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Received June 22, 1965. P.S.E.B.M., 1965, v120.

The Presence of Lymphocytes in Long Term Cultures of Newborn Mouse Thymic Epithelium.* (30566)

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Observations of *in vitro* cultures of newborn thymus fragments followed for as long as 120 days have been reported(1). We will report here the observations on cultures derived from newborn mouse thymus fragments initially explanted in a plasma clot and followed for over 22 months. The term epithelial cell, instead of that of reticulum cell employed in our previous work, will be used in the present note to describe the cells originating from the epithelial reticulum of the thymus. This appears justified both by the morphological appearance of the cells observed in culture and by the embryological derivation of the thymus(2,3,4,5,6,7).

Material and methods. Source of the thymus was a 24-hour-old MA mouse. The mouse was killed with ether and the thymus aseptically removed to a Petri dish containing Hanks' BSS. After washing, the thymus was transferred to a second Petri dish containing Hanks' BSS and finely minced. The

minute fragments were then transferred to 2 Leighton tubes (Bellco Glass Co., Vineland, N. J.) into which a coverslip had been previously inserted. Two drops of chicken plasma (Difco) were pipetted to the coverslip before inserting the thymus fragments into the tube. The plasma solidified rapidly after transfer of the fragments and the medium was then added. This procedure favored the adhesion of the fragments to the glass.

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, Bethesda, Md.). No antibiotics were used. The medium was first changed after 4 days and thereafter changed every 5-7 days. Every 2 changes, after the old medium was discarded, the culture was washed with Hanks' BSS before the fresh medium was added.

Of the original cultures, one was sacrificed

FIG. 1. 4-day-old culture. Macrophages phagocytizing lymphocytes and debris, surrounded by dead or degenerating lymphocytes. 250 $\times$.

FIG. 2. 14-day-old culture, large lymphocytes growing on background of a continuous sheet of epithelial cells. Original magnification 250 $\times$, enlarged 3 times. Phase contrast.

