Transmission and Scanning Electron Microscopy of Host Cell Entry by *Toxoplasma gondii*

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Entry by tachyzoites of *Toxoplasma gondii*, the causative agent of toxoplasmosis, into peritoneal cells was investigated with transmission and scanning electron microscopy. The process of entry is initiated by the parasite contacting the host cell with its anterior end, creating a small depression in the plasmalemma of the host cell. Occasionally, a small portion of the host cell cytoplasm protrudes and contacts the anterior end of the parasite. A cylindrical structure (35 nm in diameter) extends from the pellicle of the parasite to the host cell. Such structures appear to assist host cell entry by *T. gondii*. As the entry process progresses, pseudopods of the host cell surround the parasite and finally *T. gondii* becomes intracellular, being located in a vacuole separated from the host cell cytoplasm by a unit membrane. (Am J Pathol 87:285–296, 1977)

TOXOPLASMA GONDII, the causative agent of toxoplasmosis, can infect a variety of actively multiplying cells.[1] The mode of entry of *T. gondii* into these cells has been previously studied by light and electron microscopy.[2-5] *T. gondii* possess specific anterior end organelles; it has been speculated that these are related to the parasite's entry into the host cell.[6] Jones *et al.*[3] reported that *T. gondii* enters into the host cell by invagination of the host cell membrane. The initial contact between parasite and host cell is at the anterior end of the former. As the entry process progresses, the invagination of the host cell deepens to form a parasitophorous vacuole in which the parasite will reside; at no time do discontinuities of the host cell membranes occur. Because there is no disruption of the host cell membrane upon entry of *T. gondii*, it has been debated whether *T. gondii* enters into the host cell by phagocytosis or by active entry process. To clarify these differing views, host cell entry by motile forms (tachyzoites) of *T. gondii* was investigated by transmission and scanning electron microscopy. Such studies shed light on the mode of entry of the parasite and may contribute to control of the disease.

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Supported in part by Grant AI-10645 from the US Public Health Service; a part of this work was performed while Dr. Aikawa was a US Public Health Service Senior International Fellow FO6 TW-00012 at Osaka University, Osaka, Japan.

Accepted for publication December 21, 1976.

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Materials and Methods

The RH strain of *Toxoplasma gondii* was passed intraperitoneally at 3- or 4-day intervals in Swiss mice. The parasite-rich peritoneal fluid from infected mice was washed in PBS three times. Cells were harvested from the SD strain of rats by injecting 5 ml of 0.1% glycogen solution into the peritoneal cavities. Five days after the injection, peritoneal cells were obtained from the peritoneal cavities by lavage with heparinized medium 199. After centrifugation at 1000 rev/min for 10 minutes, the cells were suspended in medium 199 containing 10% calf serum. The suspension of peritoneal cells (1 to 1.5 $\times$ 10^6/ml) and of *T. gondii* (4 $\times$ 10^8/ml) was added to Falcon plastic dishes containing cover slips and maintained at 37 C in 5% CO_2. After a 3- to 5-hour incubation, the cells were washed in PBS, gently scraped from the cover slips with a rubber policeman, and fixed in 1.25% glutaraldehyde solution buffered with 0.1 M cacodylate buffer (pH 7.3). Transmission electron microscopy was performed as previously described.[7] Thin sections were stained with uranyl acetate and lead citrate and were examined with a Siemens Elmiskop 101 electron microscope.

For scanning electron microscopy, the specimens were fixed for 1 hour in 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at room temperatue, dehydrated through graded ethyl alcohol, and then passed into 100% Freon 113. They were then critical point dried, mounted on aluminum stubs, and coated with gold-palladium in a Hitachi sputtering system. The specimens were examined with a HFS-2S Hitachi field emission scanning electron microscope at the Department of Pathology, Kyoto University, Kyoto, Japan.

Results

Since the fine structure of tachyzoites of *T. gondii* has been reported by several investigators,[8] only a brief description of the parasite is presented here (Figure 1). The elongate tachzoite of *T. gondii* is surrounded by a pellicle composed of two membranes and a row of subpellicular microtubules. A cytostome is present in the pellicle. The nipple-like anterior end is demarcated by electron-dense polar rings. Within the anterior end is a conoid, a hollow, truncated cone-shaped structure (Figure 1). Electron-dense rhoptries are located in the anterior region and have a narrow ductule extending to the tip of the anterior end (Figure 1). Electron-dense micronemes are present in the anterior and middle portions. Mitochondria are seen usually in the region anterior to the nucleus, while the nucleus is located in the middle portion.

Scanning electron microscopy of the tachyzoite of *T. gondii* shows the organism to be crescent shaped (Figure 2). The anterior end is a truncated cone demarcated by a ring, as seen in thin sections. The surface of the tachyzoite is finely granular and slender ridges radiate from the anterior end to the posterior end (Figure 2). These ridges appear to correspond to the subpellicular microtubules seen in thin sections. The cytostome appears as an indentation in the pellicle (Figure 2). The nucleus is an elevated round structure in the midportion. The posterior end of the organism is smoothly curved and rounded.

