Effect of Altered Lighting Regimens, Time-limited Feeding, and Presence of Ehrlich Ascites Carcinoma on the Circadian Rhythm in DNA Synthesis of Mouse Spleen

E. Robert Burns, Lawrence E. Scheving, John E. Pauly, and Tien-Hu Tsai

Department of Anatomy, University of Arkansas College of Medicine, Little Rock, Arkansas 72201

SUMMARY

The objectives of the series of experiments described were (a) to determine whether there was a circadian rhythm in the incorporation of tritiated thymidine into DNA of the spleen in mice kept on a conventional light-dark cycle and fed ad libitum, (b) to study the effect that different light-dark cycles and a time-limited feeding schedule had on this circadian rhythm, (c) to ascertain what effect the presence of an 8-day Ehrlich ascites carcinoma (EAC) had on the rhythm in DNA synthesis in the spleen and what effect the EAC had on the circadian rhythm in the mitotic index in the corneal epithelium, and (d) to determine whether there was a circadian rhythm in the duration of life-span in mice bearing the EAC.

A circadian rhythm in the incorporation of tritiated thymidine into DNA of the mouse spleen consistently was characterized by a peak during the nocturnal phase and a trough during the diurnal phase of the 12-hr light-12-hr dark cycle. In animals bearing an 8-day EAC, the rhythm in DNA synthesis in the spleen was phase-shifted, its wave form was changed, and the overall 24-hr mean was increased significantly. The phasing of the rhythm in EAC-bearing mice was not reproducible. This finding demonstrated that the presence of the EAC severely altered the natural rhythm in DNA synthesis in the spleen and resulted in a rhythmic pattern which was constantly changing. The presence of an 8-day EAC, however, had no effect on the amplitude, overall level, or the phasing of the circadian rhythm in the mitotic index in the cornea. A staggered light-dark cycle of 2 weeks duration did not completely phase-shift the DNA synthesis rhythm in the spleen but did completely phase-shift the rhythm in the mitotic index in the corneal epithelium. In mice subjected to a daily feeding period limited to 4 hr, the rhythm in DNA synthesis in the spleen, in both EAC-bearing and non-EAC bearing mice, was phase-shifted such that the peak occurred during the time of feeding and the trough occurred prior to the feeding period. The rhythm in the mitotic index in the cornea was not phase-shifted or altered in any way by the feeding schedule and thus remained fixed to the light-dark cycle. The DNA synthesis rhythm in the normal spleen demonstrated a phasing very similar to the circadian rhythm in the length of survival in mice challenged with EAC and the circadian rhythm in DNA synthesis in the normal thymus.

INTRODUCTION

In tumor-bearing animals the spleen increases in weight, size, cellularity, and proliferative capacity (1, 2, 12, 22). There is a circadian rhythm in the uptake of [3H]TdR by normal spleen (20). The proliferative response of the spleen to the presence of the EAC (2, 22) has not been evaluated chronobiologically. This study was designed to investigate further the interaction between the EAC and the spleen by studying the circadian rhythm in DNA synthesis in the spleen in non-tumor-bearing mice and in mice bearing an 8-day EAC under a variety of experimental conditions: (a) in mice fed ad libitum and synchronized to a conventional light-dark cycle (12-hr light-12-hr dark), (b) in mice fed ad libitum and synchronized to light-dark cycles different from the conventional light-dark cycle, and (c) in mice synchronized to a standard light-dark cycle, but kept on a time-limited feeding schedule.

MATERIALS AND METHODS

The 1st series of experiments was designed to demonstrate the rhythm in the synthesis of DNA in the spleen of healthy mice and in mice bearing an EAC and to determine whether the phasing of the rhythm could be altered by manipulating the light-dark cycle. The mice (5 week old, random bred, Swiss-Webster) were fed ad libitum and standardized in 8 different chambers. The light-dark cycle in each chamber was regulated by an automatic timer so that all mice were subjected to 12 hr of light (6 a.m. to 6 p.m. central standard time) alternating with 12 hr of darkness. This is the "conventional" light-dark cycle. In another part of this study, animals were synchronized to a "staggered" light-dark cycle; this involved keeping different subgroups of mice in 8 chambers, each with light regulated by a timer so that 12 hr of illumination alternated with 12 hr of darkness. The situation differed from the conventional light-dark cycle in that the times of "lights on" and "lights off" were staggered so that 1 chamber had illumination from 6 a.m. to ...