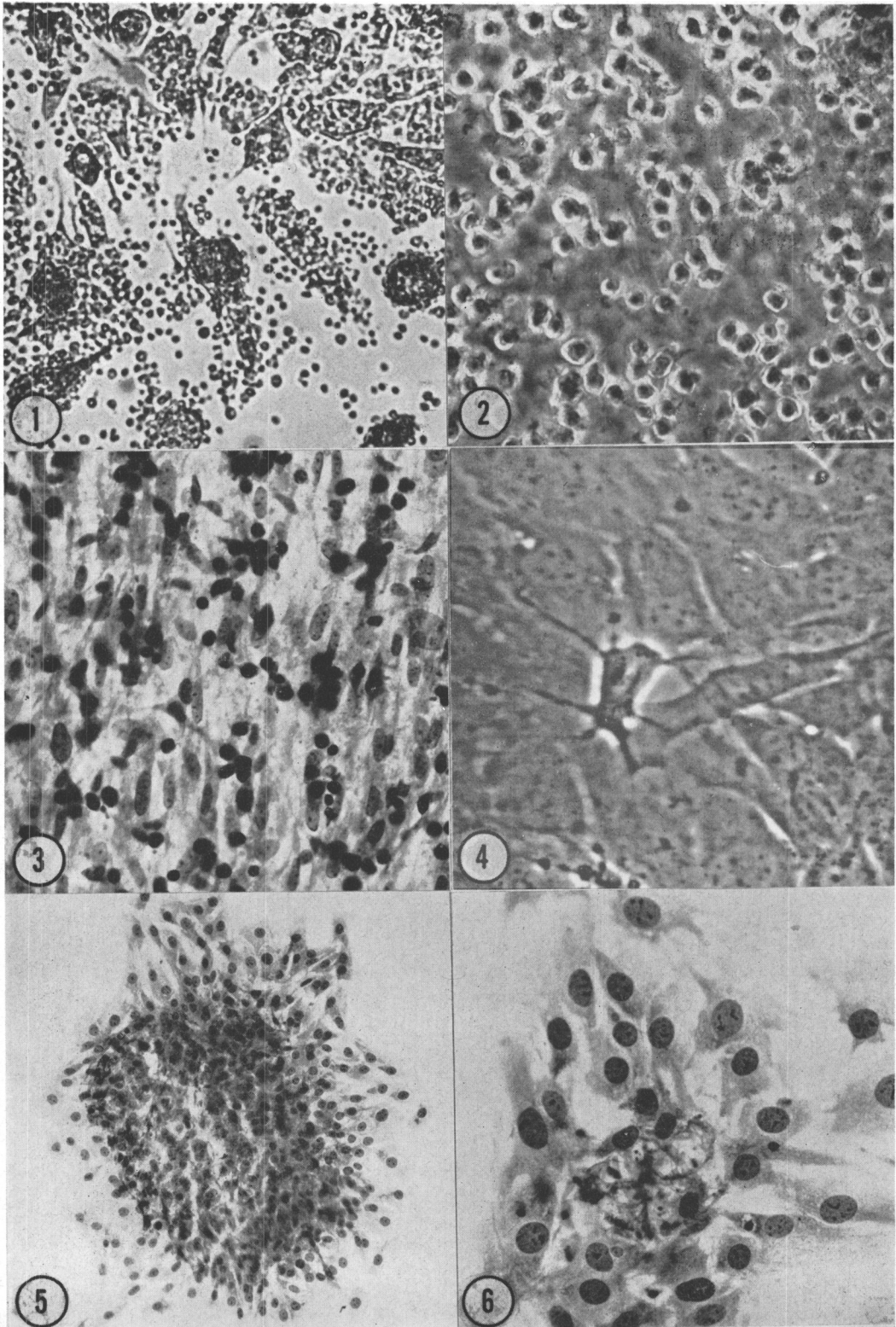
FIG. 3. 14-day-old culture. Lymphocytes growing on epithelial cells. May-Grünwald and Giemsa, 250 $\times$.

FIG. 4. 15-month-old culture. Mitosis of an epithelial cell. Original magnification 250 $\times$, enlarged 3 times. Phase contrast.

FIG. 5. 22-hour-old subculture from the 14-month-old original culture. Colony of epithelial cells. May-Grünwald and Giemsa, 110 $\times$.

FIG. 6. 18-hour-old subculture from the 14-month-old original culture. Small colony of epithelial cells. A few lymphocytes with pyknotic nuclei are visible. One is engulfed in a cytoplasmic vacuole. May-Grünwald and Giemsa, 250 $\times$.

* This work was supported by grant CA-6162 from Nat. Cancer Inst., N.I.H., U.S.P.H.S.



at the 14th day for observation of the fixed and stained material, and the other is still going on 22 months after explantation.

Subcultures were started by transferring cell sheets obtained by mechanical scraping of the original culture to other Leighton tubes and in a few cases also to T-flasks. The tubes and flasks were maintained at 37°C in an incubator without CO₂ and were observed using an inverted microscope. The thickness of the glass tubes, however, did not permit observations *in vitro* at a magnification higher than 250 ×.

The coverslips of the tubes containing subcultured cells were removed at various times, washed in Hanks' BSS prewarmed at 37°C and fixed in absolute methanol. The cells were stained by the May-Grünwald and Giemsa procedure.

Results. Lymphocytes were released in large quantity from the thymus fragments as they were inserted into the tubes and most of them were discarded at the first change of medium. Large macrophages were seen attached to the glass around the fragments after a few hours of culture. They phagocytized dead cells and debris and were surrounded by dead or degenerating lymphocytes (Fig. 1). Subsequently a vigorous growth began from the attached fragments. It was composed of large round or polyhedral cells forming a continuous sheet rapidly becoming the main component of the culture. After 3-4 days the macrophages, intermingled with scattered fibroblast-like cells, were mainly confined at the periphery of the growth. On the background of the continuous epithelial cells sheet, a few small round cells were present from the first days of culture. A new pattern however appeared after the first week of culture, when a wide-

