Circadian Bioperiodic Response of Mice Bearing Advanced L1210 Leukemia to Combination Therapy with Adriamycin and Cyclophosphamide

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ABSTRACT

Cyclophosphamide, 100 mg/kg, and Adriamycin, 5 mg/kg, were synergistic in treating L1210 leukemic mice that were inoculated with $1 \times 10^5$ L1210 cells 4 days prior to treatment. Only one course of treatment was given. There was a dramatic circadian variation in response as monitored by mean survival time or cure rate. The variation in cure rate (mice alive and apparently free of disease 75 days post-tumor inoculation) as a function of treatment timing ranged from 8 to 68% in male animals standardized to 12 hr of light alternating with 12 hr of darkness. Similarly, in female mice standardized to 8 hr of light alternating with 16 hr of darkness, the cure rate ranged from 0 to 56% depending on when the drugs were injected during a 24-hr span. No cures were obtained with either drug alone.

The maximal cure rate was recorded when the two drugs were administered during the early part of the dark portion of the light-dark cycle (whether 12 hr of light and 12 hr of darkness or 8 hr of light and 16 hr of darkness), whereas maximal mortality occurred following treatment early in the light span.

Extensive evidence also documents that maximal therapeutic advantage was obtained when the two drugs were separated by 2- or 3-hr intervals and that this effect of drug sequencing was strongly circadian stage dependent. The results indicate the need for information on endogenous temporal organization in studies of order and intervals in the administration of combination therapy.

INTRODUCTION

CTX, an alkylating agent, and ADR, an anthracycline, have pronounced effects against the L1210 transplatable leukemia. The 2 drugs are synergistic when used in combination against advanced L1210 leukemia in male C57BL/6J x DBA/2J F₁ (hereafter called BD2F₁) mice (10) and in female BD2F₁ mice (1). The incidence of cure was reported to be independent of the sequencing, i.e., of the order and interval between the 2 drugs; that is, reportedly, it did not matter whether CTX and ADR were administered together or whether one followed the other by 2, 4, 6, 8, or 24 hr (10).

The purpose of this study was to determine whether the response to these drugs is influenced by the stage of the murine circadian system at the time they are administered. Because our analyses of results reported earlier by others (10) and our own earlier work with 1-β-D-arabinofuranosylcytosine suggested that circadian rhythm effects are demonstrable with control of sequencing, we decided to reevaluate the question of sequencing using a chronobiological approach; the pattern we speak of is best described in the "Discussion."

MATERIALS AND METHODS

Study 1

On November 6, 1978, 500 male BALB/c x DBA/2 F₁ (hereafter called CD2F₁) mice, about 6 weeks old, were received from the Laboratory Supply Co., Indianapolis, Ind. Two days later, the animals were assigned randomly, with stratification by body weight, into groups as follows.

Groups on SLD 12:12. Eight groups of 25 animals each were placed in separate isolation chambers with 5 cages/chamber and 5 animals/cage. In each chamber, lights were on for 12 hr daily (LD 12:12). The times of turning the fluorescent lights on and off differed among the 8 chambers in such a way that it was possible to inject or treat all of the groups within one short span of the working day but presumably at 8 different stages of the mouse circadian system. Chart 1 diagrams the SLD 12:12 schedules.

Groups on CLD 12:12. Nineteen subgroups of 15 mice each (3 cages of 5 mice) were placed in separate isolation chambers in which the lights were turned on at 6 a.m. and off at 6 p.m. daily.

For all animals, food and water were available ad libitum. Cages were changed, and food and water were replenished once a week. We were especially careful that no isolation chamber was opened and subjected to a white light perturbation while in its dark span; when it was necessary to open such a chamber while it was dark, the room in which the isolation chambers were kept was switched to a dim red light.

Beginning on December 11, 1978, at 11 a.m. (at which time the mice were 12 weeks old, weighed about 27 to 30 g, and had been standardized for 35 days), all of the mice were given i.p. injections of $1 \times 10^5$ L1210 leukemia cells suspended in 0.2 ml of 0.9% NaCl solution. Controls in each chamber were given injections of leukemia cells last. During the 45-min injection span, cell death in the stock culture, as verified by dye exclusion, was only 5%. The tumor designation was LE59B. It was from transplant generation 04, using female DBA/2 mice received from Mason Research Clinic and implanted on December 4, 1978. Treatment was initiated 4 days later at 11 a.m.

