor cell suspensions (1 X 10^5; 0.1 ml) were injected i.p. into CDF_1 (BALB/cAnCr X DBA/2Cr) F_1 male mice, weighing 18 to 25 g. Mice were randomized and housed in plastic cages in a constant temperature facility and provided with water and laboratory chow ad libitum.

MTX (amethopterin; NSC 740) was prepared every 4 days from Lot No. 1260 X 8105 from Lederle Laboratories, Pearl River, N. Y., and was refrigerated in an opaque jar. It was dissolved in 2% sodium bicarbonate and injected i.p. Cyt (cyclophosphamide; Mead Johnson Laboratories, Evansville, Ind., Lot No. 2, man 121; NSC 26271) was freshly prepared at each injection period. It was dissolved in 0.85% NaCl solution and injected i.p. Both drugs were administered in a volume of 0.01 ml/g body weight.

RESULTS

Cross-over Experiment Involving a Single Treatment with MTX and Cyt in L1210. Mice were inoculated with 10^8 L1210 ascites cells on Day 0. The MST of untreated control mice was 8.0 days. Mice in Groups 1 and 2 were treated with MTX, 3 mg/kg, on Day 2. Group 1 then received Cyt, 40 mg/kg, on Day 4, and Group 2 received Cyt, 40 mg/kg, on Day 6. The MST of these groups were 10.6 and 11.1 days, respectively (Table 1). Two other groups received the same doses of the drugs, but the order of administration was reversed ("cross-over" Groups 1 and 2), i.e., Cyt was given on Day 2 and the MTX was given on Day 4 or 6. The MST's of the initial and cross-over groups were similar. When MTX, 30 mg/kg, was administered to mice on Day 2 and Cyt, 40 mg/kg, was administered on Day 4 or 6, the MST's were also similar to those of mice treated on the respective cross-over schedules (Groups 3 and 4).

The above schedules were repeated, with 80 in place of 40 mg of Cyt per kg. When MTX, 3 mg/kg, was administered to mice on Day 2, followed by Cyt, 80 mg/kg, on Day 4 or 6 (Groups 5 and 6), the MST's of these groups were similar to those of their respective cross-over groups.

Both groups of mice that had received MTX, 30 mg/kg, on Day 2 and Cyt, 80 mg/kg, on Day 4 or 6 had MST's of 16.4 days (Groups 7 and 8). The corresponding groups of mice on cross-over schedules had MST's that were significantly longer. Mice that received injections of a single dose of Cyt, 40 mg/kg, on Day 2, 4, or 6 showed no significant difference in MST. Mice receiving Cyt, 80 mg/kg, on Day 2, 4, or 6 also had constant MST's, as did mice receiving MTX, 3 or 30 mg/kg, on either of those days. Similar schedules with higher doses of Cyt in combination with MTX resulted in frequent toxic deaths.

Sequential Chemotherapy Experiments Involving a Single Modification in the Regularly Scheduled Treatment of L1210. The data of 3 experiments are summarized in the charts: 1 in Charts 1, 2, 6, and 7; 1 in Chart 4; and 1 in Charts 3, 5, and 8.

In Chart 1, the basic group of mice received MTX (3 mg/kg) every other day from Day 2 to 16 after leukemic inoculation. In the other groups of mice, Cyt was substituted for MTX on the day indicated. Tumor-bearing control mice died on Day 8. A single dose of Cyt, 120 mg/kg, administered on Day 2 increased the MST to 14.2 days. MTX, 3 mg/kg, administered on Day 2 increased the MST to 8.9 days. When MTX was administered every other day, from Day 2 to 16, the MST was 15.8 days.

