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Long Term Culture of Thymic Cells from Newborn Mouse Thymus Fragments.* (30039)

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Short term cultures from normal thymus cells have been used by many investigators for various purposes(1,2,3,4,5,6). Mouse lymphomatous thymic lymphocytes were grown successfully *in vitro* for over 300 days by Ioachim and Furth(7); cultures of embryonal and normal adult thymic cells, however, resulted in a good growth of only reticulum cells, while the lymphocytes disappeared completely within 2-3 days. The persistence of thymic lymphomatous lymphocytes for as long as 32 weeks was observed by Hays(8) in diffusion chambers implanted i.p. in isogeneic and allogeneic mice, whereas chambers containing normal adult thymus fargments were devoid of lymphocytes after one week. In the experiments here reported minute newborn thymus fragments were cultured *in vitro* with the purpose of obtaining a long term culture of thymic lymphocytes. Two different techniques of explanation of the thymus fragments were used.

Materials and methods. Swiss non-inbred, and inbred MA and C57 mice were used as source of the thymuses. Eighteen- to 40-hour-old mice were killed with ether, and as soon as breathing stopped the thymus was removed aseptically to a Petri dish containing Hanks' BSS. After washing, the thymuses were individually transferred to second Petri dishes containing Hanks' BSS and finely minced. The fragments were then transferred to Leighton tubes (Bellco Glass Co., Vineland, N. J.) into which coverslips had been previously inserted.

The culture medium was Hanks' BSS with phenol red as indicator, plus vitamins, glutamin, essential amino acids, and was supplemented with 11% fetal bovine serum (Microbiological Associates, Inc., Bethesda, Md.). No antibiotics were used. Two methods of culturing the fragments were employed.

In a first series of experiments one or two drops of chicken plasma (Difco) were pipetted on the cover slip before inserting the thymus fragments into the tubes. The plasma solidified rapidly after transfer of the fragments and the medium was then added. This procedure favored a rapid and firm adhesion of the fragments to the glass. The medium was first changed after 4-5 days, and at this time it was completely discarded. It seemed advantageous for the further growth of the cultures that as much as possible of the debris from dead or degenerating lymphocytes should be removed. After this first change the medium was changed regularly every week, but the old medium was no longer completely discarded; approximately ¼ of it was left in the tubes and fresh medium added.

In the second series of experiments the thymus fragments were inserted into the tubes without previously adding chicken plasma. The thymus fragments floated free in the medium and were never attached to the glass. The medium was changed the first time as described above, but 4-5 days later the fragments were transferred to a new tube. From then on the medium was changed regularly every week, always leaving a part of the old medium in the tubes.

The tubes were maintained at 37°C in an incubator without CO_2 and were observed daily using an inverted microscope. At various times the cover slips were removed from