spread growth of these small round cells became manifest (Fig. 2). They evidently corresponded to the large lymphocytes observed on the stained preparation (Fig. 3). Mitoses of the lymphocytes were seen in the first month of culture, but later became rare, and the number of lymphocytes markedly decreased until after 2 months of culture only a few were noticeable. However, redispersing in serum the sediment obtained by centrifugation of the old medium, a few readable smears were obtained, and scattered between cellular debris and degenerated cells a few well preserved large lymphocytes were always observed. Their presence was confirmed by occasional mitoses which were seen even after one year of culture. Mitoses of the epithelial cells were seen throughout the entire observation period (Fig. 4).

When the original culture was 14 months old minute cell sheets were obtained by mechanical scraping and transferred to other Leighton tubes or T-flasks. The cell sheets were excised where the epithelial cells grew as a solid sheet in order to avoid possible contamination with other cell types. Small circular cell colonies were established in less than 24 hours (Fig. 5). The cell population was composed largely of epithelial cells. In almost all the colonies, however, scattered lymphocytes were intermingled with the epithelial cells or lying on them, and in a few instances cellular and nuclear debris was present in the cytoplasm of some of the epithelial cells (Fig. 5). A few elongated fibroblast-like cells were present at the periphery of 2 subcultures. The lymphocytes were seen in larger number in some of the subcultures after 4-5 days (Fig. 7) but their number did not increase consistently thereafter. They had a

FIG. 7. 5-day-old subculture from the 16-month-old original culture. Numerous lymphocytes on and around a colony of epithelial cells. Some of the lymphocytes have pyknotic nuclei. A few elongated fibroblast-like cells are visible at periphery of the growth. May-Grünwald and Giemsa, 72 ×.

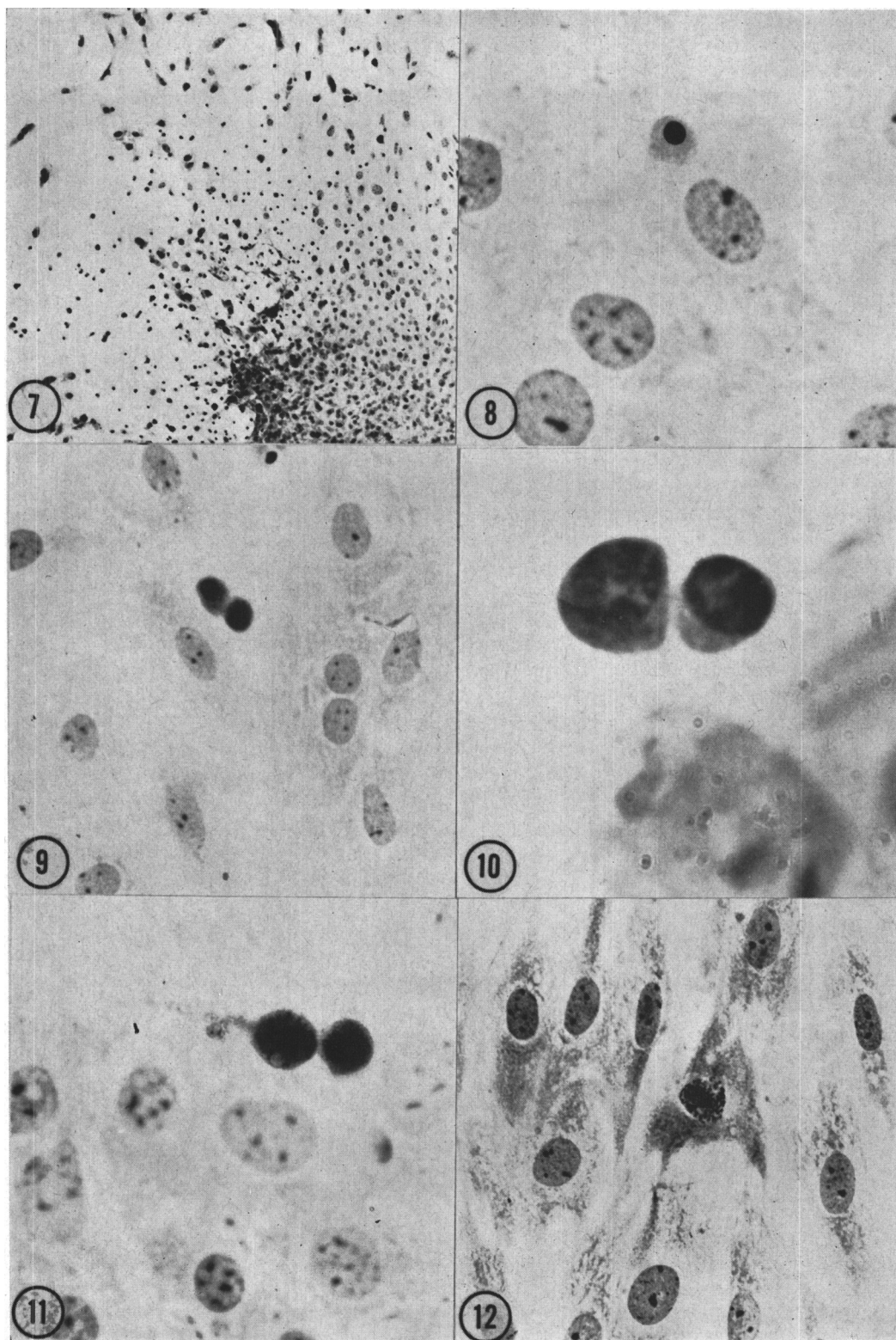
FIG. 8. 5-day-old subculture, same as Fig. 7. Plasmaecytoid lymphocyte on an epithelial cell. May-Grünwald and Giemsa, 720 ×.