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3 The abbreviations used are: [3H]TdR, tritiated thymidine; EAC, Ehrlich ascites carcinoma; PCA, perchloric acid.
In each of 4 studies, a suitable number of mice was inoculated with $0.30 \times 10^6$ live EAC cells at 1500 ± 20 min. Eight days later subgroups (usually 7 to 13 mice) of tumor-bearing mice and comparable controls were killed by cervical dislocation at 3-hr intervals over a 24-hr period. One-half hr prior to the sacrifice of each subgroup all animals received s.c. injections in the interscapular region of 25 $\mu$Ci of $[^3H]TdR$ (22 Ci/mmole; Amersham/Searle Corp., Arlington Heights, Ill.). Tumor fluid was aspirated, and the entire animal was fixed in 10% phosphate-buffered formalin after cervical dislocation at 3-hr intervals over a 24-hr time-span. One-half hr prior to the sacrifice of each subgroup all animals received s.c. injections in the interscapular region of 25 $\mu$Ci of $[^3H]TdR$. The solution was neutralized with an equal amount of 1 N HCl and then 10% trichloroacetic acid was added to make a final concentration of 5% trichloroacetic acid. The solution was vortexed and centrifuged, and the supernatant was discarded. The pellet containing protein was discarded. For liquid scintillation counting an aliquot was added to scintillation fluid that consisted of 3 liters of toluene plus 125 ml Liquifluor:Triton X-100 (2:1). The DNA concentration was determined at $A_{260}$.

The hydrolysate was centrifuged and aliquots were removed for liquid scintillation counting and DNA determination. The pellets were then hydrolyzed in 0.5 N PCA at 70° for 30 mm. The DNA concentration was determined at $A_{260}$ and the number of mitotic figures in at least 5000 cells was counted, and the mean mitotic indices of experimental and control animals from each 3-hr sampling time were computed. Results were expressed as the number of mitoses per 1000 cells (mitotic index).

The spleen of each animal was dissected out and the method of Ogur and Rosen (14) was used to isolate the DNA with the modification that the RNA hydrolysis was carried out in 1 N NaOH at 60° for 18 hr. The details were as follows. Pieces of spleen (50 to 100 mg) were placed in 1 N NaOH in covered test tubes and heated in a water bath at 60° for 18 hr. The solution was neutralized with an equal amount of 1 N HCl and then 10% trichloroacetic acid was added to make a final concentration of 5% trichloroacetic acid. The solution was vortexed and centrifuged, and the supernatant was discarded. The pellet was then hydrolyzed in 0.5 N PCA at 70° for 30 min. The hydrolysate was centrifuged and aliquots were removed for liquid scintillation counting and DNA determination. The DNA concentration was determined at $A_{260}$.

