STUDIES ON ECHINOCOCCOSIS XVIII
OBSERVATIONS ON TISSUE CULTURED GERMINAL CELLS OF LARVAL *ECHINOCOCCUS MULTILOCULARIS*

Tsukasa SAKAMOTO, Jiro YAMASHITA
and Masashi OHBAYASHI
Department of Parasitology
Faculty of Veterinary Medicine
Hokkaido University, Sapporo, Japan

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INTRODUCTION

The in vitro cultivation of *Echinococcus multilocularis* and *E. granulosus*, especially, that of the scolices has recently been attempted by many investigators. The work on the cell or tissue culture of *Echinococcus*, however, has been scanty up to the present. LUKASHENKO (1964) carried out in vitro cultivation of the minced germinative tissue of vesicles without the scolex of *E. multilocularis* in the nutrient medium containing synthetic medium 199 in combination with cotton rat embryo extract, bovine serum and lactalbumin, and he observed that the vesicles developed into those having the scolices with hooklets. However, in his report, the microscopical or cytological findings in the development of vesicles and even the characters of his minced germinative tissue were not shown in detail.

In this experiment, the tissue culture of germinal cells as an undifferentiated cell was carried out. The germinal cells propagate boundlessly in vivo and differentiate into various cells. The authors obtained some interesting cytological findings in the germinal cells, cultured in vitro.

MATERIALS AND METHODS

Daughter cysts within the mother cyst were collected aseptically from the cotton rat, infested experimentally with the eggs of the Alaskan strain of *Echinococcus multilocularis*. They were treated with 0.2% trypsin for 5 minutes and were washed well three times with HANKS' balanced salt solution (HANKS' solution). They were cut fine and were trypsinized again on a magnetic stirrer. The pieces of cuticular layer were excluded from the materials by filtration with fine mesh. The filtrate was placed in a test tube, and the tube was allowed to stand for 5 minutes. The supernatant was removed, placed in a centrifugal tube, and centrifuged for 5 minutes at 600~800 rpm. The sediment was diluted with HANKS' solution and placed in a new test tube. Those procedures were repeated three times for clearing of the admixtures.

The culture media essentially consisted of HANKS' solution with 0.5% lactalbumin and 0.1% yeast extract (YLH), medium 199 and calf serum in a proportion of 4 : 4 : 2.

Cubic culture bottles (250 ml) and Petri dishes (4 and 6 cm in diameter) with coverslips pasted on their bottoms, using polyvinyl resin (Formval), were used at 37°C.

The media were changed at intervals of 1 and 2 days, keeping their pH at 7.0 as indicated by phenol red. Each Petri dish was put in a respective can, which was then sealed with vinyl tape. Glass wares, except culture vessel, such as pipette, centrifugal tube and test tube were treated with silicone to prevent attachment of the cells.

The morphological changes of cultivating cells were observed by routine microscopy through the wall of the cubic culture bottle, and the observations of cells on the coverslips obtained from the bottom of Petri dish were carried out by routine and phase contrast microscopy, supravital staining and May-Giemsa staining.

For the examination of chromosomes, mitotic cells were arrested by colchicine treatment (50×10^{-8} M) for approximately 2 to 6 hours and were detached by trypsinization. Following water pretreatment, chromosomal slides were made by the routine air-drying method and squash method with Giemsa and dahlia violet respectively.

This experiment includes three series as follows :

Experiment 1 The morphological changes in the growth of germinal cells cultured in the medium to which 20% calf serum was added to an equivalent mixture of YLH and medium 199 were observed in process. The chromosome number of the germinal cells was investigated. Subcultures of germinal cells were repeatedly attempted.

Experiment 2 For comparion with in vitro cultured germinal cells, the cells of the connective tissue just outside of the hydatid tissue were cultured by the method described in experiment 1 and were observed morphologically. The chromosome number of the cells was investigated also.

On the other hand, the fibroblastic cells originating from the connective tissue of the host were cultured till the complete sheet was established. They were introduced into the cubic culture bottle in which the germinal cells were cultured for 10～15 days until colonies of the cells were formed. Then the morphological changes of the colonies of germinal cells surrounded by the fibroblastic cells were observed.

Experiment 3 For the purpose of studying the relationship between the multiplication of the germinal cells and composition of the media, the cells were cultured in media containing YLH, medium 199, calf serum, cotton rat embryo extract and unheated and heated cotton rat liver extracts at various rates. Then the difference between multiplication of the cells in these media was observed.

RESULTS

Experiment 1

Under the phase contrast microscope, the germinal cells sparsely lined the undeveloped daughter cysts in situ, and were asteroid shape with many long processes; the margin of cell body was smooth, the cytoplasm homogeneous, and a nucleus with one or two nucleoli was recognized distinctly. The germinal cells densely lined the developed daughter cysts

and spindly in shape, and sometimes the syncytial cells were recognized. Suparvital staining methods revealed the cells contained many neutral red granules in the cytoplasm.

The germinal cells released from the daughter cysts by trypsinization were round, but the margin of the cell body was rough. Supravital staining showed the cells contained many granules that absorbed neutral red dye. These cells were adhesive, and showed a slight degree of phagocytosis. Namely, they adhered to the glass ware and cotton fiber that strayed into the medium, they took various debris into their cytoplasm.

The cells introduced into the culture bottle with the medium, settled gradually on the bottom, and became the spindle-shaped cells which were characterized by a small and dense nucleus (2.0~7.5 μ in diameter) with one or two distinct nucleoli, and a rich and syrupy cytoplasm. With supravital staining, the cells manifested characters similar to the released germinal cells. May-Giemsa staining revealed the nucleus of the cells as exhibiting a chromatin-rich round structure with distinct nucleoli.