FIG. 9. 9-day-old subculture from 19-month-old original culture. Two plasmaecytoid lymphocytes between epithelial cells. May-Grünwald and Giemsa, 450 ×.

FIG. 10. 12-day-old subculture from 11-month-old original culture. Two lymphocytes. The nucleus of an epithelial cell is visible at the bottom. May-Grünwald and Giemsa, 1800 ×.

FIG. 11. 12-day-old subculture from 19-month-old original culture. Mitosis of a lymphocyte. May-Grünwald and Giemsa, 880 ×.

FIG. 12. 12-day-old subculture from 19-month-old original culture. Mitosis of an epithelial cell. May-Grünwald and Giemsa, 450 ×.



dense and finely granulated fairly abundant cytoplasm which stained deeply in blue. The nucleus, often shifted in an eccentric position, was round, with abundant chromatin in coarse clumps at the center and at the nuclear membrane (Fig. 8, 9, 10). The presence of bare nuclei and of cells with pyknotic nuclei suggested that many of the lymphocytes were dying or undergoing a rapid degeneration.

Mitoses of both the epithelial cells and of the lymphocytes were frequent (Fig. 11, 12, 13). Lymphocytes included within large cytoplasmic vacuoles of the epithelial cells were occasionally seen (Fig. 14) and 2 mitotic figures inside cytoplasmic vacuoles of epithelial cells were observed in 2 different subcultures (Fig. 15).

