BIOLOGICAL ACTIVITY OF SPLEEN CHALONES DETERMINED IN THE CULTURE OF LYMPHOCYTES

Department of Biochemistry, Veterinary Institute, Puławy
Head: Doc. Dr hab. Jadwiga Grundboeck-Juśko

JADWIGA GRUNDBOECK-JUŚKO

Chalones B and T isolated from bovine spleens were added to the 20-hr culture of bovine lymphocytes containing labelled precursors of the RNA-, protein- and DNA-synthesis. Their activity was established 2, 4 and 4.5 hrs later by the estimation of ^{3}H-uracil, ^{14}C-thymine, ^{3}H-thymidine and ^{14}C-leucine incorporated to the cells. The amounts of the incorporated isotopes were estimated with the scintillation spectrophotometer L-30 Intertechnique. It has been stated that both chalones reduce the incorporation of uracil, leucine and thymidine, what indicates that they inhibit the mitotic divisions influencing the lymphocytes at the stage of synthesis of RNA and protein. This stage precedes the phase of DNA-synthesis and hence a smalled quantity of labelled DNA appears in the cells. Chalones B and T inhibit the mitoses acting on the phase G_1. The differences in the effect of both chalones on the cell seem not to exist.

Hitherto performed investigations of the spleen chalones have given information about their structure, some physico-chemical properties and inhibiting by them the mitotic divisions in the lymphatic organs of mice (Grundboeck-Juśko 6, 7). The mechanism of this action has not been completely explained and contradictiory opinions have appeared in the literature. It is known that the reproductive cycle of cell division has been divided into four phases: phase of mitosis (M); "resting" phase G_1 in which the synthesis of protein, RNA and some enzymes, necessary for DNA replication takes place; phase S in which the synthesis of DNA is performed leading to the reduplication of this compound, and a short "resting" phase G_2 in which the proteins participating in the mitosis (e.g. proteins of the spindle) are synthesized, and then the next mitosis phase. Some cells after the mitotic division pass through a longer period of rest (so-called phase G_0) after which they can join the cycle in the phase G_1.

The aim of this work was the estimation, by means of labelled precursors, of the quantitative changes in the DNA-, RNA- and protein-synthesis occurring under the influence of chalone B or T, as well as an ascertainment whether those two compounds behave identically.

Material and Methods

The material for this study consisted of chalones obtained from the bovine spleens (Grundboeck-Juśko 6, 7). The activity of preparations of chalone B (fr. A-I and A-III) and T (fr. A-IV) have been checked in mice before application of them in the tissue culture. The lyophilized chalones have been used in the experiment. They were so diluted for the study that the protein content was in the range 30—100 μg/0.1 ml.

The blood collected from healthy cattle at the slaughter was used for lymphocyte culture. The blood was mixed with solution consisting of: 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 50 g EDTA in 1000 ml of redistilled water. This solution constituted 5 per cent of the collected blood. Within 30 minutes after collection, the blood was transported to the laboratory and it was centrifuged for 15 min. at 4000 rpm. After discarding the blood plasma, the buffy coat was collected and treated with 1/5 of original volume of distilled water for hemolysis of erythrocytes. After

30 sec., 1.7 per cent NaCl has been added, and the suspension was centrifuged again for 10 min. at 2000 rpm. The sediment was washed twice in PBS. Then the sediment was suspended in Parker's medium using 1/80 of the original volume. The leukocytes have been counted and diluted in the growth medium consisting of: 60 ml of Parker's medium, 20 ml of calf serum, 0.25 of phytohemagglutinin, 0.75 ml of antibiotic solution (0.5 g of streptomycin, 50 000 units of penicillin in 50 ml of redistilled water). The original number of leukocytes in the culture was 10×10^6 cells in 1 ml. Each Leighton's tube has been filled with 2.5 ml of culture suspension. Then, 50 μg of the synthesis precursor of DNA, protein or RNA, labelled with carbon or tritium, has been given to a tube. Thymine ^{14}C has been applied in the first experiment, ^{14}C-leucine in the second one, ^{3}H-uracil in the third one, and ^{3}H-thymidine in the fourth one. The isotopes were used in the following dilutions: thymine 3.75 μCi/ml, leucine 1.25 and 2.50 μCi/ml, uracil 50 μCi/ml and thymidine 25 μCi/ml of NaCl physiological solution. Volumes of 50 μl of these solutions were used for 2.5 ml of culture fluid. Some tubes without isotopes served as the control.

