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Toxoplasma gondii in Cell Cultures from Rat Retina*

By

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With 28 Figures in the Text

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The multiplication of Toxoplasma gondii and its effects on the host cell has previously been described in vitro in a wide variety of cell strains (JACOBS, COOK and JACOBS, LUND, LYCKE and SOURANDER). However, there is no information about the interaction between the parasites and cells of neuroectodermal origin in cultures from nervous tissue. The central nervous system including the retina is particularly susceptible to infection with Toxoplasma gondii (JANKU, WOLF, COWEN and PAIGE). Despite the severe clinical symptoms associated with inflammatory and necrotic changes in the nervous tissue, typical parasites are rarely seen in fixed and stained preparations of human autopsy cases (THALHAMMER). They have only occasionally been reported to be found in nerve cells and neuroglial cells in fixed preparations (MEYER, FRENKEL), and the mode of their multiplication in these cells has not been demonstrated.

The present paper reports a study of the effects of Toxoplasma gondii on cell cultures from rat retina.

Materials and methods

Culture technique. The tissue cultures were prepared as previously described in detail (HANSSON and SOURANDER). Briefly, small pieces of retinal tissue from healthy young rats of the Sprague-Dawley strain or of inbred hooded rats were explanted in plasma clots on cover glasses in Gey chambers. After the clots had set for two hours, the culture medium was added. The medium consisted of a salt solution, dye-test negative calf serum, human umbilical cord serum, and penicillin and streptomycin. In this way, nerve cells, rods and neuroglial cells grew, and were seen to be active for up to three months on the surface of a monolayer of fibroblasts and endothelial cells.

The parasites. Toxoplasma gondii of the RH strain were obtained from fresh peritoneal exudate of infected Swiss albino mice from the Municipal Virological Laboratory. The cell culture fluid was replaced by a 1:5 dilution of exudate in Hank's medium with 20% calf serum. After 1—2 hours the cultures were rinsed and new medium added. In order to test for toxic effects, the peritoneal exudate was centrifuged and the supernatant fluid without parasites was added to the cultures for up to 12 hours (LYCKE and LUND).

In all 400 retinal cultures, 3—12 at a time, suitable for cytological observations were infected. For comparisons, cultures of cerebellum, heart muscle, lung, liver and spleen tissue were also infected. The dye test for the sera in the culture medium was performed at the Municipal Virological Laboratory by the method of SABIN and FELDMAN.

Observation technique. The infected cultures were observed several times daily in an inverted Zeiss microscope with phase contrast objective. Suitable structures were photo-

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graphed with a Hasselblad 500 C camera with a Zeiss microflash as a light source. A Zeiss micro cine camera with phase contrast, and time lapse was used. To improve the resolution of the Kodak Plus-X cine film, a Braun electronic flash, EF 3 NC, was sometimes used as a light source. The time intervals between exposures were varied from 1 second to 1 minute.

Suitable cultures were fixed for histological staining. Osmium acid fixed preparations were examined in phase contrast without further staining.

Results

General effects on the retinal cultures. Toxoplasma parasites appear intracellularly in the large, thin polygonal cells of mesenchymal origin forming the monolayer of the retinal cultures within 15 minutes. Only a few of the cells are infected at first. Inside the cytoplasm the parasites increase in size and after a few hours the first binary division occurs. One cell is sometimes infected by several parasites, each giving rise to a separate clone. The parasites are found to be quite uniformly distributed in the explants. This is true also for such parts of the explant, where restricted fibrinolysis of the surrounding plasma clot has occured. No intranuclear parasites have been observed. Infected neuroectodermal cells are observed within the first hour after inoculation, but more rarely than infected fibroblasts. After 24—48 hours, the first disrupted cells are observed. Five days after the inoculation, most of the cells are destroyed. The only exception are the phagocytes, which may survive for several more days.

In principle the same results are obtained with infected cultures of all other types of cells tested. The metabolism of the infected cultures seems to be accelerated, as the pH of the culture medium becomes acid more rapidly than in uninfected cultures.

When the peritoneal exudate containing parasites is centrifuged the supernatant is free from parasites. Toxic effects of the supernatant have not for certain been demonstrated on any kind of cells in culture. In cultures with multiplying parasites cells not yet infected do not show any apparent signs of degeneration.

The properties of the intracellular Toxoplasma parasite. The Toxoplasma gondii is a crescent shaped protozoon with a pointed and a blunt end. Its length of 6,5—8,0 μ is maximal just before the binary division, and its breadth is 2,4—3,3 μ as measured in our cultures. Under phase microscopy dense organelles and sometimes refractile vacuoles moving rapidly around the spherical nucleus can be seen inside the distinct cell membrane; there is a single nucleolus in the translucent, homogenous nucleoplasm. There are one or two rod-like, phase dense organelles at the pointed end, and a more rounded organelle at the blunt end; the latter is in close connection with the nucleus, with which it may move during nuclear rotation.

