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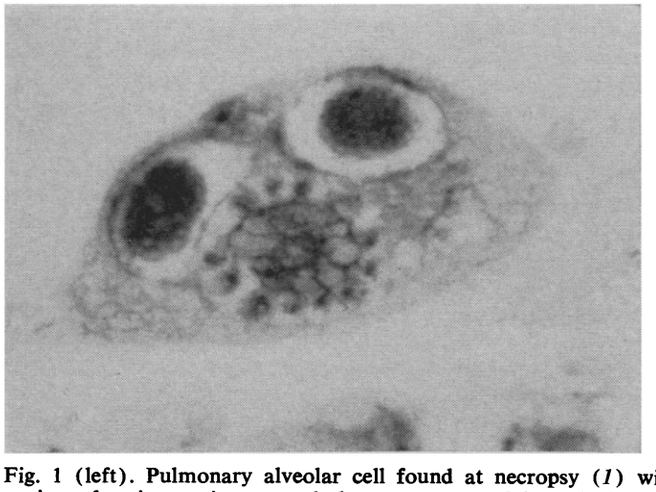
Toxoplasma gondii and Cytomegalovirus: Mixed Infection by a Parasite and a Virus

Abstract. *Human fibroblasts infected in vitro with cytomegalovirus are relatively resistant to infection by* Toxoplasma gondii *during the first 4 days of virus infection. After 5 days, however, the cytomegalovirus-infected cells become susceptible to the parasites. The toxoplasmas replicate in paracentral rosettes surrounded by host cell mitochondria. This growth configuration differs from that seen in human fibroblasts infected in vitro with toxoplasma only but resembles the pattern seen in doubly infected cells found in human necropsy tissue.*

Simultaneous infections by the obligate intracellular parasite *Toxoplasma gondii* and cytomegalovirus (CMV) occur with an unexpected frequency in patients with disseminated neoplastic disease (*1*). In necropsy tissues from several such cases, we have observed toxoplasma organisms in the same cells with CMV nuclear inclusions (*1*). The growth pattern of the parasite in some of these doubly infected cells is unusual in that the toxoplasma rosettes form a ring around the cytoplasmic body (*2*) or cytocentrum of the host cell (Fig. 1). The double infection has now been reproduced for investigation in vitro. Experiments demonstrate that virus-infected host cells, after an early period of host cell resistance to parasite invasion and suppression of toxoplasma replication, eventually become receptive to double infection. The receptive period occurs after 72 hours when CMV accumulates in the host cell cytoplasm (*2*, *3*). The conspicuous circular configuration of toxoplasma rosettes in doubly infected cells is apparently related to a rearrangement of the host cells organelles during late virus infection (*2*, *3*).

The time course of toxoplasma infection in tissue culture is relatively rapid and the host cells in culture are destroyed within 3 days by any inoculum of toxoplasma providing a multiplicity of at least one organism per cell. In comparison, a 24- to 48-hour eclipse period precedes detectable CMV replication. The in vitro studies therefore were limited to the effects of initial CMV infection on the growth characteristics of toxoplasma.

In all experiments, fibroblast cell cultures (WI-38) were infected with cytomegalovirus (Davis strain) at least 2 days before toxoplasma organisms (RH strain) were introduced. In most of the experiments the fibroblasts were grown on cover slips in Leighton tubes and infected with 7800 PFU (plaque-forming units) of cytomegalovirus per tube. At specified times, the cover slips were fixed in a mixture of acetate-buffered mercuric chloride and formalin (*4*) and stained with hematoxylin and eosin (*4*). These preparations permitted observa-



