Studies on the Management of Mouse Leukemia (L1210) with Antagonists of Folic Acid*

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The current experiments were conducted: (a) to explore the possibility of obtaining extensive survival times, by treatment with folic acid antagonists against an acute transplantable leukemia in mice; (b) to determine whether it is possible to obtain a significant number of leukemic mice which will survive as a result of antifolic therapy.

In previous studies the principle was established that the specificity of action of a drug, expressed in terms of its relative effect on host and tumor, is not static but can be altered by the manner in which the drug is employed (2-4, 6-8). Specificity of action is influenced by factors such as: (a) the time in the course of the disease at which treatment is initiated; (b) the spacing of the treatments; (c) the number of treatments; (d) the administration of a metabolite with an antagonist; (e) the age and weight of the animal. In each instance the extent of antitumor activity was dependent upon the inhibitory effect of the drug on the tumor relative to the toxic effect of the drug on the host.

Several procedures were observed to increase the antileukemic specificity of action of the folic acid antagonists when treatment was initiated up to about 5 days following tumor inoculation. It was suggested that the employment of such procedures might provide a means whereby extended antileukemic effects could be produced. The suggested procedures were: (a) the use of spaced treatment; for this purpose a 4-day spacing between treatments appeared to be an appropriate interval (4, 7); (b) the delayed administration of citrovorum factor (CF) following treatment with the antagonist (3, 7). A 24-hour delay for administration of the metabolite appeared suitable; (c) the combination of spaced treatment of antagonist plus delayed administration of CF (7); (d) an increase in the number of treatments (4, 7, 8). Various combinations of these procedures were employed in the current studies with leukemic mice.

METHODS

Experiments were conducted with the use of leukemia L1210 in CDBA hybrid male mice. Stock tumor was taken from DBA mice. In the treatment groups and in appropriate controls, a measured tumor inoculum was employed. In addition, in each experiment the tumor mash was serially diluted and inoculated into mice to determine the potency of the inoculum employed. The tumor was inoculated in the right thigh. A-methopterin and aminopterin1 were dissolved in 2 per cent sodium bicarbonate. CF,1 when employed, was dissolved in distilled water. Both the mice and the order of injection of the drugs were randomized. CF, when employed, was administered 24 hours subsequent to the antagonist. The dosage schedules are indicated with the individual experiments.

RESULTS

The results of the first experiment are summarized in Chart 1. Originally, in this experiment seventeen groups of twenty tumor-bearing (L1210) mice each received varying repeated treatments every 4 days. These included: four aminopterin groups given injections of 0.25, 0.5, 1, or 2 mg/kg aminopterin/treatment; four A-methopterin groups given injections of 5, 10, 20, or 40 mg/kg A-methopterin/treatment; four aminopterin plus delayed CF, given injections of 2.5, 5, 10, or 20 mg/kg aminopterin/treatment; four groups receiving aminopterin plus delayed CF, given injections of 0.25, 0.5, 1, or 2 mg/kg aminopterin/treatment; and five A-methopterin plus delayed CF groups given injections of 50, 100, 250, 500, or 1,000 mg/kg A-methopterin/treatment. Treatment was

1 A-methopterin, aminopterin, and citrovorum factor were kindly provided by Dr. James Smith of the Calco Chemical Division of the American Cyanamid Co., Bound Brook, N.J.

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CHART 1.—Survival patterns and 50 per cent survival times of leukemic mice (L1210) on various schedules of treatment with aminopterin or A-methopterin, with and without delayed administration of citrovorum factor. The citrovorum factor was administered 24 hours following each injection of antagonist. Lines are drawn connecting the percentages of mice surviving each 5 days following tumor inoculation. The tumor inoculum was $1.1 \times 10^6$ cells/mouse. In the tumor titration all ten untreated mice succumbed to tumor at an inoculum level of $1.7 \times 10^3$ cells/mouse. In this experiment the inoculum was prepared from ascites leukemia in DBA mice. The leukemic cells were inoculated intramuscularly. The drugs were administered subcutaneously in the axillary region. Treatment was initiated 2 days after inoculation of the leukemic cells.
initiated 2 days following implantation of the tumor. No specific time was set for discontinuing treatments.