Having penetrated the host cell membrane, the parasite moves towards the nucleus of the host cell and can soon be seen in a perinuclear position, often in the vicinity of the cytocentrum. The volume of the parasite increases and a thin membrane surrounds the vacuole with the single parasite. After a few binary divisions the parasites are enclosed in a vacuole by a more distinct membrane. There seems to be an increased density of mitochondria around the clones as compared with other parts of the infected cells.

Multiplication of the parasite follows a regular pattern and starts with the disappearance of the cell nucleolus and the nucleus becoming less distinct and bean-shaped. In addition to the bean-shaped nucleus of the mother cell, two small translucent spots become apparent, gradually increasing in size. At an intermediate stage, three nuclei connected with each other are observed, later on two distinct daughter nuclei, each with a single nucleolus, appear, while the mother cell nucleus disappears. Thereafter a membrane forms to separate the two daughter cell bodies, which frequently rotate fast within the cell membrane of the mother parasite. The organelles in the parasite cytoplasm during the division, particularly at the blunt end, increase in number and are denser and move more actively than before division. No chromosomes or chromosome-like structures can be distinguished. Finally the membrane of the mother cell ruptures, starting at the pointed end opposite the center of the clones; two new parasites are released into the vacuole after ultimately being connected only at their central parts, and remnants of the mother cell membrane disappear. The duration of the division as determined from the earliest nuclear changes to the rupture of the mother parasite cell membrane is about 60 minutes. After a few further divisions the Toxoplasma parasites form a flower-like pattern in the vacuole during the almost synchronous multiplications of the parasites. This pattern disappears at the terminal stage of the parasite division, but later returns. Between divisions the parasites may move a little in the vacuole inside the membrane surrounding the clone.

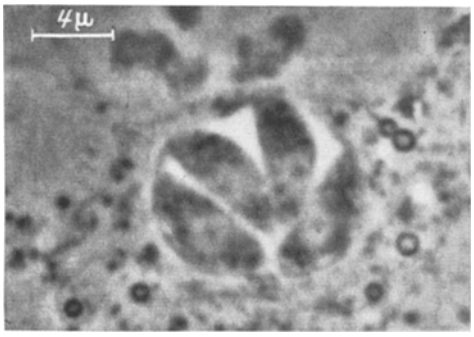
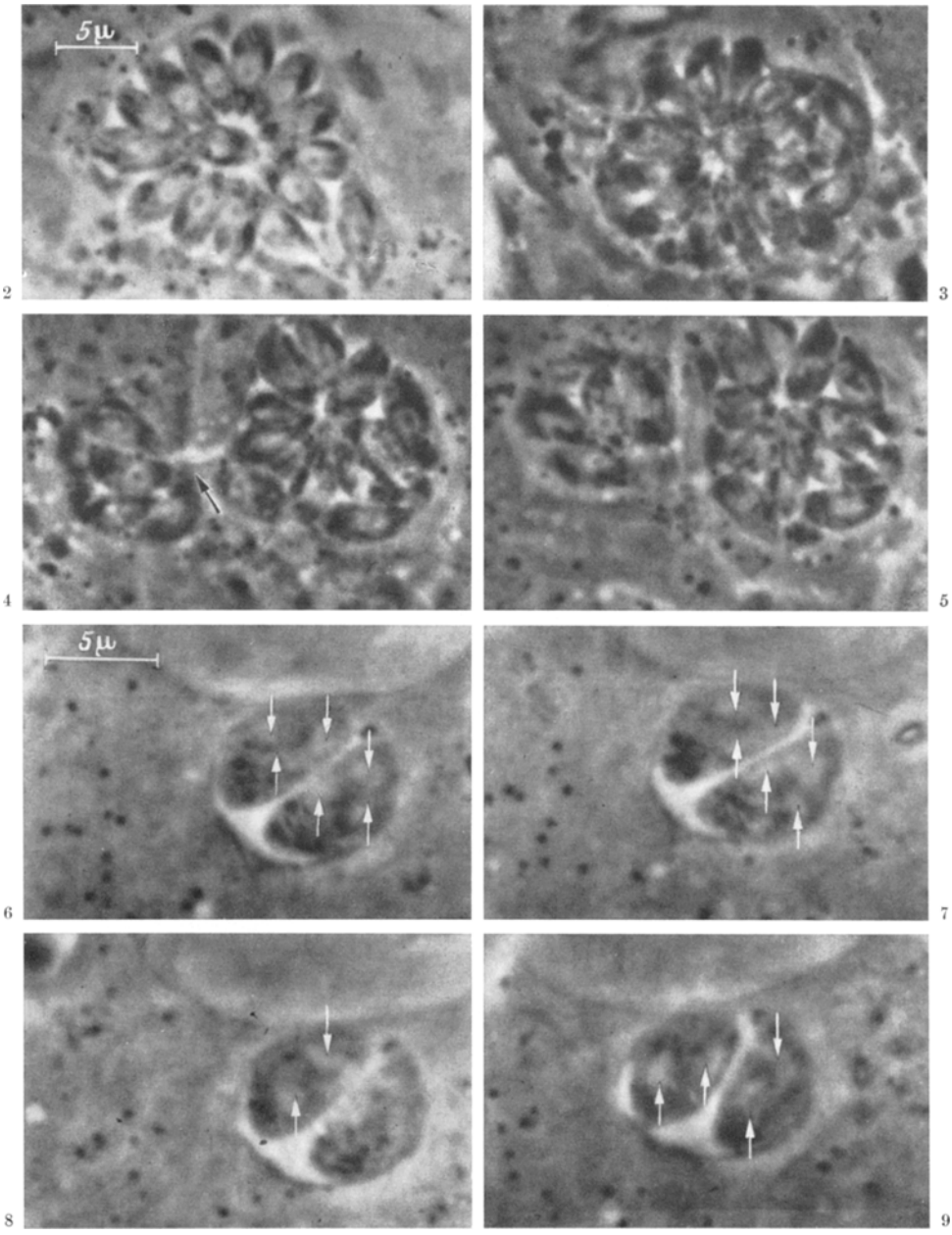