On approximately the 3rd day, a part of the cells was briskly proliferating, and they formed botryoid colonies consisting of round cells. Some round cells separated from these colonies. The desquamative round cells were collected, and were subcultured in another culture bottle. These cells settled on the bottom of bottle, and metamorphosed into the spindle-shaped cells and continued to develop afterward.

The spindle-shaped cells became asteroid with several elongated processes, and subsequently those processes branched. Under the phase contrast microscope, some cells, which were thought to have developed from the round cells, were found to be surrounded with numerous fine ciliary and spinous processes, much like the sea-urchin, and these cells were recognized for the first time. The processes of the asteroid and sea-urchin like cells elongated in straight, zigzag or weaving directions and ramified as radiated or branchy shapes. Some pointed ends of the processes were observed touching with those of other cells. Comparatively large neutral red granules and vacuoles were recognized in cytoplasm of these cells by supravital staining. The nucleus of the cells showed a chromatin-rich round structure with distinct nucleoli when stained with May-Giemsa. In due course of time, the processes of these cells spread fanwise over the surface of the bottom of the culture bottle. On approximately the 12th day, some of the asteroidal cells spread widely with numerous processes on the surface of the bottom. On about the 20th day, these cells were briskly proliferating, and they formed colonies. On the other hand, the botryoid colonies consisting of round cells, fell off and disappeared.

Moreover, the formation of a syncytium with many nuclei was recognized in many cells from approximately the 5th day. Vacuoles, beside neutral red vacuoles, appeared in the cytoplasm of some cells. In some syncytial cells, the nuclei were pressed on the cell-membrane by the enlarged vacuoles. Therefore, those cells were as vesicular as they looked, and the cells with many vacuoles were seemingly the multilocular vesicles. Up to date, those vesicular syncytial cells developed to be several times larger than normal cells, but they did not develop into a large vesicles such as that in vivo, and they fell off subsequently.

The cells in suitable condition survived for more than 100 days, but the multiplication of most cells cultured for a long time declined generally, and degenerated gradually, and for the most part, finally fell off.

For the investigation of the chromosomes of the cultured germinal cells, the air-drying and squash methods were employed. The chromosomes of the cells were granular and were too small for counting their number. The estimated number of chromosomes in mitotic cells was less than 18.

To meet the requirements of the cell strain adapted to the in vitro cultivation, cells detached from the culture bottle by trypsin or EDTA were subcultured at an interval of 7 to 10 days, but the capability of multiplication of the cells declined due to repetition of subcultures. The multiplication capability of most cells disappeared after the 3rd subculture.

Experiment 2

For comparison with the germinal cells cultured in vitro, the cells of the connective tissue just outside of the hydatid tissue were cultured and were observed morphologically.

The cells released from connective tissue by trypsinization were as round as the germinal cells. They settled on the bottom and became spindle shape. Then, the cells metamorphosed into triangular, rectoangular and polygonal forms from a spindle-shaped pattern by the formation of pyramidal processes of various sizes, and they were briskly proliferating.

These cells indicated characteristic to be flat. The cytoplasm was felt to be translucent, and the ovoid nucleus was large, flat and translucent. Under the phase contrast microscope, many definite mitochondoria were recognized in the cytoplasm. In May-Giemsa staining, the nucleus showed a chromatin-poor round structure of 10~70 μ diameter with a distinct nucleolus. Namely, the cells revealed, morphologically, the typical characteristics of the so-called fibroblastic cell. The chromosomes of these cells were large in size counting 54 diploid.

On the other hand, the fibroblastic cells obtained from the connective tissue of the host were cultured till the complete sheet was established. For observation of the change in the germinal cells surrounded by the fibroblastic cells, these cells were introduced into the culture bottle in which the germinal cells had been cultured for 10~15 days. The fibroblastic cells multiplied far beyond the growth of the germinal cells. The germinal cells were hemmed in by the briskly proliferating fibroblastic cells, subsequently degenerated and disappeared.

Experiment 3

The germinal cells were cultured in media composed of medium 199 and YLH at various rates to which calf serum, heated and unheated cotton rat liver extracts and cotton rat embryo extract were added in various amounts.

A high growth rate of the cells was seen in the medium composed of 3 parts of medium 199 and 1 part of YLH. The best result was in the basic medium to which calf serum was added at the rate of 20~30%. Of the extracts mentioned above, the addition of heated cotton rat liver extract showed considerable powers in promoting cell multiplication, other extracts acted unstably in promoting properties for the growth of the cells.

The highest growth of the cells was obtained in medium composed of 3 parts of medium 199 and 1 part of YLH to which 20~30% calf serum and 20% heated cotton rat liver extract were added. In this medium, most cells spread widely with many processes on the surface of the bottom of the culture bottle, and they formed the colonies on the 10th day.

On the 12th day, the complete sheet of the cells was established on the entire surface of the bottom of the culture bottle.

DISCUSSION

Works on the in vitro cultivation of hydatid tissue, up to the present, were reported by LUKASHENKO (1964) and LUKASHENKO et al. (1965) as far as the authors know. LUKASHENKO (1964) cut the vesicles obtained from cotton rats on the 17th~19th day after inoculation of *E. multilocularis*, into pieces with a pair of scissors and chopped them with a homogenizer. He observed the development of vesicles containing a scolex with hooklets from the minced germinative tissue of the vesicles in the nutrient medium by means of settling on concentrated blood plasma. Any cytological description of the germinal cells themselves however, during the process in which the minced germinative tissue developed into the vesicles with scolices, was untouched in his report.