Transmission electron microscopy shows that the process of entry into

host cells by the tachyzoite of *T. gondii* is initiated by the parasite contacting the peritoneal cells by its anterior region, which is oriented against the plasmalemma of the host cell, creating a small depression in the plasmalemma (Figure 3). Scanning electron microscopy on the initial stages of the entry process shows a small depression on the surface of the host cell, into which the anterior end appears to be attached (Figure 4). On occasion, however, a small portion of the host cell cytoplasm protrudes toward the anterior end of the parasite and seems to contact the anterior end or to extend inside the anterior region (Figures 6 and 7). Also to be noted occasionally are parasites with a cylindrical structure of ~35 nm diameter extending into the host cell cytoplasm (Figure 8). This cylindrical structure is continuous with the plasmalemma of the parasite and is surrounded by the plasmalemma of the host cell. No disruption of the host cell plasmalemma is seen during this process.

As the parasite enters the host cell, pseudopods of the host cell become oriented toward the parasite and extend along the pellicle of *T. gondii* (Figure 5). Scanning electron microscopy demonstrates that these pseudopods surround the parasite and interdigitate with each other, so that the parasite becomes enmeshed (Figures 9 and 10). After entry into host cells, *T. gondii* is located in a vacuole separated from the host cell cytoplasm by a unit membrane (Figure 11). About 10% of *T. gondii* contact the host cell with their posterior end or lateral aspect. In this instance, no cytoplasmic protrusion of the host cell to the parasite or the cylindrical structure can be seen.

Discussion

Intracellular organisms must enter a host cell in order to grow and to multiply. The mode of entry of the organisms has been a matter of debate for many years.[3,5] In the early 1960s, investigators noted, by electron microscopy, that the motile forms of many intracellular sporozoa possess specific anterior end organelles such as conoids, rhoptries, and micronemes, all of which were suggested to be associated with host cell entry.[6]

The conoid was suggested to have a screw-like function which aids the organisms in boring through the host cell membrane. This speculation was supported by reports in which *Eimeria callospermophili*[9] and *E. ferrisi*[10] (*Toxoplasma*-related organisms) were described as disrupting the host cell membrane at the site of entry. In contrast, Ladda *et al.*[11] showed that *Plasmodium berghei* and *P. gallinaceum* first invaginate the host cell membrane by their anterior end without disruption of this membrane. Since *Toxoplasma*-related organisms possess a conoid and *Plasmodium* does not, the different modes of parasite entry into host cell were thought

to be related to the presence or absence of the conoid.[6] It is clear from our present transmission and scanning electron microscopic study that *T. gondii*, which possesses a conoid, also enters the host cell by invagination of the host cell membrane. Similar observations were also reported on *T. gondii* by Jones *et al.*[3] and Klainer *et al.*[4] Since the earlier works did not clearly illustrate disruption of the host cell membrane by *E. callospermophili* and *E. ferrisi*, it is difficult to determine whether or not this disruption really occurs. At this moment, it seems reasonable to assume that these parasites enter the host cell by invagination of the host cell membrane regardless of the presence or absence of the conoid.

The invagination of host cell membrane around organisms can occur either by phagocytosis or by an active penetration process of the organisms. Since the erythrocyte does not possess phagocytic activity, the invagination of the membrane that occurs when *Plasmodium* enters into an erythrocyte is considered to be an example of active penetration process by *Plasmodium*. This is further supported by the fact that rhoptries of *Plasmodium* disappear after completion of the entry process, indicating active participation of the plasmodial organelles. Other examples include *Salmonella typhimurium* and *Shigella flexnei*, which invaginate the membrane of the intestinal epithelial cells during their entry. Takeuchi [12] suggested that these enter by an active penetration process.

A cinematographic study by Bommer *et al.*[2] showed that *Toxoplasma* can enter into a host cell in about 15 seconds. This fast entry process also favors active host cell entry by *Toxoplasma*. Our strong evidence in favor of active penetration of the host cell by *Toxoplasma* comes from the appearance of a cylindrical structure upon attachments of the parasite to the host cell. It appears to initiate and aid the invagination of the host cell membrane for parasite entry. The origin of the cylindrical structure is not clear but it may originate from the pellicular complex, since it is continuous with the pellicle. Because *T. gondii* possesses the cylindrical structure only when it is in contact with the host cell, such contact may be necessary for this structure to form. Although we did not observe the cylindrical structure in all of the parasites attaching to the host cell, this may be due to difficulty in sampling. Because of the small size and location of the structure, sections must be cut through the precise plane in which the exact tip of the parasite attaches to the host cell in order to see the cylindrical structure. This may be the reason why other investigators have failed to observe it previously.

