Development of Echinococcal Tissue Cultured *in Vitro* and *in Vivo*

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Introduction

The work on the tissue culture of *Echinococccus multilocularis* has been scanty up to the present. Lukashenko[2] reported that minced vesicles of *E. multilocularis* cultured in Medium 199 containing cotton rat embryo-extract, bovine serum and lactalbumin developed into those with protoscoleces. Sakamoto et al.[7] stated that the so called germinal cells, derived from the germinal layer of daughter cyst of *E. multilocularis*, proliferated briskly in primary culture, but the proliferation of the cells declined generally with the passage of time and subculture, showing a tendency of dedifferenciation. Subsequently, Sakamoto and Kotani[5] succeeded in the attempt of *in vivo* culture of larval echniococcal tissue in a diffusion chamber inserted into the abdominal cavity of cotton rat. They could not, however, make it clear whether the germinal cells developed into the vesicles or not, because the minced hydatid tissue containing brood capsule with protoscoleces was introduced into the chamber.

In the present experiment, the cells derived from germinal layer of larval *E. multilocularis* were cultured *in vitro* and *in vivo*. The author observed some interesting differences in development between the two kinds of cells cultured *in vitro* and *in vivo*.

Materials and Methods

Daughter cysts within the mother cysts were collected aseptically from the liver of cotton rats infected with the Alaskan strain of *Echinococcus multilocularis*. They were treated with 0.2% trypsin for 5 minutes and were washed well 3 times with Hanks' solution. They were cut fine and were trypsinized again on a magnetic stirrer. The pieces of cuticular layer were excluded from the material, using the sieve of 230 meshes per inch. A test tube in which the sieved material was placed was allowed to stand for 5 minutes. The upper cell suspension was removed from the test tube into a centrifugal tube, and was centrifuged for 5 minutes at 800 rpm. The sediment was dispersed in Hanks' solution. These procedures were repeated 3 times for the purpose of clearing the cells.

Basic medium used essentially consisted of NCTC 135 containing 0.5% lactalbumin hydrolysate and 0.1% yeast extract. Inactivated serum derived from fetal calf, calf, bovine, cotton rat, Wistar rat, ICR/JCL mouse or horse was added at various rates into the basic medium. For the purpose of studying the effect of tissue extracts in the media, the cells were cultured in the media added chick embryo-extract, cotton rat embryo-

extract and heated cotton rat liver-extract.

The cells were cultured in Falcon flask (30 ml in capacity) with the media as above, and with a gas phase of 90%N_2—5%CO_2—5%O_2. The morphological changes of the cells were observed by inverted microscopy. The cells growing on the coverslips pasted on the bottom of culture flask with polyvinyl resin (Formval) were observed in detail, using phase contrast microscope, supravital staining and May-Giemsa staining.

On the other hand, the germinal cells cultured *in vitro* for 3 to 7 days before using were removed from the bottom of culture flask, using 0.1% pronase P solution, and were washed three times by repetition of centrifugation and dispersion in Medium 199. The cell suspension in Medium 199 was introduced into the diffusion chamber. Two or three of the diffusion chambers were placed within the peritoneal cavity of the respective five males of cotton rats, CF-1 and ICR/JCL strain mice, Wistar and Sprague-Dawley strain rats, Mongolian gerbils and Hartley strain guinea pigs. The diffusion chamber used was made of Millipore membranes of $0.45 \pm 0.02 \mu$m in pore-size pasted on both sides of silicon gum tube, 7mm in length and inside diameter and 1.6 mm in wall thickness. The development of the cells to echinococcal tissue was examined by removing the chambers from the animals sacrified in the lapse of time. Observations were carried out extending over 7 days to 1 year, using supravital staining and phase contrast microscopy.