Most of the control mice succumbed by the 13th day following inoculation of the leukemic cells, the final death occurring on the 15th day. It soon became evident that some of the higher doses of the drugs would rapidly kill all of the mice if continued, and these were modified in an attempt to reduce lethality. After the experiment had proceeded for 20 days, approximately 70 per cent of the treated mice were still alive; 30 per cent had either succumbed to tumor, the administered treatment being too low to control the tumor, or had died of the toxic effects of excessively high treatment. Among the 70 per cent of survivors were many mice which showed no palpable evidence of tumor at the site of inoculation of the leukemic cells. In two treatment groups (50 mg/kg A-methopterin plus delayed CF; 100 mg/kg A-methopterin plus delayed CF) not a single death had occurred. In four treatment groups (10 mg/kg A-methopterin; 20 mg/kg A-methopterin; 2.5 mg/kg aminopterin plus delayed CF; 5 mg/kg aminopterin plus delayed CF) only one or two deaths had occurred. At this point it seemed unwise to continue the treatment and take the risk of killing mice through toxicity, since the possibility existed that many of the mice would no longer succumb to the leukemia. Accordingly, the last antifolic treatment was administered on the 22nd day following inoculation of the leukemia.

With the cessation of the treatment, however, mice began to succumb rapidly. Many mice died with palpable tumors at the site of implantation. Other mice died showing toxic effects of earlier treatment. Many mice died, however, without indication of toxicity or evidence of palpable tumor at the site of implantation. In 26 out of 31 such cases, leukemia developed when the spleen was transplanted into other animals. Apparently, prolonged treatment with the folic acid antagonists may cause total inhibition of growth of the tumor at the site of implantation without completely preventing the tumor cells from invading the organs of the host. There were fourteen mice surviving 148 days after tumor implantation, all among groups receiving A-methopterin, with and without delayed administration of CF. Thirteen of these fourteen mice succumbed to leukemic growth following reinoculation with the tumor. The occurrence of these survivors is notable, when it is considered that the tumor inoculum was approximately 60 times greater than that which resulted in 100 per cent mortality in control mice not receiving antifolic therapy.

When comparisons are made at the dosage levels giving the best results, it is evident that treatment with A-methopterin was more effective than treatment with aminopterin. For both drugs the use of delayed CF resulted in the achievement of somewhat more extensive antileukemic action.

A second experiment, the results of which are summarized in Chart 2, was undertaken with A-methopterin, with and without delayed administration of CF. The plan of this experiment was to modify treatment on an individual basis for each mouse, while greatly extending the duration of treatment. Because of the individual treatment given each mouse, the experiment was limited to two groups of twenty mice each. One group received 100 mg/kg of A-methopterin plus delayed CF at each treatment. The other group received 40 mg/kg of A-methopterin at each treatment. Since only two treatment groups were employed it was not possible to compare the relative effectiveness of the treatments at optimal dose levels. The following criteria were employed to...
determine whether a mouse would receive A-methopterin treatment on any particular day following the initial treatment on the 3rd day after inoculation of the leukemia: (a) a minimum of 4 days must have elapsed since the last treatment with A-methopterin; (b) weight loss must not have exceeded 10 per cent of the initial body weight; (c) the general appearance of the mouse must be satisfactory.

The control mice were all dead by the 12th day following inoculation of the leukemic cells. In general, the 4-day interval between A-methopterin treatments was maintained. In no instance did any interval between treatments exceed 6 days. By the 65th day slightly over half of the total number of mice in the two groups had died, and no A-methopterin was administered beyond this day. Thirteen of the mice treated with A-methopterin plus delayed CF and eight of the mice treated with A-methopterin alone were dead. The number of survivors at this day was substantially greater than in the previous experiment. For eight of the mice which had died up to the 65th day without evidence of palpable tumor, spleen transplants were made to other mice to test for the presence of leukemic cells. In five instances transplantation of the spleen resulted in leukemic growth.

