studies focused on the tissue of the host and involved the interaction *in vitro* of herpes simplex virus with preparations of skin fragments from newborn and more mature rabbits. The cutaneous tissue from 1-2-day-old rabbits consistently synthesized 3-10-fold more virus than tissue from 1-2-month-old rabbits. Studies indicated no apparent difference in the attachment of virus to immature and mature rabbit skin. Interferon-like-substance was elaborated in equivalent, low titers by cutaneous tissue from newborn and adult rabbits. Light and electron microscopic study of rabbit skin failed to discern apparent differences in the population, morphology, or lysosomal content of cutaneous tissue. Virus-free preparations of 2-day-old rabbit skin incorporated 100% more thymidine-2-C^{14} than skin from 2-month-old rabbits but the incorporation of uridine-2-C^{14} by these tissues was essentially equal. Thus, the cutaneous tissue of the newborn rabbit appears particularly well suited biochemically for the synthesis of DNA-containing-herpes simplex virus.

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Neoplastic Conversion *in vitro* of Newborn Mouse Thymic Cells.* (32427)

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Newborn mouse thymic cells have been cultured *in vitro* for over two years(1). In the present study their possible malignant conversion has been tested by injecting the cultured cells into newborn and adult syngeneic hosts.

Material and methods. The thymic cells were derived from minute thymus fragments of newborn MA mice. The procedure used for establishing the cell line, as well as its morphological characteristics, has been previously described(2). The thymic cultures were started in August 1963 and maintained in continuous culture until March 1966, when they were stored at −70°C. Since December 1964, subcultures from the original thymus culture were started by transferring a minute amount of cells obtained by mechanical scraping at the edges of the solid sheet of epithelial cells. Occasionally subcultures were obtained by trypsinization.

In the present experiment only subcultures obtained by mechanical scraping were used. The cultures were maintained in Leighton tubes, T-flasks or Milk bottles at 37°C in an incubator without CO_2. The culture medium

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was Hanks' BSS with phenol red as an indicator, plus vitamins, glutamin, and essential amino acids and was supplemented with 11% or 13% fetal bovine serum (GBI or Flow). The medium was generally changed every 5 days. No antibiotics were used.

For injections into mice, the cultures were digested with 0.02% trypsin solution centrifugated and resuspended in Hanks' BSS. After being counted, the cells were diluted in Hanks' and the counting was repeated to determine if the desired concentration had been obtained. Recipient mice were of the MA inbred strain, as was the thymus from which the original culture was obtained. In a first series, 18 newborns received 2.5×10^4 cells in 0.05 ml of Hanks' subcutaneously within 24 hours from birth. The same number of cells at the same dilution in Hanks' were given one month later to 12 newborn mice.

In a second series, 13 newborns received 1.2×10^5 cells in 0.05 ml of Hanks' subcutaneously within 24 hours from birth, and 5 female and 5 male 8 week-old adults received 2.5×10^5 cells in 0.05 ml of Hanks' subcutaneously.

Ten newborns were injected with 0.05 ml of Hanks' BSS as controls. The mice were weaned and separated by sex at 4 weeks of age. Groups of four to five 7- to 8-week-old MA mice were given subcutaneous transplants with a trocar from 2 of the tumors which appeared in a mouse of the second series. These tumors were maintained in transplantation and are, at present, at the 17th transplant generation.

In addition, minute fragments of the first tumor used for transplantation were cultured *in vitro*. Five 8-week-old MA mice received 2.5×10^5 of the tumor cells after one month of culturing.

Tumor-bearing animals were killed either for transplantation purposes or when in very poor condition. Sections of each tumor and of the main organs were fixed in 10% neutral formalin and routinely stained with hematoxylin and eosin. Selected special stains such as the Snook method for reticulin fibers, Masson trichrome, PAS and Alcian blue, were also used.