Fig. 1. A clone of four Toxoplasma parasites surrounded by a distinct membrane in a mesenchymal cell. The crescent-shaped parasites have a distinct nucleus and nucleolus, many phase dense organelles in their cytoplasm. Living culture, phase contrast

The membrane surrounding the parasites is osmiophilic and becomes more distinct as the clone grows. No regular contact has been found between the parasites and the vacuole membrane. The clones in infected fibroblasts and endothelial cells, which are undergoing mitotic division, are usually found only in one of the cells resulting from the division, but both cells may contain clones. Sometimes, a parasite clone may divide into two parts of different size during the host cell division (Figs. 2—5).

Toxoplasma parasites are liberated when the host cell bursts; they then rapidly penetrate into other cells in the neighbourhood. The parasites are of uniform size and show active, irregular jumping movements, which are most prominent within the first few minutes.

Parasite multiplication and cytopathogenic effects in cells of neuroectodermal origin. *1. Ganglion cells.* In retinal cell cultures it is not possible to distinguish nerve cells of the ganglion cell layer from the horizontal and the amacrine cells in the inner nuclear layer of the retina. Thus, in this study, they are all named ganglion cells. The principal characteristics of these are that they possess



Figs. 2—9

Figs. 2—5. Selected frames from a phase contrast cine record. The intervals are 36, 12 and 5 minutes respectively. Division of a clone with multiplying parasites in two separate parts of different size during mitosis of the mesenchymal host cell. The constriction of the clone membrane is marked by an arrow

Figs. 6—9. Selected frames from a phase contrast cine record. The intervals are 7, 8 and 9 minutes respectively. The binary multiplication by internal budding in the parasites in a mesenchymal cell. The nuclei of the mother and the daughter parasites are indicated by arrows

basophilic, phase dense particles — Nissl substance — in their somata and dendrites, neurofibrils, and a large, spherical, translucent nucleus with one to two prominent nucleoli. The ganglion cells show extremely slow migration compared to other cells in the cultures.

Toxoplasma parasites are observed in the cytoplasm of the ganglion cells within an hour after inoculation of the cultures. The parasites may enter the perikaryon of the ganglion cells in two different ways, either by invading through the cell membrane, or from the tips of the dendrites and then propagate towards and into the perikaryon. The latter way of invading the ganglion cells has been observed only a few times. Degenerative changes in the ganglion cell occur after the parasite invasion. About one hour before the infected cell bursts, the phase density of the cytoplasm starts to decrease and the dendrites and the axon slowly retract. Sometimes there is an increase in the amplitude and frequency of the rhytmic contractions of the infected nerve cell compared with uninfected cells. The ganglion cells usually retract all their processes before bursting, and are often in their terminal stages closely surrounded by the processes of phagocytes. The nuclear membrane becomes hyperchromatic, the nucleoplasm becomes less homogenous and the nucleus rotates more rapidly than previously. Finally the cytoplasm, and sometimes also the nucleus, swell, and the host cell membrane then bursts, and the recently divided Toxoplasma parasites are liberated. The nuclear membranes of ruptured cells do not disintegrate. After at least ten hours the multiplying parasites cause rupture of their host cell. Usually, rupture of the ganglion cells follows when their cytoplasm is completely occupied by parasites. However, it may occur after eight parasites have been formed, when only a small part of the cytoplasm is occupied by parasites.

