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Relationship between the Host Cell Endoplasmic Reticulum and the Parasitophorous Vacuole Containing *Toxoplasma gondii*

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ABSTRACT. **Tachyzoites of *Toxoplasma gondii* multiplies within the parasitophorous vacuole (PV) of the host cell. Simultaneously with parasite division growth of the vacuole takes place. Using immunofluorescence microscopy and antibodies recognizing calreticulin, a nonmuscle functional analogue of calsequestrin, and a 76 kDa glycoprotein localized in the endoplasmic reticulum (ER), we showed the incorporation of ER elements of the host cell into parasitophorous vacuole containing-*T. gondii*. In addition enzyme cytochemistry showed that glucose-6-phosphatase, an enzyme marker of ER, is also localized within the PV. These observations suggest that growth of *T. gondii* — containing PV is at least in part due to incorporation of elements of the host cell ER into the vacuole.**

Toxoplasma gondii, the agent of human and animal toxoplasmosis which has a high prevalence, is a parasitic protozoan able to infect a wide range of nucleated vertebrate cells (20, 22, 24, 25). After penetration into the cell the tachyzoites of *T. gondii* are localized within a cytoplasmic vacuole designated as parasitophorous vacuole, where they multiply until the complete destruction of the host cell. Following invasion, typical organelles from this parasite as the rhoptries, micronemes and dense granules secrete proteins into the vacuole, some of which associate with the parasitophorous vacuole membrane (PVM) while others remain free or associated with tubular structures within the vacuole (6, 7, 13, 22, 24, 26, 28). Within the PV, *T. gondii* multiplies with a generation time of 5 to 10 h. Simultaneously with the intravacuolar multiplication there is a significant import of cytoplasmic products, that include lipids (15) and other molecules (27). In the case of *T. gondii* it has been shown that the PV does not fuse with lysosomes (29). Therefore, other mechanisms may exist for products imported from the cytoplasm to the toxoplasma-containing parasitophorous vacuole. Previous morphological studies have shown that mitochondria (16, 32) and elements of the endoplasmic reticulum (16) of the host cell concentrate around the parasitophorous vacuole. We then decided to study further the association of the ER with PV analysing the distribution of three ER markers: calreticulin, a non muscle functional analogue of calsequestrin which is a highly conserved Ca^{2+}-binding protein found in the ER, a 76 kDa glycoprotein of the ER membrane (2, 5, 8, 9, 10, 18, 19, 21), and glucose-6-phosphatase, an enzyme found in the ER lumen (3, 4, 14, 35).

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MATERIALS AND METHODS

Parasites. Tachyzoites from the virulent RH strain of *Toxoplasma gondii* were maintained by intraperitoneal passages in Swiss mice and were collected in Ringer's solution at pH 7.2, 48–72 h after infection. The ascite fluid obtained from infected mice was centrifuged at 200 g for 7–10 minutes at room temperature to remove cells and debris. The supernatant, which contained the parasites, was collected and centrifuged at 1,000 g for 7–10 minutes. The pellet obtained was washed 2 or 3 times with phosphate-buffered saline solution (PBS), pH 7.2, and ressuspended to a density of 10^6 parasites/ml in 199 medium without fetal calf serum. The parasites were used within 30–40 minutes after removal from the mouse peritoneal cavity, and the viability was evaluated using a dye-exclusion test with Trypan blue.

Host cell. Vero cells (kidney fibroblast of African green monkey) were maintained in Falcon plastic flasks using 199 medium with 4% fetal calf serum and passed by trypsinization when the cell density approached a confluent monolayer. One

day before use in the experiments, approximately 2×10^5 Vero cells were placed on Linbro tissue plates that contained a round sterile coverslip, or were plated into 25 cm^2 flasks (3–5 $\times 10^5$/flask) and maintained at 37°C overnight in 5% CO_2.