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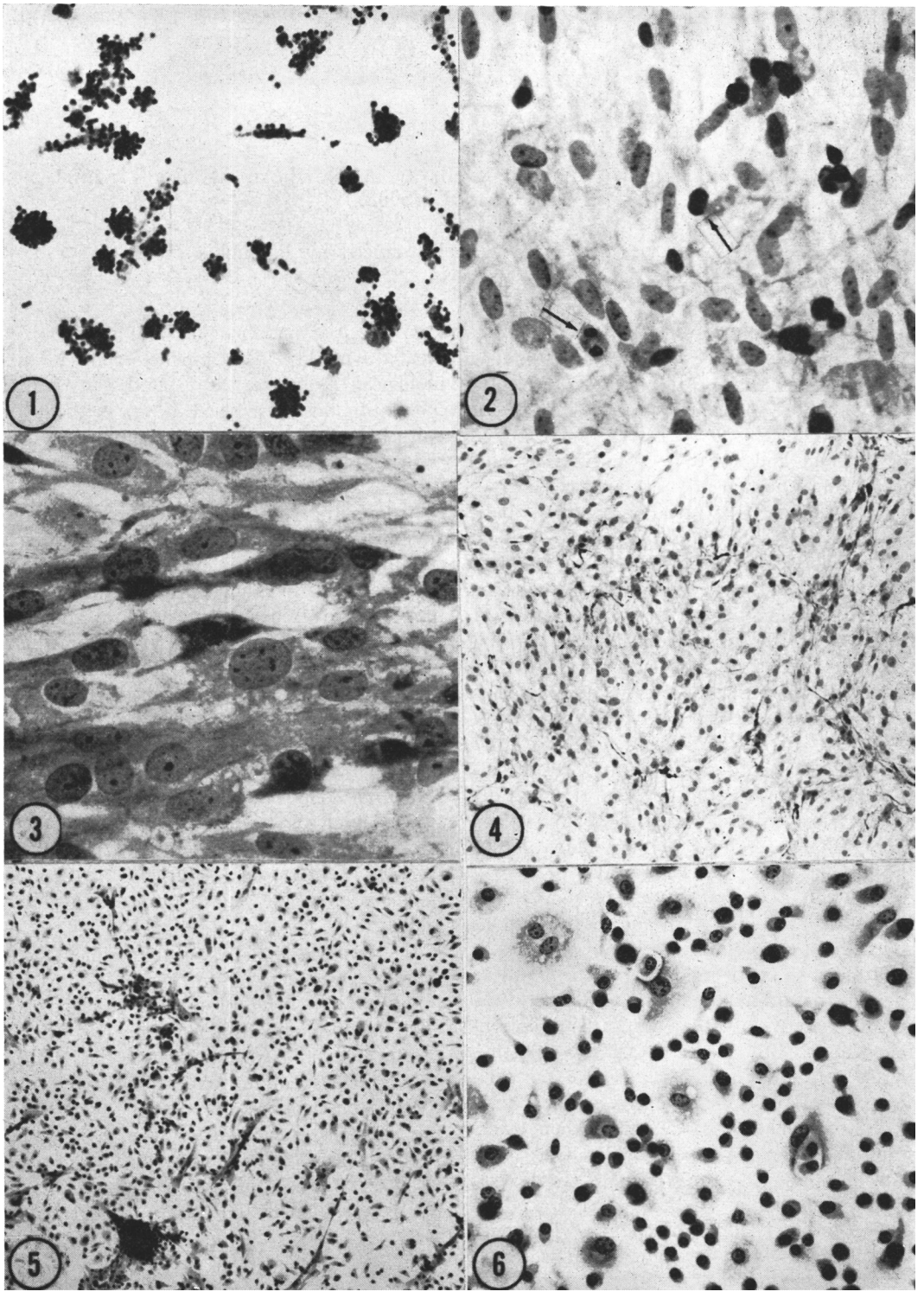

FIG. 1. 2-day-old culture. Macrophages surrounded by lymphocytes in a satellite fashion. May-Grünwald, Giemsa. 170 ×.

the tubes, washed in Hanks' BSS prewarmed at 37°C and fixed in absolute methanol. In some of the tubes of the second series a new cover slip was inserted to replace the one used for the observations of the fixed and stained material. The cells were usually stained by the May-Grünwald and Giemsa procedures, and sometimes by toluidine blue.

Results. A large number of lymphocytes were released from the thymus fragments as they were inserted into the tubes. Most of these free floating lymphocytes were discarded at the first change of the medium. A large number of macrophages attached to the glass were seen in stained preparations after one and two days of culture. Often they appeared surrounded by lymphocytes, mostly of small and medium size, either in a satellite fashion (Fig. 1) or phagocytizing dead lymphocytes and debris.