Scanning electron microscopy clearly demonstrated the formation and extent of pseudopods around *Toxoplasma*, but the extent of the pseudopods was not always clear by transmission electron microscopy. Formation

of pseudopods around the parasite may be considered evidence for phagocytosis,[13] but the pseudopods also can be seen surrounding the parasite with cylindrical structure. This suggests that the pseudopods also can be formed in active penetration.

Jones *et al.* showed that some toxoplasmas enter into host cells by their posterior end or sideways. Similarly, we also observed some parasites attaching host cells by their posterior end or by their sides. In these instances, however, no cylindrical structure or cytoplasmic protrusion of the host cell toward the parasite can be seen. This suggests that only a specific receptor site at the anterior end of the parasite is capable of inducing active penetration, whereas organisms contacting prospective host cells by other portions of their surface are phagocytosed in a similar way as inert particles.

The electron-dense rhoptries have been suggested to contain enzymes that assist entry of these organisms. Lycke *et al.*[5] extracted a protein with a molecular weight of 70,000 to 150,000 from tachyzoites of *T. gondii* and reported that the protein (penetration-enhancing factor) enhanced host cell entry by the parasite. They suggested that the protein may reside in the rhoptries. In high concentration, the penetration-enhancing factor (PEF) also caused destruction of the host cell membrane. However, the portion of the host cell membrane in close contact with the anterior end of *T. gondii* appeared to be intact in the presence of PEF. Rhoptries may contain surface-active substances that cause membrane expansion, thus bringing about membrane invagination.[6]

Bannister *et al.*[14] recently reported on the host cell entry process of *P. knowlesi* as observed by transmission electron microscopy. They demonstrated that when the parasite was in contact with erythrocytes, a part of the erythrocyte cytoplasm was engulfed by the anterior end of the parasite, and that a "narrow channel system" formed and extended deep into the cytoplasm of the erythrocyte. Our present study also shows that a part of the host cell cytoplasm was attached to the anterior end of *T. gondii*. Bannister *et al.* suggested that the entry process initially involves adhesion of the anterior end to the host cell surface and that the merozoites of *P. knowlesi* would then be pulled by their anterior end attachment to the invaginating erythrocyte membrane. A similar observation was reported by Sheffield *et al.*,[15] who reported a dense bridge of material connecting the host cell cytoplasm and the sporozoites of *Lankesteria culius*.

Our current study suggests that *T. gondii* actively enters into host cells by complicated interactions between *T. gondii* and host cells. This is indicated by formation of the cylindrical structure of *T. gondii* and its extension to the host cell, and by protrusion of the host cell cytoplasm into

the anterior region of the parasite. The fact that entry of toxoplasma organisms into host cells involves a complex and ordered chain of events gives hope to the possbility of designing a chemotherapeutic agent that can selectively interfere with these processes without interfering with ubiquitous cellular processes such as phagocytosis.

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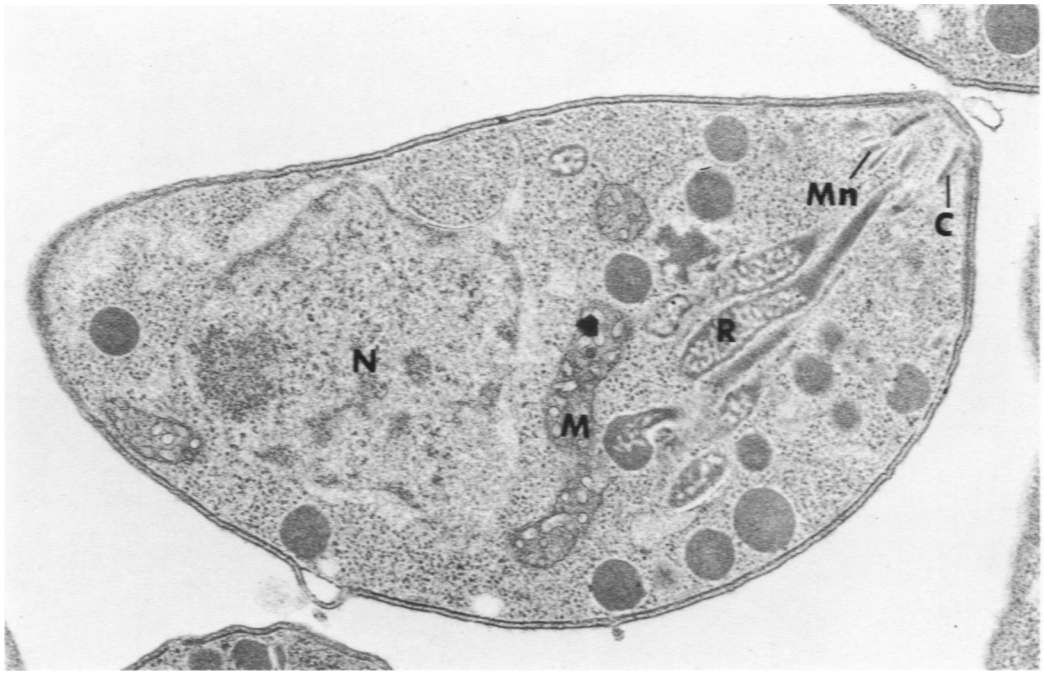
Acknowledgments

The authors wish to acknowledge, with appreciation, the help of Drs. J. Carter, P. Catanzaro, and B. Tandler of Case Western Reserve University in criticizing the manuscript and Dr. M. Fujioka of the Department of Pathology, Kyoto University, Kyoto, Japan, for the use of the Hitachi field emission scanning electron microscope.



