The Effect of Dose Interval on the Survival of L1210 Leukemic Mice Treated with DNA Synthesis Inhibitors

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SUMMARY

The survival of L1210 leukemic C57BL/6 X DBA2 F1 mice has been studied as a function of the time interval between two i.p. doses of DNA synthesis inhibitors (1-β-D-arabinofuranosylcytosine, hydroxyurea, sodium camptothecin, and 5-hydroxy-2-formylpyridine thiosemicarbazone). In general, life-span increases that were observed with single doses of these agents (i.e., a time interval of zero) were consistent with the cell kill expected (approximately 66 to 86%) for an S-phase-specific agent with a short effective contact time. The therapeutic effect appeared to be a function of the interval between the two doses. When optimal time intervals between two doses were used, cell kills in excess of 99% were achieved. In some cases, enhanced therapeutic ratios (increase in life-span compared to host weight loss) were achieved by judicious choice of the dose interval. It was possible in most cases to correlate the optimal interval for maximal cell kill with the time required for the maximal recovery of the ability of the peritoneal ascites L1210 cells to incorporate thymidine into DNA after the initial dose of the agent. The results are discussed in terms of a simple model based on cell-cycle kinetic parameters.

INTRODUCTION

The choice of treatment schedules (i.e., the temporal relationship between successive doses) can greatly influence the results of chemotherapy of neoplastic disease in experimental animals. The work of Skipper et al. (19) and Kline et al. (9) with ara-C in the treatment of LI210 leukemic mice represents a striking example of this.

With L1210 leukemia in mice, the S (DNA-synthetic) phase comprises over 50% of the total cell-cycle time (19, 21, 22). Provided that cell-cycle time and phase lengths are homogeneous, it should be possible (at least theoretically) to totally eradicate the entire LI210 cell population with only 2 optimally spaced doses of an S-phase-specific agent (i.e., an agent that kills cells only when the cells are exposed to it during the DNA-synthetic phase of the cell cycle). An initial exposure to the agent should kill the S-phase cells of the asynchronous population. One could then wait an appropriate time until the partially “synchronized” surviving cells (i.e., those in the G1, G2, and M phases during the initial exposure) had entered S phase and then “complete” the cell kill with a 2nd exposure. The studies reported below represent in part an attempt to test such a hypothesis.

In L1210 leukemic mice, the optimal interval for the 2-dose administration of an S-phase-specific agent can, of course, be determined empirically. However, we also investigated the possibility that changes in a pertinent biochemical parameter (i.e., total tumor-cell DNA synthesis) might correlate with this optimal dose interval. Such a correlation would prove of value in predicting (prior to therapy) the optimal interval for drug administration and might have important implications with respect to treatment of human neoplasia.

Analogous studies with LI210 cells growing in culture have been performed, and those results are presented in a companion paper (3).

MATERIALS AND METHODS

Agents Used

ara-C (Cytosar) was supplied by The Upjohn Company, Kalamazoo, Mich.; HU, 5-HP, and camptothecin were obtained from Drug Research and Development, National Cancer Institute, Bethesda, Md.; and TdR-3H (2.2 Ci/m mole) was obtained from New England Nuclear, Boston, Mass. Injection solutions of ara-C, HU, and camptothecin were prepared in 0.9% NaCl solution. We prepared injection solutions of 5-HP by first dissolving the agent in NaOH and then adjusting the pH to 10.0 with HCl.

