0.001), those isolated at 1 and 9 p.m. and 1 a.m. by 14% (P = 0.07; P = 0.17); the 5 p.m. extract did not cause significant inhibition of cell division in EAT.

The results of these experiments thus show that the mitosis-inhibiting activity of chalone-containing preparations of EAT depends significantly on the time of the 24-h period when they were isolated. Similar results were obtained in experiments *in vivo* with EAT chalone [1] and with hepatic chalone [2], although in these studies activity of the chalones isolated only at 4 and at 2 different times of the 24-h period was analyzed. Unfortunately, because of the low degree of purity of the chalone preparations it is impossible to give a precise answer to the question whether it is the production of chalones or their activity that changes in the course of the 24-h period.

The results point to the possibility of a role of $G_2$-chalone of EAT in the formation of the circadian rhythm of MA in this tumor by periodic changes either in the quantity of chalone produced by the tumor cells or in its activity. In addition, they are evidence that the mitosis-inhibition action of this chalone in an EAT culture may be manifested without the participation of adrenalin.

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# ACTION OF SPECIFIC PLACENTAL PROTEINS — TROPHOBLASTIC β-GLYCOPROTEIN AND CHORIONIC $\alpha_1$-MICROGLOBULIN — ON PROLIFERATION OF LYMPHOCYTES AND MALIGNANT FIBROBLASTS *in vitro*


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During the development of malignant tumors renewal of biosynthesis of embryonic and placental proteins is observed [4, 6, 8, 9], although their role in carcinogenesis has not been studied. Possibly in tumor-bearing animals, just as in pregnant women, the presence of embryonic proteins in the blood leads to the appearance of areactivity of the tumor and fetus toward transplantation antigens, for some of these proteins have the property of depressing lymphocyte proliferation in mixed cultures [10-12]. It is interesting to investigate whether embryonic proteins can inhibit the division of other intensively proliferating cells, i.e., tumor and embryonic cells. Although no such action is observed *in vivo*, one or two studies have demonstrated a possible inhibitory effect of fetoplacental factors on embryonic and tumor cells *in vitro* [1, 2, 7]. The mechanism of protection *in vivo* against the inhibitory effect of fetoplacental factors on proliferating tissues of embryo and tumor is unknown.


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The effect of two specific placental proteins, trophoblastic $\beta_1$-glycoprotein (TBG) and chorionic $\alpha_1$-microglobulin ($CAG_1$) on proliferation of malignant fibroblasts and lymphocytes activated with phytohemagglutinin (PHA) was studied.

## EXPERIMENTAL METHOD

Preparations of TBG were obtained from retroplacental serum of women in labor by the method described by Tatarinov et al. [6]. The TBG preparations had a purity of 80-90%. Preparations of $CAG_1$ were obtained from amniotic fluid in the second trimester of pregnancy by successive precipitation with 0.5% lanthanum chloride solution, ammonium sulfate (50% saturation), and lithium sulfate (60% saturation), followed by dialysis and adsorption chromatography on calcium pyrophosphate. The resulting preparation had a purity of 90%. Solutions of TBG and $CAG_1$ were sterilized by filtration through millipore filters with a pore diameter of 0.3 μ (Synpore).

Lymphocytes were isolated from peripheral blood of adult donors by centrifugation in a Ficoll–Urotrast density gradient. For the blast-transformation reaction lymphocytes were cultured in a dose of $0.5 \cdot 10^6$ in centrifuge tubes in 1 ml of medium No. 199 containing 20% inactivated AB human serum. Penicillin and streptomycin were added to the medium in a dose of 100 units/ml. The experimental tubes contained 0.1 ml of a solution of TBG or $CAG_1$ with different concentrations of protein, whereas the control tubes contained 0.1 ml of physiological saline. PHA-P (from Difco) in a dose of 10 μg/ml was used as mitogen. To investigate the combined action of TBG and $CAG_1$, 0.1 ml of a mixture of protein TBG and $CAG_1$ (60 and 30 μg/ml, respectively) was added to the experimental tubes, and 0.1 ml of either protein (30 and 15 μg/ml) was added to the control tubes. The lymphocytes were incubated for 72 h. Transformed mouse fibroblasts (cells of continuous line L) were used as malignant cells. The cells were introduced into penicillin flasks in a dose of $1 \cdot 10^5$ and cultured for 48 h in 3 ml of medium No. 199 containing 10% inactivated bovine serum. The experimental samples contained 0.3 ml of solutions of TBG or $CAG_1$ in different concentrations. When the combined action of these proteins was tested, 0.3 ml of the mixture of TBG and $CAG_1$ was added to the experimental flasks and 0.3 ml of each protein separately to the control flasks, in the same concentrations as when acting on the lymphocytes. The results were read as incorporation of $^3$H-thymidine added to the cultures in a dose of 2 μCi/ml, 4 h before the end of incubation. The cells were then deposited on filters with a pore diameter of 0.6 μ and treated with 5% TCA and 96° ethanol. The filters were placed in cuvettes with scintillation fluid and the number of incorporations of $^3$H-thymidine counted in a special counter. The viability of the cells was assessed by means of trypan blue.

## EXPERIMENTAL RESULTS

The preparations of TBG and $CAG_1$ had no toxic action on lymphocytes on malignant fibroblasts. The number of viable cells was the same as in the control after 24 h and 72 h of incubation in the presence of 60 μg/ml of each of these proteins.