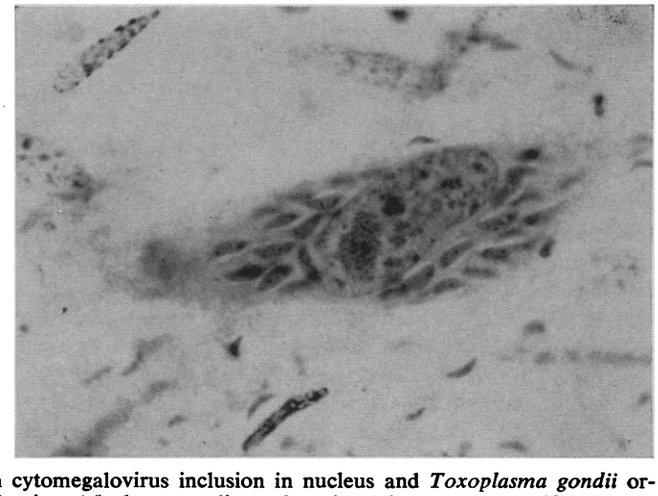


Fig. 1 (left). Pulmonary alveolar cell found at necropsy (*1*) with cytomegalovirus inclusion in nucleus and *Toxoplasma gondii* organisms forming a ring around the cytocentrum [glutaraldehyde fixation (*4*); hematoxylin and eosin; microscope magnification × 1000]. Fig. 2 (right). Nonreplicating toxoplasma in a cell infected with cytomegalovirus. The toxoplasma was inoculated 2 days after virus infection and allowed to grow for 48 hours. B5 fixation; hematoxylin and eosin stain; microscope magnification, × 1000.

tion of the whole cell. Parallel groups of cells were grown in prescription bottles, concentrated into pellets by low-speed centrifugation, and fixed with 3 percent glutaraldehyde in phosphate buffer (*p*H 7.3). After the pellets were washed in buffer and after fixation with 1 percent osmium tetroxide, they were dehydrated and embedded in epoxy resin (Epoxy 812). Ultrathin sections were stained with 10 percent uranyl acetate before examination in an RCA EMU-3F microscope. Cells infected with CMV were identified by characteristic nuclear inclusions and by electron microscopy (*2*, *3*). The toxoplasma could be identified either as replicating forms with double or rosette formations in host cell vacuoles or as nonreplicating forms with single organisms with a vacuole (Figs. 2 and 3).

When toxoplasma organisms in multiplicity of 15 to 20 organisms per fibroblast (5×10^6 organisms per milliliter) were introduced on days 2, 3, and 4 after viral infection and allowed to grow for 18 hours, no more than 6 percent of the cells with CMV nuclear inclusions contained toxoplasma organisms. Of these doubly infected cells, only one to three vacuoles per cell contained replicating parasites. Increasing the number of organisms inoculated by a factor of 10 did not increase the percentage of doubly infected cells. Longer exposure to toxoplasma (48 hours) increased the number of parasites in cells infected with virus for 4 days to a proportion seen in the non-virus-infected controls, but the toxoplasma did not show evidence of replication (Fig. 2). In contrast, fibroblasts that had been infected by cytomegalovirus for more than 6 days appeared equally as susceptible to both the toxoplasma invasion and replication (18-hour incubation) as did the non-virus-infected controls. The circular arrangement of toxoplasma rosettes in the older virus-infected cells is quite different from the uniform distribution of the rosettes in non-virus-infected cells (Fig. 3), but similar to that seen in the human necropsy tissues (Fig. 1).

In electron micrographs of late doubly infected cells, the toxoplasma rosettes are located around the central cytoplasmic body. This central body, previously described in CMV infection (*2*, *3*) is well formed after 5 days in the WI-38 cells. It contains mature virus particles and smooth membranes and is surrounded by a high concentration of mitochondria. In doubly infected cells, the mitochondria closely invest the parasitic vacuoles that contain rosettes and ring the central body region (see Fig. 4).