Survival and Toxicity Studies

The parent line of L1210 cells was obtained from Mr. I. Wodinsky (Arthur D. Little, Inc., Cambridge, Mass.) and was maintained by weekly passage (i.e., inoculation) of ascites cells in female DBA/2 mice (The Jackson Memorial Laboratories, Bar Harbor, Maine). Therapeutic studies were conducted in female C57BL/6 X DBA/2 F1 (hereafter called BDF1) mice...
from the same source, weighing 19 ± 2 g. Mice were inoculated on Day 0 with 1 x 10^6 L1210 cells per mouse. All of the agents were administered i.p. Therapy was initiated 48 hr later, on Day 2. The interval between doses was varied from 0 to 12 hr (or from 0 to 24 hr) in 1- or 2-hr increments. Eight or 10 mice per group were used. Mice were observed and their deaths were recorded twice daily. Mean survivals ± S.D. were calculated. Animals were weighed on Day 1 and again on Day 5, and average weight changes (g/mouse) were determined. The approximate percentage cell kill achieved was calculated from survival data, by the method of Schabel (17). Experiments were terminated after 45 days, and 45-day survivors were considered cured.

Recovery of DNA Synthesis.

BDF1 mice (same sex and weight as those used in therapy studies) were inoculated i.p. on Day 0 with 1 x 10^6 L1210 cells/mouse. On Day 3 (when the total tumor load was approximately 5 x 10^7 cells/mouse), mice were dosed i.p. with drug and, at various times thereafter, TdR-3H (10 µCi/mouse, 2.2 Ci/mmole) was administered (0.5 ml volume). Four to 6 mice/group were used. In addition, control mice (not treated with drug) were dosed with the same amount of TdR-3H. Control groups (2 or 3 groups, at different times during the experiment) contained 8 to 12 mice/group. Thirty min after the administration of TdR-3H, mice were sacrificed by cervical dislocation. Heparinized 0.9% NaCl solution (2.5 ml) was injected into the peritoneal cavity. This cavity was massaged, and approximately 1.5 to 2.0 ml of ascites fluid were withdrawn and added immediately to 0.1 ml of nonradioactive thymidine (10 mg/ml) in 0.9% NaCl solution. A 1:400 dilution was prepared and used for electronic particle counting (Coulter counter; Coulter Electronics, Hialeah, Fla.), and incorporation into DNA was determined by 1 of the following methods.

Test Tube Method. Cells were sedimented by centrifugation; macromolecules were then precipitated with 5 ml cold 10% trichloroacetic acid. Two washes with 5 ml of trichloroacetic acid were performed, followed by 2 washes (5 ml each), 1 with ethanol:ether (3:1) and 1 with ether. After air drying, the DNA was extracted from the cell pellet with 1 ml of 0.5 N PCA at 70°C for 20 min. After cooling, the suspension was centrifuged, and the radioactivity of 0.1-ml aliquots of the PCA supernatant was measured by scintillation techniques, with a Packard Tri-Carb Model 574 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.).

Millipore Disc Technique. Two 0.5-ml portions of ascites cell suspensions were pipetted onto Millipore discs (0.45-µm pore size; 25-mm diameter) on a filter apparatus. The liquid was removed by suction filtration. Discs were washed 3 times with 4-ml portions of 0.2 N cold PCA and once with absolute ethanol. The discs were then placed in scintillation-counting vials, 0.5 ml of 0.5 N PCA was added, and DNA was extracted by heating at 70°C for 20 min. Fifteen ml of Diotol were then added, and radioactivity was determined as described above.

Specific incorporation (cpm/10^6 cells) was determined. Incorporation rates (DNA synthesis) were compared to those observed in control animals and are expressed as percentage of control value.
interval of 9 hr, a mean survival of 12.7 ± 0.8 days (corresponding to >99.5% cell kill) was achieved. However, an interval of 12 hr was considerably less effective (mean survival, 10.3 days), and there appeared to be some indication of a 2nd optimal interval at approximately 16 hr.

Although the optimal therapeutic interval for 2 doses of ara-C was approximately 8 to 10 hr, weight loss was also particularly severe at this interval. It appeared that, with some intervals, the therapeutic index was more favorable. For example, at an interval of 3 hr, although weight loss was approximately equal to that with 2 simultaneous doses, the therapeutic effect was somewhat enhanced. Apparently, judicious choice of dose interval can result in an increased therapeutic index.