After treatment was discontinued, deaths continued to occur. By the 150th day there were three survivors out of the twenty mice that had been treated with A-methopterin plus delayed CF, and two survivors out of the twenty mice that had received A-methopterin alone. These mice are still alive and appear healthy 10 months after inoculation of the leukemia. The inoculum level was approximately 100 times greater than that which resulted in 100 per cent deaths in the control mice. Although in this experiment the 50 per cent survival time for A-methopterin was somewhat greater than that for A-methopterin with delayed CF, a strict comparison is not valid, since it was not determined whether the doses employed were optimal for this experiment.

In both experiments, the surviving mice showed little or no weight loss during the course of the experiment. Maximum weight loss in these mice was never more than 15 per cent of the initial body weight.

In several instances tumors from animals that had been treated with A-methopterin for an extended period of time were tested for resistance to the antagonist. The results of such a test are summarized in Table 1. The tumor cells came from the spleen of an animal that had been on intermittent A-methopterin treatment (40 mg/kg at each injection interval) from the 3rd through the 61st day following inoculation of the tumor (total dose, 560 mg/kg). It died on the 67th day without evidence of a local tumor at the site of implantation. It may be noted (Table 1) that the tumor retained a marked degree of sensitivity to treatment with A-methopterin, although the survival times were somewhat less than those of the sensitive strain of tumor. Similar results were obtained when the tumor material came from the local tumor at the site of implantation.

**DISCUSSION**

The experiments reported here show that it is possible, when appropriate treatment procedures are initiated several days following the inoculation of leukemia into mice, to obtain extended survival time by administration of an antimetabolite or by the employment of a metabolite in conjunction with an antimetabolite. Also, it is possible to obtain a significant number of leukemic mice which survive as the result of such treatments.

With all the known antileukemic agents the toxicity to the host is an important limiting factor in chemotherapy. This is apparently true for extended multiple treatment schedules, as well as for single treatment or multiple treatment schedules of limited duration. If the dose is too high the animals succumb primarily to drug toxicity. If the dose is too low the animals succumb primarily to tumor growth. The extent of the maximum antileukemic effect is dependent upon the extent to which the treatment may exert and maintain an inhibitory advantage with respect to the tumor, at minimum cost in lethality to the host.

### Table 1

<table>
<thead>
<tr>
<th>Total Dose (mg/kg)</th>
<th>Survival time ± S.E.</th>
<th>Strain for resistance</th>
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<tbody>
<tr>
<td></td>
<td>Sensitive leukemia</td>
<td>Strain tested</td>
</tr>
<tr>
<td>2 40</td>
<td>25.1 ± 2.3</td>
<td>23.1 ± 0.8</td>
</tr>
<tr>
<td>1 44</td>
<td>22.8 ± 1.8</td>
<td>22.6 ± 0.7</td>
</tr>
<tr>
<td>9 6</td>
<td>22.2 ± 0.6</td>
<td>20.9 ± 0.8</td>
</tr>
</tbody>
</table>

*Two mice per experimental group.*

The experimental inoculum for both strains of leukemia was 1.08 X 10⁶ cells. The drug was administered intraperitoneally on days 3 and 7 following leukemia inoculation. The leukemia tested for resistance was carried intramuscularly for several generations prior to the setting up of this experiment.
When treatment is initiated relatively early following tumor implantation, the procedures employed here tend to increase the inhibitory effect with respect to the tumor, relative to the toxicity to the host. The 4-day interval between treatments, employed alone or with delayed administration of CF, apparently permits the animal time to recover at least partially from the toxic effects of the antagonist, while the tumor is not able to do this so extensively. This permits the employment of a relatively high dose of A-methopterin during the course of therapy and permits treatment with such a dose for an extended period of time.

A possible limiting factor in the treatment of neoplasia may be the development of resistant or dependent variant forms of tumor cells (1, 9, 11). Combination drug chemotherapy has been employed in an attempt to overcome the selection of any resistant or dependent variants (10). The extensive survival times observed here, accompanied by a significant number of total recoveries from the leukemic implant on treatment with a single antagonist only, suggest that problems such as the appearance of a large population of resistant or dependent variants may not enter as a major factor until possibly quite late in the course of treatment. This is supported by the observation that the leukemic cells of several of the animals that survived under treatment for an extended period of time were tested and showed little or no evidence of resistance.

