Multiplication of Toxoplasmas in Enucleate Fibroblasts[1] (37221)

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The metabolic or other deficiencies that render the protozoan, *Toxoplasma gondii* incapable of multiplying outside of host cells remain unknown. Toxoplasmas possess pathways for aerobic glycolysis and a cytochrome system (1), and they contain the various organelles seen in cells that function and multiply independently: Golgi complex, endoplasmic reticulum, ribosomes, mitochondria and a nucleus. For many years it has been thought that the host cell nucleus might contribute some factor required for the intracytoplasmic development of the parasite. However, mitosis of the host cell is shown not necessary for parasite division, since toxoplasmas multiply normally in irradiated cells (2) or in cells treated with colchicine (3). Vischer and Suter (4) showed that toxoplasmas multiply in peritoneal macrophages, cells that do not incorporate thymidine or divide *in vitro*.

An opportunity to examine directly the role of the host cell nucleus in toxoplasma infections was presented by a new method (5) for producing enucleated cells that survive for 2–3 days *in vitro*. Carter (6) first demonstrated that nuclei could be removed from fibroblasts by exposure to cytochalasin B, and he predicted the potential value of this technique in examining various nucleus–cytoplasm interactions. We have observed the multiplication of toxoplasmas in these enucleate fibroblasts.

Materials and Methods. The RH strain of *T. gondii* was maintained by serial passage in the peritoneal cavity of mice as described previously (7). Fibroblasts (L929, American Type Culture Collection) were maintained in suspension culture in minimum essential medium (MEM) and 5% heat-inactivated fetal calf serum (HIFCS). One milliliter of the fibroblast suspension (3×10^6 cells) was placed in 35 mm plastic dishes (Falcon Plastics) and allowed to adhere to round glass cover slips 1 in. in diameter. After 1 hr the cover slips were removed from the suspending media, inverted, and placed in a 50 ml centrifuge tube (Nalge Co.) on a specially made plastic cylinder (¼ in. in thickness with an outside diameter of 1 in and internal diameter of ¾ in.), which rested on a solid Teflon platform at the bottom of the tube. Prior to addition of the cover slips, the centrifuge tube was filled with approximately 20 ml of fresh MEM–20% HIFCS which contained 10 μg/ml of cytochalasin B dissolved in DMSO (0.002 ml/ml final concentration). The specimens were centrifuged at 12,800*g* RCF at 37° for 40 min, according to the technique for enucleation of fibroblasts recently described by Prescott, Myerson and Wallace (5), and modified by Silverstein (unpublished data). The cover slips were carefully removed from the centrifuge tubes and placed in 35 mm plastic dishes, cell side up. The cells were washed twice with MEM, overlayed with fresh MEM–20% HIFCS and maintained at 37° in 5% CO_2 for either 1 or 15 hr. The medium was then removed and a suspension of toxoplasmas was added for 30 min. In the course of the enucleation procedure more than 90% of the fibroblasts were lost from the cover slips, but over 90% of the remaining cells were enucleate.

Toxoplasmas from the peritoneal cavity of infected mice were placed in MEM and centrifuged at 30*g* for 5 min to remove cells and debris. The supernate was centrifuged at

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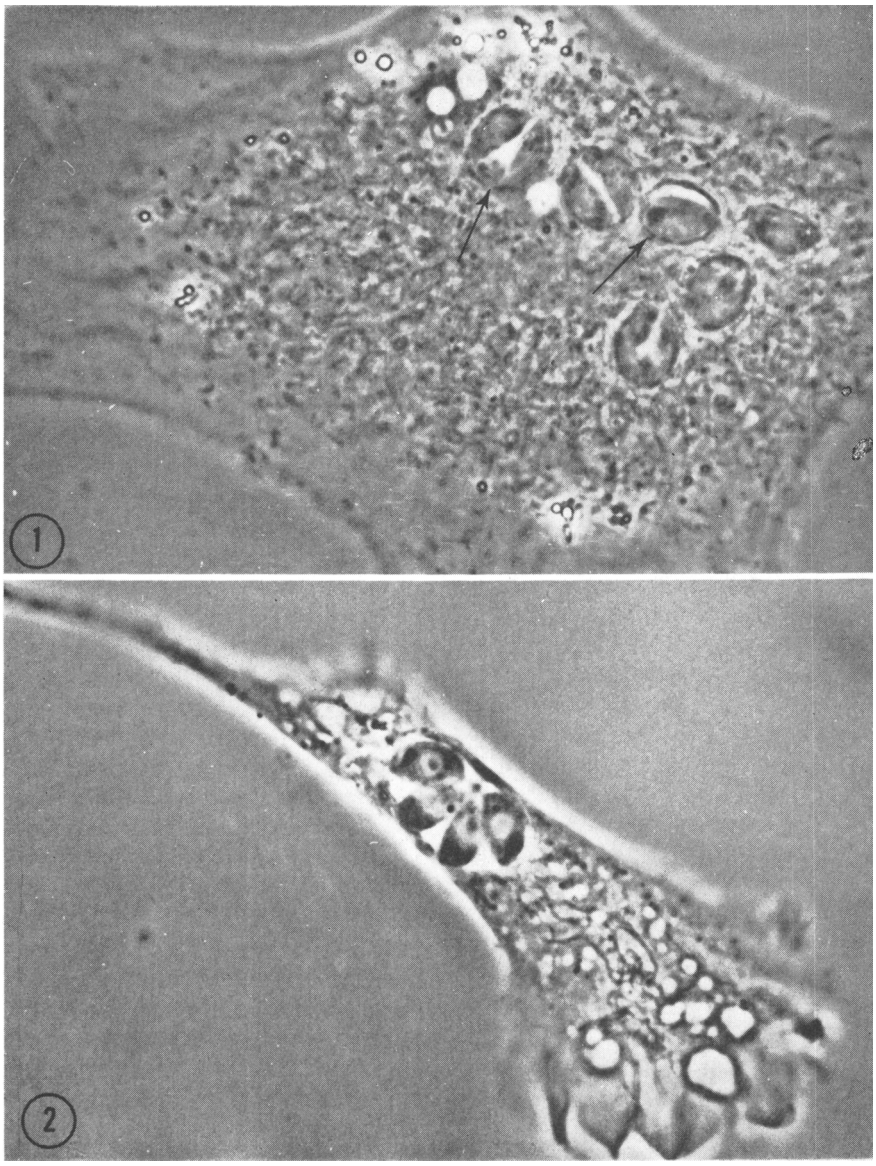