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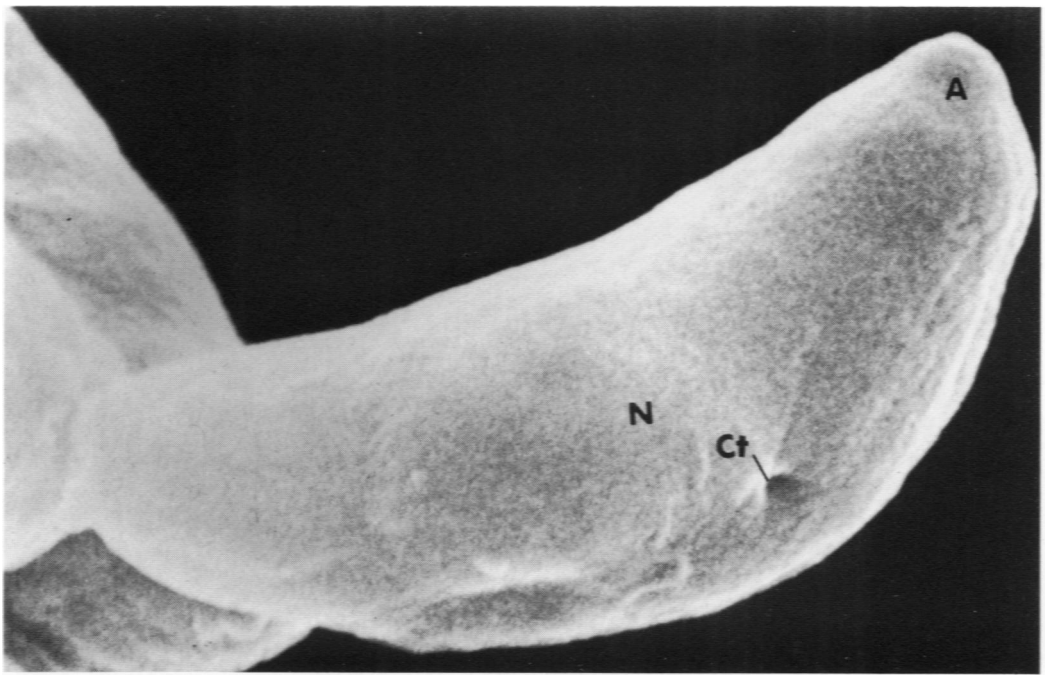

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Figure 1—Transmission electron micrograph of a tachyzoite of *Toxoplasma gondii* showing a conoid (*C*), rhoptries (*R*), micronemes (*Mn*), nucleus (*N*), and mitochondria (*M*) (×30,000). **Figure 2**—Scanning electron micrograph of a tachyzoite of *T. gondii* showing an anterior end (*A*), a cytostome (*Ct*), a nuclear region (*N*), and fine ridges along the long axis (×50,000).

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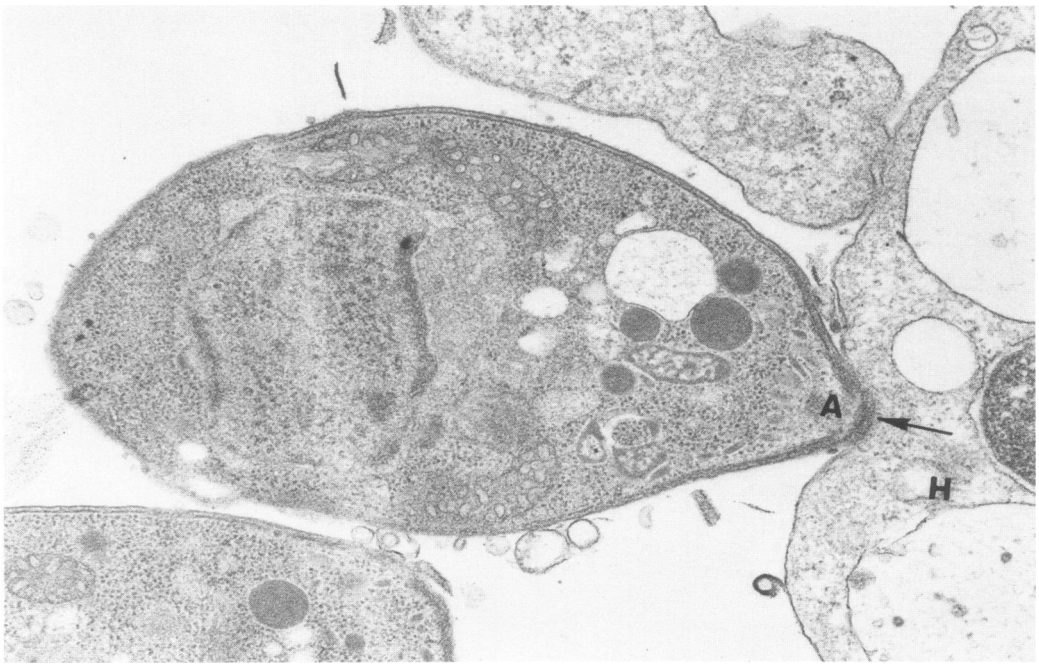