The epithelial cells had an abundant thin cytoplasm which stained faintly in blue, sometimes with large vacuoles, either optically empty or containing a pink material. They had one, sometimes 2 large round nuclei with finely dispersed chromatin and 2 or more prominent nucleoli. In the cytoplasm of the epithelial cells were occasionally seen elongated bodies which stained faintly in red and at higher magnification resembled bundles of filaments (Fig. 16). Similar or coarser bundles were also observed outside the cytoplasm. Their presence however was somehow questionable, since it was often very difficult to distinguish them from patterns determined by the overlapping of the cell borders. The finding of micronuclei was not rare (Fig. 17, 18, 19). In certain cases the nucleus of the epithelial cells showed a constriction into 2 equal (Fig. 20) or unequal portions (Fig. 21). The formation of micronuclei, or karyomeres, has been described in a large variety of normal cells(8,9) and does

not appear to be a peculiarity of the thymic epithelium. The original thymic epithelium culture is at present over 22 months old and recent subcultures do show the same characteristics described above. However, a few subcultures which were not sacrificed for observation of the fixed and stained material, were recently subcultured by trypsinization for a second and a third time. In some of the latter the rhythm of growth was accelerated, the number of mitoses increased and the border of the epithelial growth was characterized by the presence of numerous elongated fibroblast-like cells (Fig. 22). The increased mitotic rate apparently did not involve the lymphocytes, since their number was not consistently increased.