Results

The so called germinal cells released from the germinal layer of daughter cyst-wall by trypsinization were round shaped with rough margin. The cells were cultured in the media composed of the basic medium to which inactivated serum derived from fetal calf, calf, bovine, cotton rat, Wistar rat, ICR/JCL mouse or horse were added at various rates. The highest growth of the cells was obtained in the basic medium to which fetal calf serum was added at the rate of $20 \sim 30\%$.

Most of the cells introduced into the culture flask with the basic medium containing 20% fetal calf serum became spindle-shaped after being settled on the bottom. A part of the cells keeping round shape formed botryoid colonies through brisk proliferations. The spindle-shaped cells became asteroid with several elongated processes. Subsequently, those processes elongated and branched. Pointed ends of the processes were observed to be touching with those of other cells. The cells formed a net work through processes connecting each other. While the cells were subcultured at the interval of 10 days, the proliferative capability of the cells declined rapidly owing to the repetition of subculture. The proliferation of the cells stopped after the 3rd subculture.

The multiplication of the cells was observed in the medium containing 20% fetal calf serum to which the extract of cotton rat liver, cotton rat embryo or chick embryo was added. Of those extracts, the addition of heated cotton rat liver-extract showed considerable power in promoting the cell-multiplication; other extract acted unstably in the promoting properties. The processes of asteroidal cells spread fanwise over the surface of the bottom of culture flask with the medium containing embryo-extract of chick or cotton rat. Moreover, syncytia with many nuclei began to appear quite often on approximately the 5th day and later. Vacuoles appeared in the cytoplasm of those cells. In some of the syncytial cells, the nuclei were pressed on the cell-membrane by the enlarged vacuoles. Therefore, those cells appear to be vesicular, and the cells with

many vacuoles were seemingly the multilocular vesicles. Those vesicular syncytial cells developed to be several times larger than normal cells. The proliferation of nuclei in the syncytia, however, declined gradually, and those cells could not develop into such a large cyst as seen *in vivo*. Those vesicular syncytia degenerated gradually and fell off finally from the bottom surface.

On the other hand, the diffusion chambers containing the suspension of the germinal cells were placed within the peritoneal cavity of the respective five males of cotton rats, CF-1 and ICR/JCL mice, Wistar and Sprague-Dawley rats, Mongolian gerbils and Hartley guinea pigs. Echinococcal tissue developed from the germinal cells in the diffusion chamber was examined in the lapse of time.

The best growth of echinococcal tissue was found in the chamber inserted into cotton rat. On the 2nd day, the cells settled on the surface of Millipore membrane were of the round shape with the cytoplasm containing numerous neutral red granules. On the 10th day, the chamber was lined with germinal membrane consisting of the asteroid shaped cells. The initial formation of brood capsule by syncytial proliferation of the germinal cell occurred on the 30th day. Brood capsules having each one immature protoscolex appeared in the cyst on the 40th day. On the 50th day, the chambers were filled with the cysts having brood capsules containing several protoscoleces. However, the author could not find the multilocular vesiculation by budding as the one seen in the infected animal. The brisk proliferation of the cells did not decline even through the repetitions of subculture from chamber to chamber.

The echinococcal tissue in the chambers in Wistar rat developed into many small cysts about 1 mm in diameter on the 20th day. Brood capsules having protoscoleces were found barely on the 200th day. The similar development of hydatid cysts was seen in Sprague-Dawley rat also. In Mongolian gerbil, the completion of brood capsule and protoscolex was seen 3 and 4 months later, respectively. In CF-1 mouse, the chambers were filled with many well-developed cysts on the 30th day. The initial formation of brood capsule was found on the 120th day. Brood capsule with protoscolex appeared barely on the 300th day. In ICR/JCL mouse, the chambers were filled with a large number of cysts on the 30th day, but the initial formation of brood capsule was found barley on the 200th day. The complete brood capsules with protoscoleces appeared one year after explantation. In the experiment using Hartley guinea pigs insusceptible to *E. multilocularis*, several small cysts without brood capsule were found in 4 and 3 per 10 cases, 100 and 365 days after explantation, respectively.

