5. J. P. Heslop, Advances Comp. Physiol. Biochem., 6, 75 (1975).
6. H. Hyden, Acta Physiol. Scand., 6, Suppl. 17, 1 (1943).
7. P. L. Jeffrey and L. Austin, Prog. Neurobiol. Psychiatr., 2, 207 (1973).
8. Y. Komiya and M. Kurokawa, Brain Res., 139, 354 (1978).
9. R. J. Lasek, Brain Res., 20, 121 (1970).
10. P. S. Marchisio and J. J. Sjöstrand, Brain Res., 26, 204 (1971).

PRODUCTION OF PURE LINES OF THYMUS STROMAL FIBROBLASTS AND DISCRIMINATION OF FIBROBLASTS AND EPITHELIUM IN THYMUS CULTURES

A. Ya. Fridenshtein, A. A. Ivanov-Smolenskii, N. N. Kulagina, and E. A. Luriya

UDC 612.438-085.23

KEY WORDS: thymus; stromal fibroblasts; pure lines of fibroblasts.

Thymic humoral factors (thymosins) influencing differentiation of T lymphocytes are produced, it is supposed, not by thymocytes but by stromal cells of the thymus, but which stromal cells has not yet been established. It has been shown [6, 7, 8, 9] that thymosin is found in culture media of thymus explants. However, all types of stromal cells of the thymus, including macrophages, fibroblasts, and epithelium, are present in such cultures [3, 4, 7].

Accordingly the investigation described below was undertaken to determine precise criteria for discrimination in vitro between epithelial cells of the thymus and its stromal fibroblasts and to obtain pure cultures of cells of each of these types.

EXPERIMENTAL METHOD

Thymus cells of guinea pigs aged 3-4 weeks were used for explantation. The cells were isolated either mechanically or by trypsinization. In the first case the thymus was teased apart by needles in medium 199, after which the cell suspension was pipeted. In the second case the thymus was cut into fragments measuring 0.5-1 mm and agitated on a magnetic mixer for 30 min at room temperature. The medium with the liberated cells was separated and the residual fragments of tissue were covered with 0.5% trypsin and agitated on the mixer for a further 30 min. The suspensions thus obtained were sedimented by centrifugation, resuspended in medium, and filtered through four layers of Kapron. Suspensions prepared by all the methods mentioned above consisted of isolated cells, of which over 99% were thymocytes. The cells were explanted by the method in [3, 4] in plastic flasks (area of the bottom 25 cm^2), into each of which 1×10^5-5×10^5 cells were introduced, or in glass flasks (area of the bottom 40 cm^2), into each of which 1×10^6-3×10^6 cells were introduced. The cells were cultured in medium 199 with 20% embryonic calf serum. The medium was changed once a week. Some cultures were repeatedly subcultured, the first time after 2-3 weeks, and thereafter once a week. During subculture the cells were washed with medium 199, treated with 0.25% trypsin solution, and the detached cells were transferred to flasks with twice the previous area. Living cultures, and also cultures stained by the PAS and by Giemsa's methods, and after the indirect immunofluorescence test with antibodies against fibronectin (isolated from serum obtained by immunizing rabbits with electrophoretically pure fibronectin) and with antifibroblastic serum (prepared by immunizing rabbits with bone marrow fibroblasts grown in cultures [1, 2]), were used for morphological investigation. The antifibroblastic serum also was used for the complement-dependent cytotoxic test. For this purpose cultures were treated with antiserum (30 min at room temperature) 4 h after primary explantation or after routine subculture, complement was added (for 60 min at 37°C), and the cultures were then washed, covered with medium, and cultured for 12 days. The colony formation

Laboratory of Immunomorphology, N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR P. A. Vershilova.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 91, No. 1, pp. 61-64, January, 1981. Original article submitted February 20, 1980.

TABLE 1. Colony Formation Efficiency of Fibroblasts in Monolayer Cultures of Thymus Cells

Method of isolation of cells for explantation	Colony formation efficiency of fibroblasts *
Teasing thymus tissue with needles	$1:10^5$
Disintegration on magnetic mixer:	
in medium 199	$5:10^5$
in trypsin solution	$15:10^5$

*Number of colonies consisting of at least 50 fibroblasts present in 12-14-day cultures for every 10^5 explanted cells.

TABLE 2. Inhibition of Formation of Colonies of Fibroblasts by Antifibroblastic Serum in Monolayer Cultures of Thymus Cells

Dilution of serum	% inhibition of colony formation *
1:10	97,8±1,2
1:160	96,0±1,7
1:1000	97,4±2,2
1:100	95,6±2,3
1:1000	94,7±3,7

*Inhibition of colony formation in cultures treated with antifibroblastic serum and complement relative to colony formation in cultures treated with normal rabbit serum and complement.

efficiency of the fibroblasts was then compared in these cultures and in control cultures, i.e., those treated with nonimmune rabbit serum [2].