Discussion. The presence of lymphocytes, plasmacytoid lymphocytes and monocytoïd cells has been previously reported to occur in cultures of newborn mouse thymus fragments. However, where the thymus fragments embedded in a plasma clot were allowed to adhere to the glass, the final result was a pure growth of epithelial reticulum cells(1). This paper reports observations on the culture of mouse thymic epithelium for over 22 months. The findings up to 3 months of culture were comparable to those already reported: initial release of a large number of lymphocytes which were discarded at the first change of medium, rapid appearance of macrophages and scattered fibroblast-like cells, and subsequent vigorous proliferation of the epithelial cells; proliferation of large lymphocytes on the background of the epithelial cells after the first week of culture, and their gradual disappearance after the first month of culture.

Lymphocytes probably never disappeared

FIG. 13. 14-day-old subculture from 20-month-old original culture. Mitosis of an epithelial cell. May-Grünwald and Giemsa, 450 $\times$.

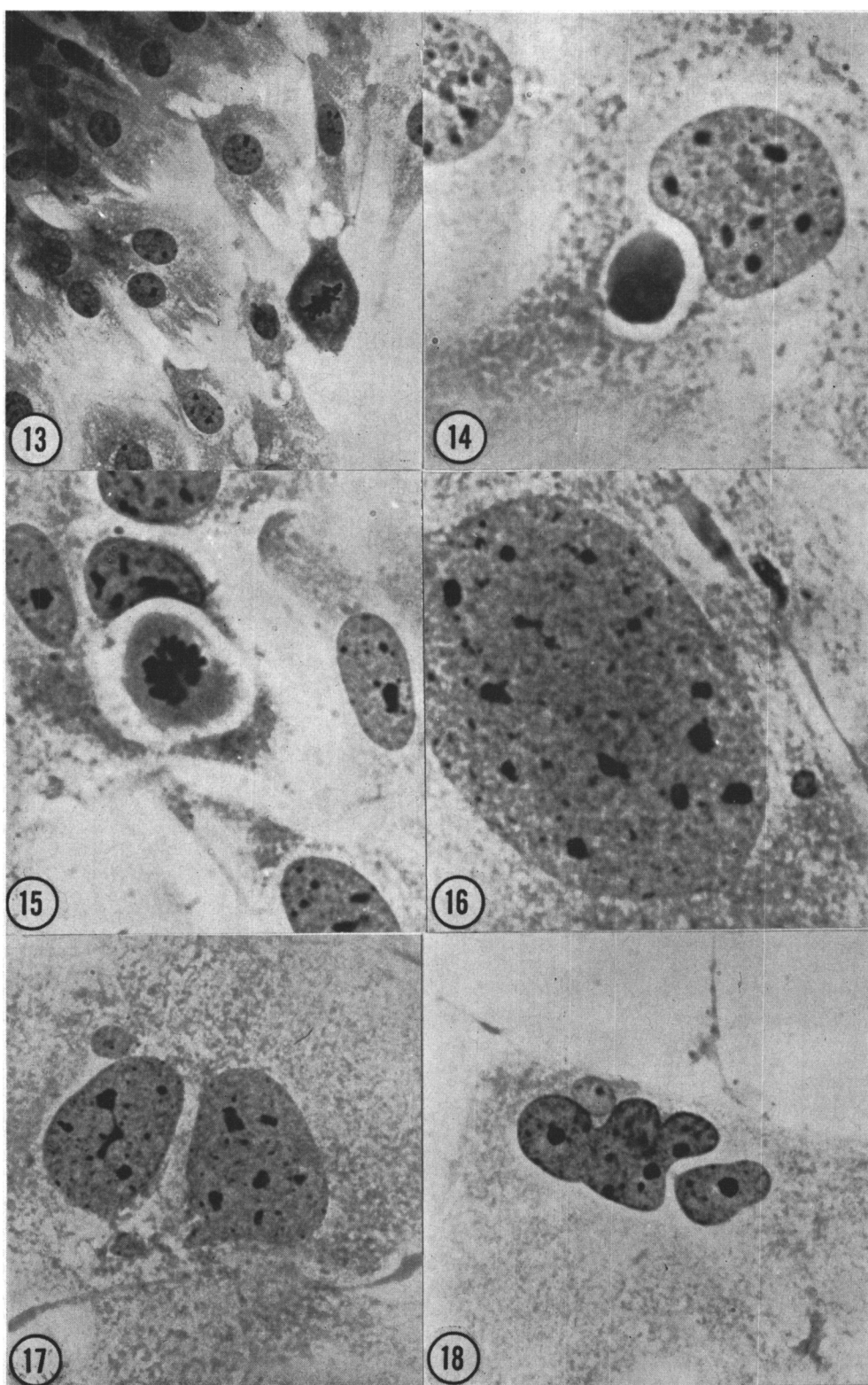
FIG. 14. 5-day-old subculture from 15-month-old original culture. A lymphocyte included in a cytoplasmic vacuole of an epithelial cell. May-Grünwald and Giemsa, 1400 $\times$.

