THYMOSIN ACTIVITY IN CULTURE MEDIA OF THYMUS FIBROBLASTS

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In cultures of the thymus produced by explantation of its fragments or cell suspensions, growth of several categories of stromal (nonlymphoid) cells, namely fibroblasts, macrophages, and epithelial cells, usually takes place and in this sense such cultures are mixed [5-7, 14]. Although thymosin activity can be detected in their culture medium [4, 5, 11-15], it has not yet proved possible to decide which stromal cells of the thymus in fact produce thymosin. In a previous paper [3] a method of obtaining pure cultures of stromal thymic fibroblasts by passage was described.

The object of this investigation was to study culture media of stromal thymic fibroblasts for the presence of activity characteristics of thymosin in them.

EXPERIMENTAL METHOD

Cells of the thymus and bone marrow of adult noninbred guinea pigs were used for explantation. The thymus was freed from surrounding tissue, divided into fragments, and cells obtained from them by teasing with dissection needles. The femora were stripped of their muscles, the two epiphyses were cut off, and the contents of the medullary canal were flushed out with a syringe. Cell suspensions were prepared in medium No. 199 by repeated drawing into a syringe with a No. 26 needle. The cells thus obtained were filtered through four layers of Kapron to obtain a suspension consisting of isolated cells. Explantation was carried out by the method described previously [1] in Roux flasks (40 cm^2) with an initial density of $0.7 \cdot 10^{-5}$-$5 \cdot 10^5$ cells/cm^2 for the thymus and $0.3 \cdot 10^5$-$1.0 \cdot 10^5$ cells/cm^2 for bone marrow.

Medium No. 199 with 20% embryonic calf serum was used as the explantation medium. The gaseous phase consisted of 5% CO_2 in air. The program of changing the medium is given during the description of the results. The first passage was carried out on the 12th-16th day, and subsequent passages after the cultures had reached a state of confluence. Before passage the cultures were washed with medium without serum and treated with 0.25% trypsin solution. The cells removed were transferred to flasks with twice the area and with fresh culture medium. Medium from cultures of the III-X passages was collected at the times indicated in the section "Experimental Results," centrifuged at 6000-8000 rpm, and the supernatant was filtered through an Amicon PM-10 filter, freeze-dried, and then concentrated 20-30 times. In some cases the media were left in a freeze-dried state for up to 1 month at −20°C before concentration. The concentrate was dialyzed through cellophane permeable to substances with mol. wt. of under 1000. The preparations thus obtained were tested for activity immediately or 1-2 days after freezing to −20°C. The preparations obtained after dialysis were assessed for their ability to cause the appearance of cells marked with θ-antigen after incubation of $5 \cdot 10^5$ bone marrow cells from CBA mice in 1 ml of the test preparation for 90 min at 37°C. Treatment of bone marrow cells with the original culture medium served as the control. The θ-positive cells were detected through the complement-dependent cytotoxic action of anti-θ-serum (AKR-anti-C3H).

Sensitivity of this activity in preparations of culture media to the temperature factor was tested in a separate experiment. For this purpose the culture medium from thymic fibroblasts at the 5th passage was kept for 18 h at 37°C before testing for activity.

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EXPERIMENTAL RESULTS

On the 10th-12th day after explantation of the thymus cells discrete clonal colonies of fibroblasts were formed in the culture with a cloning efficiency of about 10^{-5}. Foci of fibroblasts were formed in cultures of bone marrow cells on the 5th-10th day, with a colony-forming efficiency of about 10^{-4}. Details of cloning the stromal fibroblasts were described previously [1, 2]. Starting from the III passage, cultures of both bone marrow and thymus cells consisted of pure strains of growing fibroblasts without contamination with other cells. No clear morphological differences could be discovered between fibroblast cultures from bone marrow and thymus in the course of subsequent passage. The morphology of the fibroblast cultures from bone marrow and thymus during successive passages was described previously [2, 3].

The results of treatment of a suspension of bone marrow cells from CBA mice with preparations obtained from media of pure strains of bone marrow and thymus fibroblasts at the III-IX passage are given in Table 1. Media for testing were taken from cultures on the 4th-10th day after passage, when they were in a near-confluent state and contained from 10^7 to $2 \cdot 10^7$ cells. Under these circumstances the flasks contained 70-100 ml medium. It will be clear from Table 1 that treatment with preparations of medium from thymus fibroblasts caused the appearance in all experiments of an additional number of cells subject to the complement-dependent cytotoxic action of anti-θ-serum compared with treatment with preparations obtained from the original media and from culture media of bone marrow fibroblasts which did not increase the percentage of θ-positive cells. Although before primary explantation the thymus cells were washed by centrifugation, activity of the medium in which the thymus cells were suspended before being put into culture was specially tested. It was found that activity relative to induction of θ-antigen on mouse bone marrow cells was absent in these media (the media were concentrated by 20 times). The results of measurement of the content of active factor in medium of thymic fibroblasts, depending on the times elapsing after passage and change of medium, are given in Fig. 1. In these experiments 10^4 fibroblasts per 10 cm^2 were put into culture during passage, and this led to the formation of a continuous layer of cells by the 5th-7th day. It will be clear that after passage of the fibroblasts the content of active factor in the culture medium rose gradually to reach a maximum by the 5th day, after which it fell, and by the 10th day it was no longer determinable. This happened if the medium was not changed. If, however, after the cultures reached a state of confluence the medium was changed, a rapid increase of activity was recorded in the medium newly added to the fibroblasts. This was repeated during several successive changes of medium.