Discussion

The *in vitro* culture of the cells derived from germinal layer of larval *E. multilocularis* was carried out by Sakamoto et al.[6] who used the medium consisting essentially of Hanks' solution with 0.5% lactalbumin hydrolysate and 0.1% yeast extract, Medium 199 and calf serum in a proportion of 4 : 4 : 2. They stated that the cytological findings of the so called germinal cells cultured *in vitro* showed a features common with those of the cells in the germinal layer of hydatid tissue. In the present experiment in which the medium consisting of NCTC 135 containing 0.5% lactalbumin hydrolysate, 0.1% yeast extract and 20% fetal calf serum was used, the results essentially resembling to those in the previous report were obtained also.

Sakamoto and Sugimura[6] who investigated electron microscopically the histogenesis of larval *E. multilocularis* stated that the cells of germinal layer were composed of light-and dark-staining undifferentiated cells, asteroid-transforming cell, muscle cell, glycogen-storing cell and reticular interstitial cell besides syncytial cyst-wall forming cell. From the morphological resemblances between the cells cultured *in vitro* and the cells *in situ*, it is assumed that the round, asteroid-shaped and reticular cells observed in the *in vitro* culture are homologous to dark-stained undifferentiated cell, asteroid-transforming cell and reticular interstitial cell, respectively. The vesiculation in the syncytium gives us a probable assumption on the initial formation of hydatid cyst or brood capsule. It is, however, necessary to clarify electron microscopically that the microvilli are present either on the outer surface of the syncytium or on the inner surface of vacuole in the cell.

On the other hand, Sakamoto and Kotani[5] reported that the echinococcal tissue in diffusion chambers inserted into the abdominal cavity of cotton rat, developed in the same manner as that of the infective lesion. They could not, however, make it clear whether the germinal cells developed into hydatid cysts or not, because the mixture containing germinal cells and protoscoleces was introduced in the chamber. In the present experiment, it was clarified that the germinal cells were capable of developing into hydatid cysts too. The development of echinococcal tissue in diffusion chamber was observed to be varied according to the species and strains of experimental animals used. Lubinsky[2] and Lukashenko[3] reported that guinea pig was insusceptible to the infection with *E. multilocularis*. Araki[1] also found granulomatous foci containing central necrotic mass in the peritoneal cavity of guinea pigs inoculated with the protoscoleces of *E. multilocularis*. In the present experiment, however, several small cysts were found in the diffusion chambers inserted into the abdominal cavity of guinea pigs. From those findings, it is assumed that the host-specificity has an effect, in some measure, upon the development of echinococcal tissue in diffusion chamber, but the effect is weaker than that upon the hydatid tissue in the lesion of infected animals.

Summary

The cells derived from germinal layer of larval *Echinococcus multilocularis* were cultured in NCTC 135 containing 0.5% lactalbumin hydrolysate and 0.1% yeast extract to which was added inactivated serum of fetal calf, calf, bovine, cotton rat, rat, mouse or horse at various rates. The best growth of the cells was observed in the medium with fetal calf serum at 20~30%. The cells proliferated briskly, showing a tendency of dedifferenciation in the medium. On the other hand, the cells revealed the membraneous expansion of processes, the formation of syncytium and the vesiculation of the syncytium in the medium to which was added embryo-extract of cotton rat or chick.

The germinal cells were cultured in diffusuion chambers inserted into the abdominal cavity of cotton rat, CF-1 and ICR/JCL mice, Wistar and Sprague-Dawley rats, Mongolian gerbil and Hartley guinea pig. The best growth of the cells was observed in cotton rat. The chambers were filled with the cysts having brood capsules containing many protoscoleces on the 50th day. The proliferative capability of the cells was not declined by the repitition of subculture. The development of echinococcal tissue was varied according to the species and strain of the animals into which the chambers

were inserted. Several small cysts were found in the chambers of a part of guinea pig tested.