For the pure cultivation of germinal cell in this investigation, the cells were obtained from daughter cysts collected aseptically from cotton rats infested with *E. multilocularis* experimentally. SAKAMOTO et al. (1965) had already reported the morphological findings of germinal cells in the daughter cysts of *E. multilocularis* observed by the routine and phase contrast microscopes and supravital staining. In this experiment, as compared with the germinal cells of the daughter cyst in situ, the in vitro cultured germinal cells showed many morphological resemblances. Namely, the germinal cells became asteroid with many processes several days after incubation, and many large neutral red granules and vacuoles were recognized in the cytoplasm of the asteroid cells under supravital staining. The processes which elongated straight or meanderingly ramified as the radiated or branchy shape. Some pointed ends of the processes were observed to be in touch with those of other cells. These cytological findings should be recognized as common features between both germinal cells in vitro and in daughter cyst in situ. The authors suppose that these features of asteroid germinal cells are the typical characteristics of germinal cells. Those cells with branchy processes resemble, somewhat, the cells nervous system when cultured in vitro, but distinct differences of microstructure between both cells can be observed under the phase contrast microscope and supravital staining. On the other hand, some cells which had round cell bodies surrounded by numerous fine ciliary and spinous processes much like a sea-urchin, were recognized. Those cells, however, can hardly be observed in echinococcal tissue in vivo. Accordingly, those sea-urchin like cells were thought to be the cell form peculiar to the germinal cells cultured in vitro. The fact that the processes of those cells elongated and ramified with time supports a view in which such a cell form is different from the regressively changed one.

HOGUE (1919) reported that the processes of fibroblasts from the heart of the chick embryo became long and thread-like forms in hypertonic solution, and the connective tissue fibrils anastomosed with other fibrils formed thread-like processes. It can be considered that those sea-urchin like cells appeared only in an in vitro environment, differed from the in vivo condition.

Subsequently, the extreme parts of the processes spread fanwise over the surface of the bottom of the culture bottle in due course of time. The relationship between the membraneous substance on the fanwise extreme parts of the processes and the cuticular layer was not clarified in this investigation. A histochemical and electronmicroscopical investigation should be performed in connection with this subject in the future.

The formation of botryoid colonies composed of round germinal cells proliferating briskly, was recognized at an earlier stage of incubation, which was also the very image of the findings of the initial brood capsule formation. The round cells which were collected from those colonies, as they were subcultured, metamorphosed to a spindle shape and developed into asteroid cells afterward. The colonies, therefore, seem to have something in common with the massive colonies at the initial stage of brood capsule formation. Such syncytial germinal cells that appeared at times in vitro were recognized in the hydatid tissue too. In some syncytial cells, the nuclei were pressed on the cell-membrane by the enlarged vacuoles. Those cells, therefore, were as vesicular as they appeared, and the cells with many vacuoles were apparently the multilocular vesicles, but they did not develop into large vesicles such as that in vivo, and they finally fell off. Accordingly, it seems that further investigation needs to clarify what the relationship is between the above-mentioned findings and the formation of cyst. The authors could not obtain the completed vesicle. On the other hand, LUKASHENKO (1964) recognized the fact that very minute vesicles appeared at the 27th day of cultivation of the minced germinative tissue. He also observed, occasionally, the vesicles with the naked eye at the 38th day, a lamellar hyaline membrane at the 54th day and scolices having hooklets at the 99th day. The authors are unable to explain how such different results were observed. However, one of the reasons why the authors' results were obtained is that their experiment was done by means of stationary culture in which the cells multiply flatly in monolayer. Accordingly, investigation by other methods such as organ culture should be attempted for cultivation of the cells in future.

The chromosomes in germinal cells cultured in vitro are so small in size that an accurate count is not easy, however they seem to be 18 diploid. On the other hand, SMYTH (1962) examined the chromosome number in somatic cells of protoscolices of *E. granulosus*, and he stated as follows: the small size of the

chromosomes makes accurate counting difficult. Even with best available technique, many thousands of dividing cells were examined over a number of months before a reliable count was obtained. The chromosomes of *E. granulosus* numbered 18 diploid. A reliable idiogram could not derive from the somatic cell preparation used, but there appears to be one large open "U" in the haploid set of nine. LUKASHENKO et al. (1965) stated that the chromosome number of cells in the germinal layer and scolex of *E. multilocularis* incubated in vitro was 18 also. The authors, therefore, think morphological investigation in further detail should be done on the chromosomes of both echinococci.

The in vitro cultured cells of the connective tissue of the host revealed typical characteristics of the so-called fibroblastic cell. Namely, they exhibited a tendency to be flattened, spindle and angular shapes with comparatively short pyramidal processes which branched simply, and they had many definite mitochondria in the cytoplasm. Their nuclei were generally large, flat and chromatin-poor in comparison with those of germinal cells. In contrast with the chromsomes of the germinal cell, they were small and 18 diploid; those of the connective cell were large and 54 diploid. The morphological findings of these chromosomes resembled that of the texas cotton rat (*Sigmodon hispidus texianus*) reported by CROSS (1931).