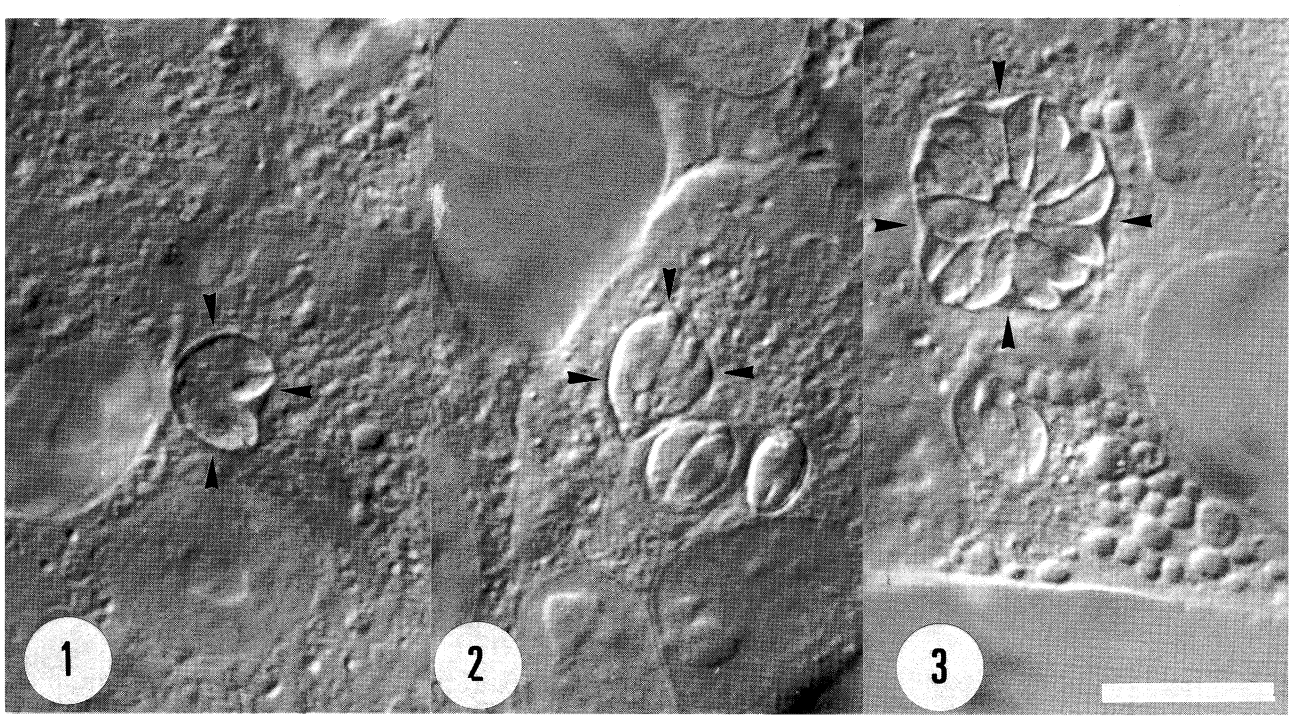
Host cell parasite interaction. Parasites suspended in 199 medium were incubated for 1 h in the presence of Vero cells using a 5:1 parasite-host cell ratio. After incubation, the cells were washed twice with PBS to remove extracellular parasites and incubated for periods varying from 24 to 48 hours at 37°C, and processed for fluorescence or electron microscopy, as described below.

Light Microscopy. After interactions the cultures were fixed with Bouin's solution and observed using Nomarski optics. All preparations were examined using Zeiss AXIOPLAN photomicroscope equiped with an 63× objective.