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Explanation of plates

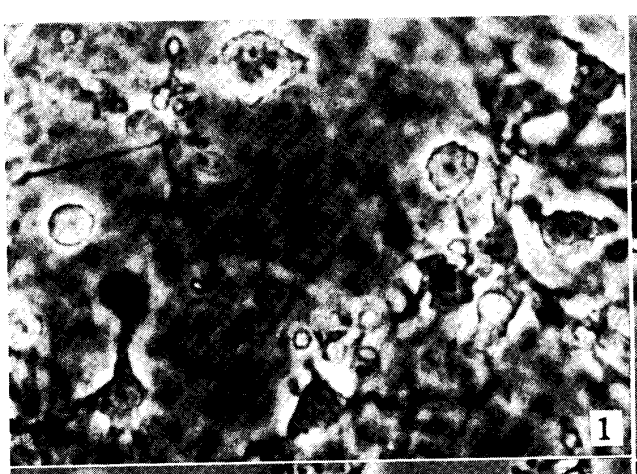
Figures 1~4 and 6 were photographed by the use of a phase contrast microscope. Figures 5 and 17 are photomicrographs of specimens stained with May-Giemsa and hematoxylin-eosin, respectively.

Plate I

Fig. 1 Asteroid-shaped cells × 350
Fig. 2 Asteroid-shaped cells with ramified processes × 1,500
Figs. 3 and 4 Asteroid-shaped cells having processes touching with those of other cells × 1,500
Fig. 5 Asteroid-shaped cells with processes × 700
Fig. 6 Reticular cell × 1,500
Fig. 7 Germinal cells with processes spreading as membranella
Fig. 8 Vesicular syncytial cell × 350