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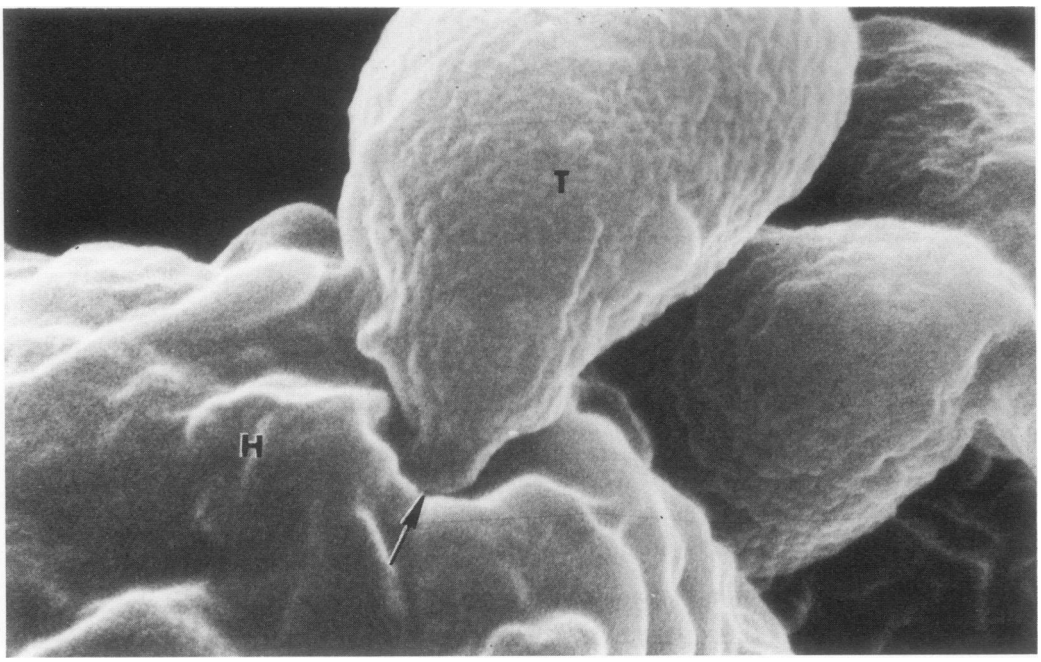
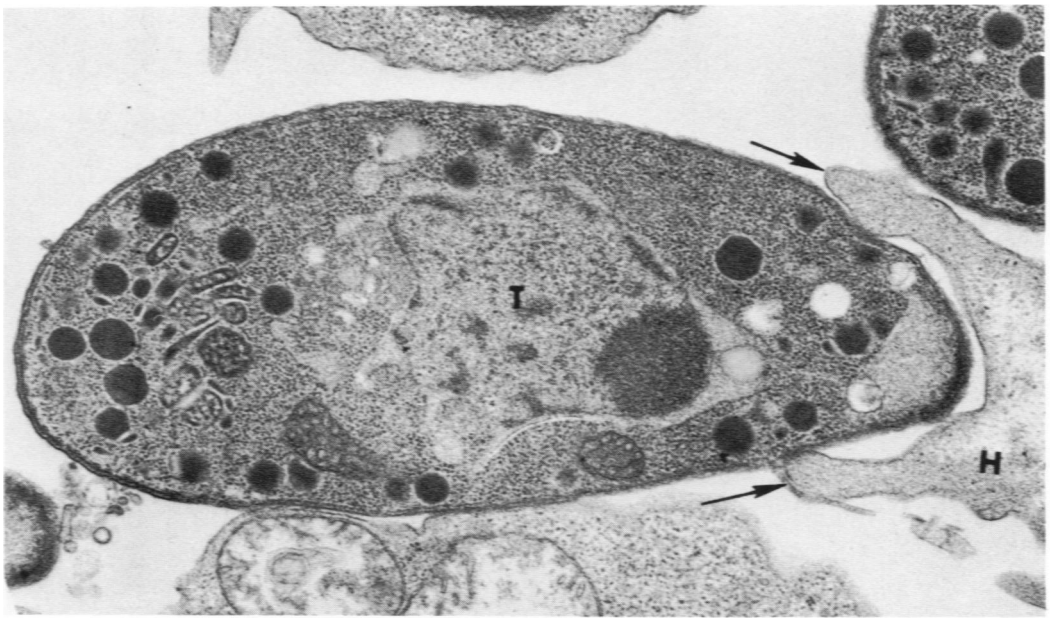