The highest growth rate of the cells was obtained in medium composed of 3 parts of medium 199 and 1 part of YLH to which calf serum was added at the rates of 20～30%. The addition of heated cotton rat liver extract usually promoted the multiplication of cells, while the actions of unheated cotton rat liver extract and cotton rat embryo extract were unstable. The proliferation of the in vitro cultured cells declined generally with time and subculture. In the mixed culture of germinal cells and fibroblastic cells of the host, the germinal cells, hemmed in by briskly proliferating fibroblastic cells, degenerated and disappeared. It seems that medium 199 and YLH, which were used as the basic medium, may be unsuitable for the cultivation of germinal cells, because these two media have been designed for the cultivation of mammalian cells. On the other hand, LUKASHENKO et al. (1965) stated that the highest cell division was observed in medium 199 containing 10% cotton rat embryo extract, 5% lactalbumin and 15% calf serum. In their experiments cotton rat embryo extract was added, and there is considerable difference between their results and the authors'.

Besides the cotton rats infested with the Alaskan strain of *E. multilocularis*, cotton rats infested with eggs obtained from the intestines of 5 native dogs and a fox from the Hokkaido, Japan were used in the present experiment also. However a small number of the daughter cysts, insufficiently developed, were obtained from these cotton rats, notwithstanding the mother cysts and scolices developed completely. The germinal cells obtained from these daughter cysts

were small, and they revealed insufficient growth in vitro. The authors have no explanatory data as to the cause of this difference of daughter cyst formation in *Echinococcus* from Hokkaido. They consider that the biological differences among the strains of *Echinococcus* should be examined in the future.

SUMMARY

The in vitro cultivation of the germinal cells from daughter cyst of *Echinococcus multilocularis* was investigated. The medium consisted essentially of equivalent HANKS' balanced salt solution with 0.5% lactalbumin and 0.1% yeast extract and medium 199 to which 20% calf serum was added. The results obtained are as follows.

The germinal cells, released from daughter cyst by trypsinization, were round at first. The cells settled gradually on the bottom of the culture bottle, and for the most part, the cells became the spindle-shaped cells. On about the 3rd day of cultivation, some of the cells were briskly proliferating, and they formed botryoid colonies consisting of round cells. The round cells which were collected from those colonies, as they were subcultured, metamorphosed to a spindle shape, and developed into asteroid cells afterward.

Several days after the incubation, the germinal cells became asteroid cells with several processes, or sea-urchin like cells with numerous fine processes. Supravital staining methods revealed these cells contained many neutral red granules and vacuoles in the cytoplasm. The processes elongated straight or meanderingly, and ramified as radiated or branchy shapes. Some pointed ends of the processes were observed in touch with those of other cells. The extreme part of the processes spread fanwise over the surface of the bottom of the culture bottle in due course of time. The asteroid cells were proliferated, and formed many colonies.

On the other hand, syncytial cells were frequently recognized among the cells mentioned above. Enlarged vacuoles appeared in the cytoplasm of some syncytial cells, and they were as vesicular as they appeared, but they did not develop into a large vesicle such as that in vivo. The multiplication of the cultured germinal cells declined generally with the passage of time and repeated subculture.

Of the media used in the investigation, the highest growth rate of the cells was obtained in medium composed of 3 parts medium 199 and 1 part 0.5% lactalbumin and 0.1% yeast extract in HANKS' balanced salt solution combined with 20~30% calf serum and 20% heated cotton rat liver extract.

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EXPLANATION OF PLATES

Figures 1, 3, 7~12 & 18 were photographed with the use of a phase contrast microscope. Figures 2, 5, 6, 14, 16 & 17 are photomicrographs of specimens stained with May-Giemsa.