Leukocytes were cultivated for 19—20 hrs in 37°C and then some cultures were treated with chalone T and other with chalone B. Ten μg of colchicine in 0.1 ml of PBS or only 0.1 ml of PBS have been given to the control tubes. The protein content in the chalone preparations was within the range of 30—160 μg/0.1 ml. The culture was maintained for further 2.4—4.5 hrs and then the tubes were chilled.

In the fourth experiment with ^{3}H-thymidine, an additional variant has been applied in which the isotope and chalone were introduced to the leukocyte culture at the same time. The samples were refrigerated after 4.5 hrs and then they were utilized like in experiments with other isotopes.

Preparation of samples for the scintillation spectrophotometry. — 2, 4 or 4.5 hrs after application of chalone, the cultures have been chilled in an ice bath. Then, analogical samples in groups of three were put together and centrifuged for 15 min. at 4000 rpm. The supernatant was discarded and the sediment was washed twice with PBS and centrifuged after each washing. Then, the remaining fluid was drained off from the tubes which were placed on the filter paper upside down, and then the sediment was suspended in 0.5 ml of PBS. 100 μl of the suspension of each sample has been transferred on the filters of glass fibre paper Whatman GF/C, size 1×1 cm. The filters were dried in a stream of hot air and placed in 20 ml of scintillation fluid containing 4 g PPO, 100 mg POPOP in 1 l of toluen. The samples ready for use were placed in the scintillation spectrophotometer (SL-30 Intertechnique). Each sample was counted three times, 10 min. each time. The controls consisted of the filters by themselves, the filters with istotope without culture and the filters with the culture without isotope. Each version was examined twice at least.

The remaining culture has been diluted with 3 ml of PBS, the leukocytes were counted in a Bürker chamber and the density of the suspension was estimated by means of a Coleman Junior colorimeter, diameter of tubes 1 cm, wave length 655 nm. The obtained figures were reduced to equivalents of 1×10^6 cells and were presented in disintegrations per minute (dpm).

Results

In the presented experimental scheme, it has been observed the inhibition of incorporation of the labelled thymine to DNA by the fraction A-I in 5 among 6 performed experiments (Table 1). The assimilation of the isotope was lowered by the chalone 10 to 22.5 per cent in comparison with the control culture. Using the labelled thymidine, it has been shown the inhibition of DNA-synthese by three fractions of chalones: A-I, A-III and A-IV (Table 4).

The synthesis of protein, as measured by the incorporation of leucine, has been inhibited by the fraction A-III in 5 among 6 experiments. The decrease of assimilation of the isotope induced by the fraction A-I (Table 2) was 7—54 per cent in 3 among 7 performed experiments and in 3 among 8 experiments with the fraction A-IV.

Table 1

Influence of the chalone (fr. A-I and A-IV) on the incorporation of ^{14}C — thymine to DNA of leukocytes in vitro

Experiment	Time of action (hrs.)		Background in cpm	Activity of thymine in dpm 1×10^6 cells			
	of isotope	of chalone or colchicine		Not treated cells (control)	Cells treated with		
					colchicine	chalone fr. A-I	chalone fr. A-IV
1	19	4	26	79.4	35.75 (55)	72.07 (10)	72.39 (9)
2	20	4	26	42.3	41.30 (3)	43.15 (2)	35.76 (16)
3	19	4	27	52.0	51.08 (2)	42.93 (18)	50.00 (4)
4	19	4	27	30.7	29.23 (5)	25.54 (17)	25.98 (15)
5	21	4	26	29.2	22.83 (22)	23.26 (20)	28.91 (1)
6	21	4	26	20.0	18.48 (8)	17.50 (22)	20.00 (0)

Figures in parenthesis indicate the incorporation decrease in comparison with control (in per cent)

The incorporation of uracil to the lymphocytes and thus the synthesis of RNA, was inhibited by the fractions A-IV, A-I, and A-III in all experiments. The decrease of the assimilation of uracil was 22—68 per cent (Table 3).