Figure 3—Transmission electron micrograph of a tachyzoite with its anterior end (*A*) directed toward a host cell (*H*). A small invagination (*arrow*) is formed at the surface of the host cell. (×27,000) **Figure 4**—Scanning electron micrograph of a tachyzoite (*T*) with its anterior end extending into an invagination (*arrow*) of the host cell membrane (*H*) (×52,000).



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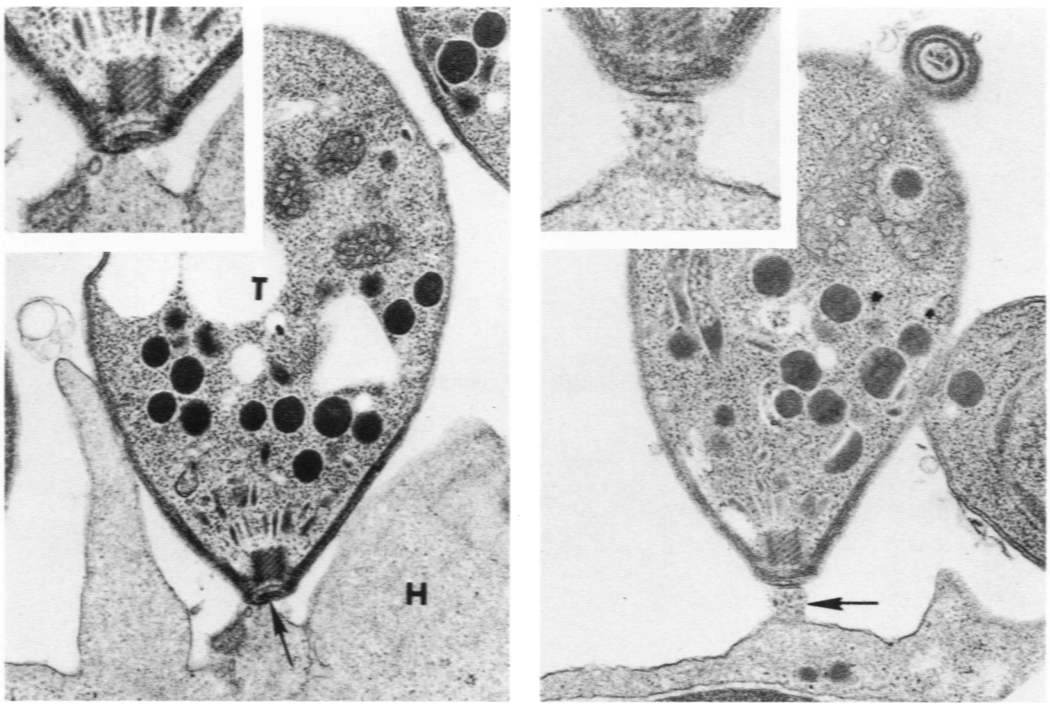

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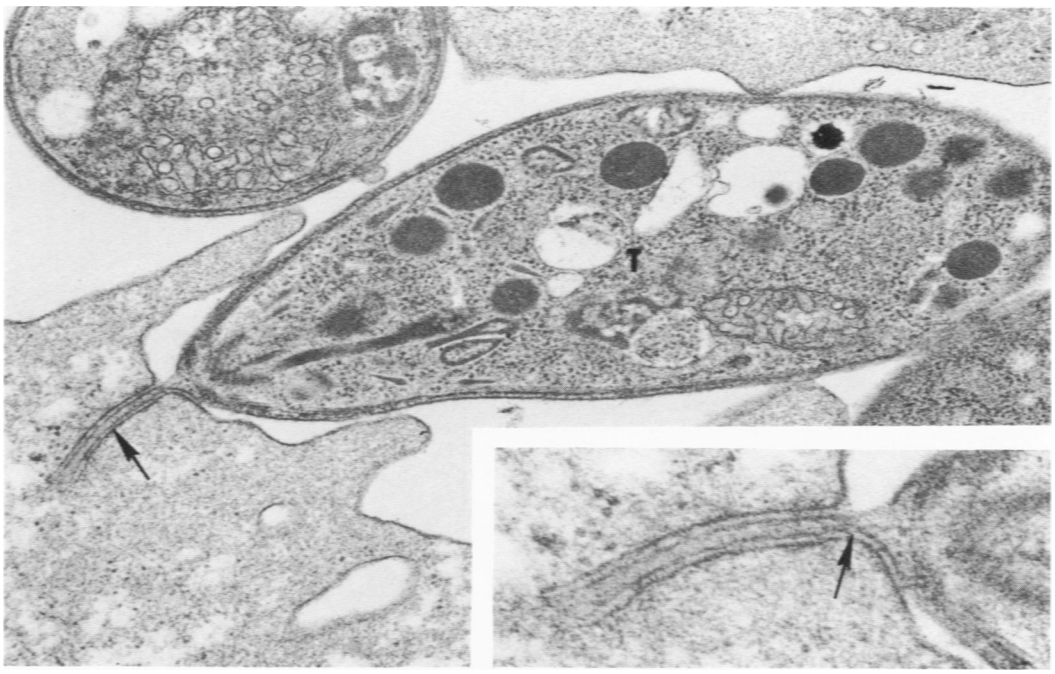
Figure 5—A tachyzoite (*T*) entering a host cell (*H*). Pseudopod (*arrow*) of the host cell extend toward *T. gondii*. (×25,000) **Figure 6**—A tachyzoite (*T*) entering a host cell (*H*). A small part of the host cell cytoplasm protrudes into the anterior end (*arrow*). (×18,000) **Inset**—Higher magnification showing a protrusion of the host cell cytoplasm into the anterior end (×35,000). **Figure 7**—A contact between the parasite and the host cell (*arrow*) (×25,000). **Inset**—Higher magnification of a portion of 7 (×47,000).