FIG. 15. 5-day-old subculture from 15-month-old original culture. Mitotic figure included in a cytoplasmic vacuole of an epithelial cell. May-Grünwald and Giemsa, 1100 $\times$.

FIG. 16. 14-day-old subculture from 20-month-old original culture. Elongated body with the appearance of a bundle of filaments in cytoplasm of an epithelial cell. One micronucleus is also visible. May-Grünwald and Giemsa, 1800 $\times$.

FIG. 17. 4-day-old subculture from 16-month-old original culture. Binucleated epithelial cell with 2 micronuclei. Nucleus on the right shows a nuclear budding at lower left pole. May-Grünwald and Giemsa, 1200 $\times$.

FIG. 18. 12-day-old subculture from 16-month-old original culture. An epithelial cell with 2 nuclei of unequal size and one micronucleus. Several buddings of the larger nucleus are visible. May-Grünwald and Giemsa, 1150 $\times$.



completely from the culture since occasional mitoses were seen even after one year of culture, and a few lymphocytes were observed in the sediment obtained by centrifugation of the old culture medium. It is therefore possible that a few free floating lymphocytes

were discarded at each change of medium and a few of them that might have been loosely attached to the epithelial meshwork, were detached when the culture was washed with Hanks'.

Starting from the 14th month of culture

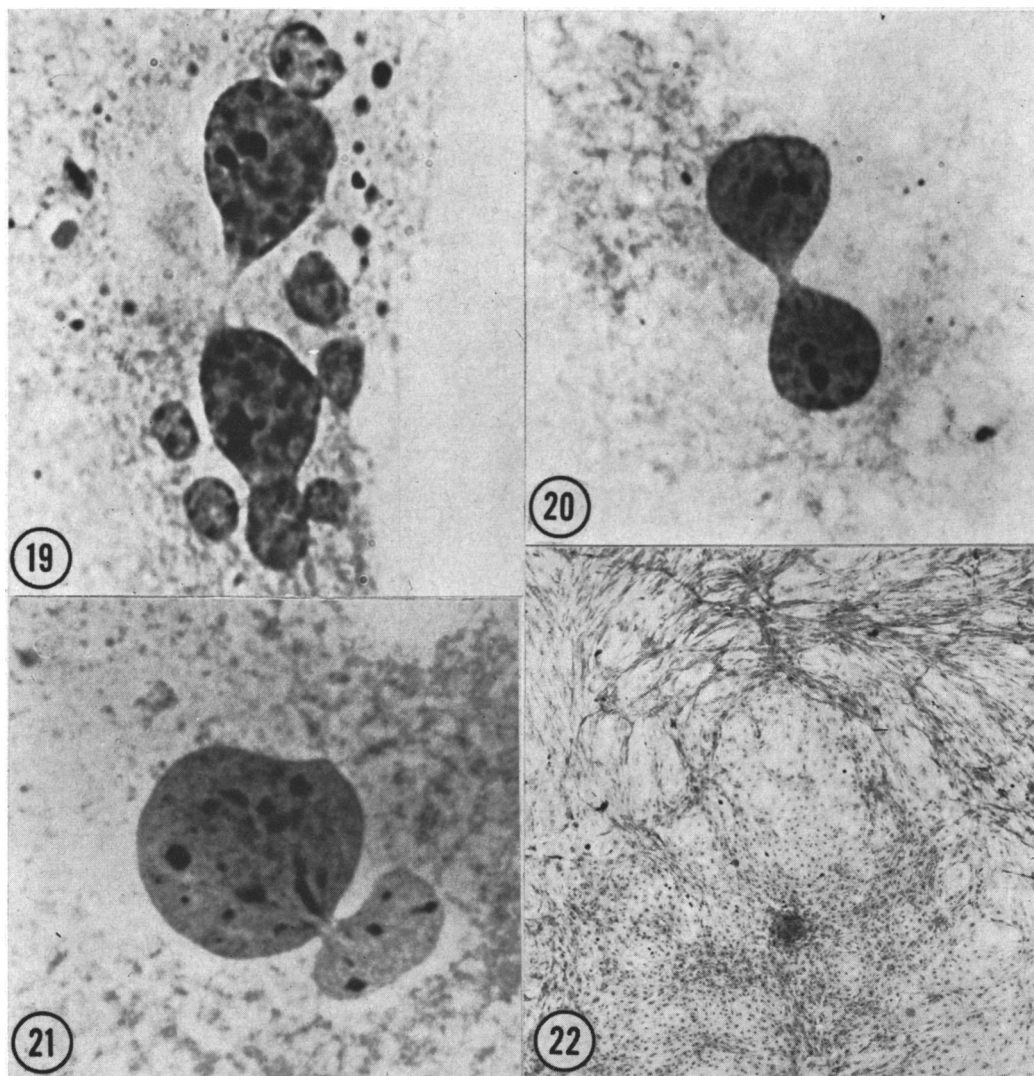


FIG. 19. 14-day-old subculture from the 20-month-old original culture. A possibly degenerating epithelial cell with 2 nuclei connected by a very thin filament. At least 6 micronuclei are visible. May-Grünwald and Giemsa, 1800 $\times$.

FIG. 20. 14-day-old subculture from 20-month-old original culture. An epithelial cell with a constriction of the nucleus into 2 equal portions. May-Grünwald and Giemsa, 1500 $\times$.

FIG. 21. 10-day-old subculture from 20-month-old original culture. An epithelial cell showing constriction of the nucleus into 2 unequal portions. May-Grünwald and Giemsa, 1800 $\times$.

