EFFECT OF CYCLOPHOSPHAMIDE INJECTION TIME ON CHANGES IN RHYTHMS OF SERUM α-FETOPROTEIN LEVELS IN MICE WITH HEPATOMA 22a AND OF TUMOR CELL PROLIFERATION

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UDC 616.36-006-092.9-085.277.3-036.
96-053.1+616.36-006-018.15

KEY WORDS: fetoprotein; hepatoma; cyclophosphamide.

It was shown previously that administration of cyclophosphamide (CP) to mice with hepatoma 22a at different times of tumor development causes different changes in the α-fetoprotein (α-FP) level in the animals' blood serum. The cytostatic was most effective on the 4th day of the growth cycle of the hepatoma [5]. Meanwhile investigations have shown that the biological action of antitumor agents depends on the time of their administration in the course of the 24-h period [1, 2, 6-8].

The object of this investigation was to study changes in the rhythm of the blood serum α-FP level and of proliferation of hepatoma 22a in mice after administration of CP at different times of the 24-h period.

EXPERIMENTAL METHOD

Experiments were carried out on 300 male C3HA mice weighing 23-25 g, into which a hepatoma 22a was transplanted after adaptation for 14 days to particular conditions of illumination (alternation of light and darkness every 12 h, daylight from 8 a.m. to 8 p.m.) and to a particular feeding rhythm (food was given *ad lib.* at 4 p.m.). The strain of hepatoma 22a was obtained from the Laboratory of Experimental and Tumor Strains, Oncologic Scientific Center, Academy of Medical Sciences of the USSR.* A suspension of tumor cells in physiological saline (1:3) in a volume of 0.5 ml (10^8 tumor cells) was injected subcutaneously into the animals' flank. In the experiments of series I dependence of the time of appearance of α-FP in the blood of mice with hepatoma 22a on the time of injection of CP on the first day of tumor development was studied. There were five groups of animals (24 mice in each group): 1) control, 2) receiving CP at 11 a.m., 3) at 5 p.m., 4) at 11 p.m., and 5) at 5 a.m. The α-FP concentration was determined in mice of all groups from the 8th through the 11th day of the experiment daily at 11 a.m.-12 noon. The mice of series II were divided into the same five groups but received CP on the 4th day of tumor growth and the α-FP concentration and parameters of proliferation were determined on the 12th-13th day of the growth cycle of the hepatoma, at different times of the 24-h period (11 a.m., 2, 5, 8, and 11 p.m., 2, 5, and 8 a.m. in 48 control animals and at 11 a.m., 5 and 11 p.m., and 5 a.m. in the 96 experimental animals). In series I and II CP was injected subcutaneously in a single dose of 150 mg/kg. The control and experimental animals of series II were given an injection of [^{3}H]thymidine, in a dose of 0.7 mCi/g body weight (specific activity of the isotope 12.5 Ci/mmole) 1 h before sacrifice. The animals of different groups, taken at a particular time of the investigation (from 11 a.m. on the 12th day to 8 a.m. on the 13th day) were killed simultaneously by decapitation. The serum α-FP concentration was determined by double immunodiffusion in agar, using a standard test system, by Ouchterlony's method in Khramkova and Abelev's modification [3]. The sensitivity of the test system was 0.5 mg %. Pieces of tumor for histological investigation were kept in Carnoy's solution, dehydrated, and embedded in paraffin wax. To prepare autoradiographs, sections 3 μ thick were coated with type M (Photographic Chemical Research Institute Project) emulsion and exposed for 22 days at 4°C, after which the developed sections were stained with Mayer's hematoxylin. To assess proliferation of the hepatoma cells the mitotic

*The authors are grateful to E. S. Revazova for providing the strain of hepatoma.

Department of Biology, Medico-Biological Faculty, N. I. Pirogov Second Moscow Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR N. P. Bochkov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 93, No. 4, pp. 73-75, April, 1982. Original article submitted October 13, 1981.

TABLE 1. Time Course of Changes in α-FP Level (in mg %) in Blood Serum of Control Mice with Hepatoma 22a and Experimental Mice Receiving CP on the 4th Day (at 11 a.m., 5 and 11 p.m., and 5 a.m.)

Group of animals	Day of tumor development				
	8th	9th	10th	11th	12th
1 (control)	1,2±0,07	1,0±0	1,0±0	1,8±0,57	4,16±1,08
2	0	0	0,1±0,1	1,5±0,69	0,62±0,24
		$P<0,001$		$P<0,001$	
3	0	1,0±0,76	0,3±0,07	1,2±0,71	1,0±0,22
				$P=0,034$	
4	0	0	0	0,5±0,39	1,0±0,25
				$P=0,034$	
5	0	0	0	0,8±0,07	1,0±0,42
				$P=0,034$	

Legend. Values of P given compared with control.