Discussion

On the basis of presented results, it may be concluded that the fraction A-IV regarded as chalone T inhibits the synthesis of RNA, DNA and protein. It is known that the synthesis of RNA occurrs in the phase G_1 of the cell reproduction cycle and therefore it may be accepted that chalone T acting on the lymphocytes inhibits them in the phase G_1. The third fraction A-I regarded as the chalone B inhibits markedly the RNA synthesis diminishing the incorporation of uracil, however, it does not influence the synthesis of protein. However, exerting an influence on synthesis of RNA it inhibits the proliferation of cells arresting them in the phase G_1. The big decrease of the RNA synthesis reveals the drop of normal metabolism of leukocytes in the phase G_1. The lowering of the DNA synthesis may be explained by the decrease of the cell number in the phase S, what is a natural consequence of the cell inhibiting in the phase G_1. Distinct differences have not been revealed between the activities of both chalones.

Natural inhibitors of mitosis isolated from bovine spleens restrained the incorporation of thymidine in the leukocyte culture from people affected with leukemia (Haucke 8, 9). However, analogical facts have not been observed concerning the ^{14}C-uracil and the phenylalanine. Similar effect found Attallah et al. (1) treating the cultures of human lymphocytes with the chalone from calf spleens of molecular weight 30 000—50 000 D. After an incubation of 66 hrs, 1 μCi of ^{3}H-thymidine was added and the cell cultivation was continued for additional 6 hrs. The authors found 51 per cent inhibition of thymidine incorporation within 72 hrs. This inhibi-

Table 2

Influence of the chalone (fr. A-I, A-III and A-IV) on the incorporation of ^{14}C — leucine to protein of leukocytes in vitro

Experiment	Time of action (hrs)		Activity of leucine in dpm 1×10^6 cells				
				Cells treated with			
	of isotope	of chalone or colchicine	Not treated cells (control)	colchicine	chalone fr. A-I	chalone fr. A-III	chalone fr. A-IV
1	20	4	77.0	75.0 (3)	99.5 (—)	— (—)	166.2 (—)
2	20	4	117.6	95.8 (19)	125.9 (—)	— (—)	159.7 (—)
3	19	4	149.6	132.7 (12)	182.2 (—)	192.4 (—)	125.6 (17)
4	19	4	56.0	54.8 (3)	60.7 (—)	35.2 (38)	54.5 (3)
5	19	4	246.0	225.7 (9)	106.2 (57)	180.0 (27)	131.7 (47)
6	20	4	470.5	385.9 (18)	396.2 (16)	419.1 (11)	641.6 (—)
7	19	4	735.6	731.9 (1)	727.9 (2)	688.2 (7)	702.6 (5)
8	19	4	3115.5	2993.3 (4)	2130.8 (32)	1429.1 (54)	1053.2 (66)

Note. 0.3125 μCi of leucine in 50 μl have been used in experiments 1 —4.
0.625 μCi of leucine in 50 μl have been used in experiments 5 — 8.
Figures in parenthesis indicate the incorporation decrease in comparison with control in %

Table 3

Influence of the chalone (fr. A-I, A-III and A-IV) on the incorporation of ^3H — uracil to RNA of leukocytes in vitro