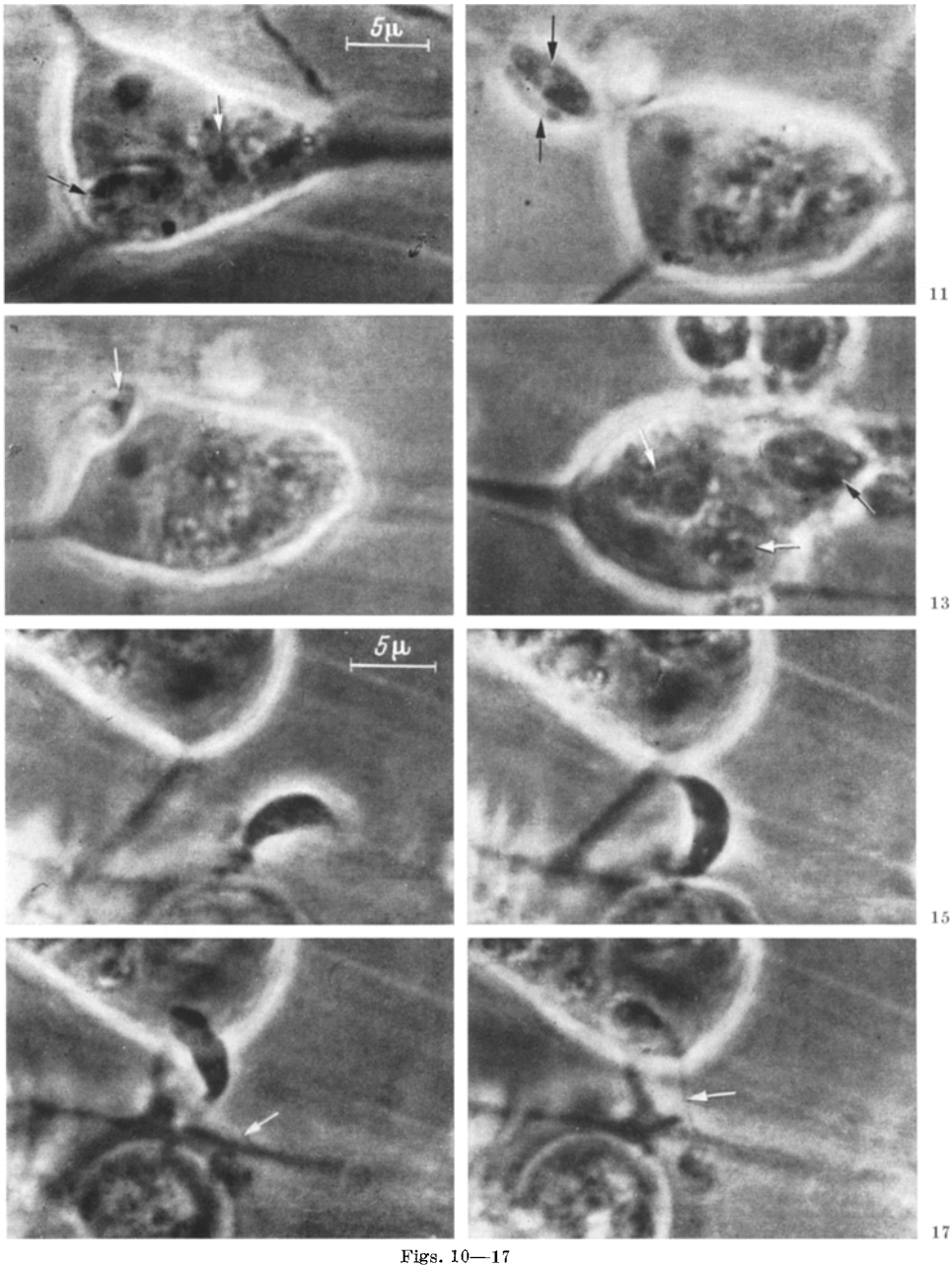
2. Bipolar nerve cells. The bipolar nerve cells are small and oval with polar, branching processes. They have a round, eccentric nucleus with small chromatine granules and a distinct nucleolus.

The mode of infection follows the same pattern as that described for ganglion cells. However, in contrast to the ganglion cells, the bipolar nerve cells usually contain only one to four parasites when they burst, although occasionally eight have been observed. This smaller number of parasites fills only a part of the host cell.

3. Rods. The cells from the outer nuclear layer of the rat retina consist almost exclusively of rods (TANSLEY). They have a thin rim of cytoplasm surrounding a round nucleus with a single nucleolus and several chromatin granules. During the first ten days in vitro the rods form rosettes with a central lumen bordered by rod-like processes. The rosettes later disintegrate and isolated rods are easily observed.

The rods show the same reaction to infection as the other nerve cells. However, only one or two Toxoplasma parasites have been observed in bursting rods.