Of the groups of mice scheduled to receive MTX, 3 mg/kg, every other day from Day 2 to 16, the group with the earliest Cyt substitution, 120 mg/kg (Day 2) survived the longest; MST, 23.4 days. There was 1 mouse "cured" in this group of 20 mice, which was counted as a 60-day survivor. The group with the next earliest Cyt substitution (Day 4) survived 2nd longest; MST, 21.7 days. The MST of mice scheduled to receive Cyt on Day 16 was 16.4 days, which approximates the 15.8-day MST of mice that received MTX every other day. The earlier the substitution was made, the longer the mice lived. This is indicated by a plot of MST's of all of the groups of mice (Chart 1).

When a dose of Cyt, 40 mg/kg, was similarly substituted for MTX (Chart 2), the group of mice receiving Cyt substitution earliest had an MST of 17.6 days. This was not significantly longer than the MST of the last group, 16.8 days, which had received Cyt on Day 16. The MST of mice that received a single dose of Cyt on Day 2 was about 1 day longer than that of the mice receiving a single dose of MTX. A plot of MST's suggests a possible advantage in earlier Cyt substitution.

When Cyt (120 mg/kg) was substituted for a higher dose of MTX (4 mg/kg; Chart 3) the advantage in substituting Cyt

<table>
<thead>
<tr>
<th>Injection of MTX or Cyt (mg/kg)</th>
<th>Survival time</th>
<th>Cross-over survival time</th>
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<tr>
<td>Mouse group</td>
<td>Day 2</td>
<td>Day 4</td>
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<tr>
<td>1 MTX 3 Cyt 40</td>
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<td>3 MTX 30 Cyt 40</td>
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<td>5 MTX 3 Cyt 80</td>
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</tr>
<tr>
<td>6 MTX 3 Cyt 80</td>
<td>16.3</td>
<td>0.4</td>
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<td>7 MTX 30 Cyt 80</td>
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<tr>
<td>9 MTX 30 Cyt 80</td>
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<tr>
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<tr>
<td>20 Cyt 80</td>
<td>16.0</td>
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</tr>
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</table>

a Mean ± S.E.
b The order of injection of the drugs was reversed. Example: in Group 1, Cyt, 40 mg/kg, was administered on Day 2 and MTX, 3 mg/kg, on Day 4.
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Chart 1. Sequential chemotherapy experiments involving a single modification in the regularly scheduled MTX treatment of L1210 leukemic mice. CDF, male mice, 18 to 25 g, were inoculated with $10^5$ L1210 ascites tumor cells i.p. on Day 0. Treatment with drugs was i.p. $\bullet$, the individual survival time of mice; $\circ$, MST of a group of mice. This legend applies also for Charts 2 to 8. q2d, every 2nd day, q4d, every 4th day.

early was more evident. The MST of the group that received the Cyt substitution on Day 2 was 25.9 days, with 1 mouse cured. Delaying the substitution to Day 4 lowered the MST 4 days, to 22.1 days. The MST of the group with a Day 16 substitution was 15.8 days. The MST of mice that received a single dose of Cyt, 120 mg/kg, was about 6 days longer than that of mice that received a single dose of MTX, 4 mg/kg.

When MTX, 36 mg/kg, was substituted at various times for MTX, 4 mg/kg, a plot of MST's suggests some advantage in earlier substitution (Chart 4). However, there was no significant difference in the MST’s of the individual groups. The MST’s of groups receiving a single dose of MTX, 36 or 4 mg/kg, differed by only 1 day.

Cyt, 120 mg/kg, was again (as in Chart 3) substituted on different days for MTX, 4 mg/kg (Chart 5). However, after the last day of substitution, each group continued to receive MTX, 4 mg/kg, every other day until death. Again, the group that received Cyt on Day 2 survived the longest; MST, 29.0 days. There was progressive decrease in MST with increasing delay in Cyt substitution. Thus, the MST of the group that received
Cyt earliest was 13.7 days longer than that of the group scheduled to receive Cyt last.

Utilizing a similar design, we increased the interval between doses to 4 days (Charts 6 to 8). When Cyt, 120 mg/kg, was substituted for MTX, 15 mg/kg, and was administered every 4th day from Day 2 to 14, there was an advantage in early substitution (Chart 6).