It would appear from these experiments that the eventual cause of death of leukemic mice which survived for an extended period of time, rather than being attributable specifically to the development of marked resistance of the tumor cells to treatment, is governed by the various factors which may influence the host-tumor-drug relationship. The eventual death of leukemic mice may be associated with: (a) the slow but persistent growth of the tumor in the face of treatment. This could occur where the treatment was not sufficiently effective to keep the tumor in check. Despite any set-backs to the tumor from treatment, the tumor may still grow progressively, infiltrate the host, and deplete the host of essential stores of metabolite (12). This may give the appearance of a developing tumor cell resistance to treatment. In earlier studies it was observed that, although a single dose of aminopterin extended the survival time of leukemic mice when administered several days following tumor implantation, it was ineffective when administered several days prior to the time of death controls (4). This diminution of the effectiveness of aminopterin against an advanced tumor, while giving the appearance of tumor cell resistance, could, instead, be related to increased number of tumor cells and decreased tolerance of the host; (b) the gradual development of toxic effects to the mouse, when treatment damage to the mouse is just greater than the ability of the mouse to recover. If the mouse eventually dies of cumulative toxic effects, but the cause of death is not recognized as such, this, too, may be mistakenly taken as evidence for developing tumor resistance; (c) both of the above phenomena could be occurring simultaneously. With present procedures, the distinction between a safe treatment for the mouse and an adequate treatment for the tumor was narrow, but nevertheless sufficient to permit extended survival times and the occurrence of survivors from leukemia. In such a situation, small changes in factors such as the degree of sensitivity of the tumor cells, tolerance of the host, metabolite levels, rate of growth of tumor and host, etc., could influence the effectiveness of treatment. If, with respect to a drug, tolerance of the host to treatment developed proportionately to the appearance of resistant tumor cells, it would be merely necessary to increase the dose level of the drug to maintain a therapeutic effect. Since the host-tumor relationship may change during the course of the disease (4), it is considered likely that the optimal schedule of treatment, the appropriate dose of drug, the time of administration of a metabolite with an antimetabolite, and the choice of drug all could be altered by the status of the animal and tumor at any particular time during the course of the disease. Preliminary studies indicate that, when treatment with A-methopterin is begun late in the course of the disease, a shorter interval between treatments may be more effective and, further, that the optimal level of treatment may be considerably reduced. The current studies emphasize the desirability, in extended treatment, of studies of the influences of such factors on the host-tumor relationship in chemotherapy.

The host-tumor-drug relationship should also be taken into consideration in treatment with combinations of drugs. It may not be necessary for a combination treatment to overcome resistant or dependent variant tumor cells in order to give additive or synergistic antitumor effects. For a drug combination to be more effective than the individual drugs it may merely be necessary that the drug combination have a proportionately greater effect against the tumor than against the host. In an experiment conducted in this laboratory, in which combination treatment with A-
methopterin and 6-mercaptopurine was employed, the advantage resulting from combination treatment with respect to the tumor was canceled out by the increased toxicity of the combination treatment for the host (5). However, the possibility exists that, either with this combination of drugs on some other schedules of treatment or with combinations of other drugs, the combination treatment will result in a more extensive effect with respect to inhibition of tumor growth as compared with the toxicity of the combination for the host. In the investigation of tumor chemotherapy it would be desirable to obtain such a combination treatment. Once such a combination treatment has been obtained, it would then be possible to study its influence: on tumor and host; on the possible emergence and significance, in treatment, of resistant and dependent variants; and on phenomena such as sequential and concurrent blocking of enzymatic systems (13–16) with respect to both the tumor and the host.

SUMMARY

By the employment of appropriate procedures of administration of folic acid antagonists, with or without delayed administration of CF, it was possible, when treatment was initiated several days following implantation, to demonstrate extended survival time of leukemic mice. It was also possible to obtain a significant number of survivors of leukemia. The inoculum level was well above the level which gave 100 per cent takes in untreated animals, and the occurrence of survivors of leukemia apparently resulted from the treatment. Leukemic cells from mice that succumbed after an extensive period of treatment with A-methopterin showed little or no evidence of resistance. The efficacy of treatment on extended multiple treatment schedules, as was formerly observed on limited treatment schedules, appears to be governed primarily by the host-tumor relationship. Factors which may govern this relationship are discussed.

REFERENCES
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