Figure 8—A tachyzoite (*T*) showing a cylindrical structure which extends from the anterior end into the host cell cytoplasm (arrow) (×30,000). **Inset**—Note that the cylindrical structure is continuous with the plasmalemma of the parasite (*arrow*) (×65,000).

Figure 9—Scanning electron micrograph of *T. gondii* (*T*) entering a host cell (*H*) (×21,000).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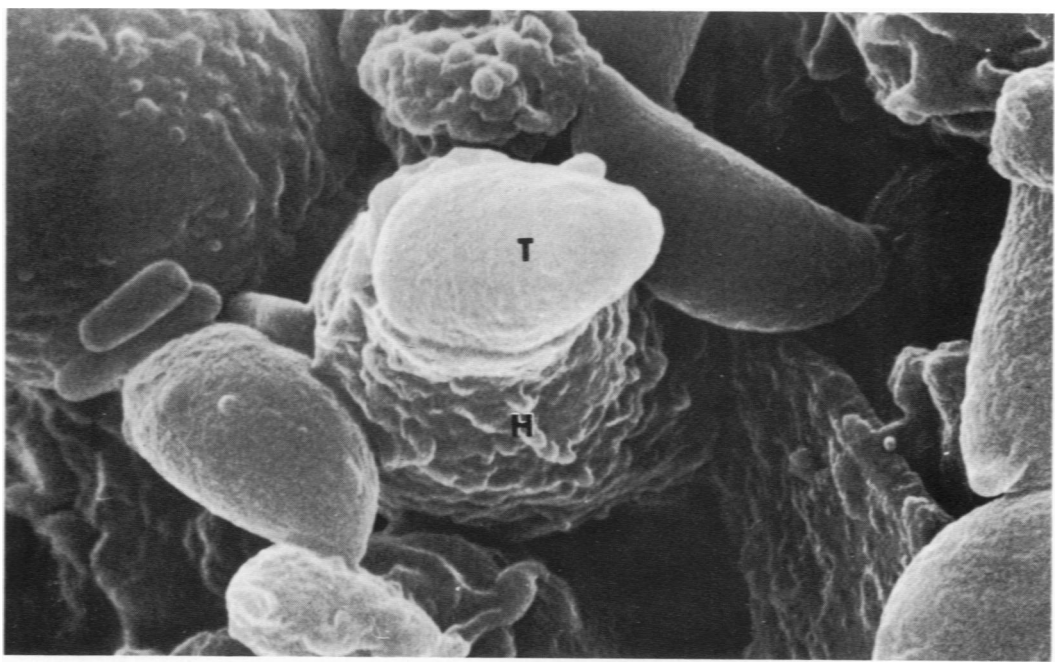
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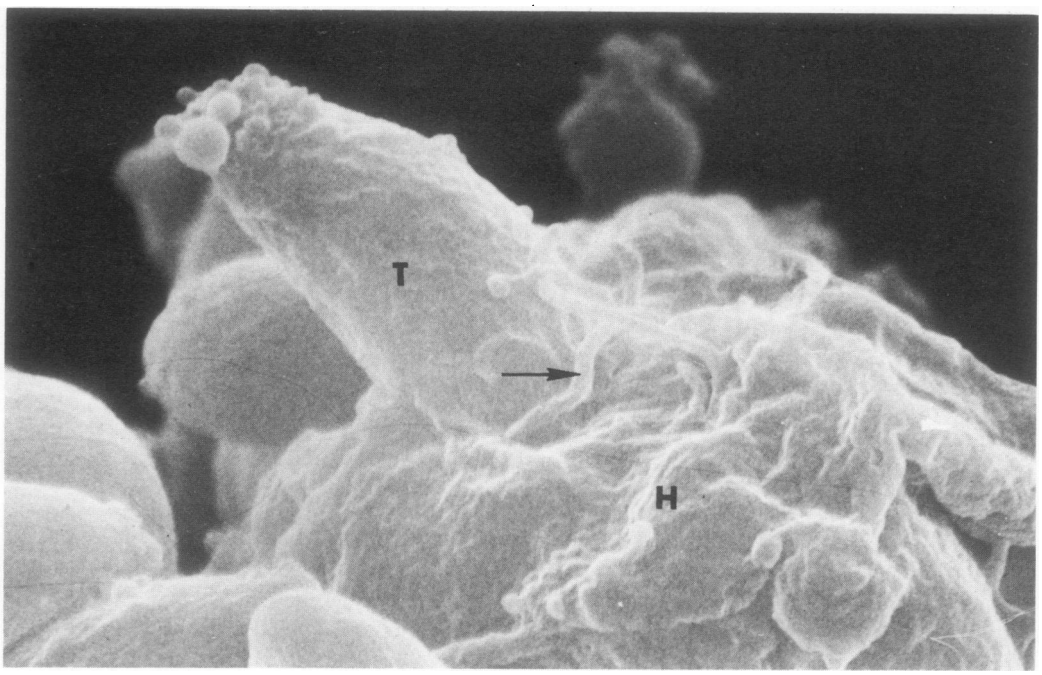