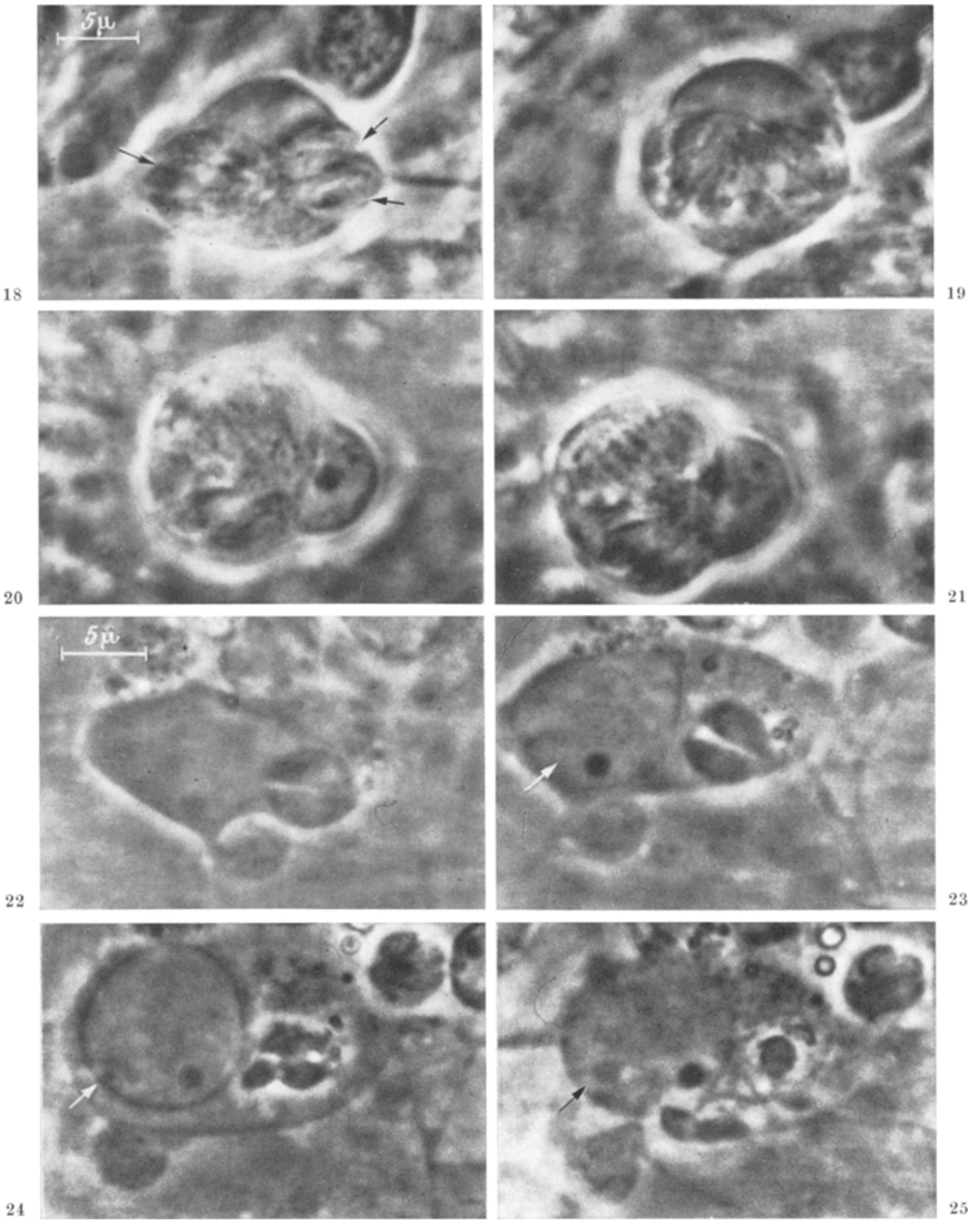
4. Neuroglial cells. Fibrous astrocytes of somewhat different shape and size than those in other parts of the central nervous system are found in quite large number in all retinal explants. The cell body has a rapidly changing shape, and many processes with thin undulating membranes at the points of junction.