When the dose of Cyt was decreased to 40 mg/kg and substituted at various times for MTX, 15 mg/kg, the MST’s of the mice were not altered, regardless of the day of substitution (Chart 7). The MST of mice that received a single dose of Cyt, 40 mg/kg, on Day 2 was slightly less than the MST of mice that received MTX, 15 mg/kg, on Day 2.

When the dose of MTX was increased to 20 mg/kg (Chart 8), the MST of the group of mice receiving a dose of Cyt, 120 mg/kg, on Day 2 was 25.7 days. This was the longest survival
time observed in the 3 experiments involving treatment with MTX every 4th day. When substitution was delayed until Day 18, the MST was reduced to 12.5 days.

DISCUSSION

The current studies suggest that if one is to use 2 classes of agents in sequential chemotherapy, 1 being a CCSN agent such as Cyt and the other being a CCSS agent such as MTX, then the optimal sequence of administration of the drugs would be to give the CCSN agent first, provided that it is given in a high enough dose.

Thus, when 1 dose of Cyt and 1 dose of MTX were administered in sequence to groups of L1210 leukemic mice, the mice that received Cyt prior to administration of MTX had longer MST’s than did mice on cross-over schedules when the doses of Cyt and MTX were high. The single dose of Cyt (80
mg/kg) in this instance increased life-span more than did the single dose of MTX (30 mg/kg).

Similarly, when 1 of a series of doses of MTX was replaced by a high dose of Cyt (120 mg/kg), the earlier was the substitution, the longer was the MST (Charts 1, 3, 5, 6, and 8). This effect was noted with several dose levels of MTX and with intervals of 2 and 4 days between treatments.

When a low dose of Cyt was used for substitution which was equally effective with the single dose of MTX, no effect of replacement was noted. When a high loading dose of a CCSS agent (MTX, 36 mg/kg) was substituted for a low dose (MTX, 4 mg/kg), earlier substitution increased chemotherapeutic effectiveness.

A number of studies have suggested a rationale for improved survival with sequential schedules. Increased survival has been obtained in sequential chemotherapy studies when a priming dose of 1 drug was followed by a series of treatments with a 2nd drug (12). The principle involves the use of priming...
therapy to decrease the number of tumor cells, and this in turn permits the drug that is used sequentially to be more effective (7).

Priming dose schedules of MTX were demonstrated to increase its antileukemic effectiveness (20). In a series of experiments, it was indicated that MTX, a CCSS agent, exhibited a decreasing percentage of kill of tumor cells as the population of tumor cells increased. Kinetic considerations suggested that the priming dose of MTX may lower the tumor cell population sufficiently for subsequent doses of the drug to kill tumor cells more effectively. Schabel has suggested that, in sequential therapy, utilization of an alkylating (a CCSN agent), prior to administration of an antimitabolite (CCSS agent) would result in increased chemotherapeutic effect. Treatment with the alkylating agent would constitute priming therapy. He proposed, also, that with progressive tumor growth the sensitivity to antimitabolites decreases. Laster et al. (17) treated CA-755 tumor-bearing mice with a single dose of Cyt (CCSN agent) followed by daily doses of 6-mercaptopurine (CCSS agent) and noted a higher percentage of cures.

In the current experiments, the priming dose of Cyt or MTX apparently also lowered the tumor cell population so that the percentage of tumor cell kill of subsequent doses of MTX (CCSS agent) was increased. The earlier the priming dose was given, the greater were the number of subsequent doses of MTX that could exert increased effect by acting against a reduced tumor population. The beneficial effect of a priming dose of Cyt was also observed when it was followed by even a single dose of MTX (30 mg/kg).

In addition to the current study on the importance of the sequence of treatment with 2 drugs, Goldin (6) has published data which indicate that, when single doses of 4 drugs are administered sequentially, the order in which the drugs are given may alter the life-span of leukemic mice. The concept of using drugs in specified sequences may have relevance to planning multiple drug clinical chemotherapy designs.

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