Ultrastructural Study of the TG180 Murine Sarcoma Cell Invasion by *Toxoplasma gondii*: Comparison between *in Vivo* and *in Vitro* Cell Cultures

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Infection of non-adherent TG180 murine sarcoma cells with Toxoplasma gondii *was compared, at the ultrastructural level, in both* in vivo *and* in vitro *conditions. Suspensions of 3.0 x 10^6 TG180 cells infected* in vitro *with 1.0 x 10^6 parasites of the RH strain were harvested between the first and 6th day post-infection and processed for transmission electron microscopy.* In vivo *infection was made by intraperitoneal inoculation in mice of 1.0 x 10^6 TG180 cells, that were co-inoculated with a parasite suspension at the same cell concentration. Cells were harvested 10, 20, 30 min and 24, 48 h post-inoculation and processed for transmission electron microscopy at the same conditions of the* in vitro *culture. It was observed TG180 murine sarcoma cells with intense and equivalent intracellular parasitism in both conditions. Host cells with parasitophorous vacuoles containing up to 16 parasites, as well as parasites undergoing mitoses or presenting a bradyzoite-like morphology, were frequently seen in both culture methods.*

Key words: *Toxoplasma gondii* - TG180 murine sarcoma cells - *in vitro* culture - transmission electron microscopy

Toxoplasma gondii is an intracellular parasite of a wide range of vertebrate hosts, including the human host. The infection caused by this parasite may be asymptomatic in healthy adults, but it can be an important illness to non-immune pregnant women by inducing embryopathy. In immunocompromised hosts, as in patients suffering of Aids or submitted to transplants, this parasite can originate encephalitis.

The most effective treatment currently used is a combination of pyrimethamine and sulphadiazine, which does not eliminate the encysted form of the parasite (Bunetel et al. 1995). Alternatively, it can be used spiramycin, a macrolide antibiotic, which shows no evidence of harmful effects on foetal development or toxicity (Desmonts & Couvreur 1974, Bunetel et al. 1995). There is a low number of toxoplasmicidic drugs on clinical use, but several new drugs have been proposed. New methods are necessary to test these drugs. According to Bunetel et al. (1995), the infection of TG180 murine sarcoma cells *in vitro* allows an easy, rapid, accurate and economical method to determine the effects of drugs against *T. gondii*.

It has been described in the literature the study of the interaction of *T. gondii* and host cells by *in vivo* or *in vitro* models (Chang et al. 1972, Shepp et al. 1985, Pavesio et al. 1992, Saffer et al. 1992, Speer et al. 1997).

The major aim of the present investigation was to study the infection of TG180 cells by *T. gondii* by comparing cultures submitted to *in vivo* and *in vitro* conditions. In addition, it was verified the adequacy of this method, emphasizing the characteristics of the *in vitro* infection, by using the transmission electron microscopy to evaluate the results.

MATERIALS AND METHODS

T. gondii - The RH strain used was obtained from animal facilities of our laboratory. The tachyzoites were cultured by intraperitoneal inoculation of female Balb/C mice. The parasites were harvested from mice by rinsing of the peritoneal cavity with 5 ml of Eagle modified minimum essential medium (MEM).

TG180 murine sarcoma cell cultures - The TG180 cell line was derived from the ATCC (CCRFS-180 II) sarcoma 180 in 1958 (Sartorelli

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& Booth 1961). A sub-population of this cell line adapted to *in vitro* culture (Couatarmanach et al. 1991) was used in our experiments. For the *in vitro* experiments the cells were grown in MEM supplemented with 10% fetal calf serum, 40 µg/ml gentamycin and 25 µg/ml amphotericin B. The cultures were incubated at 37ºC in an atmosphere of 95% air and 5% CO_2 in 5 ml-tissue culture flasks. The cells were observed daily by inverted light microscopy, growing in suspension as non-adherent polymorphic cells.

Then, $3.0x10^6$ TG180 cells were inoculated into flasks containing 5 ml of MEM culture medium and used after 8-14 passages. Cell countings were made with the use of "Trypan blue".