Results. The data are summarized in Table I. Six tumors appeared in the 30 mice injected when newborn with 2.5×10^4 cultured thymic cells, and 4 tumors in the 13 mice injected when newborn with 1.2×10^5 cultured thymic cells. No tumors appeared in the 10 mice injected with 2.5×10^5 cultured thymic cells when 8 weeks old.

The times of appearance of the tumors were 16 weeks, 16, 21, 24, 49, and 64 weeks for the 6 tumors which were observed in the 2 groups of mice receiving 2.5×10^4 cells. It was 25, 46, 46, and 47 weeks for the 4 tumors which appeared in the group receiving 1.2×10^5 cells. The time when a subcutaneous nodule became palpable, usually when it was approximately 5 mm in diameter, was considered to be the time of appearance. From then on the tumors grew fast, reaching a size of up to 3.5 cm in diameter in about 2 weeks. The mice injected with the cultured cells gained weight as did the controls in-

TABLE I. Results From Injection Of Cultured Thymic Cells.

	No. of mice inj at birth	No. Alive at weaning	No. of mice inj at 8 wk of age	No. of cell injected	No. of mice with tumor
1.	18	6	—	2.5×10^4	—
		12			3
2.	13	4	—	2.5×10^1	1
		8			2
3.	13	8		1.2×10^5	3
		5			1
4.	—	—	5	2.5×10^5	—
			5		—

jected with the Hanks' solution and did not show signs of sickness until the appearance of the tumors. Nine of the tumors occurred subcutaneously in the upper back or upper flank. One apparently originated in the retroperitoneum and involved the mesentery. All of the 10 tumors had identical gross and microscopical characteristics. They were soft, whitish masses fairly easily detachable from the surrounding tissues. When allowed to grow to a size of 3 cm in diameter or more, they had a tendency to ulcerate the skin, and necrotic areas were always found in their central portions.

There was a consistent uniformity in the morphology of the 10 tumors. The cells were round or polyhedric, in limited areas slightly elongated but never to the point of being spindle shaped. The cytoplasm was acidophilic with poorly defined margins, due to the closeness between cells. The nuclei were round with minutely dispersed chromatin, somehow more condensed at the periphery. They often contained one, rarely two, nucleoli (Fig. 1). Clear patterns of invasion toward the surrounding tissues were observed only when the tumors were allowed to grow to a very large size. No metastases were seen. Mitoses were numerous. Reticulin stain revealed the presence of fibrils in limited areas of the tumors (Fig. 2). In most instances the fibrils could be related to vessels or to the host's stroma; in some instances, however, their presence as minute fibrils surrounding individual tumor cells seemed to indicate a strict relation with the tumor cells themselves. As for the identification of these tumors, some cytological features, considered with their origin from cultures whose main component was thymic epithelial-like cells, suggested a diagnosis of undifferentiated carcinoma. However, the presence of reticulum fibers, as indicated above, has made the differential diagnosis, with poorly differentiated reticulum-cell sarcoma, rather difficult.

The first subcutaneous transplantation by trocar of the tumors to adult MA mice was followed by growth of a palpable tumor in about 50% of the animals in 3 to 4 weeks. The second transplant generation resulted in 90-100% takes in 2 to 3 weeks, and 100% takes were observed from the third transplant generation. The histological and morphological characteristics of the transplanted tumors were essentially similar to those observed in the original tumors, although a more pronounced invasiveness was noted. Moreover, after the 5th transplant generation, peculiar whorl-like arrangements of the cells, which were not seen in the original tumors, became a rather frequent finding (Fig. 5).