Further evidence that the toxicity of ara-C is dependent on dose interval is shown by the data in Table 1. Although a total dose of 4000 mg/kg i.v. (in 2 equal doses) could be administered without lethality to male albino mice if the interval between the doses was less than approximately 4.5 hr, 2 doses of 400 mg/kg, with an interval of 9 hr between them, resulted in 60% mortality. Two doses of 2000 mg/kg, with an interval of 9 hr, were lethal to all mice.

After the administration of a single 100-mg dose of ara-C per kg to L1210 leukemic mice, ascites-cell DNA synthesis (as measured by TdR-3H incorporation) is rapidly inhibited and remains at less than 1% of control level for approximately 4 hr. DNA synthesis recovers after this time, reaching a maximum (of approximately 15 to 25% of control) after about 10 hr. This is approximately the same interval at which a 2nd dose of ara-C gives optimal therapeutic effects.

Results of similar studies with HU are shown in Chart 2. In this and the following cases, the intervals studied ranged from 0 to 12 hr. With HU, 2 simultaneous doses of 250 mg/kg yielded very little therapeutic effect (mean survival, 8.4 ± 0.3 days compared with 8.0 ± 0.4 days for the control). This corresponded to less than 50% cell kill. However, at an interval of 4 to 5 hr, survivals of approximately 12 days were observed (corresponding to approximately 99.5% cell kill). Survival then decreased as the interval increased (to approximately 7 hr). At an interval of 10 to 12 hr, another peak of activity was observed. No particular pattern was observed with regard to toxicity (as measured by weight change), again indicating that the therapeutic index could be improved by the judicious choice of interval. With HU, the optimal interval for 2 doses of the agent correlated extremely well with the interval at which DNA synthesis recovered to its maximum after the initial inhibition, following 1 dose of HU. A 2nd recovery of DNA synthesis was also observed at approximately 10 to 12 hr, corresponding to what appears to be another good interval for therapeutic results.

Results with 5-HP are shown in Chart 3. Two simultaneous doses of 250 mg/kg yielded less than 60% cell kill (mean survival, 8.6 ± 0.5 days compared with 8.0 ± 0.4 days in the control group). Survival was again a function of the dose interval, with a peak (mean survival, 11.8 ± 0.7 days; cell kill, approximately 99.3%) occurring at approximately 6 hr. Little change in toxicity (as measured by weight loss) was observed as a function of dose interval. With 5-HP, recovery of DNA synthesis was also observed at approximately 10 to 12 hr, corresponding to what appears to be another good interval for therapeutic results.

Table 1

<table>
<thead>
<tr>
<th>Dose interval (hr)</th>
<th>ara-C, 400 mg/kg/dose</th>
<th>ara-C, 900 mg/kg/dose</th>
<th>ara-C, 2000 mg/kg/dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.5</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>7.5</td>
<td>30</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>60</td>
<td>80</td>
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<tr>
<td>10.5</td>
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<td>50</td>
<td>90</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

*a* All but 2 of the 57 deaths occurred by Day 7.
synthesis appeared to lag significantly behind the optimal interval for 2 doses.

Results with camptothecin are shown in Chart 4. In this case, there was no obvious relationship between the dose interval and the survival of L1210 leukemic mice. Two 20-mg/kg doses administered simultaneously were quite effective (mean survival, 13.0 ± 0.7 days, compared with 8.8 ± 0.6 days with controls; cell kill, approximately 99.3%) and gave results similar to those observed when the interval between doses ranged from 1 to 8 hr. A slight decrease in activity was observed when the interval was 9 to 12 hr. However, with this agent, toxicity appeared to increase rather regularly as the interval between the 2 doses increased. The decrease in therapeutic activity observed at intervals of 9 to 12 hr may reflect this rather severe toxicity. No recovery of DNA synthetic capacity was observed after a single dose of camptothecin at intervals up to 12 hr.