Confocal Microscopy. For visualization of the endoplasmic reticulum the purified polyclonal antibody against calreticulin (kindly supplied by M. Opas, University of Toronto) was used at a 1:50 dilution in phosphate-buffered saline (PBS). Polyclonal antibodies against calreticulin were produced in goat as described earlier (9), and shown not to cross react with calsequestrin (9, 33). We also used a monoclonal antibody against a ER glycoprotein of 76 kDa. The monoclonal antibody was obtained from Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences (University of Maryland, Baltimore, MD, USA). FITC-conjugated secondary antibody against goat or rabbit (Sigma Chemical Company, USA) was used at 1 : 20 in PBS. Control or infected cells grown on 12 mm-diameter glass coverslips were incubated in solution containing 3.7% paraformaldehyde, 0.1% glutaraldehyde and 0.1% Triton X-100 for 3 min, washed in PBS for 5 min, and then processed for labelling with the antibodies. The specimens were then examined in a Zeiss Confocal Laser Scanning Microscope (CLSM), using a 488 nm argon laser. Photographs were taken on Tri-X (ASA 100) film using the automatic exposure control camera. Tri-X film was developed for 5 min in Kodak HC 110 (dilution B).

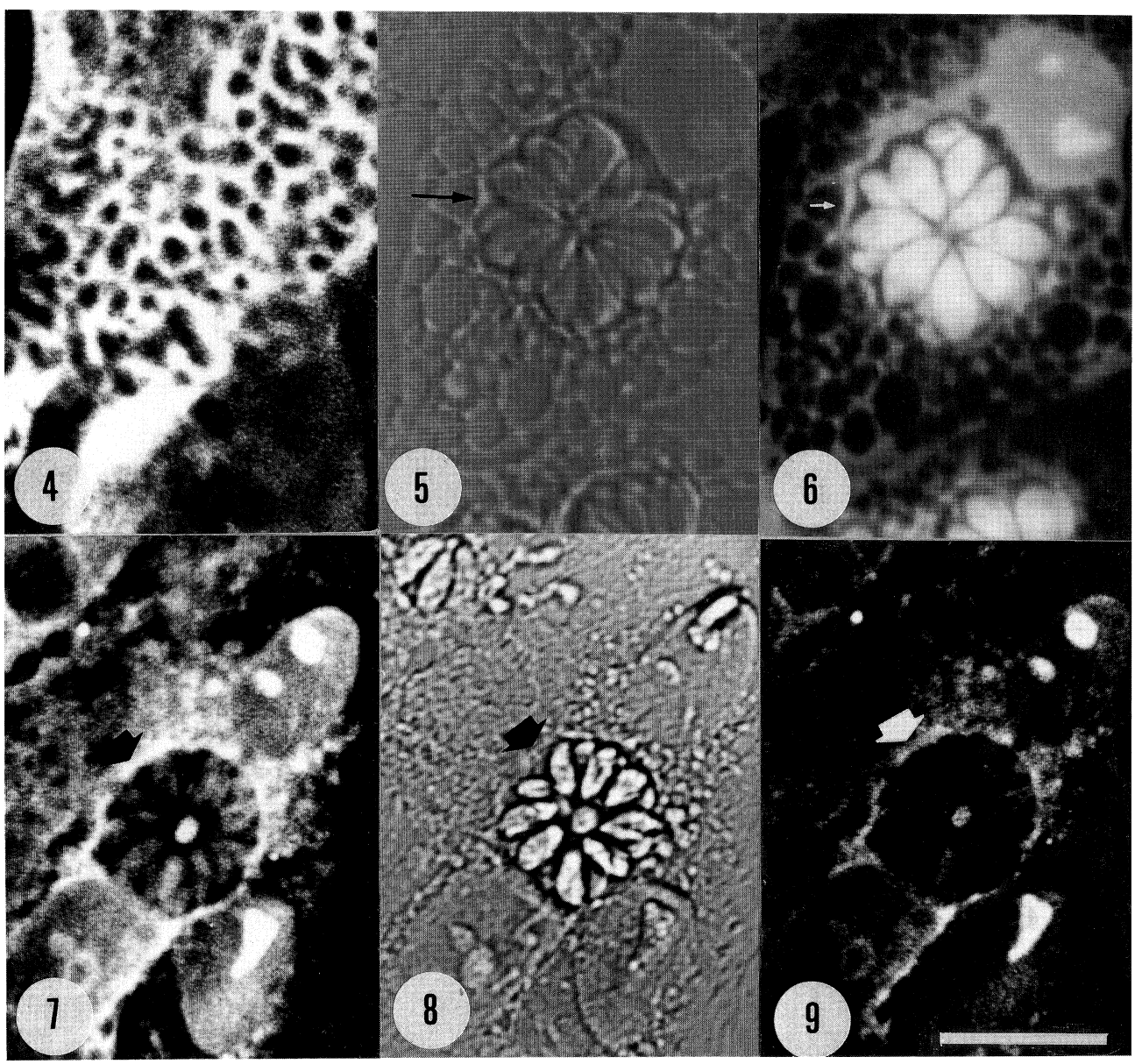
Electron microscopy. Vero cells were plated in culture flasks, cultivated as described above and allowed to interact with the parasites. After interaction the cultures were washed with PBS and fixed for 1 h at room temperature in a solution containing 1% glutaraldehyde, 4% paraformaldehyde, 5 mM $CaCl_2$ and 5% sucrose in 0.1 M cacodylate buffer, pH 7.2. Then, they were washed with cacodylate buffer with 5% sucrose and postfixed for 1 h in a solution containing 1% OsO_4, 0.8% potassium ferrocyanide and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2. The cells were rinsed with cacodylate buffer, dehydrated in acetone and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate, and observed with a Zeiss 902 Electron Microscope.