The cell colonies obtained by culturing minute fragments of the first tumor which occurred in a mouse after the injection of the original thymic cell culture, showed morphological characteristcis similar to those observed in the original thymic cells culture. They were mainly composed of large, epithelial-like cells outgrowing as a solid sheet (Fig. 3-4). In some of the colonies, however,

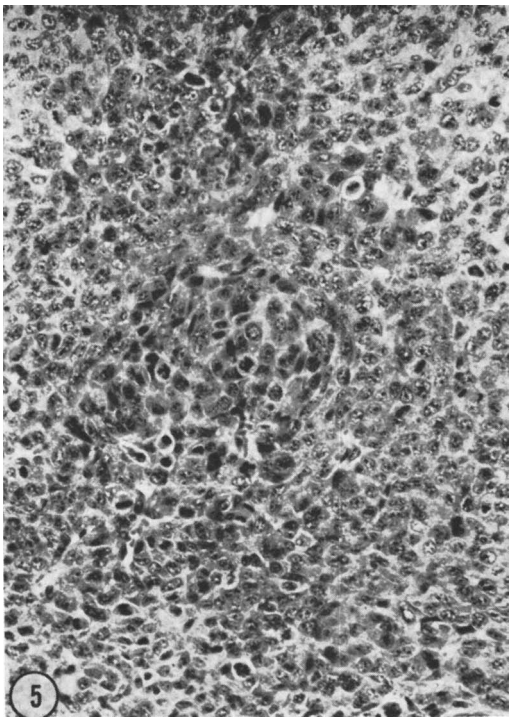

FIG. 5. Sixth transplant generation of a tumor originating from s. c. injected cultured thymic cells. Whorl-like arrangement of the cells. Hematoxylin and Eosin, 113 ×.

the sheet-like growth of the epithelial-like cells was surrounded or even penetrated by 3-cornered cells displaying an apparent lack