For *in vivo* experiments, TG180 sarcoma murine cells were grown as ascitic tumors in adult female Balb/C mice in our animal facilities. For the experiments, mice were intraperitoneally inoculated with $1.0x10^6$ TG180 cells.

In vitro infection - Six culture flasks were infected with $1.0x10^6$ tachyzoites each. This 3:1 TG180:*T. gondii* ratio was used as it has been already shown to yield large numbers of intracellular parasites (Couatarmanach et al. 1991). Cell suspensions were collected from the flasks daily from the first to the sixth day post-infection by centrifugation at 1,000g for 10 min. To avoid cell damage due to medium acidification, MEM culture medium was added on the second and third day post-infection. Cell suspensions were fixed overnight with 2.5% glutaraldehyde/2% paraformaldehyde (Karnovsky 1965) diluted in 0.1M phosphate buffer, pH 7.2, washed three times in 5 ml of phosphate buffered saline (PBS) pH 7.4 and processed for transmission electron microscopy as described below. On day 6, culture flasks of uninfected cells were also fixed and used as controls.

In vivo infection - Female Balb/C mice were intraperitoneally inoculated with a suspension of 1.0×10^6 TG180 cells. Subsequently, these animals were also inoculated with another cell suspension containing 1.0×10^6 *T. gondii* by the same route. Mice were sacrificed after 10, 20, 30 min and 24 and 48 h post-infection and their ascitic fluids were collected. This material was fixed with 2.5% glutaraldehyde/2% paraformaldehyde, washed three times in PBS and processed for transmission electron microscopy as described below.

Transmission electron microscopy - Briefly, the material was post-fixed for 1 h in 1% OsO_4, dehydrated in increasing concentrations of ethanol and embedded in Epon. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and examined in a Zeiss EM 109 transmission electron microscope.

RESULTS

Infection of TG180 cells in vivo - The TG180 murine sarcoma cells present a spherical morphology, with finger-like extensions at their periphery after 24 h of infection. In the cytoplasm, it was observed a large amount of free ribosomes and poorly developed rough endoplasmic reticulum. Mitochondria, lipid droplets and multivesicular bodies were also present. The centrally located nucleus is frequently spherical and with chromatin distributed in groups of electron-dense masses. One or two irregularly shaped nucleoli could be observed. After 24 h, *T. gondii* could be found both in contact with the TG180 cell surfaces and starting the penetration process through invaginations of the host cell plasma membrane (Figs 1a, d). The parasites were also found inside parasitophorous vacuoles after 24 h (Fig. 1b). Vacuoles, free ribosomes, Golgi complex, dense granules, rhoptries and mitochondria of several sizes were present in the intracellular parasites (Fig. 1c). It was also observed TG180 cells with vacuoles containing up to sixteen newly-divided *T. gondii* in the material collected after 48 h of infection (Fig. 1c).

Infection of TG180 cells in vitro - The tumor cells presented similar cellular and subcellular structures as observed in the *in vivo* condition. We observed the presence of intracellular parasites exhibiting spherical or elongated nuclei with evident nucleoli (Figs 2a, b). Vacuoles, free ribosomes, Golgi complex, dense granules, rhoptries and mitochondria of several sizes were also present (Figs 2b, 2d, 3b, 3c), as observed in *in vivo* cultured parasites. Parasites undergoing mitotic processes were frequently found in *in vitro* cultures (Figs 2a, d). There was a low number of intracellular parasites one to two days post-infection and the host cells were seen with single or two parasitophorous vacuoles containing two or four parasites (Figs 2b, d). Furthermore, these vacuoles had their external surface in close relation with mitochondria, which were distributed surrounding them (Fig. 2b). A more accentuated parasitism was seen from three to six days after infection of the tumor cells. After five or six days of infection, it was observed host cells presenting up to eight parasitophorous vacuoles that contained heterogeneous numbers (1 to 16) of parasites (Figs 2c, 3a). These parasitophorous vacuoles had dispersed structures inside them that seemed microtubules (Fig. 3a). We found tachyzoites and cell debris in the extracellular environment coming from the disrupted TG180 cells (Fig. 3c). Parasites morphologically similar to bradyzoites could be also observed (Fig. 3d).