Fifteen cultures were carried out using the first technique described above, where the fragments, after being embedded in a plasma clot, adhered to the glass. One of the cultures was discarded after a few days because of contamination. Eleven cultures were sacrificed between the 1st and the 80th day of culture and 3 were sacrificed at the 100th, 102nd, and 108th day of culture. In the cultures of this series reticulum cells began to proliferate vigorously from the 2nd day and within 4-5 days they had formed interwoven networks on a large area around the fragments. The periphery of the growth was composed mainly of macrophages phagocytizing lymphocytes. However, lymphocytes were still released from the fragments; they either floated free in the medium and were progressively discarded, or they adhered to the network formed by the reticulum cells, growing on it as on a feeder layer. Mitoses of both lymphocytes and reticulum cells were observed quite frequently in the first 8-14 days (Fig. 2-3). After the 25th day, mitosis of lymphocytes was no longer seen in most of the cultures and the number of lymphocytes decreased notably. This resulted in the complete disappearance of lymphocytes, leaving a pure colony of reticulum cells in the majority of the cultures of this series (Fig. 4).

In 3 cultures only, the growth of the reticulum cells did not prevail. In these the reticulum cells grew in groups, forming small islands, both adjacent and scattered at various distances from the original fragment. After 20 days a striking feature was the progressive appearance of large lymphocytes. In spite of an evident increase of the cell population up to the 40th day, lymphocytes were only rarely seen in mitosis. After the 40th day the cultures appeared to be stabilized (Fig. 5). In 2 of these 3 cultures, a common feature was the finding of one or more lymphocytes inside the cytoplasm of macrophages. In the stained preparations the lymphocytes appeared perfectly preserved within large cytoplasmatic vacuoles, surrounded by a clear halo (Fig. 6). In certain cases these vacuoles seemed to be open to the exterior; we were unable to decide whether this was merely an artifact. Mitosis was never observed in these engulfed lymphocytes.

In the second series of experiments 13 cultures were started. Ten cultures were sacrificed between the 5th and the 81st day of culture, and 3 were sacrificed at the 102nd, 116th, and 120th day of culture. The thymus fragments did not adhere to the glass and floated free in the medium throughout the entire observation period. In these cultures no prominent growth of reticulum cells was observed. In the stained preparations, one day after the transfer of the fragments to a new tube, colonies of few cells and numerous scattered single cells were already noted on the cover slip. Rate of growth of these cell populations was very low. Although mitoses were seen up to 40 days after the

FIG. 2. 14-day-old culture. Lymphocytes are growing on reticulum cell network as on a feeder layer. Two lymphocytes in mitosis (arrows). May-Grünwald, Giemsa. 375 ×.

FIG. 3. 9-day-old culture. A mitosis of a reticulum cell is shown. May-Grünwald, Giemsa. 450 ×.

FIG. 4. 104-day-old culture. Pure culture of reticulum cells. May-Grünwald, Giemsa. 55 ×.

FIG. 5. 68-day-old culture. Cell population is composed of large active lymphocytes and a few scattered macrophages and reticulum cells. May-Grünwald, Giemsa. 55 ×.

FIG. 6. 64-day-old culture. Two macrophages have lymphocytes included into cytoplasmatic vacuoles. An opening of vacuoles to the exterior is visible. May-Grünwald, Giemsa. 250 ×.