For cytochemical studies, the cells were plated in culture flasks, cultivated as described above and allowed to interact with the parasites. After interaction the cultures were washed with PBS and fixed for 1 h at 4°C in a solution containing 1% glutaraldehyde, 1% paraformaldehyde, and 5% sucrose in 0.1 M cacodylate buffer, pH 7.2. Then, they were washed with



Figs. 1–3. Light microscopy (Nomarski optics) of Vero cells infected with tachyzoites of *T. gondii* for 9 (Fig. 1), 12 (Fig. 2) and 24 hours (Fig. 3). The arrowheads show the limits of the parasitophorous vacuole which increases in size. Bar = 8 μm. N = nucleus, T = *Toxoplasma*.

0.06 M Tris-malete buffer, pH 6.5, with 5% sucrose at 4°C. The cultures were pre-incubated in a solution containing 5 mM $MgCl_2$, 4 mM $CeCl_3$ in Tris-malete buffer with 5% sucrose for 30 min at 37°C. The cells were rinsed two times in buffer and then incubated in a solution containing 5 mM glucose-6-phosphate (monossodic), 5 mM $MgCl_2$, 4 mM $CeCl_3$ in Tris-maleate buffer, pH 6.5, with 5% sucrose for 30 min at 37°C. The cells were postfixed for 1 h in a solution containing 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.2 at 4°C. The cells were rinsed with cacodylate buffer, dehydrated in acetone and embedded in Epon. Thin sections were quickly stained with uranyl acetate and lead citrate, and observed with a Zeiss 900 Electron Microscope. Analysis were carried out in a Zeiss EM912 Omega transmission electron microscope fitted with an energy dispersive system for X-ray microanalysis (Link ISIS Oxford).