PLATE I

Figs. 1 & 2 Germinal cells of daughter cyst in situ ×1,670

Fig. 3 Germinal cells being released from daughter cyst ×1,670

Fig. 4 Botryoid colonies consisting of round germinal cells ×630

Figs. 5 & 6 Asteroid germinal cells ×340


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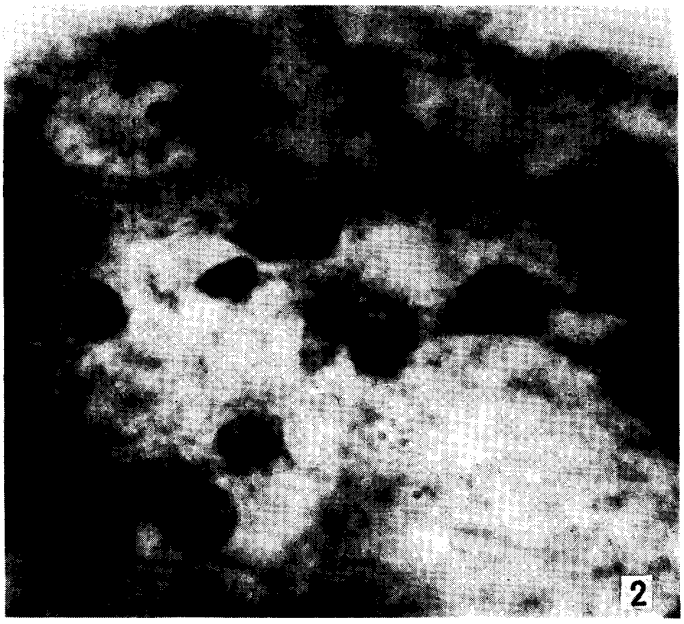
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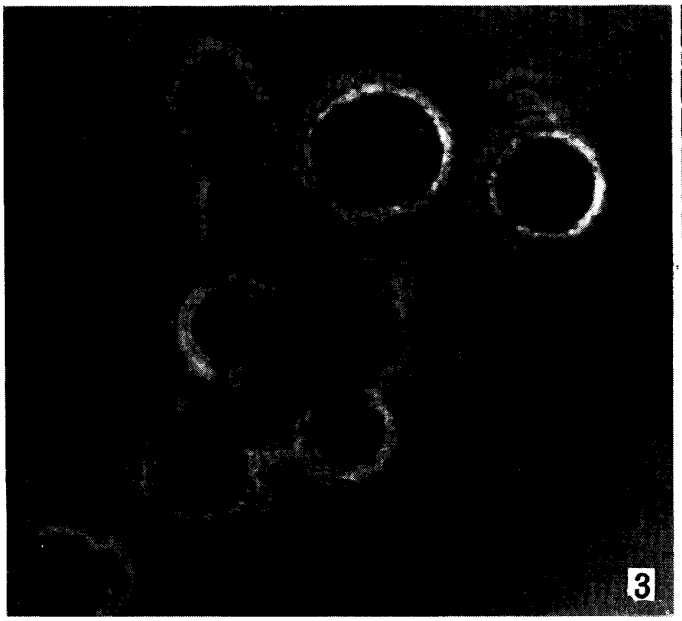
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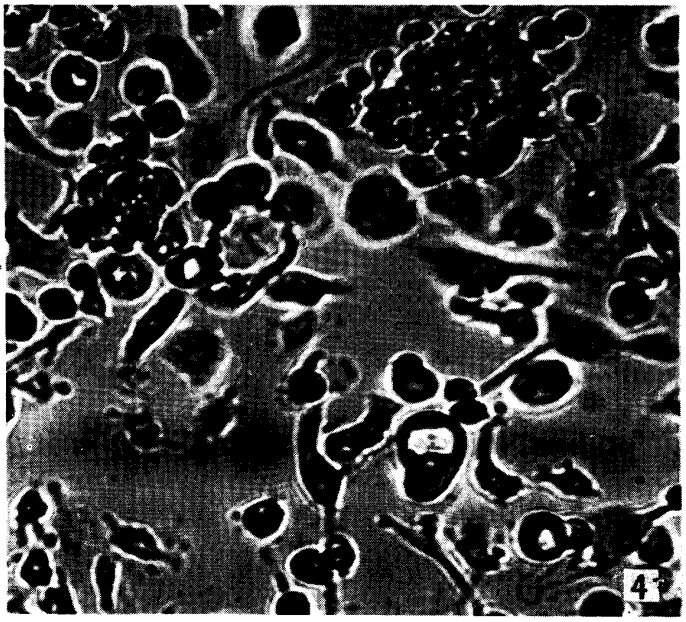
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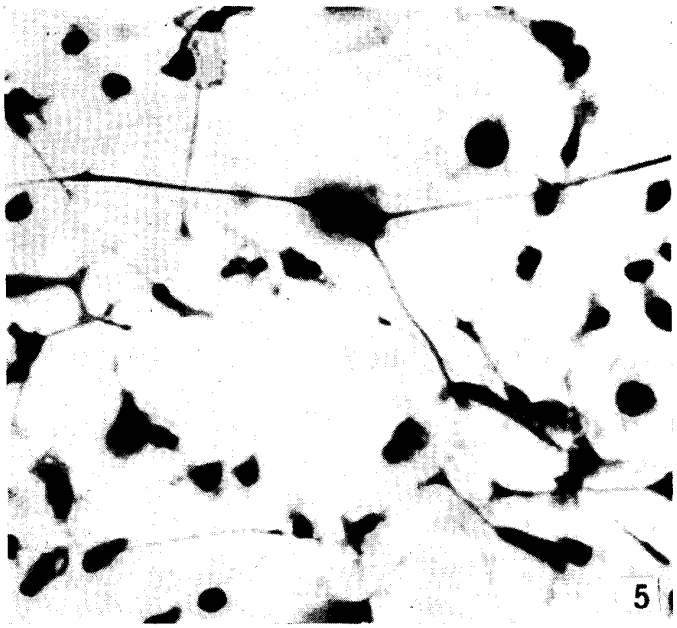
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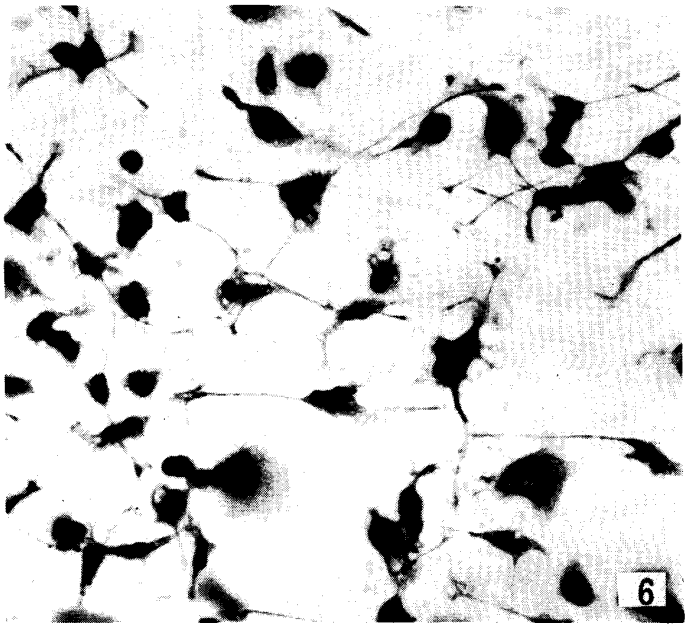
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PLATE II