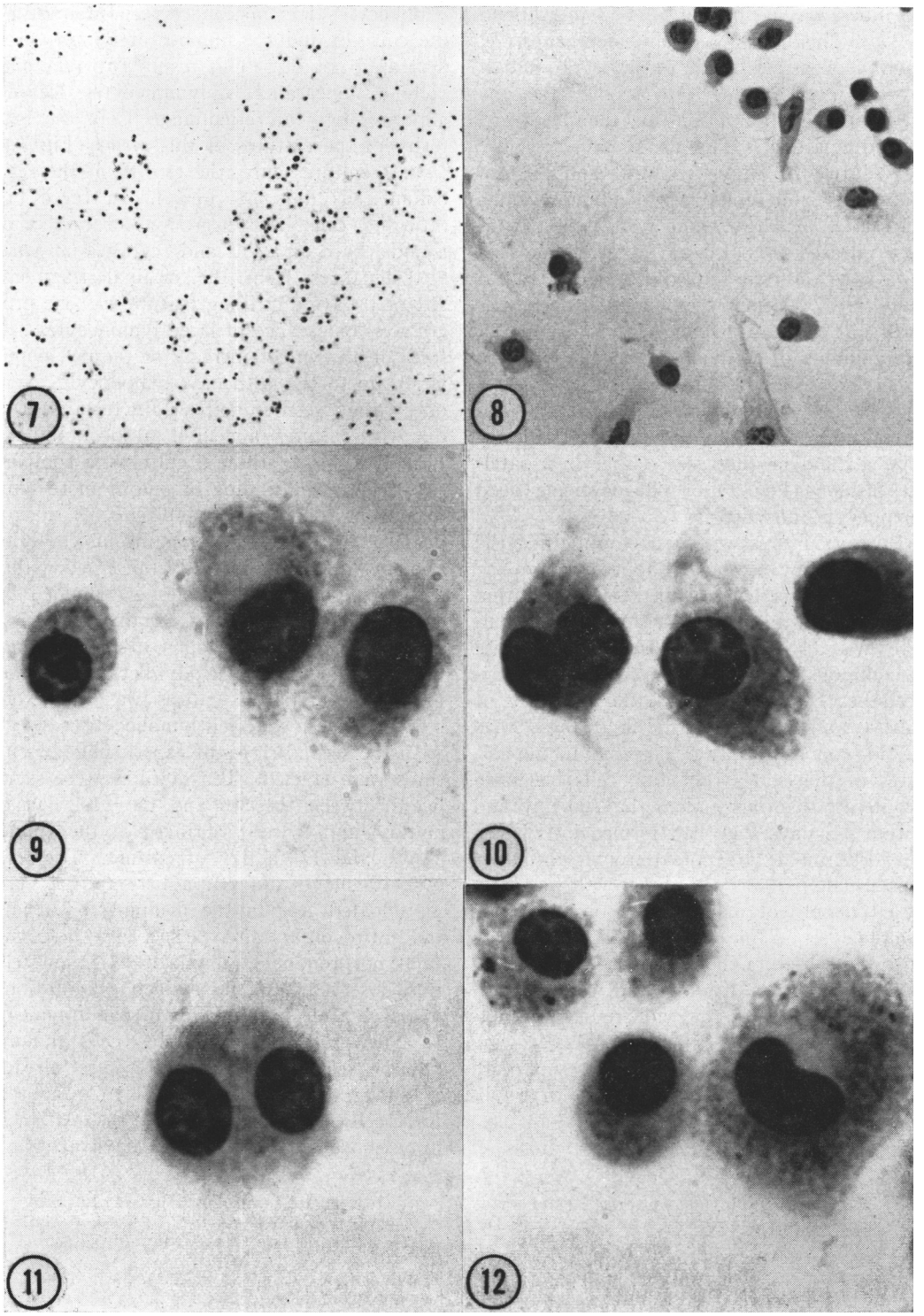

FIG. 7. 102-day-old culture. Cell population is composed of large lymphocytes and plasmacytoid lymphocytes, and a few macrophages and reticulum cells. May-Grünwald, Giemsa. 72 ×.

transfer, and even a single one at 102 days, the populations appeared more or less stabilized after 30 days from the transfer until the end of the observation period at 120 days. The cell population was composed mainly of large lymphocytes, plasmacytoid lymphocytes and monocyte-like cells, and a few large epithelial-like cells and macrophages (Fig. 7-8). The large lymphocytes and plasmacytoid lymphocytes ranged between 9-18 μ in greatest diameter, the cytoplasm was generally dense, deeply basophilic, finely granulated, and occasionally contained vacuoles of various size. The nucleus was round, the chromatin abundant and distributed in coarse clumps in the center and at the nuclear membrane. The eccentric position of the nucleus and the presence of a perinuclear halo allowed identification of some cells as plasmacytoid lymphocytes (Fig. 9-10). Large lymphocytic cells of intermediate morphology were common. The few binucleated cells noted were morphologically similar to binucleated plasma cells (Fig. 11). The monocyte-like cells had a size varying from 17-22 μ and possessed a large kidney-shaped nucleus (Fig. 12).