The extent and time course of DNA synthesis recovery of ascites L1210 cells is shown as a function of ara-C dose (Chart 5). In this chart, results are plotted semilogarithmically to emphasize the extent of inhibition of DNA synthesis. At a dose of 10 mg ara-C per kg, recovery of DNA synthesis was somewhat more rapid and achieved higher levels than at doses of 30 or 100 mg/kg. The peak of this recovery, however, still occurred approximately 8 to 10 hr after drug administration.

DISCUSSION

All of the agents examined in this study (ara-C, HU, 5-HP, and camptothecin) inhibit DNA synthesis of L1210 cells growing in culture more than they inhibit either RNA or protein synthesis (3). Bhuyn et al. (4) have shown with synchronized DON Chinese hamster cells that all of these agents are S-phase specific; that is, they are maximally cytotoxic to cells in the S (DNA-synthetic) phase of the cell cycle. Results with asynchronous L1210 cells in culture (3) are...
also consistent with with the S-phase specificity of these agents.

If one makes a number of assumptions, it is possible to construct a very simple model to describe the cell kill one might expect with 2 short exposures of L1210 cells to S-phase-specific agents. The assumptions involved are the following. (a) The time of exposure to cells to the agent (contact time) is short with respect to the cell-cycle time, (b) All S-phase cells (but no non-S-phase cells) are killed (i.e., rendered incapable of further proliferation) by an instantaneous exposure to some minimally effective level of the agent in question. (c) The L1210 cell population is homogeneous with respect to cell-cycle times and the lengths of the various phases. (d) The exposure to the agent does not affect the progression of non-S-phase cells into the S phase (i.e., the agents are not "limiting").

The phase and cell-cycle lengths for L1210 cells in vivo have been determined (G. L. Neil and T. E. Morley, unpublished results, our laboratories) and are listed below with estimations of the fractions of cells in any particular phase. These fractions are roughly approximated by division of the phase length by the total cell-cycle time (TC).

<table>
<thead>
<tr>
<th>Phase</th>
<th>Length (hr)</th>
<th>Approximate % of total no of cells in a particular phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>1.9 (TG1)</td>
<td>19</td>
</tr>
<tr>
<td>S</td>
<td>6.6 (TS)</td>
<td>66</td>
</tr>
<tr>
<td>G2 + M</td>
<td>15 (TG2 + M)</td>
<td>15</td>
</tr>
<tr>
<td>Total cycle</td>
<td>10.0 (TC)</td>
<td>100</td>
</tr>
</tbody>
</table>

Using the above assumptions and cell-cycle parameters, one may calculate that the fractional cell kill (expressed as a fraction of those cells present at the time of administration) expected for a single administration of an S-phase-specific agent would equal (TS + length of effective exposure time)/TC = (6.6 + length of effective exposure time)/10.0. For the ideal case in which exposure time is infinitely short, the cell kill achievable with a single dose would simply equal the fraction of cells in the S phase (approximately 66%). For an effective exposure of 2 hr, the cell kill would be approximately 86%.

The cells unaffected by the initial exposure (i.e., those in G1, G2, or M during that time) will then become most sensitive to a 2nd exposure when they have all progressed into the S phase. The optimal time for a 2nd dose will correspond to the time when all of these cells are in the susceptible S phase. For an agent with infinitely short contact time, this dose may be administered 3.4 (Ts) hr after the 1st dose. After 6.6 hr, the cells will begin to enter the G2 phase and the population will become less sensitive to the agent. For an agent with an effective contact time of 2 hr, a 2nd dose would be optimal anywhere from 3.4 to 8.6 hr after the 1st dose.