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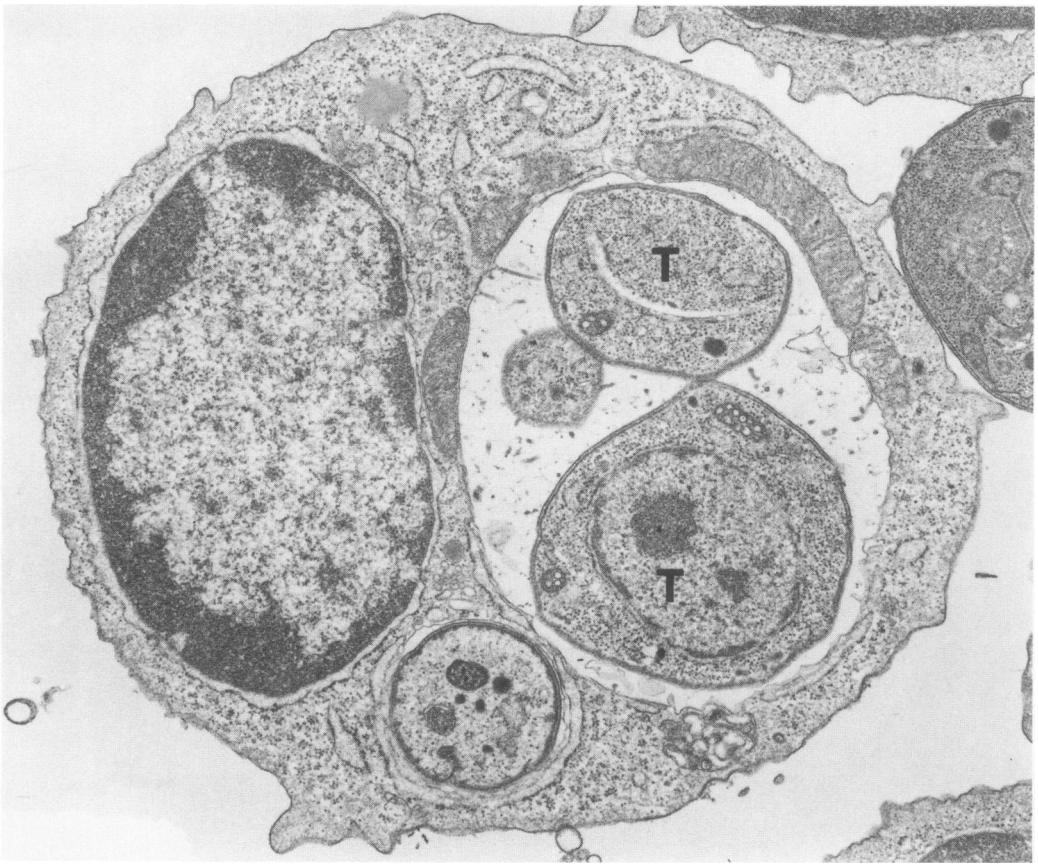

Figure 10—Scanning electron micrograph showing *T. gondii* (*T*) entering a host cell (*H*). Note pseudopods surrounding the parasite (*arrow*). (×30,000) **Figure 11**—Transmission electron micrograph showing two *T. gondii* (*T*) within a vacuole of a host cell (×16,000).