A 0.2% suspension of carmine was added to some of the cultures 4-24 h before fixation. These cultures were vigorously washed before fixation.

EXPERIMENTAL RESULTS

Explantation of thymus cells isolated by mechanical means (by teasing the tissue of the gland with needles or on a magnetic mixer without trypsin) gave similar results. Discrete colonies – clones of fibroblasts – were formed in the cultures on the 10th-12th days, as was described previously [3, 4]. The colonies also included a few macrophages; by this time the thymocytes had died and particles of cell debris adhered in large numbers to the surface of the fibroblasts, leaving the space between the colonies free. Data on colony formation efficiency of the fibroblasts are given in Table 1.

After the addition of carmine to the cultures particles of the dye were found to be firmly adherent to the surface of the fibroblasts. During subculture of the primary cultures, each containing scores of colonies of fibroblasts, cell strains which morphologically appeared to be pure cultures of fibroblasts after the third subculture readily appeared.

In primary cultures of thymus cells isolated by trypsinization colonies of fibroblasts also appeared but, in addition, epithelial islets were formed. They had smoother edges than the fibroblast colonies and consisted of polygonal, and not of elongated cells. These differences, however, were not always equally clear. A more definite feature was the absence of particles of debris or carmine on the surface of the epithelial cells. In cultures which attained a state of confluence, foci of epithelium could be distinguished by this feature among continuous sheets of fibroblasts as regions of translucency ("holes") in the sheet. As a rule the epithelial islets differed from the surrounding fibroblasts in their greater content of PAS-positive material. However, the clearest differences related to binding of the immunologic preparations used in the study – antifibronectin antibodies and antifibroblastic serum – by the fibroblasts and epithelium.

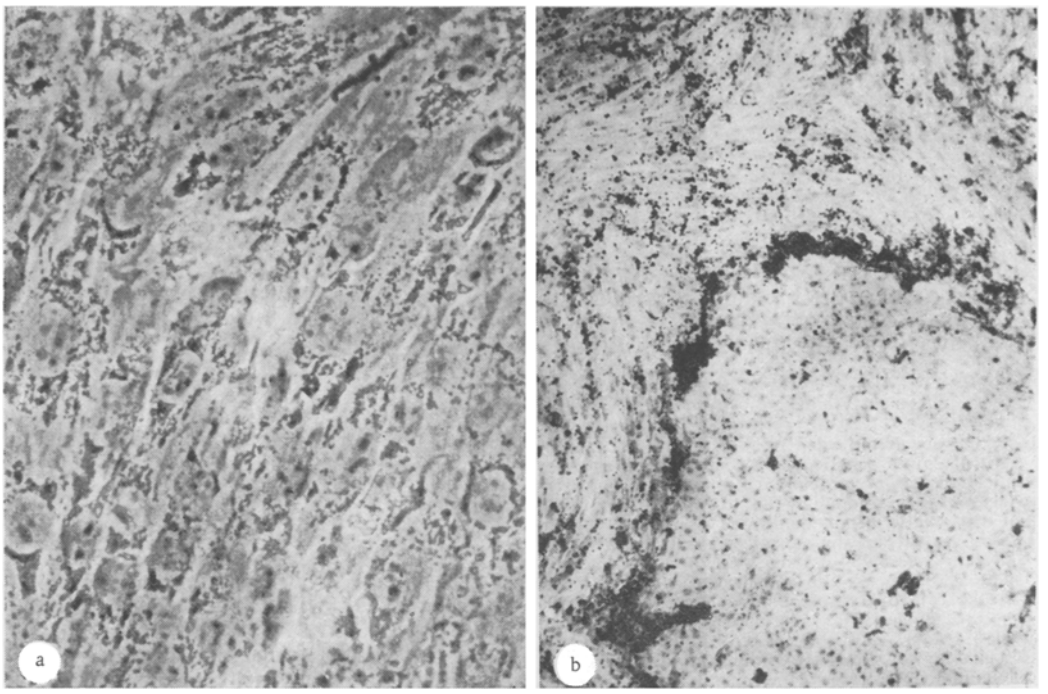

Fig. 1. Colonies of fibroblasts in cultures of thymus cells: a) 14-day culture of thymus cells isolated by mechanical disintegration. Colonies of fibroblasts, 320×; b) 16-day culture of thymus cells isolated by means of trypsin. Boundary between colony of fibroblasts and epithelial focus. Culture after addition of carmine, 160×.