Plate II

Fig. 9 Cysts on the 30th day in ICR/JCL mouse × 3
Fig. 10 Cysts on the 100th day in CF-1 mouse × 3
Fig. 11 Cysts in diffusion chamber on the 60th day in ICR/JCL mouse × 3
Fig. 12 Cysts with a few of brood capsules on the 200th day in Wistar rat × 3
Fig. 13 Cysts with many brood capsules on the 200th day in cotton rat × 3
Fig. 14 Initial formation on the 120th day in CF-1 mouse × 600
Fig. 15 Brood capsules each with an immature protoscolex on the 120th day in ICR/JCL mouse × 200
Fig. 16 Cysts with many brood capsules on the 300th day in Mongolian gerbil × 5
Fig. 17 Cysts with brood capsules containing protoscoleces on the 300th day in CF-1 mouse × 70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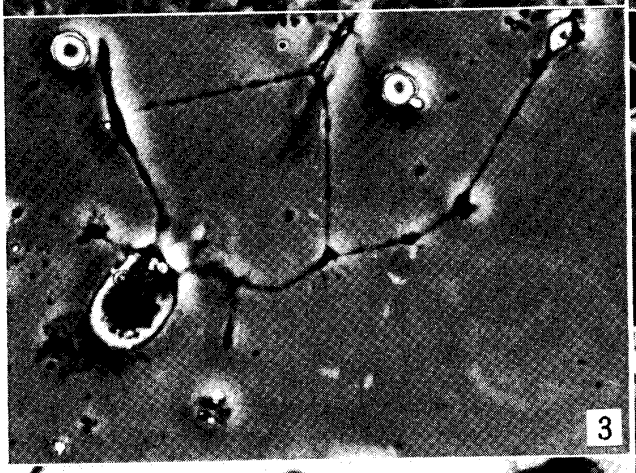