Cells surviving the 1st dose will of course be least sensitive to a 2nd dose when they have all progressed through the S phase. The optimal time for a 2nd dose will correspond to the time when all of these cells are in the susceptible S phase. For an agent with infinitely short contact time, this dose may be administered 3.4 (Ts) hr after the 1st dose. After 6.6 hr, the cells will begin to enter the G2 phase and the population will become less sensitive to the agent. For an agent with an effective contact time of 2 hr, a 2nd dose would be optimal anywhere from 3.4 to 8.6 hr after the 1st dose.

Assumption 1. The time of exposure of target cells to the agent will be determined by the pharmacokinetic behavior of the agent. The pharmacokinetic behavior of ara-C (5, 14, 16), HU (13), camptothecin (7), and 5-HP (20) in mice has been studied. All of these drugs have relatively short plasma half-lives and, after the administration of doses similar to those used in our study, effective levels would be expected to persist in the plasma for no more than 1 to 3 hr. In some cases, however, body fluid levels may not accurately reflect the levels in the target cells themselves. Metabolism (both anabolic and catabolic) within the cell itself is often of utmost importance. For example, phosphorylation of ara-C (to its corresponding nucleotides) can serve as a concentration or "trapping" mechanism which may not be reflected in body fluid levels.

As mentioned above, for a nonlimiting agent (i.e., one that does not affect the progression of non-S-phase cells into the S phase), an increase in the effective contact time will increase the period of time during which administration of a 2nd dose

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*These results are in reasonable agreement with other published data (19, 21, 22).
*The actual percentages in each phase, calculated by the more rigorous method of Okada (15), are: G1 + M, 11%; G2, 23%; S, 66%. For simplicity, the estimates based directly on phase length (see above) are used in "Discussion."

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Chart 5. Dose-interval studies with ara-C in L1210 leukemic mice; dose response and DNA synthesis recovery. L1210 leukemic BDF, mice were treated with a single i.p. dose of ara-C (arrow). At various times, TdR-3H (10 μCi/mouse; 2.2 Ci/mmole) was injected i.p. Incorporation into DNA of peritoneal ascites cells (cpm/10^6 cells) was determined 30 min later. The interval shown does not include the 30-min labeling period. Results are expressed as percentage of incorporation in control (untreated) mice.
will be optimal. However, the earliest time (3.4 hr) at which a 2nd dose will be optimal will not be changed. With a limiting agent, the optimal dose interval may be longer than that described above and will depend on the contact time of the agent and on the time required for the non-S-phase cells to recover their ability to progress into the S phase.

Assumption 2. Cells at different points in their progression through the S phase may well have different sensitivities towards agents interfering with DNA synthesis. There is, of course, also some finite exposure time necessary so that the cytotoxic activity of an agent can manifest itself. This is particularly likely for an agent such as ara-C, which requires anabolic transformation to manifest its lethal activity. Although all of the agents studied may rightfully be considered S-phase specific, this specificity is probably not absolute, and killing of non-S-phase cells may be possible at appropriate concentrations. This appears to be particularly true for camptothecin; with this agent (although maximal cytotoxicity is seen for S-phase cells), cells in G₁, and G₂, and M are also killed (4).

Assumption 3. The cell population is, of course, not completely homogeneous with respect to cell cycle and phase lengths; indeed, the cell-cycle parameters referred to above represent only average values.

Assumption 4. It is well documented that agents such as ara-C (6) and HU (1, 18), for example, are self-limiting to some extent, in that they affect the progression of non-S-phase cells through the rest of the cell cycle and, in particular, slow the progression of G₁ cells into the susceptible S phase. This is discussed in more detail in a companion article (3).

The simple model described above would also predict that DNA synthesis (Tdr-3H incorporation) should increase after the initial inhibition, as non-S-phase cells enter S. The time for maximal recovery of DNA synthetic capacity would then coincide with the time for optimal administration of a 2nd dose. It has been shown by Sinclair (18), however, that for at least 1 of the agents studied (HU), S-phase cells, destined to die after exposure to the drug, do recover (to a limited extent) the ability to incorporate Tdr-3H. The magnitude and kinetics of such incorporation may well perturb any expected correlation of optimal dose interval and time for recovery of DNA synthetic capacity.