Discussion. In the experiments here reported, cultures of newborn mouse thymic cells were followed as long as 120 days. While the strain of mice had no influence on the culture patterns, the technique of explantation did have significant consequences. Whereas in cultures derived from fragments embedded in plasma clot the end result was a pure colony of reticulum cells, in other cultures from unattached thymus fragments the cell population consisted mainly of lymphocytes, monocytoid cells and plasmacytoid cells. We did not observe cells which could be identified as mast cells. According to Ginsburg and Sachs(9), who obtained a pure suspension of mast cells in mouse thymus cultures, the continuous presence of a feeder layer is an essential factor for the differentiation of lymphocytes into mast cells to take place.

The survival of lymphocytes *in vitro* for at least 120 days is in keeping with the recent report of Chang(10) who noted the presence of lymphocyte-like cells for more than 100 days in long term cultures of mouse peritoneal macrophages. The plasmacytoid cells observed in our cultures may originate either by transformation of lymphocytes or directly from reticulum cells. Further studies are in progress in the attempt to elucidate this point. The origin of plasma cells, directly or through intermediate stages, both from reticulum cells or from lymphocytes derived from either blood or lymph nodes, has been extensively demonstrated(11,12,13,14). There is some support in the literature for the concept that thymic lymphocytes can give rise to plasma cells. That transformation of thymic lymphocytes into plasma cells or eosinophil myelocytes may occur has been stated (15), and transformation *in vitro* of rabbit thymocytes into plasma cells or plasmacytoid cells has been described by earlier investigators(16,17). The thymus, which normally contains but a few plasma cells, does not undergo the cellular changes mainly represented by massive plasma cell proliferation shown by the other lymphatic organs after parenteral injection of an antigen. This lack of reactivity of the thymus is apparently due to the presence of a blood barrier preventing the antigen to penetrate the organ. If the antigen is injected directly into the thymus, plasma cells containing specific antibodies do appear(18). Stutman and Zingale(19) showed that autografted thymuses, by contrast with the usually located glands, responded to antigenic stimulation with the appearance of a striking number of plasma cells involved in specific antibody production.

FIG. 8. 116-day-old culture. Large lymphocytes and 2 reticulum cells. May-Grünwald, Giemsa. 460 ×.

FIG. 9. 116-day-old culture. Plasmacytoid lymphocytes. May-Grünwald, Giemsa. 1800 ×.

FIG. 10. 102-day-old culture. Same as Fig. 9; and one binucleated plasmacytoid lymphocyte. May-Grünwald, Giemsa. 1800 ×.

FIG. 11. 102-day-old culture. Binucleated plasmacytoid lymphocyte. May-Grünwald, Giemsa. 1800 ×.

FIG. 12. 120-day-old culture. Large monocyte-like cell and 3 plasmacytoid lymphocytes. May-Grünwald, Giemsa. 1800 ×.

Our results seem to give further support to the concept that at least part of the thymic cells are immunologically competent and can give rise to plasma cells. It is possible that the serum added to our culture medium contained substances effective as antigenic stimuli to the cultured cells. In view of recent findings that phytohemagglutinin(20, 21,22,23,24,25) or leukocytes of unrelated origin(26,27) as well as tuberculin and tetanus and diphtheria toxoids(28,29) induce cultured lymphocytes to actively synthesize DNA and undergo mitosis, the role played by the serum supplementing our medium in the *in vitro* growth of mouse lymphocytes for long periods, and possibly in their transformation into plasma cell forms, requires further investigation.

Summary. *In vitro* cultures of newborn mouse thymic cells were followed for as long as 120 days. Depending on the technique of explantation, two distinct types of cell populations were obtained: whereas in cultures derived from thymic fragments embedded in plasma clot the final result was a pure colony of reticulum cells, in cultures derived from unattached thymus fragments the cell population mainly consisted of lymphocytes, monocytoid cells and plasmacytoid lymphocytes.

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