Fig. 7 Germinal cell with elongated processes ×3,340

Fig. 8 Germinal cells forming cellular net with stretched processes which contact the processes of neighboring cells ×340

Figs. 9 & 10 Germinal cells with branched processes ×1,670

Fig. 11 Germinal cell with numerous fine ciliary processes ×1,670

Fig. 12 Germinal cells with ciliary processes ramified radially ×1,670


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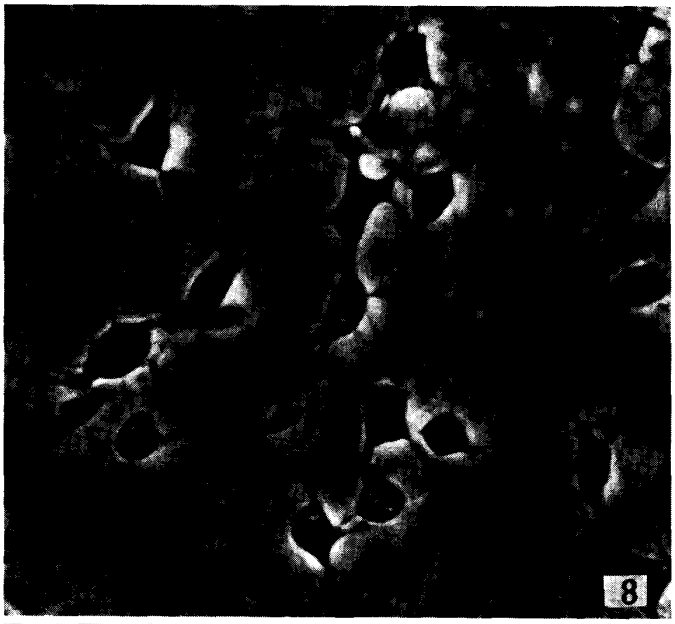
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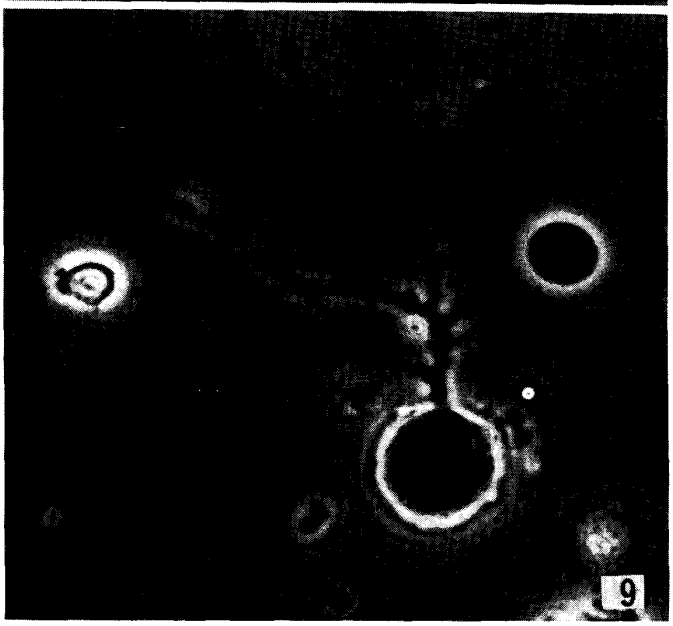
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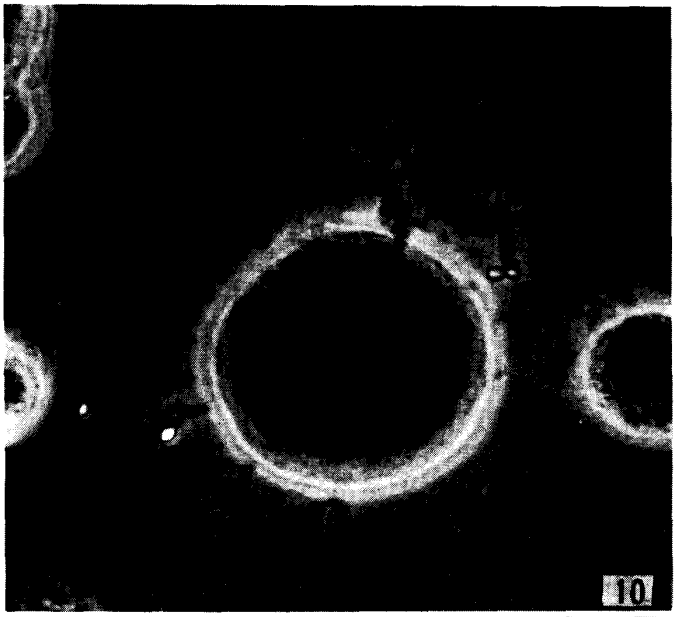
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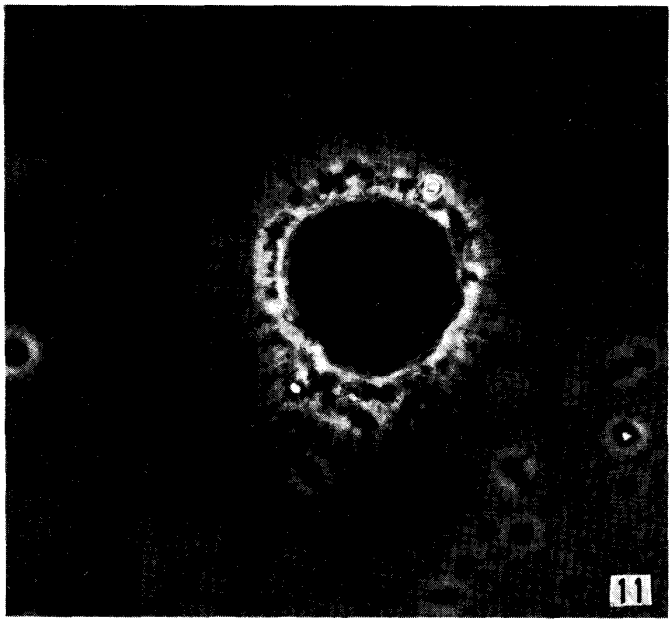
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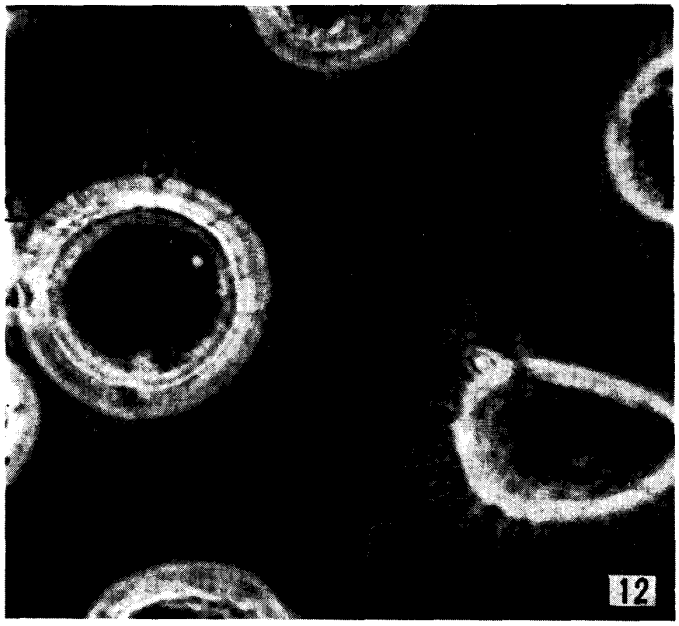
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PLATE III