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RESULTS

There was significant variation along a 24-hr time scale in the incorporation of $[^3H]TdR$ into the DNA of mouse spleen in the control, non-tumor-bearing mice (Chart 1, solid line, closed circles, experiment completed on February 28, 1974). The maximum incorporation of $[^3H]TdR$ was sustained and occurred between 7 p.m. and 4 a.m.; lowest incorporation of $[^3H]TdR$ occurred between 10 a.m. and 1 p.m. When the lowest and highest means were compared, the variation represented a 45% change. In this February 28, 1974, experiment there was significant variation along the 24-hr time scale in the incorporation of $[^3H]TdR$ into the DNA of mouse spleen in the mice bearing an 8-day EAC (Chart 1, dashed line, closed circles). When mice bearing an 8-day EAC were compared to the controls in the February 28, 1974, experiment, the wave form of the 2 rhythms was similar, but there were higher S.E.'s of the mean in the data from the EAC mice. At all time points except one, 4 a.m., there was significantly greater incorporation of $[^3H]TdR$ into DNA of the spleen in the EAC mice. The overall 24-hr mean level of $[^3H]TdR$ incorporation into DNA of the spleen was...
significantly ($p < 0.0005$) greater in the EAC-bearing mice (see also Chart 5). Results obtained from a 2nd comparable study (differing only in that it was completed on May 16, 1974) confirmed that an 8-day EAC caused a significant increase ($p < 0.002$) in the amount of DNA synthesis in the spleen (Chart 1, dashed line, open circles) when compared to the controls (Chart 1 solid line, open circles; see also Chart 5). In this May 16, 1974, experiment there were 4 time points (4 a.m., 7 a.m., 10 p.m., and 1 a.m.) when no significant statistical difference could be detected between control and EAC mice. The phasing of the rhythm in DNA synthesis in the spleen in the EAC animals (dashed line, open circles) was almost the inverse of the rhythm in the controls (solid line, open circles). The rhythm in the control animals reached a peak around the time of the transition from dark to light and a trough occurred from 1 p.m. to 5 p.m. The rhythm in the EAC animals had a sustained peak during the middle of the light period and a trough near 12 p.m. In both the February 28, 1974, and May 16, 1974, experiments the lowest and highest daily means in each rhythm (Chart 1) were significantly different in the EAC and control animals ($p < 0.01$ and $p < 0.005$, respectively). The percentage increase from the lowest to the highest daily mean in the May 16, 1974, study was 46% for the controls and 137% for the tumor-bearing animals. For non-tumor-bearing animals the May 16, 1974, study demonstrated the general reproducibility of the phasing (trough during the light span, peak during the dark span) of the rhythm in DNA synthesis in the spleen when compared to the February 28, 1974, study. However, the rhythm in DNA synthesis in the spleen from the EAC-bearing mice was not reproducible and demonstrated a completely different phasing between the February 28, 1974, and May 16, 1974, experiments.

As part of the May 16, 1974, study, other groups of mice (controls and EAC-bearing) had been subjected to a staggered 12-hr light-12-hr dark cycle. If the rhythm in DNA synthesis in the spleen of non-tumor-bearing animals completely phase-shifted to the staggered light-dark cycle during the 2 weeks the mice were kept on this lighting regimen, the rhythm (Chart 2, solid line) should have a phasing very similar to that seen in the controls in Chart 1. A comparison

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**Chart 1.** Rhythmic patterns in the uptake of [3H]TdR (cpm/μg DNA) into the DNA of the spleen in control or non-tumor-bearing mice (solid lines) and in mice bearing an 8-day EAC (dashed lines). ○, data from the experiment completed on February 28, 1974. □, data from an identical experiment repeated on May 16, 1974. In the February 28, 1974, experiment each point on the solid line represents the mean ± S.E. of 3 to 5 mice and each point on the dashed line represents the mean ± S.E. of 9 mice. In the May 16, 1974, experiment each point on the solid line represents the mean ± S.E. of 13 mice and each point on the dashed line represents the mean ± S.E. of 12 mice. The abscissa contains the time of day and the light-dark cycle. These experiments were completed under a conventional light-dark cycle. 20th, 8 p.m.

**Chart 2.** Rhythmic patterns in the uptake of [3H]TdR (cpm/μg DNA) into the DNA of the spleen in control or non-tumor-bearing mice (solid line) and in mice bearing an 8-day EAC (dashed line). All mice were kept on a staggered light-dark cycle (see "Materials and Methods"). Each point in the dashed line represents 12 mice and each point on the solid line represents 13 mice. Since the staggered light regimen was used, all animals received injections of [3H]TdR at one time and all were killed 0.5 hr later, but this single time point actually represented the 8 different sampling time points during one 24-hr period. The animals were fed ad libitum. 20th, 8 p.m.
of these data demonstrated that this was not the case. The natural, endogenous rhythmicity in DNA synthesis in the spleen was present (Chart 2, solid line), but it was not synchronized to the new (staggered) light-dark cycle. The slight differences in amplitude between the control rhythms in Charts 1 and 2 can be explained as natural biological variability. A comparable study completed under similar staggered light-dark conditions (April 5, 1974) confirmed that such a lighting regimen, at least within a span of 2 weeks, did not completely phase-shift the rhythm (data not illustrated). A comparison of the rhythms (Chart 2) in DNA synthesis in spleen in control (solid line) and EAC-bearing animals (dashed line) showed a significant increase ($p < 0.02$) in the incorporation of $[^{3}H]$TdR into the DNA of the spleen in the EAC animals at 6 of the time points. At 1 p.m. the difference was not significant, and at 10 a.m. the EAC animals showed a significant decrease in DNA synthesis ($p < 0.01$).