Figs. 10—17

Figs. 10—13. Selected frames from a phase contrast cine record. The intervals are 100, 20 and 210 minutes respectively. A ganglion cell with a large nucleus and a prominent nucleolus contains clones of Toxoplasma gondii, which are marked by arrows. The two overlapping parasites enter the perikaryon in the dendrite at the upper left in Fig. 11. The same two parasites are in Fig. 12 located in the perikaryon and deforming the nucleus. In Fig. 13 the nucleus is dislocated and appears shrunken. Notice the signs of cytoplasmic degeneration

Figs. 14—17. The same ganglion cell as in Figs. 10—13. The time intervals are 15, 90 and 30 seconds respectively. The parasite in the center of the field is invading the nerve cell by direct penetration of the cell membrane. Notice the filament between the parasite and the dendrite (arrow)



Figs. 18—25

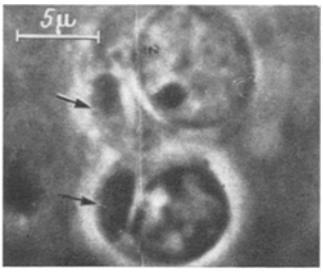
Figs. 18—21. Selected frames from a phase contrast cine record. The intervals are 45, 95, and 160 minutes respectively. A ganglion cell with a distinct nucleus and three clones (arrows) of Toxoplasma gondii. The multiplying parasites dislocate the nucleus, which shows increasing signs of degeneration

Figs. 22—25. Selected frames from a phase contrast cine record. The intervals are 47, 22 and 6 minutes respectively. An astrocyte with two parasites dividing into four. Both the nucleus (arrows) and the cytoplasm show extensive swelling. The last picture shows rupture of the cell membrane and the liberated four parasites

The eccentric oval nucleus is well marked and contains a few nucleoli and a fine-grained chromatine structure.

Toxoplasma parasites multiply in the astrocytes and cause them to rupture. In cine records a swelling both of the cytoplasm and the nucleus is often observed before cell rupture. Nuclei from burst neuroglial cells, however, seem to have intact nuclear membranes. A few parasites, usually two to eight, cause rupture of quite large astrocytes although they only fill a fraction of the cytoplasmic volume.

5. Cells from the retinal pigment epithelium. The cells from the retinal pigment layer are observed as a part of the monolayer. They are fairly large, polar cells



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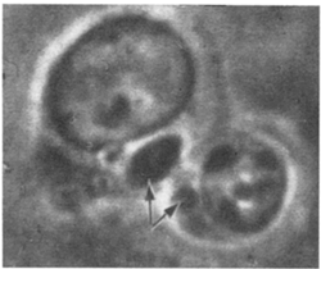

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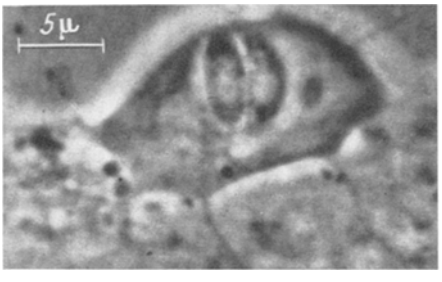

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Figs. 26 and 27. A bipolar nerve cell and a rod below to the right, each with a single parasite. The bipolar nerve cell shows terminal degeneration, and the parasites change their position

Fig. 28. A neuroglial cell with two Toxoplasma parasites in an early stage of division deforming the astrocyt nucleus. Phase contrast, living culture

with dark brown, rod-shaped, almost uniform melanin granules regularly distributed, in the central parts of the cytoplasm, except in a narrow perinuclear zone. Their nuclei are oval and have several nucleoli.

The parasites invade these cells and form large clones in the cytoplasm before the host cell bursts. As in cells of mesenchymal origin terminal swelling has so far not been observed.

Multiplication of parasites and cytopathogenic effects in cells of mesenchymal origin. Fibroblasts and endothelial cells from the retinal blood vessels grow out in cultures to form the monolayer, on the surface of which cells of neuroectodermal origin are seen. Mesenchymal cells have a variable form, changing from spindle to large, thin, polygonal shapes with one or more nuclei and several nucleoli. Mitosis are often seen and the cells have a lot of perinuclear granules and thin, broad expansions.

Toxoplasma parasites are found in these cells within 15 minutes after inoculation. As the parasites divide, they occupy an increasingly large part of the cytoplasm. The clones often dislocate and deform the nuclei. The infected mesenchymal cells frequently undergo mitotic division. After about 24 hours,

rupture of the cells can be observed for the first time and all the cells are destroyed within a few days.

Numerous phagocytes are found in the explants a few hours after inoculation and at an early stage of infection, a few of them also are invaded by Toxoplasma parasites. Parasite multiplication occurs in their cytoplasm and they burst. Sometimes it is difficult to recognize the parasites among the material engulfed in the phagocytes.