Fig. 13 Germinal cells with processes spreading as membranella ×170

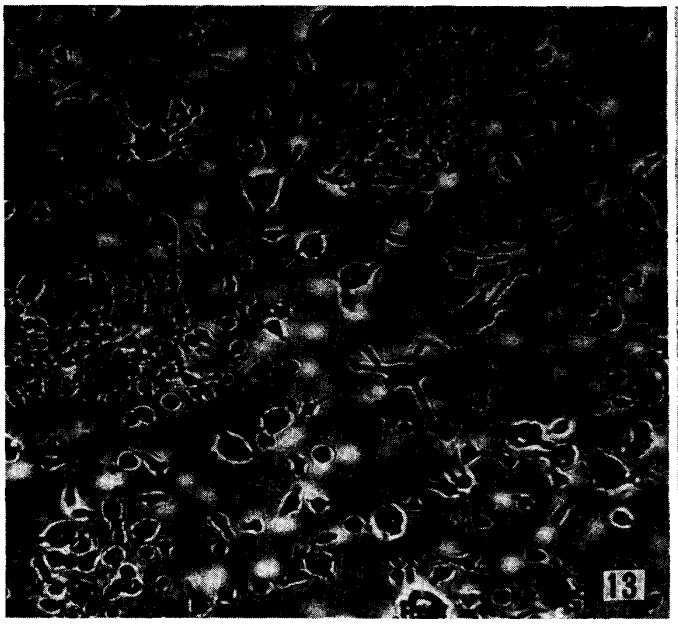
Fig. 14 Syncytial germinal cell ×630

Fig. 15 Vesicular syncytial cells ×340

Fig. 16 Vesicular syncytial cell ×630

Fig. 17 Fibroblastic cells ×170

Fig. 18 Fibroblastic cells ×340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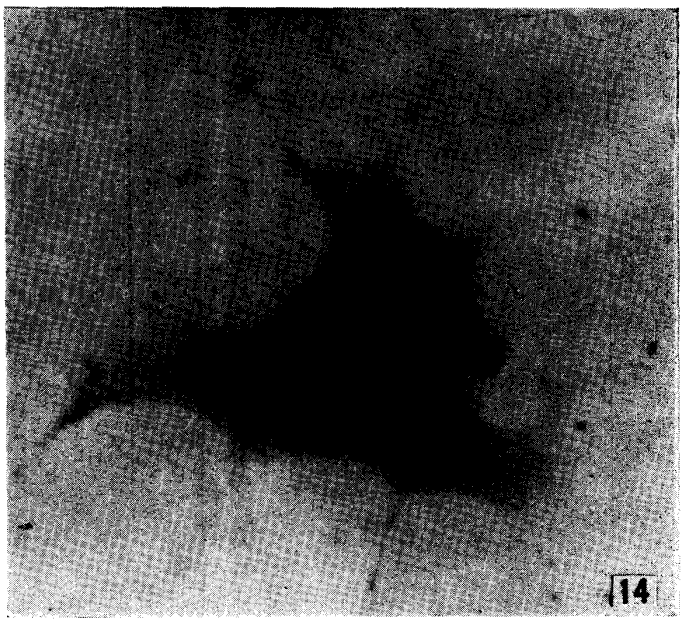