In both staggered light experiments (April 5, 1974, and May 16, 1974), the rhythm in the mitotic indices in the corneal epithelium in control animals was, however, completely phase-shifted, i.e., it demonstrated a phasing identical to that seen in the conventional light-dark cycle: the peak occurred early in the light period and the trough occurred early in the dark period. The cornea data from the May 16, 1974, staggered light experiment are illustrated in Chart 3. It also is evident in Chart 3 (dashed line) that the presence of an 8-day EAC had no effect on the rhythm in the mitotic index of the corneal epithelium.

In another phase of this investigation, EAC-bearing and non-EAC-bearing mice were subjected to a limited feeding period (12 p.m. to 4 p.m.) for 21 days (Chart 4). EAC-bearing mice fed ad libitum served as controls for the EAC-bearing mice on the time-limited feeding schedule. This study was concluded on July 11, 1974. Again significant variation was seen in DNA synthesis in the spleen in the EAC-bearing mice fed ad libitum; in this case the circadian difference represented a 148% change (Chart 4, dashed line). In both the EAC-bearing (dotted line) and non-EAC-bearing mice (solid line) subjected to the time-limited feeding schedule the peak in the uptake of $[^{3}H]$TdR by the spleen occurred during the time of feeding and the trough occurred prior to feeding. The mice on the time-limited feeding schedule gained significantly less weight from the weighing on June 19 to the weighing on July 3. The ad libitum-fed mice gained an average of 3.6 ± 0.3 g, whereas the mice subjected to the time-limited feeding schedule lost an average of 2.5 ± 0.2 g. These differences in body weight gain were statistically significant ($p < 0.003$). Subsequent to the inoculation of the EAC, the weight gain over the 1st 7 days was 2.3 ± 0.3 g for the time-limited-feeding animals whereas the ad libitum-fed animals demonstrated a weight increase of 6.0 ± 0.4 g. These differences in body weight gain were significant ($p < 0.003$).

The circadian rhythm in the mitotic indices in the corneal epithelium remained fixed to the 12-hr light-12-hr dark cycle and was not affected by the time-limited feeding schedule.
This observation confirms earlier data (21) and, therefore, is not illustrated again here.

Chart 5 summarizes the effect that an 8-day EAC has on the 24-hr mean [\(^{3}\)H]Tdr incorporated into the DNA of the spleen under 3 different experimental conditions: conventional light-dark, staggered light-dark, and time-limited feeding. In the April 5, 1974, study there were several time points along the 24-hr scale when the [\(^{3}\)H]Tdr incorporation was significantly higher in the EAC mice.

To determine whether mice inoculated with \(0.30 \times 10^6\) EAC cells demonstrate any circadian differences in the length of survival in days, separate groups of mice received injections of EAC cells at 4 a.m., 7 a.m., etc. (To rule out the unlikely possibility of circadian influences in the donor cells subsequently causing a rhythm in host survival time after EAC transplantation, some donor mice were kept on a conventional light-dark cycle and some were kept on a reversed or dark-light cycle. For each EAC inoculation a donor was taken from the light-dark cycle and its EAC cells were mixed, by volume, with an equal amount of EAC cells obtained from a donor on the dark-light cycle.) The number of days after EAC cell injection when death occurred was recorded. There was a circadian rhythm in the length of survival in the EAC-bearing mice (Chart 6, dashed line) with the shortest survival time occurring during the middle of the light period.

Chart 7 demonstrates the remarkable synchrony between the rhythms in DNA synthesis in the spleen and the thymus (16) obtained from the same non-tumor-bearing animals.