Experiment	Action of (in hrs)		Background in cpm	Activity of uracil in dpm 1×10^6 cells				
				Not treated cells (control)	Cells treated with			
	Isotope	chalone (colchic.)			colchicine	chalone fr. A-I	chalone fr. A-III	chalone fr. A-IV
1	20	2	19	31185	17980 (43)	16675 (47)	20215 (36)	12615 (60)
2	19	2	18	21190	11330 (47)	8715 (60)	15085 (29)	6880 (68)
3	19	2	18	7075	6700 (6)	5044 (29)	5585 (22)	4915 (31)
4	20	2	20	4700	3290 (30)	3695 (22)	4135 (17)	3430 (28)
5	20	2	19	7315	6330 (14)	4565 (38)	6295 (14)	6170 (16)

Figures in parenthesis indicate the incorporation decrease in comparison with control (in per cent)

Table 4

Influence of the chalone (fr. A-I, A-III and A-IV) on the incorporation of ^3H — thymidine to DNA of leukocytes in vitro

Experiment	(in hrs) Action of		Background in cpm	Activity of thymidine in dpm 1×10^6 cells				
				Not treated cells (control)	Cells treated with			
	Isotope	chalone (colchic.)			colchicine	chalone fr. A-I	chalone fr. A-III	chalone fr. A-IV
1	4.5	4.5	24	246.10	241.04 (3)	155.28 (37)	213.64 (14)	183.92 (26)
2	4.5	4.5	24	800.75	727.64 (10)	788.70 (2)	— (—)	533.00 (34)

Figures in parenthesis indicate the incorporation decrease in comparison with control (in per cent)

tion was more distinct in lymphocytes T. A lowered incorporation of ^{3}H-thymidine to lymph node and thymus cells of guinea pigs was observed in vitro by Ernstrom (3). The chalone was applied on the beginning of the experiment and the isotope 30 minutes before the termination of the culture. The largest decrease of the incorporation was observed in the 8-hr culture. An inhibiting effect has not been found in the 4-hr cultures. Only the cultures older than 5 hrs showed this effect. The molecular weight of the compound causing this effect was 30 000 D. The decrease of thymidine incorporation may demonstrate a smaller DNA synthesis. Similar observations were described by Moorhead et al. (12). The major part of pre-cited authors studied the thymidine incorporation. In our investigations, we have also noted an inhibition of thymidine assimilation, however, the inhibiting effect was considerably higher at the estimation of uracil incorporation.

In all our experimental schemes, similar conditions were applied, however, they were different from those described by other authors. The istotope had been introduced to the cell culture at once and the chalone 2, 4 — 4.5 hrs before the termination of the culture, in fear of its cytotoxic activity. The above technique was also motivated by the fact that the action of chalone may be inverted in connection to the dose (Lazzio et al. 10, 11). Thus, we considered that the time of influence of the chalone on cell culture should not be too long, however, it has to be appropriate to the duration of the longest phase. According to Frindel et al. (4) the G_1 phase of the bone marrow cells of mice lasts 2 hrs., G_2 one lasts the same time, and the phase S 4.5 hrs.

It is evident from the experiments of Balazs et al. (2) that lymphocytes cultivated *in vitro* excrete to the culture fluid a factor called division cycle inhibitor (DCI). It is possible that the lymphocytic chalone is involved here. This compound, isolated by Stejrnholm (13) from human blood, inhibited mitoses of human lymphocytes *in vitro* blocking also the synthesis of DNA, RNA and protein.

In our experiments, we have applied chalone B (fr. A-I) and chalone T (fr. A-IV). The chalone T induced a greater inhibition when thymidine and uracil had been used. It may be explained by the fact that the whole population of lymphocytes of bovine peripheral blood consists of lymphocytes T in 81.4—87.4 per cent, what has been shown in experiments performed by Garcia de Lima and Mitscherlich (5).

The results obtained in this study corroborate the earlier observations concerning the inhibition of thymidine incorporation. They demonstrated that diminuation of uracil assimilation occurs too, what can suggest that the inhibition takes place in the G_1 phase, i.e. in the phase of RNA synthesis, and not during DNA synthesis as one considered previously.

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