Circadian Variation of α- Amanitine Sensitive RNA-Synthesis in Normal Human Lymphocytes

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A major aspect of successful cancer chemotherapy is the dose-time schedule of application of cytostatic drugs. Clinical trials have proven the superiority of intermittent high-dose combination chemotherapy, the intervals between administrations being of paramount importance. In spite of a number of studies in animal models demonstrating the additional importance of the day-time of application[1, 2], these aspects have not been taken into account for human cancer chemotherapy so far. Since RNA synthesis by DNA-dependent RNA polymerases is one of the sites of action of cytostatic drugs, e. g. Actinomycin D, we have studied RNA synthesis by α-amanitine sensitive RNA polymerase B in isolated nuclei from human normal and chronic lymphocytic leukemia (CLL) lymphocytes over a 48 h period of observation.

All patients (8 controls, 2 CLL patients) were hospitalized and obeyed to a constant pattern of life (sleep from 22.00 to 6.30 h, meals at 8.00, 12.00, and 18.00 h). Plasma cortisol concentrations were determined every 6 hours in 4 patients proving the regularity of this important body rhythm. No medical treatment was given during and at least two weeks prior to the investigation. 20 ml of citrated venous blood were taken every 6 hours (00.00, 6.00, 12.00, 18.00 h) and lymphocytes isolated using a modified standard procedure[3] (cf. Legend to Fig. 1). Contamination by non-lymphoid cells was less than 3 % in all patients studied as determined by cytochemical criteria[4].

Nuclei were prepared according to Wiegers and Hilz[5] and assayed for RNA polymerase activity after sonication (for incubation conditions see legend to Fig. 1)[6]. Absolute lymphocyte counts in all 8 normal patients exhibited diurnal variations following a rhythm of approximately 24 h, as described by Sharp[7]. Data were fitted to a cosine function using Fast Fourier Transformation[8]. The computed mean cosine function was

$$f(t) = 3081 + 760 \cos(\omega t - 20.7)$$

(t = time in hours, ω = angular frequency fixed at 15 °/h) with the acrophase at 01.23 h and lowest values at 13.23 h (Fig. 1). Differences between lymphocyte counts at 00.00 and 12.00 h are highly significant ($p < 0.01$).

Variance analyses as well as trend analyses proved the high significance of the fitted cosine function. A definite rhythm of RNA polymerase B activities, i. e.

Fig. 1: Absolute lymphocyte counts in peripheral blood in normals and in patients with chronic lymphocytic leukemia (CLL)