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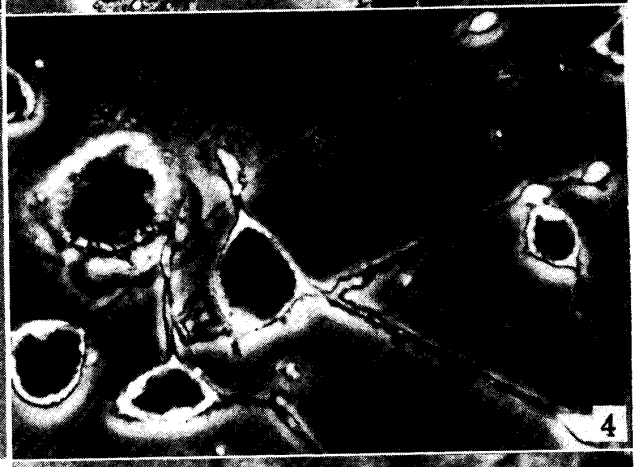
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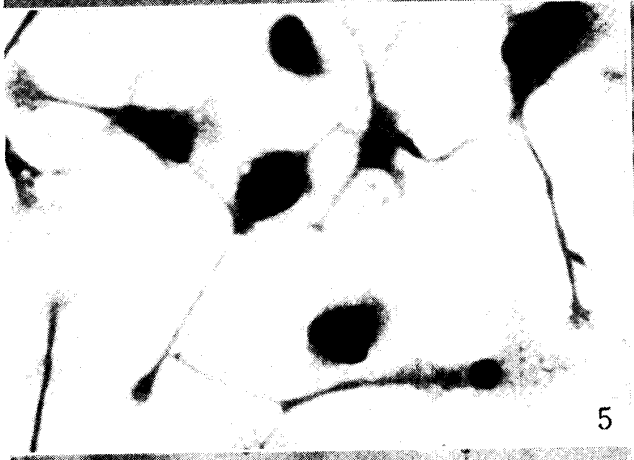
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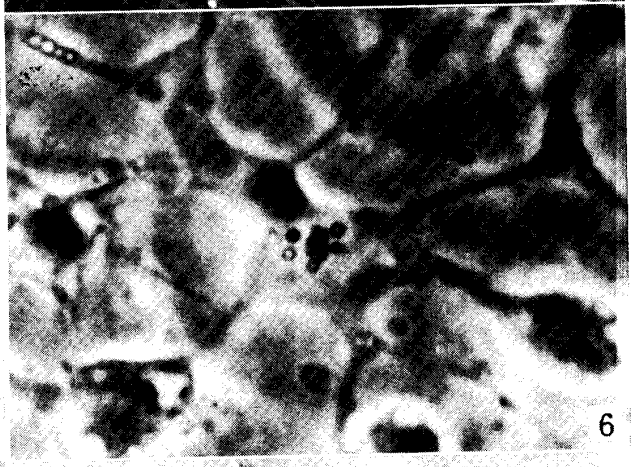
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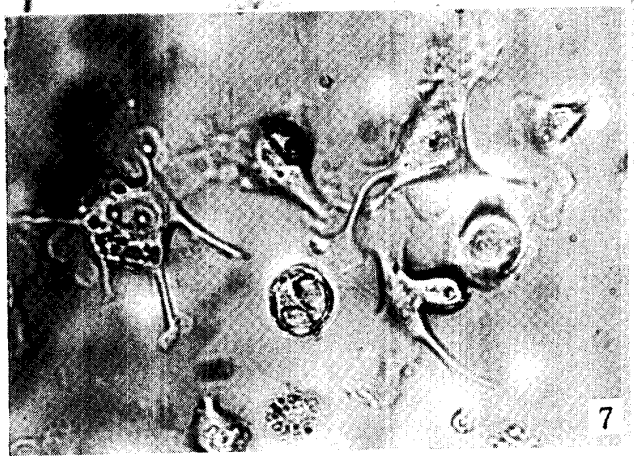
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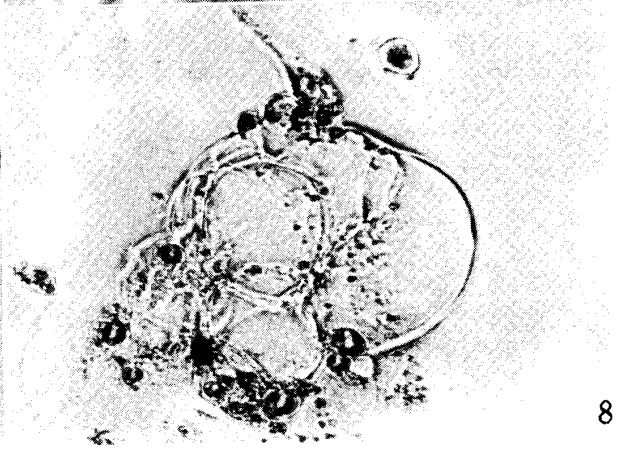
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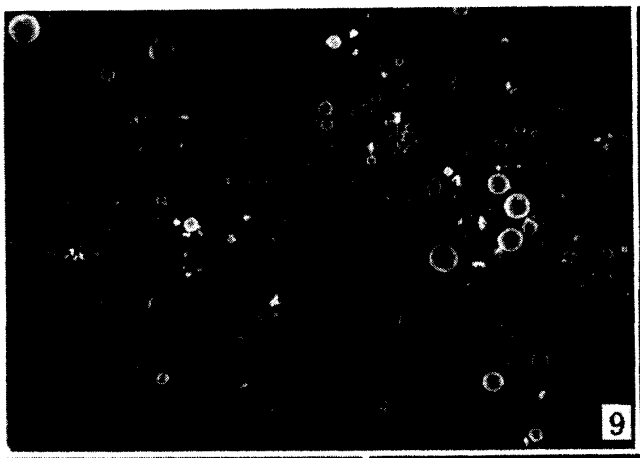