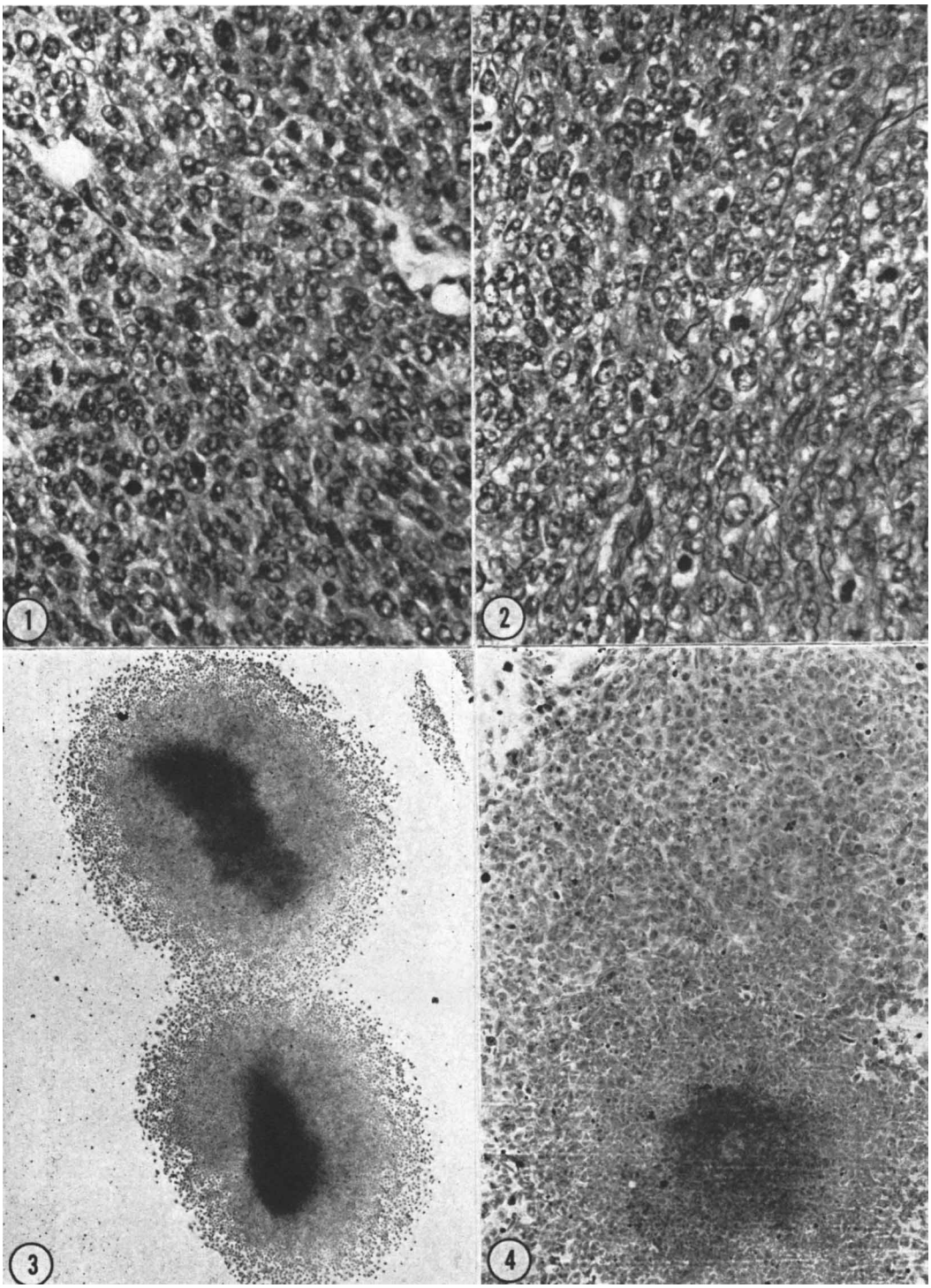

FIG. 1. Subcutaneous tumor arising at site of injection of cultured thymic cells. Hematoxylin and Eosin, 338 ×.

FIG. 2. Same as Fig. 1. A few reticulin fibers are present in limited areas. Snook, 338 ×.

FIG. 3. One-week-old culture of minute fragments derived from a tumor originating from s. c. injected cultured thymic cells. May-Grünwald and Giemsa, 17 ×.

FIG. 4. Same as Fig. 3, larger magnification. The growth is composed of large epithelial-like cells. May-Grünwald and Giemsa, 38 ×.

of cohesion. This pattern was more evident after one or more subcultures.

The subcutaneous injection into adult mice of 2.5×10^5 tumor cells cultured *in vitro* for one month resulted in the growth of a tumor in all of the 5 injected mice in 3 to 4 weeks. The tumors so obtained were morphologically identical to the tumor from which the cultured cells were derived.

Discussion. The injection into syngeneic adult and newborn mice of newborn mouse thymic cells which were cultured *in vitro* for over 2 years, has been used in the present experiment with the purpose of investigating their possible malignant conversion *in vitro*. As previously reported(1), morphological changes and an increased multiplication rate had been observed in cell colonies that were subcultured several times by trypsinization. Such changes did not occur in subcultures obtained by mechanical scraping, and in the present study only cells derived from the latter were used. The cells which were injected into the syngeneic hosts were therefore exposed to the action of trypsin only once, at the time when they were dispersed to obtain a suspension of cells suitable for injection into animals. The possible occurrence of a malignant conversion of normal cells after various periods of culture *in vitro* has been widely demonstrated(3). It has been shown that cells cultured *in vitro* may have acquired neoplastic potentialities without displaying morphological changes(3,4).

The cell colonies used for the injection were composed of epithelial-like cells with a scanty population of lymphocytes(1). The tumors were basically composed of one type of cell which can safely be assumed to have derived from the epithelial-like cells which were the main component of the cultures. We should assume that the lymphocyes observed in the original cultures, in constrast with the epithelial cells, either did not undergo a malignant conversion, or that the lymphocytes underwent the same basic changes as the epithelial cells but that their neoplastic potentialities were hindered by the overwhelming population of epithelial cells.

Summary. Newborn mouse thymic cells were cultured *in vitro* for over 2 years and their possible malignant conversion was tested by injecting the cultured cells into newborn and adult syngeneic hosts. While no tumors grew in the adults, one tumor grew subcutaneously at the site of injection in 9 of the 40 newborns and in one newborn a tumor grew in the retroperitoneum. All tumors had identical morphological characteristics. Two of the tumors so obtained were transplanted and are at present at the 17th transplant generation.

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