DISCUSSION

In vitro assays are very important to evaluate the effects of new drugs against intracellular microorganisms, particularly when there are some associated *in vivo* factors, such as the host immune system, which can interfere in the effects at the intracellular level. *T. gondii* is an obligatory intracellular parasite that can infect all mammalian cells so far studied. *In vitro* culture of this parasite can be performed by tissue culture using a broad range of cells (Butenel et al. 1995), including the adherent and non adherent cells. Park et al. (1993) described that un-

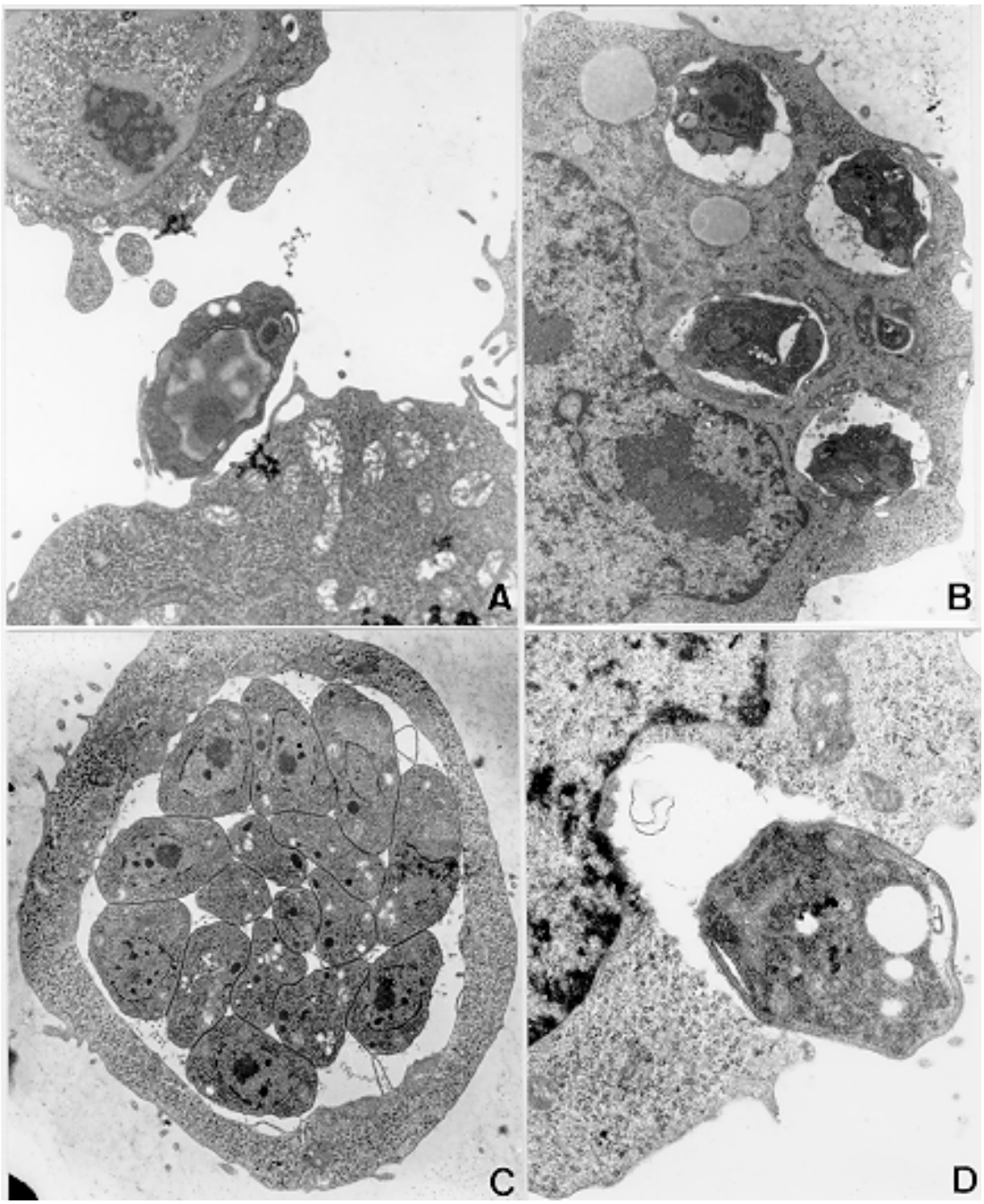

Fig. 1: TG180 sarcoma murine cells after *in vivo* infection by RH strain of *Toxoplasma gondii*. A: TG180 host cell with parasite attached to its plasma membrane, after 24 h of infection, 4,400X; B: host cells presenting four parasitophorous vacuoles with parasites in mitotic process after 48 h of infection, 7,000X; C: cell presenting at least sixteen parasites inside a parasitophorous vacuole, after 48 h of infection, 7,000X; D: cell being invaded by tachyzoite 24 h after infection, 12,000X.

der *in vitro* conditions tumor cells are the most suitable lines for the growth and multiplication of *T. gondii*. The TG180 murine sarcoma cells are commonly utilized to produce *T. gondii* by co-inoculation in the peritoneal cavity of mice. Couatarmanach et al. (1991) demonstrated that the culture of TG180 *in vitro* could produce large amounts of *T. gondii*, up to 259 parasites/cell. Therefore, this method of culture can be very useful to evaluate the effect of new drugs against *T. gondii*.

We observed by transmission electron microscopy that TG180 murine sarcoma cells are very