Discussion

In the retinal cultures all the different types of cells of neuroectodermal origin, nerve cells, rods, neuroglial cells, and retinal pigment epithelium cells, are invaded by Toxoplasma parasites, which multiply and finally cause rupture of the infected cells. No extracellular parasite multiplication or clone formation has been observed. In contrast to what has previously been described for cells of mesenchymal and neoplastic origin (SOURANDER, LYCKE and LUND), signs of progressive degeneration prior to bursting are observed in cells of neuroectodermal origin. Terminal swelling of the cytoplasm and sometimes also of the nucleus is observed in small ganglion cells, bipolar nerve cells, rods and astrocytes. The parasites are suddenly discharged from the ruptured cells into the surrounding medium and in a few seconds actively penetrate adjacent intact cells. The parasites show the same pattern of active movements as previously reported (MANWELL and DROBECK, WESTPHAL). The ganglion cells like the mesenchymal cells may be completely filled by multiplying parasites, but unlike the mesenchymal cells the ganglion cells show increasing signs of degeneration before bursting. The small ganglion cells, bipolar nerve cells, rods and astrocytes always contain only a few parasites, occupying a small part of the cytoplasm. However, severe degenerative changes in the nucleus and in the cytoplasm are also seen in these cells. In phase contrast cine records there seems to be an increased density of mitochondria around the clones compared with other parts of the infected cells and of uninfected cells. The same observation has been made by electron microscopy (LUDVÍK).

The greater vulnerability of nerve cells and neuroglial cells as compared with retinal pigment epithelium cells and cells of mesenchymal origin may reflect differences in the metabolic interaction between the Toxoplasma parasites and the different types of host cells. The observations on infected cells from cultures made for comparison from cerebellum, heart muscle, lung, liver and spleen show the same type of reactions to infection as described for retinal cultures.

This study has demonstrated that Toxoplasma gondii not only multiplies in cells of mesenchymal origin but also in those derived from the neuroectoderm and destroyes both. The multiplication rate and the invading capacity of the parasites appear to be the same for all different types of cells in the cultures. A few parasites may cause rapid destruction of nerve cells, rods and neuroglial cells. This is in contrast to the large number of parasites seen in retinal pigment epithelium cells and in cells of mesenchymal origin. The remarkably short survival time of infected neuroectodermal cells in cultures may explain the fact that the occurrence of Toxoplasma parasites in nerve and neuroglial cells of fixed and stained tissue has rarely been reported in the literature.

As previously described for other types of cultured cells (LUND, LYCKE and SOURANDER) the large clones of proliferating parasites with their surrounding membrane show a strong resemblance to the "vegetative pseudocysts" in tissue sections from Toxoplasma infected animals and humans. The large "vegetative pseudocysts" in the retina probably arise in endothelial cells or in fibroblasts of the walls of the blood vessels. When comparing the maximal size of the nerve cells, rods and neuroglial cells infected with Toxoplasma gondii with that of cells of mesenchymal origin only the clones in the latter reach a size equal to that of the "vegetative pseudocysts" in chronically infected animals (FRENKEL, NAKAYAMA and MATSUBAYASHI). This hypothesis of the origin of the "vegetative pseudocysts" is supported also by the observations of early and progressive degeneration of infected cells derived from the neuroectoderm while cells of mesenchymal origin survive for a longer time and do not reveal any degenerative changes before bursting. Further support is gained from the earlier observations of FRENKEL and also NAKAYAMA and MATSUBAYASHI that the pseudocysts only occur in the inner part of the retina. This suggests, that the distribution of the pseudocysts corresponds to the retinal vascular pattern. In pigeons, which lack retinal blood vessels, infections with Toxoplasma gondii results in uveitis but not in retinitis (FRENKEL). Thus, the available evidence indicates, that the "vegetative pseudocysts" are formed and maintained in cells of mesenchymal origin in the walls of the retinal and cerebral blood vessels.

Toxoplasma parasites have been shown to be able to multiply in colchicine-treated cells and in irradiated cells, which have lost their mitotic capacity (COOK and JACOBS, LUND, LYCKE and SOURANDER). No mitotic activity occurs in the cultured nerve cells. The pattern of parasite multiplication is the same in these different kinds of non-mitotic cells. Thus, the conclusion that the multiplication of Toxoplasma gondii is not dependent on the mitotic capacity of the host cell, also applies to the nerve cells.

The nerve cells have been observed to be invaded by the parasites directly through the cell membrane in the same way as described for other cells (SOURANDER et al., LUDVÍK). In addition the parasites may sometimes invade the ganglion cells through the tips of the dendrites, and then move in the cytoplasm of the processes towards and into the perikaryon (Figs. 11 and 12). This mode of invasion and transport of infective agents has previously been reported only in neurotropic viral infections (for review see SABIN). It seems possible that intraneuronal parasites may spread also in this way in the living animal.