DISCUSSION

These studies demonstrate for the first time that the incorporation of [\(^{3}\)H]Tdr into DNA in the spleen of young adult mice subjected to a 12-br light-12-hr dark cycle and fed \textit{ad libitum} is characterized by significant circadian variation. This observation confirms a similar finding for the uptake of [\(^{3}\)H]Tdr into the DNA of the rat spleen (20); however, there are differences between the mouse and the rat in the phasing of this rhythm. In the mouse the peak levels are associated with the dark phase and a sharp drop off occurs just after the transition from dark to light with the lowest levels occurring around the middle of the light span. The daily upward swing begins toward the end of the diurnal phase. [\(^{3}\)H]Tdr incorporation into the DNA of the thymus (16) from these same animals had a phasing that is remarkably similar to that described for the spleen; both organs are characterized by a sharp decline during the light phase (Chart 7). However, the overall magnitude of DNA synthesis in the thymus was much less than that seen in the spleen. In the spleens taken from the EAC-bearing mice, DNA synthesis was significantly increased (4 out of 5 experiments) whereas in the thymus the opposite situation prevailed (16).

The wave form and phasing of the circadian rhythm in DNA synthesis in the spleen (and thymus) does not seem to follow any predictable pattern in EAC-bearing animals fed \textit{ad libitum} (Charts 1 and 4); the peaks and troughs do not...
Circadian Rhythm in DNA Synthesis in Spleen

The rhythm in DNA synthesis in the spleen can be synchronized to a standard light-dark cycle in *ad libitum*-fed animals. An attempt was made to phase-shift the rhythm in DNA synthesis in the spleen in *ad libitum* fed mice by using a set of staggered light-dark cycles. The results of these studies indicate that if one wishes completely to phase-shift the rhythm in DNA synthesis in the spleen by the light inversion technique (staggered light studies), it will, if indeed it is possible, require a standardization of longer than 2 weeks. We recognize that in this respect these studies are incomplete, because we have not determined just how long it does take to phase-shift the rhythm completely, if it is biologically possible; however, that was not one of the original objectives of the investigation. This aspect of the study demonstrates that, although variables such as the mitotic index in corneal epithelium, body temperature, and serum corticosterone levels may be completely phase-shifted in 7 to 10 days in the rodent (for review see Ref. 19), other variables such as DNA synthesis may take a much longer time. This latter point is not always recognized, and therefore such information may be important to anyone who wishes to investigate the rhythm in DNA synthesis in the spleen. Also, the results caution against the not infrequent practice of generalizing about phase-shifting from information on only a limited number of variables.

The rhythm of serum corticosterone levels, liver glycogen, body temperature (13), and eosinophil levels (15) will synchronize to time-limited feeding periods. These data demonstrate that in certain cases food intake can override the synchronizing effect of the light-dark cycle. However, Scheving et al. (21) demonstrated that the rhythm in the mitotic index of the corneal epithelium of the mouse would not synchronize to a variety of 4-hr feeding periods; Philip-

Chart 6. A comparison of the phasing of the circadian rhythm in the uptake of \[^3H\]TdR into the DNA of the spleen in non-tumor-bearing mice (solid line); same data as in Chart 1 from the experiment concluded on February 28, 1974, with the circadian rhythm in the length of survival time in days (dashed line) for separate groups of mice inoculated with \(0.30 \times 10^6\) EAC cells at different points in the circadian period (1 group received \(0.30 \times 10^6\) EAC cells at 4 a.m., a different group received \(0.30 \times 10^6\) EAC cells at 7 a.m., etc.). All animals were kept on a conventional light-dark cycle and fed *ad libitum*. 20°', 8 p.m.

consistently occur in the same area of the light-dark cycle as the rhythms in the non-tumor-bearing, *ad libitum*-fed animals did. In 4 studies on *ad libitum*-fed, EAC-bearing mice, some of which are illustrated, the troughs occurred at 1 p.m., 9 p.m., 11 a.m., and 1 a.m., respectively. This inconsistency in the phasing of the rhythm is one of the major findings of this work. The presence of tumor in the host does not abolish fluctuation in DNA synthesis in the spleen, it causes a change in the phasing and wave form of the rhythm as well as stimulating an overall increase DNA synthesis. Single time point sampling in such a changing system will result in data never taken from the same point in the rhythm (see Ref. 18). Such a single time point sampling technique could lead to 3 different conclusions, i.e., tumor significantly stimulates, inhibits, or has no effect on DNA synthesis in the host spleen.