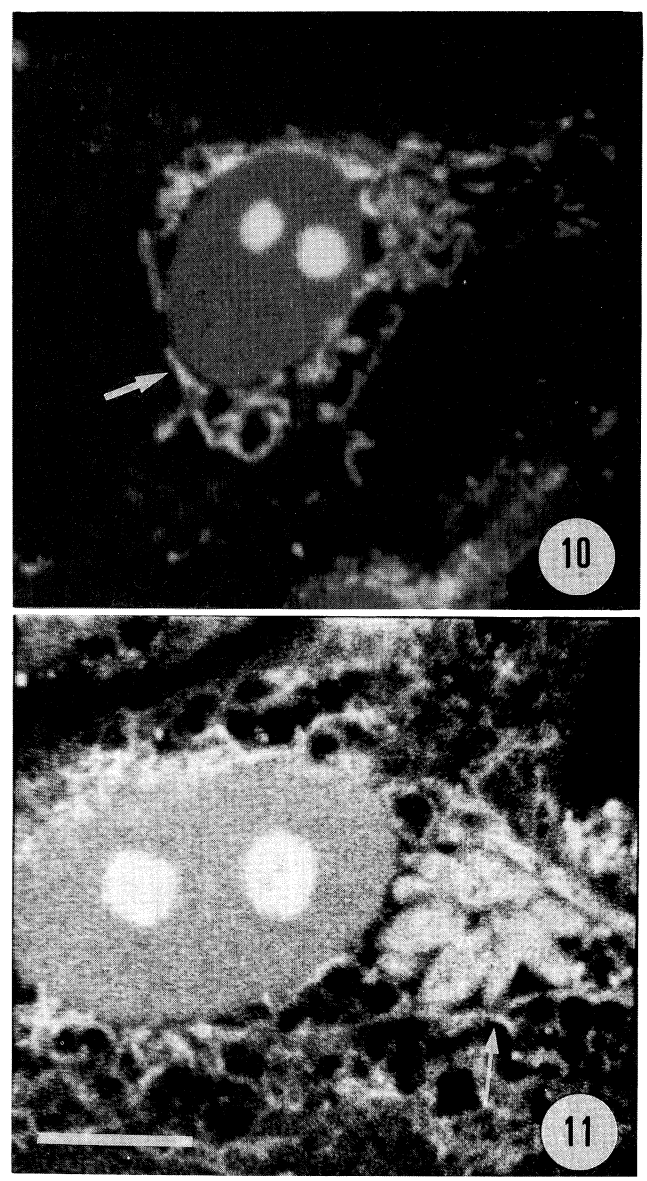
Figs. 4–9. Confocal laser scanning microscopy of Vero cells initially incubated in the presence of polyclonal anti-calreticulin antibodies and subsequently with FITC-labeled goat anti-rabbit IgG. A reticulated fluorescent pattern is observed in the cytoplasm of unifected (Fig. 4) and infected (Fig. 6) cells. Photographs taken at different focal planes show staining of the membrane lining the parasitophorous vacuole and of the intravacuolar parasites (Figs. 5–6, arrows; Figs. 7–9, arrowheads). Figures 5 and 6 as well as 8 and 9 are from the same field. Bar = 10 μm for all figures. T, *Toxoplasma*; V, parasitophorous vacuole.

RESULTS AND DISCUSSION

Light microscopy. Light microscopy observations of cultures of Vero cells allowed to interact for various periods with *Toxoplasma gondii* showed clearly that the parasitophorous vacuole increased in size with the intravacuolar replication of the tachyzoites (Figs. 1–3).

Immunofluorescence localization of calreticulin. Immunofluorescence microscopy of control Vero cells incubated with antibodies which recognize calreticulin showed a typical reticulated staining pattern distributed throughout the cell (Fig. 4). Dark areas, which contained other cytoplasmic structures, appeared surrounded by intense light areas which correspond to the endoplasmic reticulum components. This staining pattern was basically similar to that reported for other cells (11, 18, 33). Possibly the large dark areas seen in control cells correspond mainly to mitochondria.