The multiplication of Toxoplasma gondii is binary and follows a regular pattern (NICOLLE and MANCEAUX, CROSS, SCHMIDT-HOENSDORF and HOLZ, LUND et al.). The mode of division has been described as mitotic by HOLZ, who based his opinion on observations of six chromosomes in parasites obtained from colchicine-treated mice. In living retinal cultures the multiplication of the Toxoplasma parasite is preceded by the formation of two daughter nuclei and small organelles inside the mother cell; the latter is finally completely filled and destroyed as the two daughter parasites are formed. This appearence is similar to the mode of reproduction by internal budding in the parent, producing two daughter parasites, as described in fixed, silver stained smears by GOLDMAN, CARVER and SULZER. In recent electron microscopic studies (LUDVÍK, GAVIN,

WANKE and JACOBS) are described various stages of internal division of Toxoplasma gondii. According to these studies the formation of the two daughter parasites takes place inside the mother parasite. At the beginning of the division the nucleus assumes an U-form, each of its arms being directed towards the parasite's pointed end. The arms give rise to the two new nuclei, and the middle part of the mother nucleus disintegrates. In the final stage two well-developed daughter individuals fill the shell of the mother parasite. Our observations on living parasites are in agreement with these latter three reports on internal division of Toxoplasma gondii, and do not support the opinion of the mitotic nature of multiplication.

The binary division of the parasite has been described as occuring by fission in earlier reports (NICOLLE and MANCEAUX, FRENKEL, GANGI and MANWELL, GAVIN et al., LUND et al.). However, the results of the present study as those of GOLDMAN, CARVER and SULZER and of LUDVÍK clearly indicate that the binary division of Toxoplasma gondii results in the formation of two new parasites inside the mother individual. This mode of reproduction disagrees with the definition of fission (SMITH and CONANT).

In the cytoplasm of the infected cells, the parasites form clones surrounded by distinct membranes (HOGAN, YONEDA, FEENEY, ZWEIGART and LEWIS, GARNHAM, BAKER and BIRD, LAINSON), although such membranes have not been seen in another electron microscopic study (MEYER and MENDONCA). This discrepancy may be due to different treatments of the tissue. When mitosis occurs in infected cells of mesenchymal origin, a clone may either find its way into one of the two cells resulting from the division, or divide into two parts, one in each cell. In the latter case the clone membrane becomes constricted and two clones with different numbers of parasites are formed, each surrounded by its own membrane. LUDVÍK observed, that the host cell reacts to the invading parasites by forming a membrane around them. It thus seems likely, that the clone membrane is a real structure keeping the Toxoplasma parasites together, and preventing them from being diffusely spread in the cytoplasm of the host cell.

The occurrence of locomotor organelles has been discussed by WESTPHAL. He was able to demonstrate a filament originating from the Toxoplasma parasite. In the present study a filament possibly identical with the structure mentioned by WESTPHAL has been demonstrated (Figs. 14—17).

Summary

Retinal cultures from young rats were inoculated with the RH strain of Toxoplasma gondii, and observed by phase contrast microscopy and time lapse cinematography. Parasite multiplication followed by destruction of the host cell was demonstrated in ganglion cells, bipolar nerve cells, rods, neuroglial cells, retinal pigment epithelium and cells of mesenchymal origin. The retinal pigment epithelium cells and the cells of mesenchymal origin were filled with a large number of Toxoplasma parasites and showed no degenerative changes, while the nerve cells, rods and neuroglial cells contained only a few parasites and showed terminal degenerative changes both in the cytoplasm and the nucleus. The parasites invaded the ganglion cell soma also through the dendrites. The

binary division of the parasite resulted in the formation of two daughter parasites inside the ultimately destroyed mother individual. During mitosis of the host cell a clone might be divided into two parts, each with its own membrane, or may reappear intact in one of the daughter cells. Evidence is presented indicating that the membrane surrounding the Toxoplasma clone is formed by the host cell.

Zusammenfassung

Netzhautkulturen von jungen Ratten wurden mit Toxoplasma gondii (RH-Stamm) infiziert und unter dem Phasenkontrastmikroskop sowie mittels Zeitraffungskinematographie beobachtet. In Ganglienzellen, bipolaren Nervenzellen, Stäbchen, Neurogliazellen, Pigmentzellen der Retina und Zellen mesenchymalen Ursprungs war eine Vermehrung der Parasiten mit nachfolgender Zerstörung der Wirtszellen zu sehen. Die Pigmentzellen der Retina und die Zellen mesenchymalen Ursprungs waren angefüllt mit einer großen Zahl von Parasiten und zeigten keine degenerativen Zeichen vor dem Platzen, während die anderen Zellen nur wenige Parasiten enthielten und im Cytoplasma und am Zellkern terminale degenerative Veränderungen aufwiesen. Die Parasiten dringen in die Ganglienzellen auch durch die Dendriten ein. Die binäre Teilung des Parasiten führt zur Bildung von zwei Tochterzellen innerhalb des schließlich zerfallenden Mutterindividuums. Während der Mitose der Wirtszelle kann sich ein Clon der Parasiten in zwei Teile teilen, jede mit seiner eigenen Membran oder unverändert in eine der Tochterzellen übernommen werden. Es werden Belege dafür angeführt, daß die Membran, welche einen Toxoplasmaclon umgibt, von der Wirtszelle gebildet wird.

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