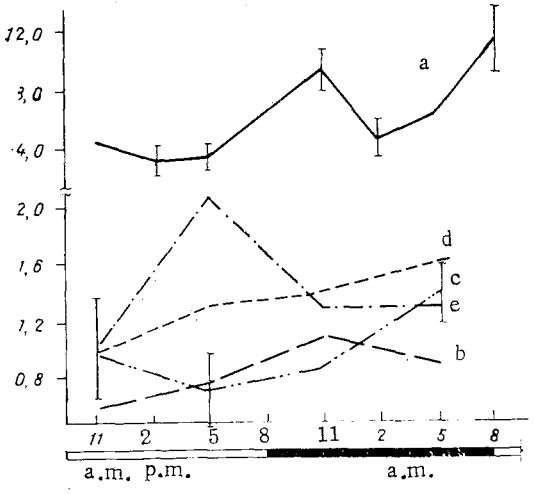

Fig. 1

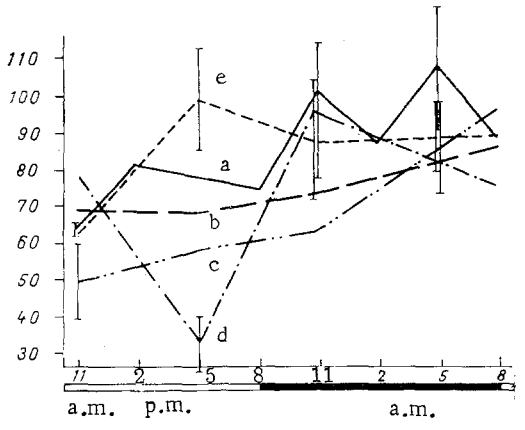

Fig. 2

Fig. 1. Daily fluctuations in serum α-FP level in mice with hepatoma 22a depending on clock time of injection of CP. Abscissa, clock time; ordinate, α-FP concentration (in mg %). a) Without CP, b) injection of CP at 11 a.m., c) at 5 p.m., d) at 11 p.m., e) at 5 a.m. Here and in Figs. 2 and 3, vertical lines on curve show value of m.

Fig. 2. Daily fluctuations in number of DNA-synthesizing cells of hepatoma 22a in mice depending on clock time of injection of CP during 4th day of tumor development. Abscissa, clock time; ordinate, value of RI. a) Control, b) injection of CP at 11 a.m., c) at 5 p.m., d) at 11 p.m., e) at 5 a.m.

index (MI) and the index of DNA-synthesizing cells (RI) were calculated after examination of 7000-10,000 cells in preparations from each animal, and the results were expressed in promille. The cells were taken to be labeled with [^{3}H]thymidine if four or more grains of reduced silver were observed above their nucleus. The results were subjected to statistical analysis by Fisher's and Student's tests, at a level of significance of $P \leq 0.05$.

EXPERIMENTAL RESULTS

As Table 1 shows, α-FP appeared in the blood serum of the control animals on the 8th day of tumor development. Injection of CP on the 4th day of the growth cycle of the tumor delayed appearance of the protein by 1-3 days depending on the clock time of injection of the compound. The greatest change in the time of appearance of α-FP in the blood was observed after injec-

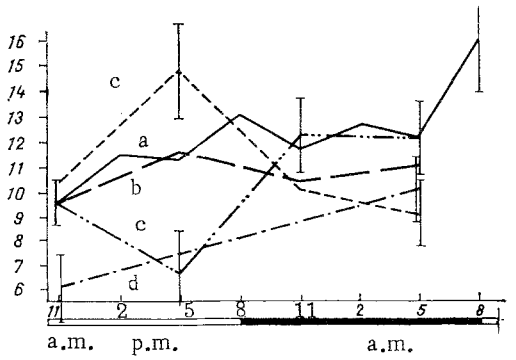

Fig. 3. Daily fluctuations in number of dividing hepatoma 22a cells in mice depending on time of injection of CP during 4th day of tumor development. Abscissa, clock time; ordinate, value of MI. a) Control; b) injection of CP at 11 a.m., c) at 5 p.m., d) at 11 p.m., e) at 5 a.m.

tion of CP at 11 p.m. and 5 a.m., the smallest when it was injected at 5 p.m. When CP was injected on the 4th day of tumor development, it lowered the α-FP level considerably (by 76-85%) compared with the control on the 12th day of growth of the hepatoma (Table 1).

The results of the experiments of series II show that the serum α-FP level of the control mice differed at different clock times on the 12th-13th day of development of hepatoma 22a (Fig. 1a). Throughout the period studied, this parameter was observed to increase twice (at 11 p.m. on the 12th day and at 8 a.m. on the 13th day) compared with its values at 2 p.m. and 1 a.m. on the 12th day and at 2 a.m. on the 13th day ($P \leqslant 0.02$). The relative amplitude of the fluctuations was 2.5-2.8 and the mean value over the 24-h period was 6.1 ± 1.0.