Vero cells infected with tachyzoites of *T. gondii* change the distribution of mitochondria and endoplasmic reticulum in the cytoplasm. However, only ER elements were seen in close association with parasitophorous vacuole membrane. The dark areas were significantly reduced in control cell (Fig. 4), but they were enlarged in infected host cells (Fig. 6). Previous studies showed that host cell mitochondria concentrate around the parasitophorous vacuole (16). Examination of calreticulin localization in several focal planes, using a confocal laser scanning microscope, showed labeling of the parasitophorous vacuole, as well as of intravacuolar tachyzoites (Figs. 5–6). The labeling of the PV membrane (Figs. 7–9) strongly suggests incorporation of elements of the host cell endoplasmic reticulum into the membrane lining the PV. The endoplasmic reticulum (ER) is the largest continuous membrane structure in the cytoplasm (33), with several functional and structural damains (1, 12, 36). Calreticulin, a major Ca^{2+} binding protein found in the ER (17, 18) is a conserved protein of 60 kDa molecular mass and possesses low affinity Ca^{2+}-binding sites and a single high affinity Ca^{2+}-binding site (2). In Ptk_2 cells both smooth and rough ER spreading throughout the cytoplasm could be observed using antibody against calreticulin (11). However, there was no labeling of the mitochondria, Golgi complex membranes, plasma membrane or nuclear matrix, thus indicating that it is a specific marker of the ER. Calreticulin is common in different tissues of vertebrate and some plant tissues (18). However, little is known about the calreticulin in ER of Protozoan parasites. Some studies (18, 23) suggest that calreticulin may be confined to, or enriched in, the rough endoplasmic reticulum. In addition, to the endoplasmic reticulum localization, in some cell types calreticulin can also be detected in the nuclear envelope. This is not surprising as endoplasmic reticulum is continuous with nuclear membrane. The intense labeling in the peripheral position of the parasite (Fig. 6) is probably due to the inner membrane complex which seems to be a specialized endoplasmic reticulum (17). Our present observations of labeling of the parasitophorous vacuole using anti-calreticulin



Figs. 10–11. Confocal laser scanning microscopy of Vero cells initially incubated in the presence of monoclonal antibody recognizing a glycoprotein of the membrane of the rough endoplasmic reticulum and subsequently with FITC-labeled goat anti-mouse IgG. The projections radiating toward the cell periphery are observed in uninfected (Fig. 10) and infected (Fig. 11) cells. Some regions of the nucleus and parasitophorous vacuole (arrows) membranes are observed. Figs. 7 and 9 were taken from the middle and the lower position of the parasitophorous vacuole. Bar = 10 μm for all figures. T, *Toxoplasma*; N, nucleus.