FIG. 22. 6-day-old subculture. Cells were obtained by mechanical scraping of original culture when it was 22 months old and thereafter subcultured twice by trypsinization. Large epithelial cells colony which shows at periphery a growth of elongated fibroblast-like cells. Scattered lymphocyte and several mitoses are visible. May-Grünwald and Giemsa, 35 $\times$.

minute cell sheets were transferred to new tubes and subcultured. Scattered lymphocytes and plasmacytoid lymphocytes appeared in newly formed epithelial colonies. The presence of well preserved lymphocytes and of a few dividing cells within cytoplasmatic vacuoles of the epithelial cells suggest that the lymphocytes can survive and multiply inside the epithelial cells. Mitoses of lymphocytes outside the epithelial cells were however more frequent. The multiplication of leukemic lymphoblasts within reticular cells was observed by Ioachim and Furth(10) in cultures of thymic lymphoma. They did not observe mitoses inside the reticular cells in cultures of normal thymus, although isolated non-dividing lymphoblasts were seen in a few reticular cells of a normal embryonal thymus culture. Thymic lymphocytes inside thymic epithelial cells were seen by Murray(7) who, however, never observed mitosis of the engulfed lymphocytes.

There are 3 possibilities as to the origin of the lymphocytes and plasmacytoid lymphocytes observed in our cultures: (1) lymphocytes may survive and multiply indefinitely in culture; (2) they may originate from a few scattered non-epithelial cells, or (3) may originate from the epithelial cells. It has been recently reported that lymphocytes in women have a lifetime of over 500 days(11); their survival *in vitro* for a very long period seems therefore conceivable. The presence of lymphocytes, as well as the presence of mitotic figures, inside the epithelial cells, might represent the condition of survival and multiplication in adverse conditions. Emperipoiesis, however, has been described to occur quite frequently(12,13,14). Mitoses of the lymphocytes in our cultures were not a rare event and the presence of lymphocytes throughout the observation period can be explained by both a long survival time of at least some lymphocytes, and their multiplication. The rapid degeneration and death of part of the lymphocyte population seen in our subcultures after 4-5 days might be related to the presence of a lymphocyte component with a shorter life span, as was suggested by Ottesen(15).

That the lymphocyte could originate from

a few non-epithelial cells cannot be disregarded, since we had no way of definitely excluding the presence of cells other than epithelial and lymphocytic from our cultures.

The origin of the thymic lymphocytes has been long debated(7,16,17,18,19,20,21,22) and the same question is open insofar as the repopulation of a damaged postnatal thymus is concerned(23,24). Evidence has been produced that under certain experimental conditions the repopulation of the thymus occurs through the infiltration of cells from other sources(25,26). The production of an humoral factor by the epithelial reticulum of the thymus, which stimulates lymphocytosis and enables thymectomized animals to establish immunologic competence has been also demonstrated(27,28,29,30,31,32,33,34).

There is some experimental support in the literature for the origin of the thymic lymphocytes from the thymic epithelial cells(7, 18,19,21,22). We could never observe, however, a clear cut transitional form between an epithelial cell and a lymphocytic cell. The establishment of cell clones from single thymic epithelial cells may clarify this point.

The changes observed in the cell colonies which were subcultured more than once by trypsinization are reminiscent of some of the characteristics of malignant cells, although abnormal mitoses and relevant alteration of the nucleo-cytoplasmatic ratio were not seen. However, since the malignant transformation of different cell types after various periods of cultivation *in vitro* is known to occur(35,36, 37,38,39,40), the possibility of a malignant conversion in our latest subcultures will be investigated.

Summary. Thymic epithelial cells derived from newborn mouse thymic fragments were cultured *in vitro* for over 22 months. Subcultures were started by transferring minute cell sheets obtained by mechanical scraping of the original culture when it was 14 months old or older. Lymphocytes, many of them with the morphological appearance of plasma cells, were seen in the subcultures. Intact lymphocytes, as well as mitotic figures were seen within cytoplasmatic vacuoles of the epithelial cells. The possible bearing of these

observations on the origin of the thymic lymphocytes is briefly discussed.

The authors wish to thank Dr. P. Shubik for his interest in this work and to acknowledge the valuable assistance of Mr. Andrew J. Washington in preparation of the photographs.

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Received June 24, 1965. P.S.E.B.M., 1965, v120.