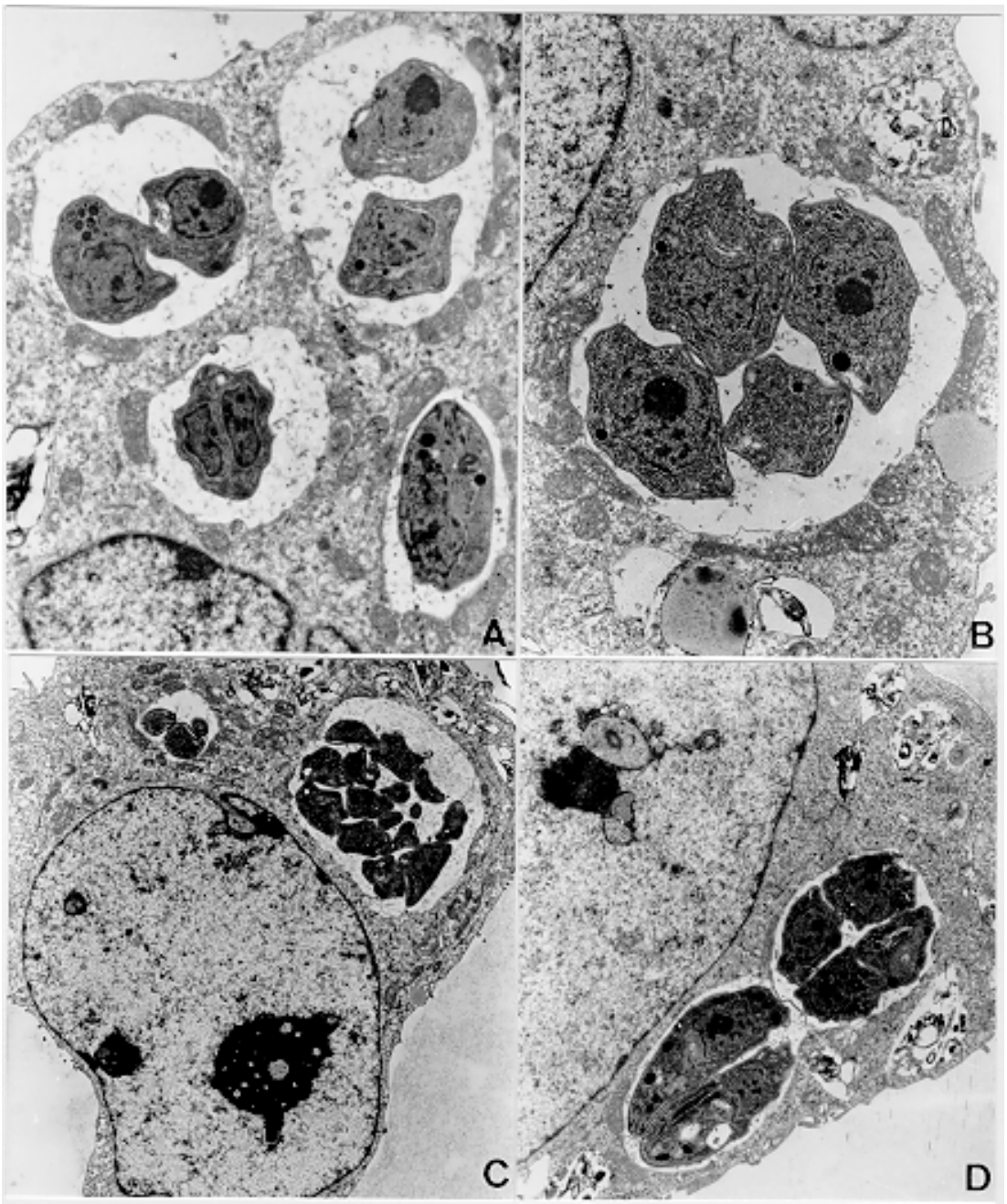

Fig. 2: TG180 sarcoma murine cells after *in vitro* infection by RH strain of *Toxoplasma gondii*. A: host cell presenting four parasitophorous vacuoles with parasites undergoing mitosis, after 24 h of infection, 7,000X; B: cell presenting at least four parasites inside parasitophorous vacuole, after 48 h of infection, 12,000X; C: cell presenting two vacuoles, one of those containing at minimum of 16 parasites inside a large parasitophorous vacuole, after five days of infection, 4,400X; D: cell presenting two and four parasites inside parasitophorous vacuole, after 48 h of infection, 7,000X.

susceptible to *T. gondii* infection. Basically, the same parasitism degree was observed in both *in vivo* and *in vitro* cultures. We found parasites in the intracellular environments, forming large parasitophorous vacuoles containing up to 16 parasites. Furthermore, extracellular parasites were found close to cellular debris, probably arising from heavily infected cells that released these parasites to the extracellular environment.

Ultrastructural analysis of TG180 sarcoma cells showed that these cells, when submitted to *in vitro* culture, have basically the same cytoarchitectural