FIG. 1. Phase-microscopic appearance of a mouse fibroblast 9 hr after enucleation with cytochalasin B and 8 hr after infection with toxoplasmas. The cell is well spread. Phase dense mitochondria and granules are scattered throughout the cytoplasm. Phase lucent vacuoles and refractile lipid bodies are also seen. No nucleus is present. Several vacuoles contain one or two toxoplasmas (arrows). Phase contrast; glutaraldehyde fixation; ×1600.

FIG. 2. Phase-microscopic appearance of a fibroblast 17 hr after enucleation and 16 hr after toxoplasma infection. The cell morphology suggests membrane ruffling and active pinocytosis at the time of fixation. Thread-like mitochondria and phase lucent vacuoles are seen. In the center of the cell there is a large vacuole containing four toxoplasmas. The parasites are attached at one end forming a rosette, a characteristic of dividing toxoplasmas. Phase contrast; glutaraldehyde fixation; ×1600.

350*g* for 10 min to sediment the toxoplasmas. The parasites were resuspended in MEM–20% HIFCS (1–20 × 10^6/ml in different experiments) and overlayed cover slips containing fibroblasts which had been exposed to cytochalasin B 1 or 15 hr previously. After 30 min the parasite suspension was removed, the plates were washed twice with MEM, and fresh MEM–20% HIFCS was added. The plates were maintained in 5% CO_2 and 95% air at 37°. Cover slips were removed at varying times from 3 to 24 hr after infection, fixed in 2.5% glutaraldehyde in Na cacodylate (pH 7.4) for 10 minutes at 4°, inverted on 1 drop of water, rimmed with paraffin–Vaseline and examined by phase-contrast microscopy. The number of toxoplasmas per vacuole was recorded and the generation time was calculated using previously described methods (8). Control observations were made on fibroblasts treated in a similar manner, but not exposed to cytochalasin B, and on fibroblasts which retained their nucleus following the cytochalasin-centrifugation procedure.

Results. Figures 1 and 2 show enucleate fibroblasts infected 1 hr after cytochalasin B exposure and fixed in glutaraldehyde 8 and 16 hr after toxoplasma infection. Several vacuoles in the 8 hr specimen show two parasites, indicating they have divided once. The micrograph of a specimen fixed 16 hr after infection shows a vacuole containing four parasites, indicative of two parasite divisions. By counting the number of toxoplasmas in 50 or 100 vacuoles the mean number of toxoplasmas per vacuole was determined. Table I shows the results of several experiments comparing the generation times of toxoplasmas in normal fibroblasts, in fibroblasts that had retained their nuclei after the cytochalasin-centrifugation procedure, in enucleate fibroblasts infected 1 hr after cytochalasin, and in enucleate fibroblasts infected 15 hr after cytochalasin. The results show no significant difference in the generation time of the toxoplasmas in these different fibroblasts.

Discussion. The effects of enucleation on mammalian cell function other than cell division have been but little studied. Prescott, Myerson and Wallace (5) demonstrated that enucleate fibroblasts incorporate ^{3}H-leucine at a normal rate for up to 90 min. Under the conditions employed by us for cytochalasin exposure and for cell maintenance, many enucleate fibroblasts remained intact and appeared morphologically "healthy" for up to 40 hr. These enucleate cells, however, have not been observed to survive longer than 3 days *in vitro* (5).

If toxoplasma multiplication were to depend on some factor directly or indirectly connected with nuclear function, then one might have expected to observe a prolonged parasite generation time in the enucleate cells, particularly when the infection was initiated fifteen hours after enucleation (generation time of the fibroblasts employed is about 15 hr). No alteration in parasite survival or multiplication pattern was detected, however. We therefore conclude that the intracellular factor or factors necessary for toxoplasma multiplication are produced or stored in the cytoplasm without continued participation of the cell nucleus. The nature of these factors remain unknown.

Summary. Enucleation of fibroblasts was accomplished by centrifugation of glass adherent cells in media containing cytochalasin B. *Toxoplasma gondii* entered the enu-

TABLE I. Generation Times of Toxoplasmas in Fibroblasts Under Varying Conditions.

Cell conditions	No. of toxoplasmas/vacuole; after infection (hr): 3	8	16	21	24	Calc generation time (hr)
Normal fibroblasts	1.00 ± 0.0	1.26 ± 0.4		7.72 ± 2.3		5.0[a]
Cyto-B nucleated cells		1.26	3.04			5.5
Enucleated cells, 1 hr		1.34	3.25			5.5
15 hr		1.65			10.2	5.7

[a] Calculated by the slope on semilogarithmic graph.

cleate cells and multiplied in them in a manner identical to that seen in normal fibroblasts. The results establish that the cellular factor required for multiplication of toxoplasmas is not derived from the nucleus.

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