The resistance of the active factor to a temperature of 37°C also was tested. For this purpose the sample with medium was kept at 37°C for 18 h. Under these conditions activity of the medium was found to be lost (the initial medium caused the appearance of 9% of cells sensitive to the cytotoxic action of anti-θ-serum, compared with 2.3% after the same medium had been kept at 37°C.

Factors leading to the accumulation of cells sensitive to the cytotoxic action of anti-θ-serum in bone marrow are regarded as active components of thymosin, which induce the appearance of θ-antigens on the surface of some bone-marrow cells. Activity of culture medium of thymic fibroblasts with respect to induction of θ-antigen indicates that this medium contains at least some of the factors with which thymosin's activity is connected. These factors are formed in culture of growing thymic fibroblasts. This is shown by the absence of activity immediately after passage and by the dynamics of accumulation of activity in the medium during growth of fibroblasts. Meanwhile, bone marrow fibroblasts growing in culture do not form factors with thymosin activity. Data [5, 7, 9, 11, 12, 14] showing that thymosin is synthesized by the epithelial cells of the thymus cannot be accepted as sufficiently convincing, for the cultures used in those investigations were a mixture of different types of adhesive thymic cells. In sections of the thymus treated with antithymosin serum [10], it is difficult to distinguish epithelial and reticular cells reliably.

To judge from the data described above, the production of liberation of thymosin by fibroblasts into the medium evidently ceases after cells in confluent cultures cease to divide and it is resumed when they begin to proliferate again after a change of medium. Disappearance of activity in media of confluent cultures can probably be explained by the fact that the active factor undergoes inactivation when kept in culture medium at 37°C. Data showing that strains of thymic fibroblasts during passage are not contaminated by other cells were given previously [3]. Besides morphological criteria, this is also shown by the presence of fibronectin and tissue-specific fibroblast antigen on the surface of all cells of these

TABLE 1. Appearance of θ-Antigen on Mouse Bone Marrow Cells after Treatment with Preparations of Culture Media from Strains of Stromal Fibroblasts

Medium from strains of thymus fibroblasts			Medium from strains of bone marrow fibroblasts			Original culture medium (% of θ-positive cells)
no. of passage	day after change of medium	% of θ-positive cells*	no. of passage	day after change of medium	% of θ-positive cells*	
II	8	9,1	III	5	0	0
III	10	10,5	III	7	1	0
III	10	7,8	IV	10	0	0
III	10	8,9	IV	14	0	0
III	10	10,8	V	4	0	
IV	8	11,0	V	6	0	
V	4	6,6				
V	5	14,7				
V	5	9,4				
V	6	10,0				
V	7	13,5				
VI	5	9,2				
VI	7	9,0				
VII	7	12,0				
VIII	6	11,0				

*Percent of additional θ-positive cells appearing after treatment of bone marrow cells from CBA mice with preparations of culture media, compared with bone marrow cells treated with medium No. 199 with 20% embryonic serum. θ-Positive cells revealed as number of dead cells (by the trypan blue exclusion method) after treatment with AKR-anti-C3H serum in a dilution of 1:100 and complement; treatment with normal mouse serum and complement served as the control. Each number represents mean of three or four determinations.

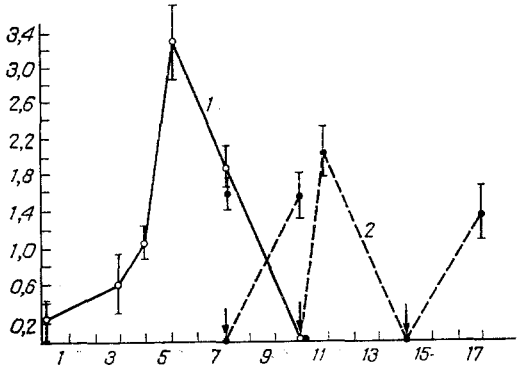

Fig. 1. Thymosin activity in culture medium from strains of thymic fibroblasts. Abscissa — days of culture; ordinate — thymosin activity (in units). Strains of thymic fibroblasts at III, IV, and VI passages (1) kept in cultures without change of medium for 10 days. Strains of thymic fibroblasts at V and VI passages (2) were kept in culture without change of medium for 7 days, after which the medium was changed at times indicated by arrows. An increase of 5% in the number of θ-positive cells during treatmentof bone marrow cells of CBA mice with preparations of culture medium was taken as 1 unit of thymosin activity.

strains; collagen of types I and III is synthesized in these cultures. According to preliminary data, thymosin activity in the induction of θ-antigen on bone-marrow-cell test can also be detected in media from monoclonal cultures of thymic fibroblasts.

Thymic fibroblasts growing as a cell strain *in vitro* first synthesize substances which evidently have activity of one of the thymosins. This observation raises the question of which of the stromal cells of the thymus are responsible for the production of other thymosins and, in particular, whether the thymic epithelium is active in this respect.

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