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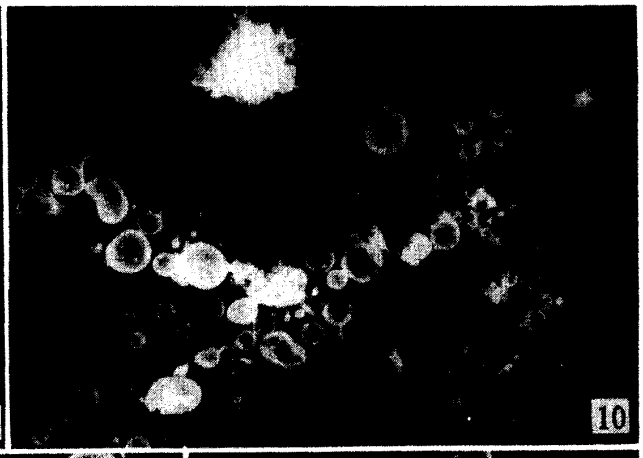
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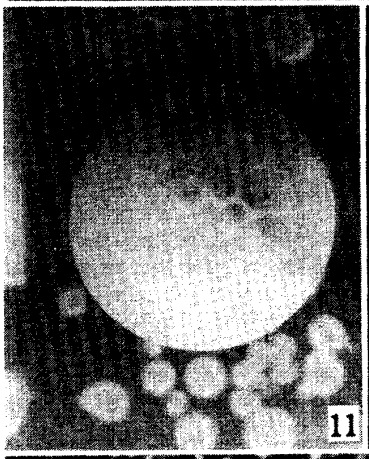
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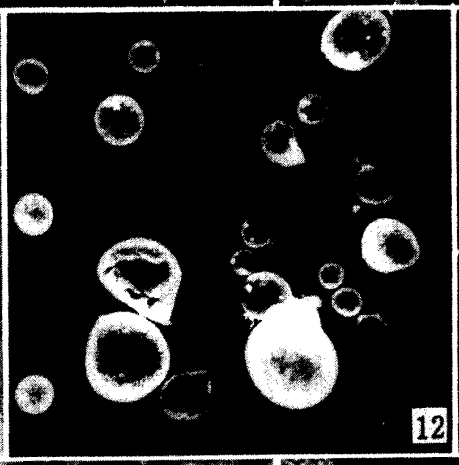
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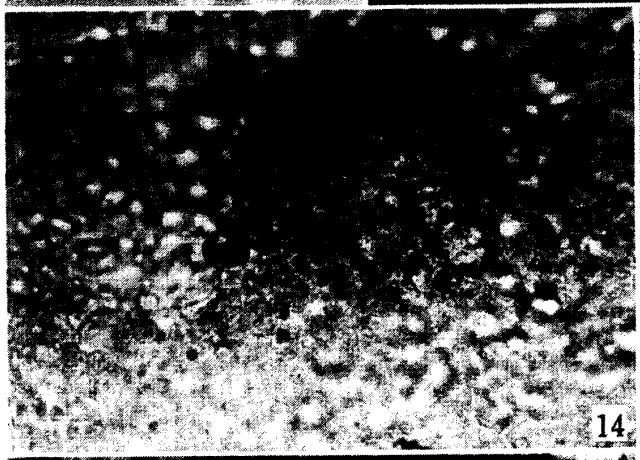
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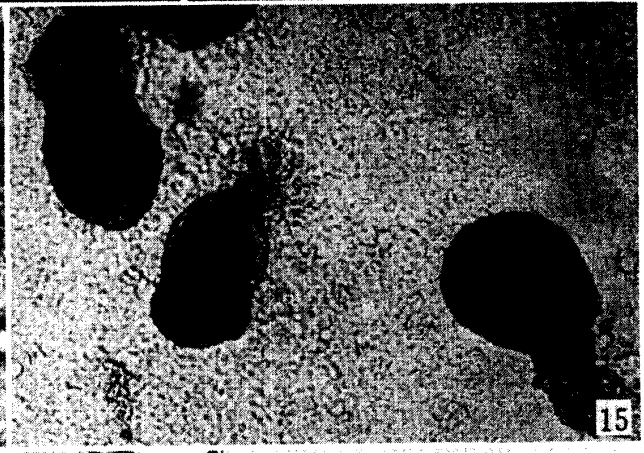
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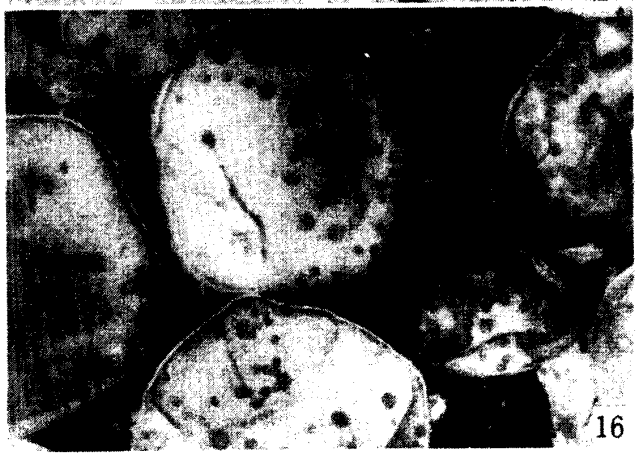
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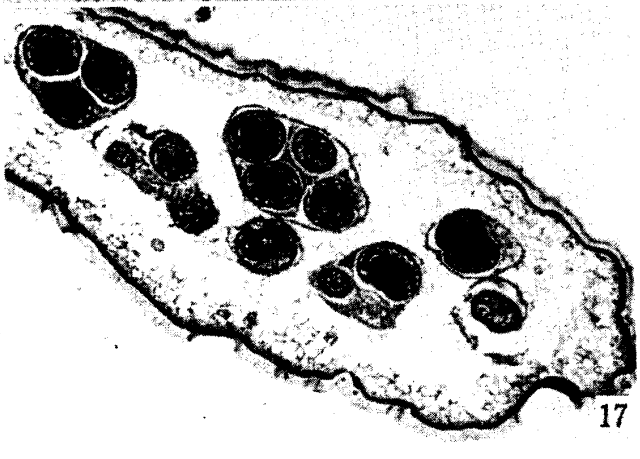
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