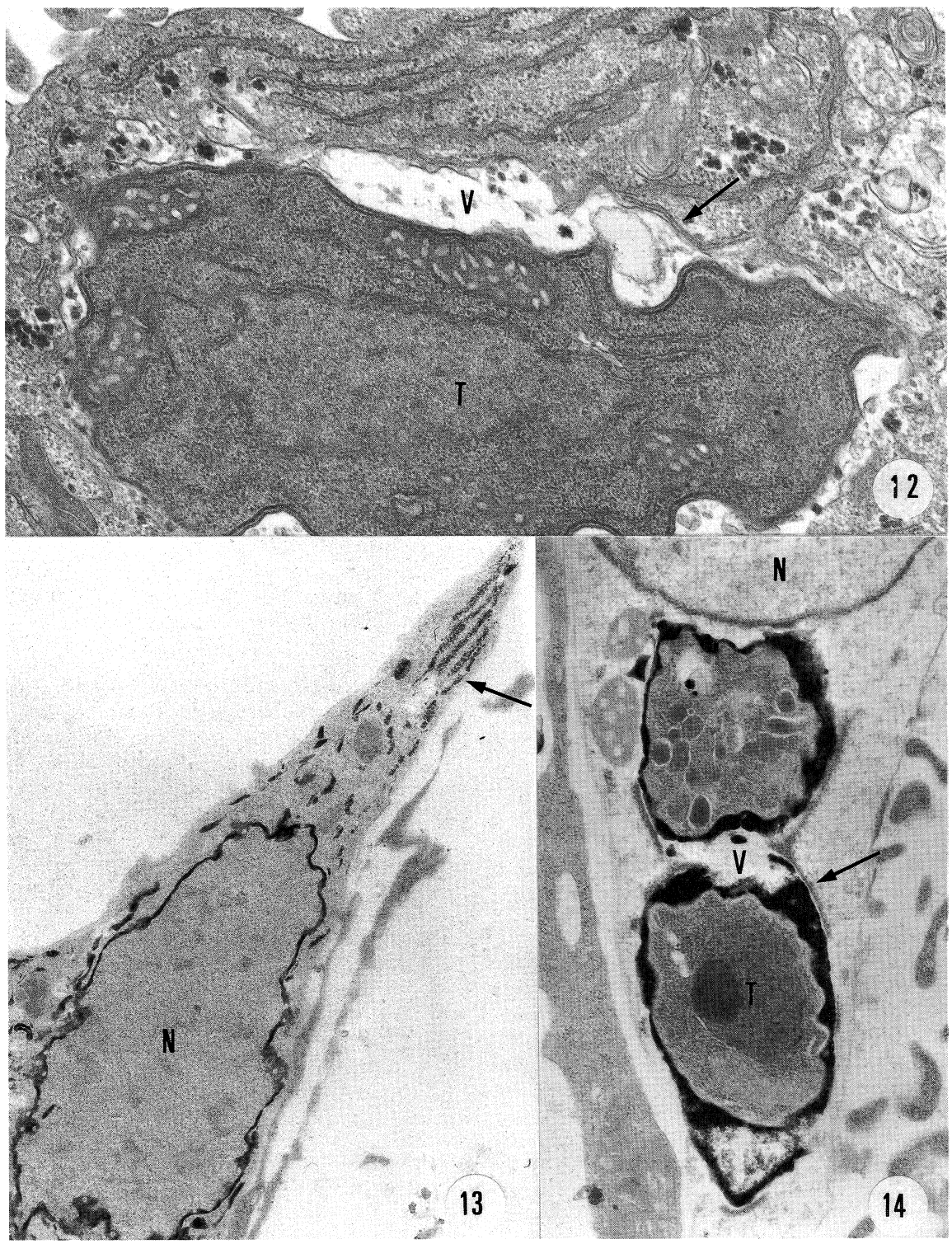


Fig. 12. Transmission electron microscopy of a thin section of a Vero cell infected with *Toxoplasma gondii* (T). Profiles of the endoplasmic reticulum (arrows) are seen in close contact with the membrane lining the parasitophorous vacuole (V). ×36,000

Figs. 13–14. Transmission electron microscopy of Vero cells incubated in the presence of a medium designed for the localization of glucose-6-phosphatase. Electron-dense reaction product, indicative of enzyme activity, is seen in association with profiles of the endoplasmic reticulum distributed throughout the cytoplasm and the nuclear membrane of uninfected cells (Fig. 13) and within the parasitophorous vacuole of infected cells (Fig. 14). N, nucleus; T, *Toxoplasma gondii*; V, parasitophorous vacuole. Fig. 11, ×8,700; Fig. 12, ×15,000

antibodies strongly suggest either fusion of the host cell ER with the vacuole or release of ER components of the parasite into the vacuole. If the first situation occurs, the incorporation of ER elements could explain the growth of the vacuole during evolution of the intracellular parasitism. Recent studies have shown a close association of host cell ER with vacuoles containing *Legionella pneumophila* (31).

Immunofluorescence localization of an RER glycoprotein. We also used a monoclonal antibody that recognizes a 76 kDa structural glycoprotein of the rough endoplasmic reticulum membrane (10). Immunofluorescence observation of control cells showed intense labeling of the perinuclear region, with projections radiating toward the cell periphery (Fig. 10). The nucleus was also stained. Vero cells infected with tachyzoites of *T. gondii* showed the same labeling pattern observed in control cells. However, the nuclear and parasitophorous vacuole membranes were stained in a slightly different pattern (Fig. 11) as observed with anti-calreticulin antibodies. The slight difference in the staining pattern of infected cells with the two antibodies used may be due to the fact that the ER is not a homogenous structure.