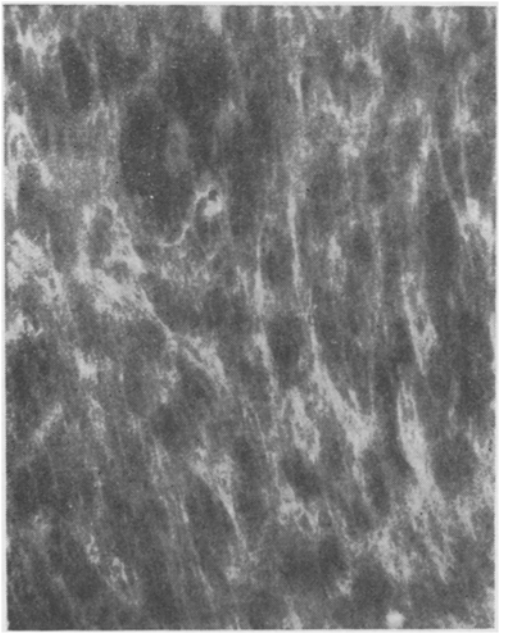

Fig. 2. Binding of antifibronectin serum by fibroblasts in thymus cultures. 16-day culture of thymus cells isolated by means of trypsin, 720×.

The antifibronectin antibodies, as shown by the results of the precipitation test [1, 2], were monospecific; antifibroblastic serum, after exhaustion with erythrocytes, liver powder, and lymphocytes, according to the results of the immunofluorescence, precipitation, and complement-dependent cytotoxicity tests, was specific for fibroblasts. As Table 2 shows, the formation of foci of fibroblasts in primary cultures of thymus cells was

sharply inhibited by antifibroblastic serum. Meanwhile this serum had virtually no effect on the formation of epithelial islets. Fibroblasts of the subcultures preserved their sensitivity to the cytotoxic action of antifibroblastic serum. In the immunofluorescence test cells of the epithelial islets bound neither antifibronectin antibodies nor antifibroblastic serum; on the other hand fibroblasts, of both primary cultures and subcultures, bound both these preparations specifically, as shown by the results of the corresponding [1, 2] control tests. Under these circumstances the structures revealed by antifibronectin antibodies formed a thin network on the surface of the fibroblasts or they had the appearance of fibrils between the cells, whereas structures found with the aid of antifibroblastic serum were uniformly distributed in the cytoplasm of the fibroblasts.

During subculture of primary cultures obtained as a result of explantation of thymus cells, isolated by trypsinization, islets of epithelium disappeared as early as after the first subculture, and these cultures became pure strains of fibroblasts. Correspondingly all colonies in primary cultures of cells isolated mechanically and all cells in all subcultures bound antifibronectin antibodies and antifibroblastic serum in the immunofluorescence test. Treatment with antifibroblastic serum and complement, carried out 2 h after subculture of the strains caused death of all cells in culture. Stromal fibroblasts of the thymus, like fibroblasts of bone marrow, spleen, and lymph nodes [9, 10], are thus already liberated from the tissue by gentle mechanical treatment. They can reproduce intensively in cultures and give rise to strains of fibroblasts capable of prolonged subculture, and synthesizing collagen of types I and III [5]. Epithelial cells capable of growth in cultures can be isolated from the thymus by treatment with trypsin. Under the conditions of culture epithelial cells of the thymus differ from fibroblasts by being less adhesive, by not producing fibronectin, and by deficiency of specific fibroblastic antigen. According to these features epithelial cells and fibroblasts can differ significantly in cultures. As a result of subculture of primary explants the epithelium is eliminated from thymus cultures, and for that reason pure strains of thymic fibroblasts appear even in cases when the primary cultures contained epithelium. The results show that it is possible to obtain pure continuous strains of stromal fibroblasts in order to study whether their cells synthesize thymosin and whether they transfer the thymic microenvironment.

LITERATURE CITED

1. A. A. Ivanov-Smolenskii and A. G. Grosheva, Byull. Éksp. Biol. Med., No. 4, 451 (1978).
2. A. A. Ivanov-Smolenskii and A. G. Grosheva, Byull. Éksp. Biol. Med., No. 10, 454 (1978).
3. A. Ya. Fridenshtein, R. K. Chailakhyan, and K. S. Lalykina, Cell Tissue Kinet., 3, 393 (1970).
4. A. Ya. Fridenshtein, Yu. F. Deriglazova, N. N. Kulagina, et al., Exp. Hematol., 2, 83 (1974).
5. A. Ya. Fridenshtein, Int. Rev. Cytol., 47, 327 (1976).
6. M. E. Gershwin, R. M. Ikeda, W. L. Kruse, et al., J. Immunol., 120, 971 (1978).
7. J. M. Goust, D. H. Vesole, and H. H. Fudenberg, Clin. Exp. Immunol., 38, 348 (1979).
8. A. M. Kruisbeck, G. C. Astaldi, M. J. Blankwater, et al., Cell. Immunol., 35, 134 (1978).
9. H. Wekerle, I. R. Cohen, and M. Feldman, Eur. J. Immunol., 3, 745 (1973).
10. H. Wekerle, B. Paterson, U.-P. Ketelsen, et al., Nature, 256, 493 (1975).