The writers showed previously [4] that the **α-FP** level in the blood serum of mice undergoes rhythmic changes on the 15th-16th day of development of hepatoma 22a. The results of the present investigation show that these fluctuations also take place at earlier times of tumor development. Just as on the 15th-16th day of the growth cycle of the tumor, they have a **period of** under 24 h.

Changes in the values of RI and MI were observed in tumors in the control animals on the 12th and 13th day of their development (Figs. 2 and 3). Minimal values of MI and RI in the control were observed at 11 a.m. on the 12th day and maximal values at 8 and 5 a.m. on the 13th day, respectively ($P = 0.029$; $P = 0.034$). The relative amplitude of the rhythms was 1.7 and the mean diurnal values of RI and MI were 85.16 and 12.24%, respectively.

No significant fluctuations in the **α-FP** titer in the blood serum of animals of groups 2 and 4 were found during the 24-h period (Fig. 1b, e). The mean diurnal values of **α-FP** in these groups were 0.8 and 1.3, or 79 and 87% below the control ($P = 0.001$). In the animals of group 3 rhythmic changes in the **α-FP** level were observed, with a minimum at 5 p.m., and a maximum at 5 a.m. ($P = 0.05$).

In the animals of group 5 fluctuations in the **α-FP** level likewise took place during the 24-h period with a maximum at 5 p.m. and a minimum at 11 a.m. ($P = 0.05$). The relative amplitude of the rhythm of **α-FP** in the mice of these groups was 1.5 and its mean diurnal values 1.0 and 1.6 (74 and 84% respectively, below the control; $P = 0.001$).

Injection of CP into mice with hepatoma 22a at different times on the 4th day of tumor development thus was accompanied not only by delay in the appearance of **α-FP** in the blood, the duration of which varied, but also by disturbances of the structure of the rhythmic waves of the protein level and a fall in its mean diurnal values. Besides disappearance of the rhythmic changes in the **α-FP** concentration in the blood serum, there were also characteristic phase shifts of the rhythms during the 24-h period with a decrease in the relative amplitude of the waves compared with the control.

In the animals of group 2 no rhythmic changes in RI and MI were found in the tumor (Figs. 2 and 3). The mean diurnal values of these parameters were 72.14 and 10.58%, respectively (difference from control not significnat). Rhythms of RI and MI (Figs. 2 and 3) were observed in the tumors in the mice of group 3. Minimal values of RI were observed at 11 a.m. on the 12th day and maximal values at 5 a.m. on the 13th day ($P = 0.023$), the relative ampli-

tude was 1.9, and the mean diurnal value of RI was 65.79%. The lowest value of MI was noted at 5 p.m. and its highest values at 11 p.m. and 5 a.m. (P = 0.047); the relative amplitude was 1.8 and the mean diurnal value 10.03%. Although the mean diurnal values of RI and MI were lower than the control values by 23 and 18%, respectively, these differences are not significant.

The number of DNA-synthesizing cells in tumors in the animals of group 4 reached a maximum at 11 p.m. and a minimum at 5 p.m. (P = 0.017); the relative amplitude of the fluctuations was 2.8 and the mean diurnal value of RI 70%. The number of mitoses in the tumors in these animals was increased at 5 a.m. and reduced at 11 a.m. (P = 0.05); the relative amplitude of the rhythm was 1.7 and the mean diurnal value 8.08%. The mean diurnal value of RI in these animals did not differ significnatly from the control, whereas the mean diurnal value of MI was 34% below the control value (difference statistically significant, P = 0.006).

In the animals of group 5 high values of RI were observed between 5 p.m. and 5 a.m., and minimal values at 11 a.m. (P = 0.047-0.05). The relative amplitude of the waves was 1.6 and the mean diurnal value 88.78%. The highest values of MI in these animals were found at 5 p.m. and the lowest at 5 a.m. (P = 0.032). The relative amplitude was 1.6 and the mean diurnal value of MI 11.11%. On average for the 24-h period neither RI nor MI differed significantly from the control.

The action of CP, injected into animals at different clock times during the experiment, was thus manifested chiefly as changes in parameters of rhythms of the number of DNA-synthesizing and dividing hepatoma cells. Depending on the clock time at which CP was injected, the rhythms of RI and MI either remained intact or were suppressed, or underwent phase shifts.

The results of this investigation are evidence that CP, when injected into animals on the 4th day of growth of a hepatoma 22a, induces a subsequent significant fall in the blood α-FP level, whereas its inhibitory effect on proliferative processes in the tumor is much weaker. Meanwhile the times of appearance of α-FP in the blood, the character of rhythmic fluctuations in the protein concentration, and the number of DNA-synthesizing and dividing tumor cells all depend on the clock time of injection of the cytostatic.

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