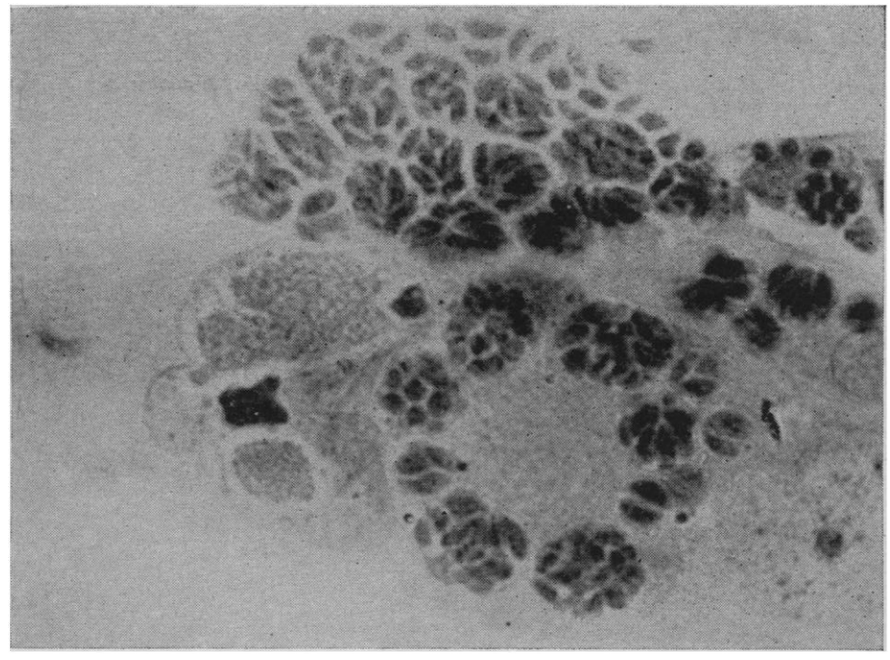


Fig. 3. Comparison of toxoplasma replicating in a non-virus-infected cell (top) and a virus-infected one (below). The cell was infected by virus for 7 days and toxoplasma for 18 hours. B5 fixative; hematoxylin and eosin stain; microscope magnification, × 1000.

Since *Toxoplasma gondii* have grown readily in WI-38 fibroblasts and in every other diploid cell line studied, the observed interval of suppression of growth in virus-infected fibroblasts is of interest. A possible explanation of the suppression of parasite growth would be production of an inhibitor by the host

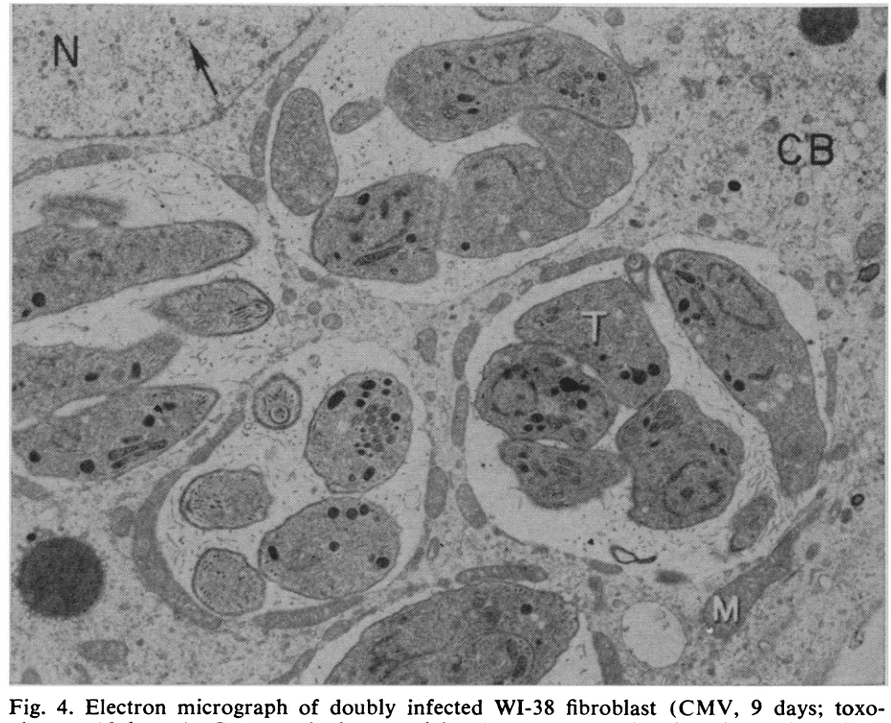


Fig. 4. Electron micrograph of doubly infected WI-38 fibroblast (CMV, 9 days; toxoplasma, 18 hours). Cytomegalovirus particles (arrows) are abundant in nucleus (*N*). Toxoplasma organisms (*T*) form rosettes within large cytoplasmic vacuoles at edge of central body (*CB*). Note prominent ring of mitochondria (*M*) around each parasite-filled vacuole. Uranyl acetate and lead citrate stain; microscope magnification, × 8400.

cells as a reaction to cytomegalovirus. The presence of an interference factor during the first 8 to 72 hours of in vitro CMV infections has been postulated by Glasgow (*5*) on the basis of experiments that demonstrated a lack of host cell susceptibility to invasion by a second virus. Another hypothesis is that CMV infection depresses DNA-dependent RNA synthesis, in the manner of closely related herpes viruses (*6*), and thereby deprives the toxoplasma of a vital protein supply. The latter possibility is not supported by preliminary experiments. Fibroblasts that were incubated with mitomycin C (50 μg/ml), actinomycin D (0.5 μg/ml), or puromycin (20 μg/ml) for ½ hour and washed prior to inoculation of parasites or infected in the presence of these antimetabolites sustained growth of the *Toxoplasma gondii* in a manner similar to control cultures.