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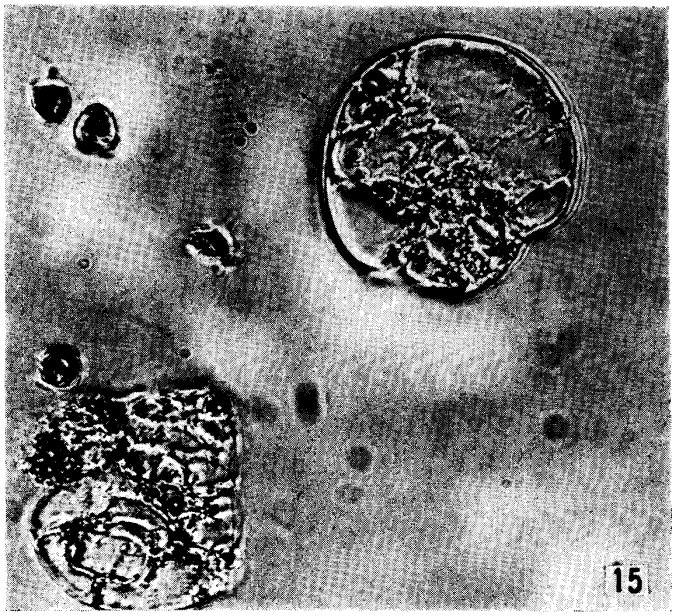
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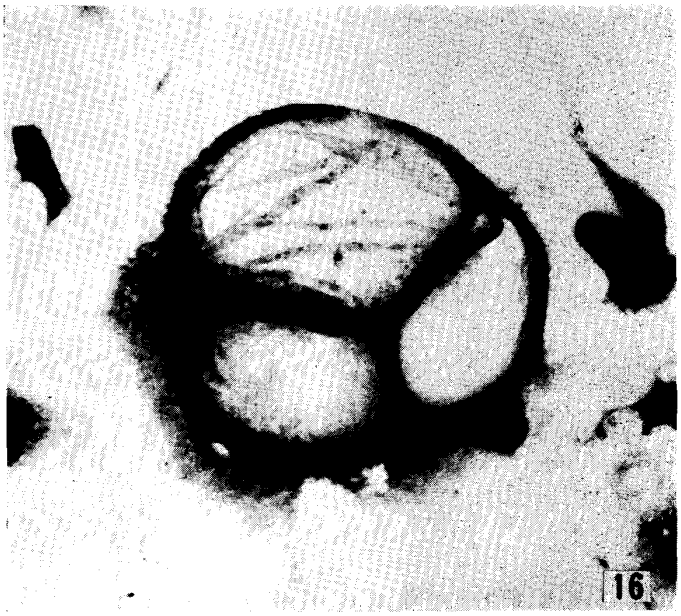
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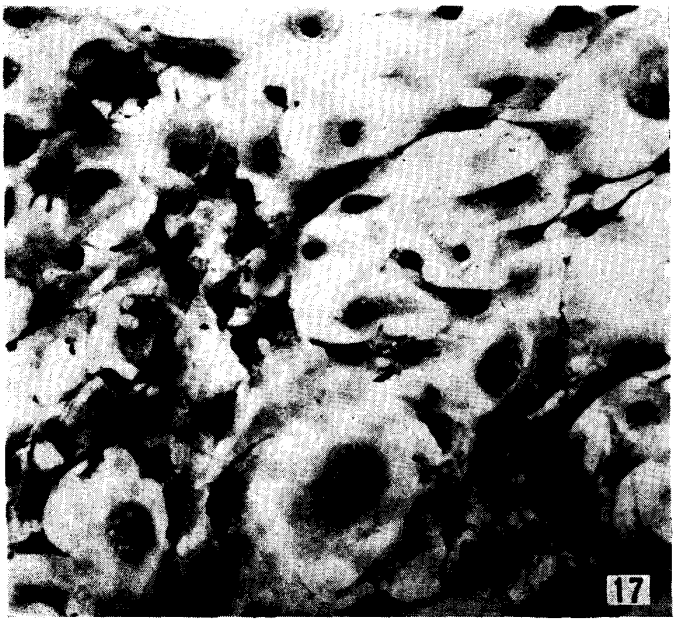
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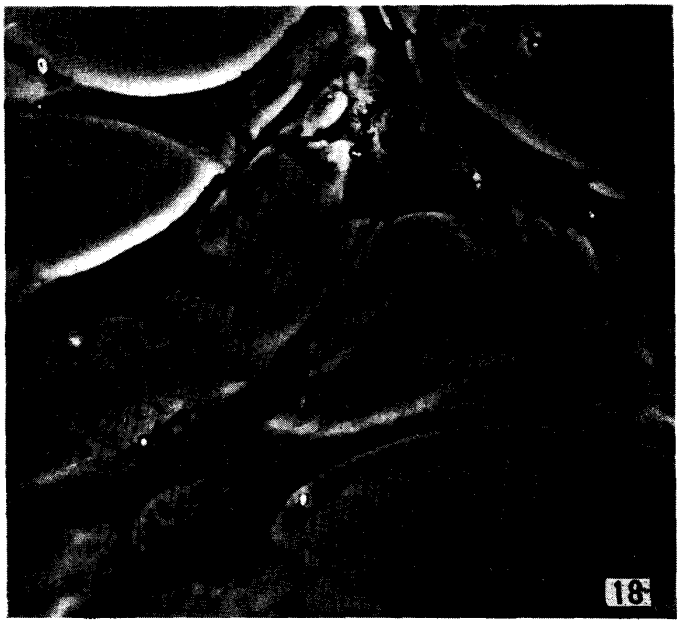
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