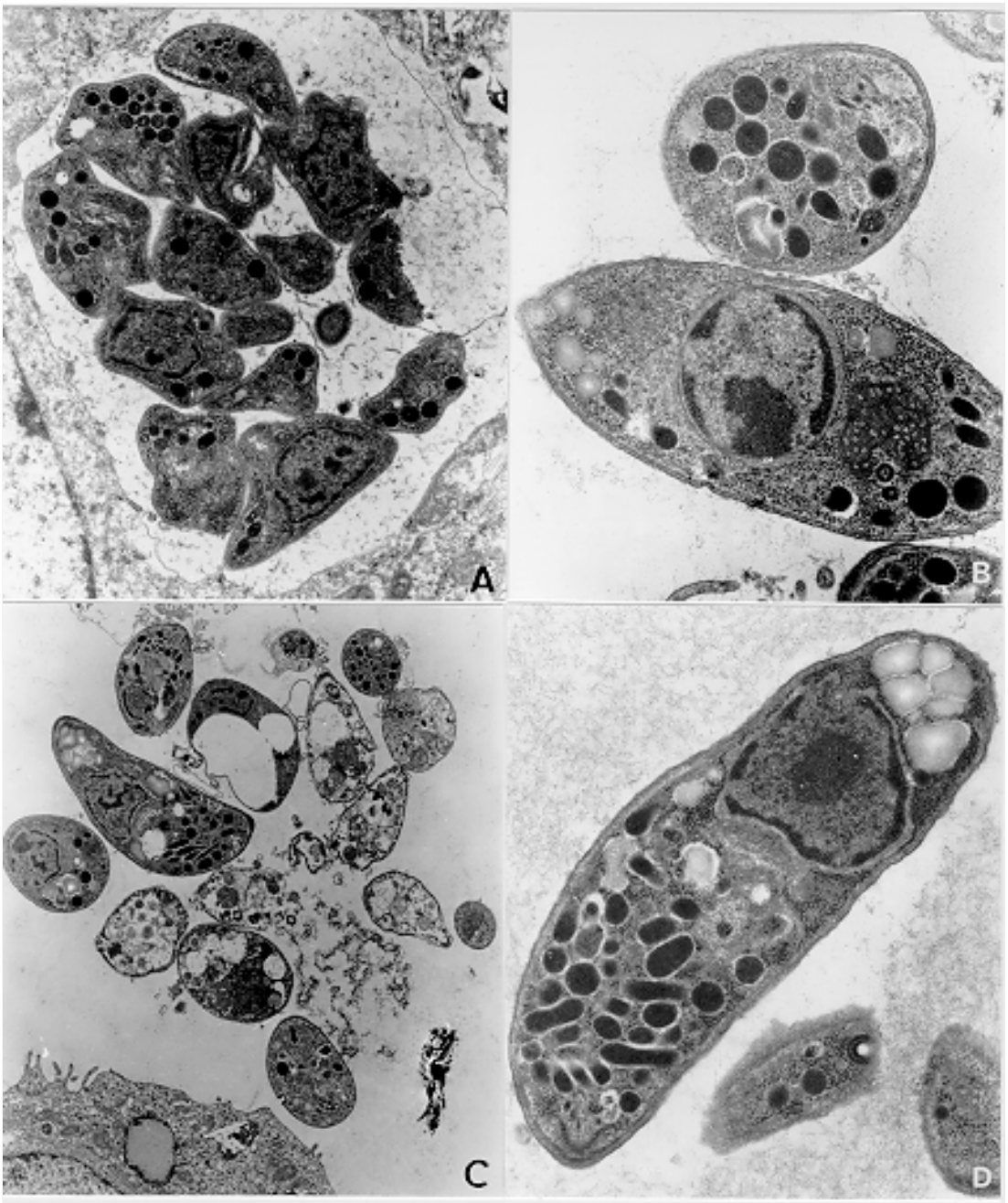

Fig. 3: TG180 sarcoma murine cells after *in vitro* infection of by RH strain of *Toxoplasma gondii.* A: detail of host cell presenting at minimum 16 parasites in mitotic division inside parasitophorous vacuole, after six days of infection, 12,000X; B: detail of the apical complex of an extracellular parasite from a recently lised host cell, after six days of infection, 20,000X; C: newly-released extracellular parasites and cellular debris from a disrupted host cell, after six days of infection, 7,000X; D: a parasite with vacuoles that seem amilopectin storage normally found in bradyzoite forms of *T. gondii*, 20,000X.

aspects of the *in vivo* cultured cells. As a cellular substrate for *T. gondii* infection, the TG180 can be utilized for diagnostic purpose, or to test new drugs with antitoxoplasmic activity. The TG180 cells can be a good option, as this cell line presents fast growth, is economically viable and is very susceptible to the *T. gondii* infection.

Lindsay et al. (1993) observed in culture of human foreskin fibroblast cells (Hs 68) the presence of host cell mitochondria surrounding the *T. gondii* tissue cyst wall and suggested that this may contribute directly or indirectly to the formation of the vacuole membrane. Interestingly, we observed frequently the presence of uncountable mitochondria neighboring the parasitophorous vacuoles, some of them completely enclosed by a net of hypertrophied mitochondria, and similar to those described by Lindsay et al. (1993).

Another remarkable observation was the presence of parasites in the extracellular environment, some of them harboring an electron-dense substance in their anterior portion that resembled bodies of amilopectine. These parasites presented morphology similar to bradyzoite forms. However, it will be necessary to search for bradyzoite specific antigens in these preparations to confirm these findings. One of the major challenges nowadays is the study of new drugs that can cross the toxoplasmic cyst wall and show toxic effect against parasites located inside these structures. The TG180 murine sarcoma cells may be useful to test new drugs that have their toxoplasmicidic effect just against the bradyzoites.

Our results demonstrated that the TG180 murine sarcoma is a suitable cellular substrate to *T. gondii* culture in both *in vivo* and *in vitro* conditions. Thus, this cell type might be used as a potential tool to isolate this parasite with diagnostic aims or as an alternative approach to test new toxoplasmicidic drugs *in vitro* against both tachyzoites and bradyzoites.

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