Treatment of SLD 12:12 mice. All animals in the 8 isolation chambers subjected to the staggered light-dark cycle received ADR, 5 mg/kg, followed immediately by CTX, 100 mg/kg. The treatment was administered to all animals between 11 a.m. and 11:30 a.m. beginning 96 hr (4 days) after tumor inoculation; it took 30 min to inject all animals. See Chart 1 for the timing of

1 Supported in part by Grant CA-14388 from the National Cancer Institute.
2 The abbreviations used are: CTX, cyclophosphamide; ADR, Adriamycin; MST, mean survival time; CLD, conventional light-dark schedule; SLD, staggered light-dark schedule; LD, light-dark cycle; ANOVA, analysis of variance; SLD 8:16, staggered-light-dark schedule; LD 12:12, light-dark cycle.
injection in relation to the 8 light-dark cycles.

**Treatment of CLD 12:12 Mice.** Also on Day 4 at 11 a.m., one half of the mice housed on the conventional light-dark cycle were given injections of CTX, 11 mg/kg, and the other half were given injections of ADR, 5 mg/kg. The second drug (ADR or CTX, respectively) was then administered as shown in Table 1. There were 15 mice in each of the subgroups above, or a total of 135 mice/group. In addition, at 11 a.m., one group of 10 mice was given ADR only, 5 mg/kg, and a second group of 10 was given CTX only, 100 mg/kg.

Death of animals was recorded for 75 days after the inoculation of tumor. The data on survival time include only those mice that died on or before 75 days. Cure rate data (75-day survival) were transferred to punch cards and analyzed for a statistically significant (F = 69.6; p < 0.001) MST of Animals Dying. An ANOVA indicated that the variation in MST, as a function of treatment timing along the 24-hr time scale, was statistically significant (F = 69.6; p < 0.001). The longest MST [27 ± 3.2 (S.D.) days] was recorded for mice treated 11 hr after lights on or the equivalent of 5 p.m. for animals standardized to the conventional LD 12:12 cycle; the shortest MST (20.5 ± 1.1 days) occurred in animals treated 2 hr after the lights went on, or the equivalent of 8 a.m. for animals on a conventional LD 12:12 cycle.

Long-Term (75-Day Postinoculation) Survivors (Cures). Chart 2 illustrates the pattern of cures for mice on SLD in both studies. In the case of Study 1, 68% of the animals treated at 1 hr into the dark span were cured (apparently free of the disease). For those animals treated 1 hr after the light went on, only 8% were cured. When the highest and lowest number of cures were compared, they were found to be highly significant (p < 0.005). No animals were cured with either drug alone: with CTX, the MST was 13.6 ± 2.5 days; with ADR, it was 10.7 ± 0.8.

**Sequencing of the 2 Drugs in Animals on CLD**

From the data illustrated in Chart 3, it appears that it made little difference whether CTX was administered to all animals at 11 a.m. followed by ADR or whether ADR was administered first followed by CTX. There is some suggestion from the data that a better therapeutic efficiency might be achieved if the second drug were administered 3 hr after the first. However, a *χ²* test performed on data from each order of drug administration separately indicated no statistically significant difference between the 2 groups (drug together or 3 hr apart). On the other hand, if the first drug was administered 3 hr after the first, the cure rate was highest when the second drug was administered 3 hr after the first. If the second drug was administered 3 hr after the first, the cure rate was highest when the second drug was administered 3 hr after the first. If the second drug was administered 3 hr after the first, the cure rate was highest when the second drug was administered 3 hr after the first.
concomitant treatment with CTX and ADR. Study 1 on male mice in LD 12:12; Study 2 on female mice in LD 8:16. HALO, hr after lights on.

...that found when the drugs were given concomitantly. The data have a statistically significant difference (p < 0.001) between the two. However, if data from the 2 orders were pooled, there was a statistically significant difference in the cure rate when the second drug was given during a span of 9 hr in the dark span, no cures were observed, irrespective of the order of the 2 drugs. The data show a remarkably good fit to a 24-hr cosine curve, thus strongly supporting the rhythmic nature of the response; (c) results presented in the right and left polar plots as a whole suggests that the response to interval and order manipulation, i.e., to the sequencing (in the case of CTX and ADR therapy), depends critically on circadian system stage and hence should be optimized accordingly.