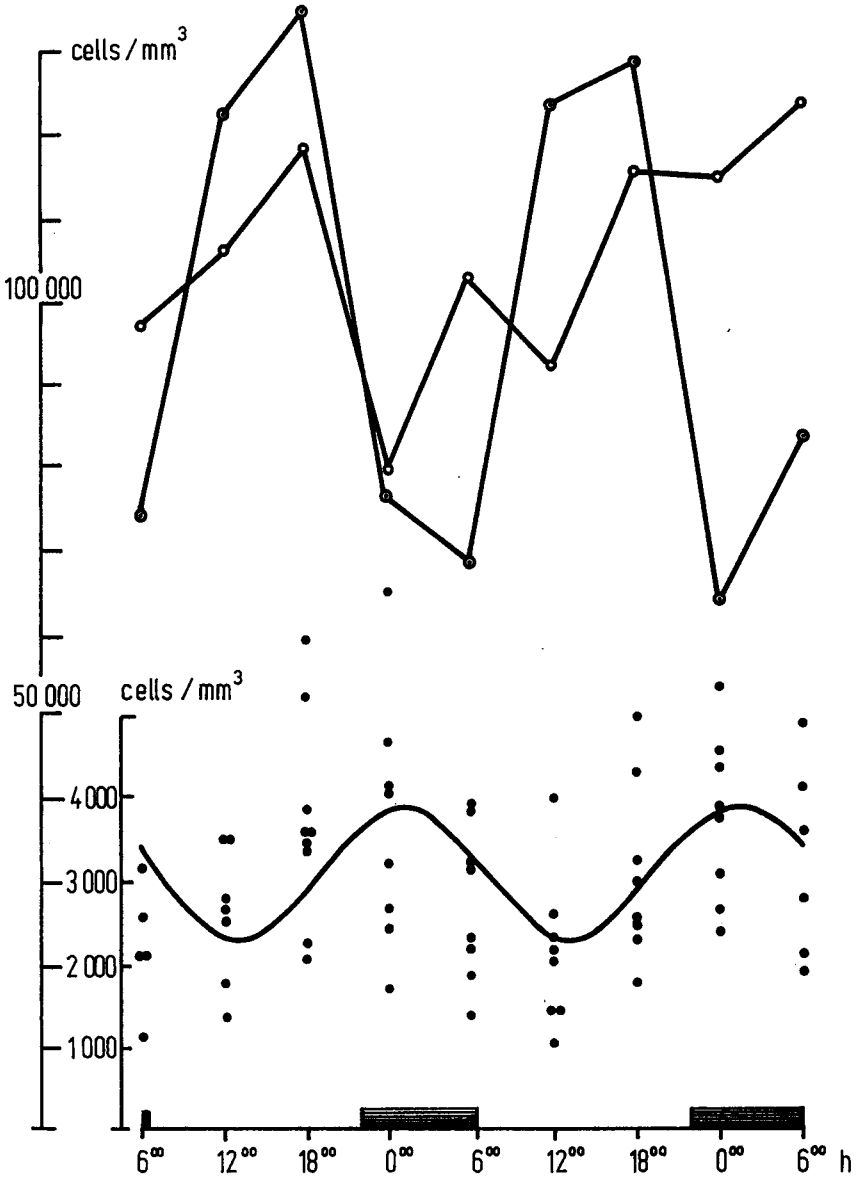

Fig. 1: *Absolute lymphocyte counts in peripheral blood in normals and in patients with chronic lymphocytic leukemia (CLL).*

Absolute cell counts were determined using a NEUBAUER counting chamber and differential counts of stained blood smears.

Lymphocyte counts (.) of 8 normal subjects and their fitted cosine function (-) are represented in the lower graph.

The upper graph contains the data from 2 patients with CLL. The computative acrophase of the regular curve was 15.00 h.

α-amanitine sensitive incorporation of ^{3}H-UMP into acid precipitable material, was observed in all normal subjects during the period of investigation. These variations followed a regular 24 hour rhythm in 6 of 8 normal subjects with maximum acitivty at 00.00 h and minimum incorporation of ^{3}H-UMP at 12.00 h (Fig. 2). Differences between enzyme activities at 00.00 and 12.00 h are highly significant ($p < 0.001$). The mean level of activity was 42.2 pmoles (^{3}H) UMP in-

Fig. 2: Activities of RNA polymerase B (α-amanitin sensitive) in normal and leukemic lymphocytes during a period of 48 hours