The light-dark cycle is the dominant synchronizer of the circadian system in rodents fed *ad libitum*. Evidence for this includes a demonstration that a number of rhythmic variables such as body temperature, the number of circulating eosinophils, serum corticosterone levels, and the mitotic index of corneal epithelium can be phase-shifted 180° within a period of 7 to 10 days simply by inverting the light-dark cycle (5, 6, 19). The data in this paper demonstrate that

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Chart 7. A comparison of the phasing of the circadian rhythms in the uptake of \[^3H\]TdR into DNA of the spleen (dashed line) and thymus (solid line) from the same non-tumor-bearing mice. All mice were kept on a conventional light-dark cycle.
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pens et al. (17) confirmed these results in rats. Some rhythmic variables will synchronize to time-limited feeding schedules; some will not; and others, perhaps a greater number, demonstrate an interaction between the 2 synchronizing agents. In both EAC-bearing and non-EAC-bearing mice, limiting food to a specific span of time did (Chart 4) synchronize the peak of the rhythm in synthesis of DNA in the spleen to the feeding span. There appears to be some interaction between the synchronizing effect of the light-dark cycle and that of the meal schedule, because the waveform of the rhythm becomes more or less bimodal. A more detailed study of the interaction between the light-dark cycle and the meal schedule on the rhythm in the uptake of \([3H]TdT\) by the normal mouse spleen has been completed in our laboratory (unpublished data). In brief, regardless of the timing of the meal (6 to 10 a.m., 12 to 4 p.m., 6 to 10 p.m., or 12 to 4 a.m.) the peak in the uptake of \([3H]TdT\) occurred during or immediately before the feeding period. It is apparent that the rhythm in DNA synthesis in the spleen in non-tumor- and tumor-bearing mice can be synchronized to a time-limited feeding period.

There is a circadian rhythm in the length of survival time in different subgroups of mice that received injections of identical doses of EAC cells (0.3 \( \times 10^6 \) cells) at 3-hr intervals (different subgroup/time point) and left untreated. The phasing of this rhythm in survival time coincides with the phasing of the rhythm seen in DNA synthesis in the spleen. The shortest survival time (measured in days) is associated with the lowest level of DNA synthesis in the control rhythm. The same phase relationship also applies to thymus DNA synthesis and survival time (16). A coincidence in timing between 2 rhythmic variables does not necessarily imply a causal relationship. These data indicate that the defense system of the host is rhythmic in its response to and/or interaction with the tumor. Similar circadian rhythmicity exists in the rejection of skin homografts (4) and in the production of antibodies to sheep RBC in mice (3).

In mice bearing the EAC, the spleen undergoes hypertrophy and hyperplasia (2, 12, 22) which has been interpreted as an indication of hyperactivity of the defense system of the host (2, 12). Recently, Lala and Lind (8) demonstrated that lymphoid cells, primarily derived from bone marrow, accounted for most of this cell proliferation. While splenomegaly is occurring in response to the EAC, the host is incapable of rejecting primary or secondary homografts or heterografts (10). This immunosuppression also has been obtained by injecting cell-free fluid from the EAC into the host (7, 11, 23). The EAC induces an immunosuppression which is apparently specific for the thymus-dependent cell system. In fact, the EAC has been shown to cause a depletion of the thymus-dependent population of lymphocytes (9). Thus, the thymus-dependent cell system may be significantly inhibited whereas the bone marrow-dependent cell system may be stimulated by the presence of the EAC. This may help explain why DNA synthesis in the spleen is increased, but DNA synthesis in the thymus (16) is decreased. However, in both the "depressed" thymus and the "stimulated" spleen in mice bearing the EAC, DNA synthesis continues to follow a rhythmic pattern although it is apparently constantly changing in response to the presence of the EAC.

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