In view of the many uncertainties involved in the assumptions forming the basis of the simple model described, it perhaps somewhat presumptuous to use it to predict (or explain) the data obtained. However, in some cases, the agreement was surprisingly good. With 3 of the agents (ara-C, HU, and 5-HP), the cell kill achieved with a single dose (approximately 50 to 85%) is consistent with that predicted for S-phase-specific agents of short duration.

Dose intervals are also found at which a 2nd administration of the agent greatly increases the cell kill. Cell kills in excess of 99.5% were achieved. With HU and 5-HP, the optimal dose interval (5 and 6 hr, respectively) is consistent with that predicted (approximately 3.4 to 6.8 hr) by the simple model. The optimal interval for a 2nd dose of ara-C is somewhat longer (approximately 9 hr) than the predicted value. Similar results were observed in studies in vitro (3). Karon and Shirakawa (8) showed, with DON cells, that after a finite exposure to ara-C, subsequent progression of G₁ cells into S was slowed (8), a phenomenon that might explain the results observed in our studies.

With ara-C, HU, and 5-HP, DNA synthesis did recover after an initial dose, and the time for maximal recovery with ara-C and HU corresponded closely to the optimal time for administration of a 2nd dose. The lag in DNA synthesis recovery with 5-HP may reflect DNA synthesis resumption by S-phase cells that are destined to die.

Results obtained with camptothecin are entirely inconsistent with the model presented. Single doses (20 mg/kg) apparently killed more than the S-phase population, no dose interval effect was observed, and DNA synthesis did not recover. Results of studies mentioned above (4), indicating that camptothecin kills a significant number of cells in phases other than S, may represent at least a partial explanation for this discrepancy.

The synchronization of mammalian cells in vivo by treatment with DNA synthesis inhibitors has been reported previously. Bertalanffy and Lindsey-Gibson (2) observed partial synchrony with ara-C in B16 murine melanoma. Madoc-Jones and Mauro (13) described partial synchrony of mouse lymphoma cells with HU. Using a spleen colony assay, they studied the effects of the interval between a dose of HU and either a 2nd dose of HU, a dose of a Vinca alkaloid, or a dose of X-rays, on the survival of the lymphoma cells. Life-span data were not reported, however.

The results of this study have some clinical implications. Judicious choice of dose interval in the treatment of human neoplasia with agents such as those used in our study may result in enhanced therapeutic effects. Measurements of the time course of recovery of DNA synthesis of leukemic cells, for example, after an initial dose of 1 of these agents should have some predictive value in determining such intervals.

Indeed, Lampkin et al. (10-12) studied the recovery of DNA synthesis in leukemic cells of patients after treatment with ara-C and found evidence for a considerable degree of synchronization. These authors used a more rigorous measure of DNA synthesis (labeling index) which allows one to determine the number of cells actively incorporating Tdr-3H. The incorporation method used in our study measures only the total rate of DNA synthesis. However, because of the great time savings effected, this latter method might prove more useful in a clinical situation wherein autoradiographic techniques may be impractical. Lampkin et al. (12) have also studied the effects of several phase-specific chemotherapeutic agents (including ara-C) administered to patients after initial synchronization with ara-C, and they concluded that a greater therapeutic advantage was achieved over that seen with administration of the 2nd agents themselves.

The determination of optimal schedules for the clinical treatment of neoplastic disease with chemotherapeutic agents is an exceedingly important and difficult task. Hopefully, the studies described above, particularly those relating changes in a pertinent biochemical parameter (DNA synthesis recovery) to optimal dose intervals in an experimental animal system, will encourage further studies of a similar nature at the clinical level.
ACKNOWLEDGMENTS

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REFERENCES

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