Study 2 (Animals on an LD 8:16 Schedule)

MST of Animals Dying. The longest MST (27.7 ± 3.2) was found in mice treated in the middle of the dark span, and the shortest MST (17.8 ± 0.8) was found in mice treated 1 hr into the light span (Chart 5). An ANOVA indicated significant variation in MST as a function of treatment time (F = 4.41; p = 0.001). The MST without drug treatment was 8.5 ± 1.2 days.

Long-Term (60-Day Postinoculation Survivors (Cures). The cure rate ranged from 0 to 52.4% among groups treated at the 6 different time points (Chart 5). The variation yielded a $\chi^2$ of 13.1 (p < 0.0005). The reason for terminating this particular study at 60 days rather than at 75 days as in Study 1 was that it is generally agreed that very few animals are likely to die after 60 days; thus, it is difficult to justify the additional cost of keeping the animals longer.

DISCUSSION

The data on MST and cure rate indicate that the response of...
leukemic mice to the concomitant administration of CTX and ADR is strongly circadian stage dependent. This is not surprising when one considers that variation in host toxicity to CTX alone (2, 6) and to ADR alone (7, 8) is strongly circadian stage dependent. Also, it has been demonstrated that CTX in combination with 1-β-d-arabinofuranosylcytosine is influenced by the stage of the circadian system at which the drugs are administered (9). However, consideration should be given to the fact that mice on the SLD schedules were inoculated with tumor at the same circadian stage at which they were treated. Thus, at least part of the variation seen in Chart 2 and summarized in Chart 4 (left) could be a result of differences in tumor growth as a function of circadian stage at the time of tumor inoculation. Work with assessment of all pertinent factors must be reserved to larger projects involving thousands (rather than hundreds) of mice. The present results suffice to indicate, however, the importance of circadian stage, whatever the extent to which factors other than sequencing are concerned.

In the study of sequencing, all mice received the tumor at the same circadian stage. The results in Chart 3 (Study 1) suggest that the administration of CTX and ADR with a 3-hr interval (the first drug being given at 11 a.m.) constitutes a better schedule than administering the 2 drugs concurrently. This impression was supported by a statistically significant result from a χ² test if data from the 2 orders of injection were pooled. Tobias et al. (10) carried out a similar study on a similar mouse leukemia model and concluded that there was no effect of sequencing. However, they obtained a 55% and a 65% cure for the 2 sequences when the drugs were administered 2 hr apart and only a 35% cure (in both of these studies) when the ADR and CTX were administered concomitantly. We performed a χ² comparison of their data from the 2 groups in question in
each of the 2 separate studies and found (as they had reported) no statistical significance. However, when their data from the 2 studies were pooled, there was a significant difference dependent on whether the drugs were given concomitantly or with a 2-hr interval ($\chi^2 = 4.511; 0.05 > p > 0.025$). We believe that failure to find statistical significance in both our own and the studies of Tobias et al. without pooling simply resulted from having used too few animals per time point. In our own study, 15 animals/time point were used; in their study, 14 animals/time point were used. What makes this even more convincing is that, in one of our earlier studies, which unfortunately was complicated by some of our animals being subjected to an excessive environmental heat shock at a critical stage of the study, a similar phenomenon was still strongly suggested by the data that could be considered reliable in spite of the accident. For example, in this earlier experiment, we found that for animals (in light daily from 6 a.m. to 6 p.m.) to which CTX was administered concomitantly with ADR at 11 a.m., 12% of the animals were cured; whereas if ADR was given during the same 9 hr described above during the dark span, only 5% were cured at each time point. There were again more survivors at 11 a.m. on the second day (21%). Moreover, in this earlier study, the second drug was administered at intervals up to 48 hr rather than 24 hr, and we found no cures after the 24-hr interval. Thus, there seems to be no advantage in separating the 2 drugs beyond 24 hr, under the specific conditions and for the scope of this study.

We conclude from data now available that the best way of administering these 2 drugs in combination is to separate them by 2 to 3 hr. One might argue that this may hold true if the first drug is given near midlight but may not be the case if the first drug is administered at another circadian stage (for example, during middark). Admittedly, the outcome might be completely different. The circadian variation in response should be considered in any study of sequencing (3).

The second study was restricted to a concomitant administration of CTX and ADR, with MST and cure rate as end points. The results extended the above conclusions to female mice and demonstrated the reproducibility of the results from one study to another.

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REFERENCES

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