Electron microscopy. Examination of thin sections of infected cells showed the presence of profiles of the smooth ER as well as the rough ER in close association with the membrane lining the parasitophorous vacuole (Fig. 12). In order to determine more precisely a close association between these two membranes, we used enzyme cytochemistry, to localize sites containing glucose-6-phosphatase, a well known marker of ER in mammalian cells (4, 25, 35). In control cells reaction product, indicative of enzyme activity, was seen in association with the nuclear membrane, and typical profiles of the ER distributed throughout the cell (Fig. 13). In Vero cells infected with *T. gondii* intense deposition of reaction product was seen within the parasitophorous vacuole (Fig. 14). The reaction product was specific and contained cerium precipitate due to interaction with phosphate ions released following enzyme hydrolysis, as shown by X-ray microanalysis (Fig. 15). Glucose-6-phosphatase is a luminal protein involved at the terminal step of glyconeolysis and glyconeogenesis in liver and other mammalian cells (4). The presence of reaction product of glucose-6-phosphatase in the membrane and the intravacuolar space of the PV, again suggest the establishment of a close relationship between ER and the vacuole membrane. No reaction product was seen when the substrate was omitted from the incubation medium, or when β-glycerolphosphate was used as substrate thus excluding the possibility we detected another phosphatase. Previous studies have shown that intracellular tachyzoites of *T. gondii* release into the vacuole a potent nucleoside triphosphate hydrolase (30).

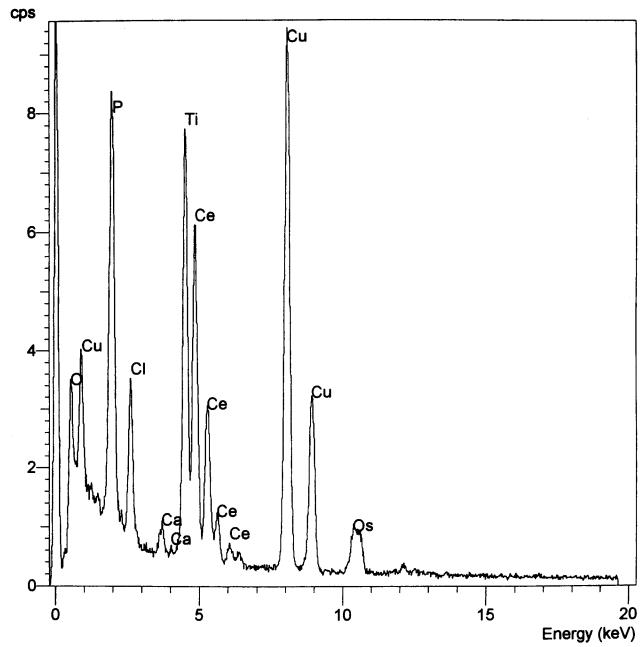


Fig. 15. X-ray microanalysis spectrum of the electron dense reaction product found within the parasitophorous vacuole. Peaks indicative of phosphorous (P) and cerium (Ce), are clearly seen, confirming the specificity of the cytochemical medium.

Taken together these observations suggest that the host cell endoplasmic reticulum, recognized by the presence of calreticulin and a 76 kDa glycoprotein, associates with the parasitophorous vacuole membrane, probably releasing some of its components into the parasitophorous vacuole, as indicated also by the presence of glucose-6-phosphatase within the vacuole. This process could also be involved in the process of growth of the vacuole. Further studies are necessary to determine if components of the ER secreted into the vacuole play, in association with other molecules secreted by the tachyzoites, some role in the metabolism of the protozoan.

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