The curious replication pattern of toxoplasma parasites in cells doubly infected with CMV may offer some clue as to their obligate nutritional requirements. In late CMV infection, there appears to be an overall increase in cytoplasmic volume, and fibroblasts assume many ultrastructural characteristics of macrophages. The Golgi zone hypertrophies, dense lysosomal bodies are numerous, and there is a measurable increase in the total content of acid phosphatase (*2*, *3*). The organelle structure of CMV-infected cells is also similar to macrophages in that mitochondria tend to accumulate around a cytocentrum (*7*). Toxoplasma parasites are confined to vacuoles within the cytoplasm of the host cell. Host cell mitochondria investing the parasite-filled vacuoles (Fig. 4) may be required by the parasites as a source of energy or they may serve to maintain a metabolic gradient within the parasite vacuole.

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7 February 1968

Dendritic Spikes and Their Inhibition in Alligator Purkinje Cells

Abstract. *Alligator Purkinje cells generate action potentials in the peripheral dendritic tree, after synaptic depolarization via superficial parallel fibers. These action potentials are inhibited at the dendrite level by preceding parallel-fiber volleys at close intervals. We conclude that this inhibition is produced by the activation of the inhibitory interneurons of the molecular layer, the stellate cells, which establish synaptic contacts with the dendrites of the Purkinje cells.*

Ramón y Cajal first hypothesized that neuronal dendrites receive and channel incoming information toward the axon, the neuron's output system (*1*).

In studying the anatomy of the Purkinje cell, one wonders how the distal region of the dendrites can act upon the soma and axon of this neuron. The two known mechanisms by which such distant dendrites can influence the activity of the cell are (i) by direct electrotonic spread from the distal dendrite to the soma, or (ii) by the initiation of action potentials or local responses which can be conducted either in an all-or-none manner or in a decremental fashion down to the axon.

The possibility that the large dendrites of Purkinje cells could generate action potentials has been suggested because they can be invaded antidromically after electrical stimulation of the underlying cerebellar white matter (*2*). We now present evidence that, in the alligator cerebellum, action potentials are initiated in peripheral dendrites after synaptic depolarization. Spike initiation, which can occur 50 to 150 μm from the dendritic terminals, is followed by a full-size action potential which is conducted towards the soma, possibly in a noncontinuous manner. Furthermore, this type of dendritic action potentials can be blocked at the dendritic level, most probably by inhibition mediated through the stellate interneurons of the molecular layer.

South American alligators (*Caiman sclerops*) ranging in size from 35 cm (200 g) to 67 cm (2 kg) were anesthetized with sodium thiopental (10 mg/km). The cerebellum was exposed by a craniotomy which left visible most of the medial lobe, the fissura secunda, and part of the posterior lobe (Fig. 1A). After removal of the dural membrane and the arachnoid tissue, the surface of the cerebellar cortex was accessible to electrical stimulation and recording. The molecular layer, the outermost layer of the cerebellar cortex, was activated by means of a bipolar metal electrode insulated to its tip (Fig. 1A). The field potentials generated in the cerebellum were recorded with micropipettes filled with 4*M* NaCl having an average d-c resistance of 2 to 3 megohms. The average response computation of the field potentials was carried out by means of a Fabri-Tek 1064 computer.

The alligator cerebellar cortex is uniquely suited for electrophysiological studies. It is in this animal, the only surviving member of the ruling reptiles (the Archeosaurus, from which stem the "dinosaurs" as well as the birds), where the first single-cell layer of Purkinje cells seems to appear in evolution (*3*).

The dendritic tree of alligator (Fig. 1B) grows out of the soma as a single stem, about 100 μm long, which then subdivides into two or three secondary branches which, in turn, bifurcate several times. Most of this tertiary bifurcation takes place in the superficial 250 μm of the molecular layer. In many cases, branches grow sideways or even downward towards the granular layer; but even in these instances, the length of the dendritic segment between the soma and the first bifurcation is about the same for the descending as for the ascending branches. The tertiary dendrites do not seem to decrease in diameter as much as those in other species, so that fairly thick dendrites can be found up to the surface of the cortex (Fig. 1B). The parallel fibers are