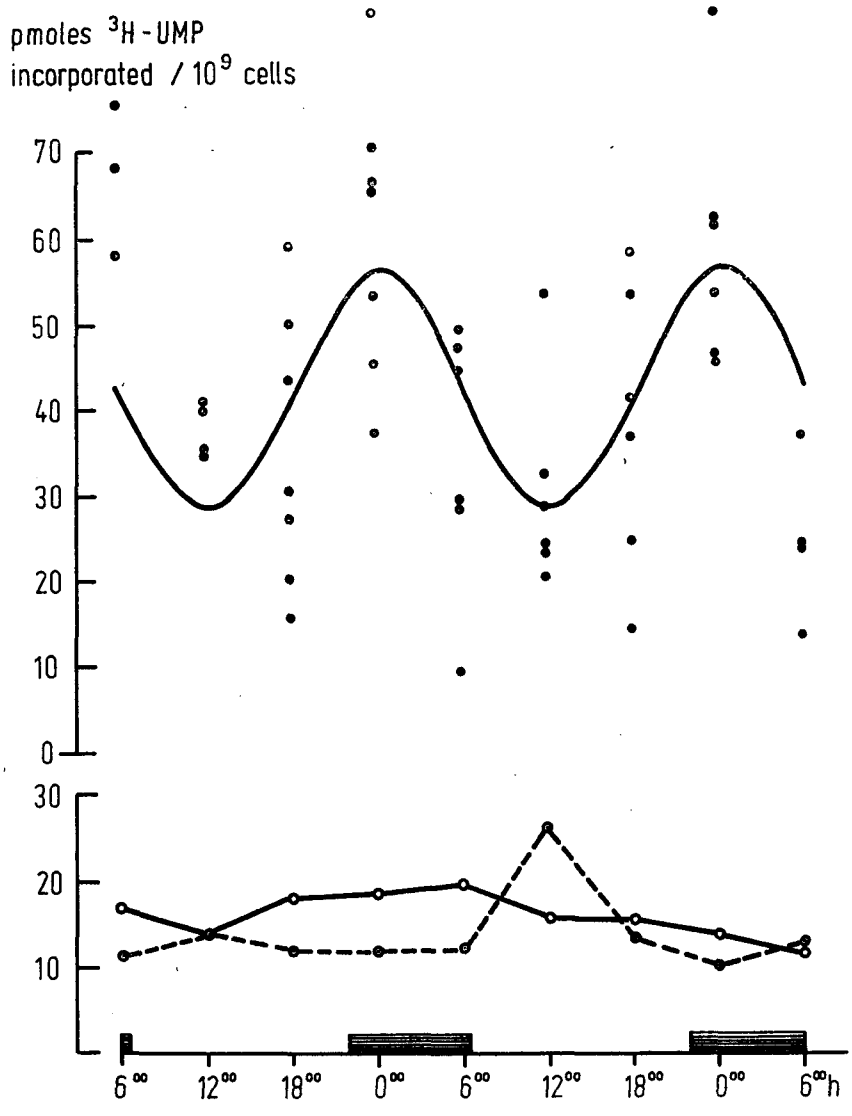

Fig. 2: *Activities of RNA polymerase B (α-amanitine sensitive) in normal and leukemic lymphocytes during a period of 48 hours.*

Lymphocytes were prepared using a modified standard procedure[3]. Citrated venous blood was layered on top of a Ronpacon^R-Ficoll-mixture (10 % Ronpacon w/v, 6.4 % Ficoll w/v) and centrifuged for 20 minutes at 1200g at room temperature. Lymphocytes were removed from the interphase, resuspended in isotonic buffer and sedimented at 175g for 20 minutes. RNA polymerase activity was determined by incubation of 50 µl of enzyme solution (sonicated nuclei[6]) in a final volume of 100 µl under the following conditions:

ATP, CTP, GTP 1 mM each, 1 µM (^{3}H) UTP (specific activity 10 or 49 Ci/mmole), 225 mM Tris HCl pH 8.5, 0.12 M $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 2.5 mM $MnCL_2$, 50 mM K-EDTA, 3 mM ß-mercaptoethanol, 2 µg of denatured calf thymus DNA, 15 % glycerol (v/v). After incubation for 30 minutes at 37 °C the reaction was terminated by pipetting duplicate aliquots of 30 µl on Whatman GF/FA filter discs, followed by TCE precipitation[6].

RNA polymerase activity is expressed in pmoles (^{3}H)-UMP incorporated per 10^9 cells The values from 6 normal subjects are shown in the upper part of the figure. The data obtained from 2 patients with chronic lymphocytic leukemia are represented in the lower part of the figure. No significant circadian variation of the enzyme activity could be demonstrated in CLL.

corporated /10^9 nuclei (range 32.9–50.0) with an amplitude of 14.3 (range 5.5–27.0). Single cosine functions were computed for each subject, computative acrophases being between 22.26 and 02.58 h. The mean cosine function was

$$f(t) = 42.2 + 14.3 \cos(\omega\tau - 6.1)$$

with the acrophase at 00.24 h (Fig. 2). Statistical analysis (variance analyses, trend analyses) proved the high significance of fitted cosine functions.

Variation of RNA polymerase B activities of two normal subjects were not compatible with a circadian rhythm, in spite of the regular circadian variation exhibited by these patients' lymphocyte counts. Mathematical analysis demonstrated an ultradian rhythm (2 periods/24 h) in one and a reversed rhythm (lowest values at 00.00 h) in the other. In two patients with CLL, no rhythmicity of α-amanitine sensitive RNA synthesis could be detected. Mean values of RNA polymerase B acitivty were 16.2 and 13.9 pmoles (^{3}H) UMP incorporated/10^9 nuclei, being significantly lower as compared to normal controls ($p < 0.05$). In contrast to the constant RNA polymerase activity, white blood cell counts exhibited extreme variations, 79,400–123,800/mm^3 in one and 62,00–135,000/mm^3 in the other. The patient with the more stable disease, requiring no specific therapy, exhibited a remarkably regular circadian rhythm of peripheral blood lymphocyte counts (Fig. 1).

Comparing our results with the circadian variation of RNA contents of total human white blood cells described by Kohler et al.[9], an inverse relationship with the amount of total RNA as well as of 5S-RNA is observed, while 4S-RNA contents follow a pattern similar to that of RNA polymerase B. Provided that activity of RNA polymerase B, the enzyme responsible for heterogenous nuclear RNA (pre mRNA) synthesis, is representative of overall RNA synthetic activity[6] of the cell, 5S-RNA would be a fraction of slow turnover accumulating at times of even low RNA synthesis, while 4S-RNA would be a fraction of high turnover. It has to be taken into account, however, that RNA synthesis is only one facet of RNA metabolism. No definite conclusions can be drawn from our observations for a dose-time schedule for application of cytostatic drugs. The existence of a circadian rhythm of RNA polymerase activity in normal and its absence in CLL lymphocytes, however, allows a rational approach towards a chronotherapy. Taking into account pharmacokinetic aspects, drugs, which exert their effect via inhibition of RNA synthesis, certainly should not have their maximum cytostatic activity during the time when the host's lymphocytes are most actively synthesizing RNA, a probable prerequisite for immunological defence mechanisms.

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