The effect of TBG and $CAG_1$ on PHA-induced proliferation of lymphocytes is shown in Fig. 1A, in which the absolute number of incorporations of $^3$H-thymidine in the control cultures, namely 68,000 ± 1733 cpm, is taken as 100%. In cultures without the mitogen the number of incorporations was 925 ± 190 cpm (index of stimulation 73). TBG in a dose of 60 μg/ml caused almost total inhibition of the proliferative response. This concentration of the protein is physiological, for approximately the same level of TBG is observed in the serum of women during the second trimester of pregnancy, whereas in the third trimester it is four times higher [5]. A decrease in the TBG concentration led to a decrease in the inhibitory effect, which was completely abolished by a dose of 3 μg/ml. Not all series of the TBG preparation had equal suppressive activity. Protein of one series, in a dose of 60 μg/ml, inhibited the proliferation response by only 50%. $CAG_1$, in the same doses as TBG, did not depress stimulation of lymphocytes.

Similar results were obtained when the effect of TBG and $CAG_1$ on proliferation of transformed fibroblasts was studied (Fig. 1B). TGB inhibited proliferative activity of L-cells with a marked dose dependence of the effect. In a protein concentration of 60 μg/ml the number of $^3$H-thymidine inclusions in the experimental cultures was 20.3 ± 4.4% of the control ($P < 0.01$). $CAG_1$ in the same concentrations had no effect on division of malignant fibroblasts.

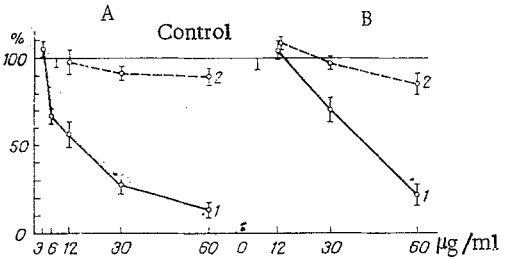


Fig. 1

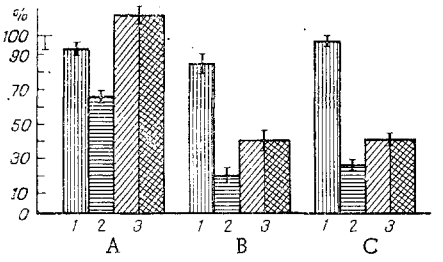


Fig. 2

Fig. 1. Effect of TBG and $CAG_1$ on PHA-induced proliferation of lymphocytes (A) and proliferation of malignant fibroblasts (B). Abscissa, protein concentration in culture (in μg/ml); ordinate, number of incorporations of $^3H$-thymidine in experimental cultures, expressed as percentage of control. 1) TBG; 2) $CAG_1$.

Fig. 2. Combined action of TBG and $CAG_1$ on proliferation of lymphocytes stimulated by PHA (A, B) and on mitotic activity of malignant fibroblasts (C). Horizontal axis) protein concentration in culture (in μg/ml); vertical axis) number of incorporations of $^3H$-thymidine in experimental cultures, expressed as percentage of control. 1) $CAG_1$ (15 μg/ml); 2) TBG (30 μg/ml); 3) mixtures of TBG and $CAG_1$ preparations in the same doses.

TBG thus depressed proliferation of malignant cells *in vitro*. Probably its inhibitory effect was not exhibited *in vivo* because of some protective mechanisms. We know that TBG is secreted intensively by the placenta into the maternal blood stream and, under normal conditions, it is practically never found in amniotic fluid [5]. Conversely $CAG_1$ in the highest concentrations is present in the amniotic fluid in the first trimester of pregnancy, but is not found in the serum of pregnant women [3]. It was therefore postulated that the biological function of $CAG_1$ is opposite to that of TBG.

Accordingly, the effect of TBG on proliferation of lymphocytes and malignant fibroblasts was studied when mixed with $CAG_1$ (Fig. 2). The dose of TBG in the mixture was twice the dose of $CAG_1$ since the maximal concentration of TBG *in vivo* is about twice that of $CAG_1$. Complete abolition of the inhibitory effect of TBG on PHA-induced lymphocyte proliferation was observed in the presence of one batch of the $CAG_1$ preparation (Fig. 2A). The TBG preparation used in these experiments in a concentration of 30 μg/ml inhibited the proliferative response of the lymphocytes to PHA to 65.7 ± 3.1% of the control ($P < 0.05$); the $CAG_1$ preparation in a dose of 15 μg/ml did not affect mitotic activity of the lymphocytes (92.3 ± 3.1%). A mixture of these preparations in the same concentrations actually had a slight stimulating effect (114.7 ± 5.4%; $P \geqslant 0.05$). When the action of a mixture of other batches of TBG and $CAG_1$ on proliferation of lymphocytes and also of malignant fibroblasts was studied, only a decrease in the inhibitory effect of TBG was observed. TBG reduced the proliferative activity of the cells to 22.0 ± 4.0% (Fig. 2B) and 27.5 ± 2.7% (Fig. 2C) of the control. Mixed with $CAG_1$ it increased proliferative activity to 45.2 ± 6.3% (Fig. 2B) and 40.0 ± 3.1% (Fig. 2C). The differences are statistically significant ($P < 0.05$).

On the basis of these results it is difficult to decide whether the inhibition of abolition observed was due to the $CAG_1$ protein itself or to other impurities in the preparation. Further investigations are needed with the use of different batches of preparations and with choice of optimal concentrations of both proteins.

However, the results do suggest that besides suppressive factors, fetal and tumor cells may secrete substance abolishing the action of inhibitors. This is possibly the way in which intensively proliferating embryonic and tumor tissues are protected *in vivo*.

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