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Lytic Cycle of *Toxoplasma gondii*

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INTRODUCTION

Before discussing the *Toxoplasma* lytic cycle itself, we will provide brief overviews of its taxonomy, life cycle, pathogenesis, genetics, and ultrastructure. A basic understanding of these aspects will help appreciate how the lytic cycle fits into the overall biology of the parasite and the technical aspects of studying it. Following this introduction, we will describe our current understanding of the five components of the lytic cycle: attachment, invasion, vacuole formation, replication, and egress. Because of the special role of calcium signaling in these processes, a sixth section devoted to this aspect will also be included.

Taxonomy

Toxoplasma gondii is an obligate intracellular protozoan pathogen that was first described in 1908 by Nicolle and Manceaux working in North Africa and by Splendore working in Brazil. The species designation originated from the name of the North African rodent (*Ctenodactylus gondi*) from which this parasite was isolated. The genus name is derived from the Greek work *toxon*, meaning "bow" and referring to the crescent shape of the organism.

Toxoplasma belongs to the phylum Apicomplexa, which consists of intracellular parasites that have a characteristically po-

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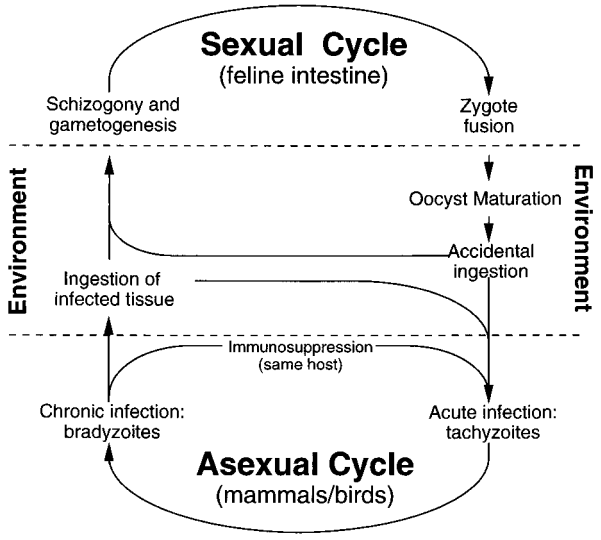

FIG. 1. Diagram of the *Toxoplasma* life cycle. The sexual cycle (top section) is initiated when a member of the feline family ingests either oocysts or tissues that are infected with bradyzoite cysts. This cycle is limited to the feline intestine and results in the shedding of oocysts in the cat's feces (middle section). Following oocyst maturation (activated after being excreted from the cat), the oocysts become highly infectious and survive in the environment for months and possibly years. Any warm-blooded animal that ingests these infectious oocysts becomes a host for the asexual cycle (bottom section). The sporozoites that are released from the oocyst will infect the intestinal epithelium and differentiate into the tachyzoite stage. After an acute infection, characterized by the dissemination of tachyzoites throughout the body, tissue cysts arise as a result of differentiation to the bradyzoite stage. Upon ingestion of these tissue cysts in raw or undercooked meat from a chronically infected host, the bradyzoites will infect the intestinal epithelium of the next susceptible host and differentiate back to the tachyzoite stage to complete the asexual cycle. If the ingesting animal is a cat, the bradyzoites can differentiate into the sexual stages, thereby completing the full life cycle. The sexual and asexual components of the life cycle are potentially independent; in particular, the asexual phase can theoretically cycle between intermediate hosts ad infinitum. The degree to which the sexual and asexual portions of the overall life cycle feed into one another in nature is not known.

larized cell structure and a complex cytoskeletal and organellar arrangement at their apical end (42). Other members of this phylum include the human pathogens *Plasmodium* (the cause of malaria) and *Cryptosporidium* as well as the animal pathogens *Eimeria* (the cause of chicken coccidiosis) and *Sarcocystis*.

Life Cycle

Toxoplasma is capable of infecting and replicating within virtually any nucleated mammalian or avian cell (41, 182). Its life cycle is divided between feline and nonfeline infections, which are correlated with sexual and asexual replication, respectively (Fig. 1). The sexual part of the cycle is outside the scope of this review. The asexual component consists of two distinct stages of growth depending on whether the infection is in the acute or chronic phase. The tachyzoite stage defines the rapidly growing form of the parasite found during the acute phase of toxoplasmosis. Tachyzoites are approximately 5 μm long and 2 μm wide (159). They replicate inside a cell with a generation time of 6 to 8 h (in vitro) until they exit the cell to infect neighboring cells, usually after 64 to 128 parasites have accumulated per cell (129). In the infected animal, tachyzoites differentiate into bradyzoites and form tissue cysts that first appear 7 to 10 days postinfection. These cysts are found predominantly in the central nervous system and muscle tissue, where they may reside for the life of the host. The development of tissue cysts throughout the body defines the chronic stage of the asexual cycle. Cysts that are ingested through eating infected tissue are ruptured as they pass through the digestive tract, causing bradyzoite release. These bradyzoites can then infect the epithelium of the intestinal lumen, where they differentiate back to the rapidly dividing tachyzoite stage for dissemination throughout the body, thereby completing the asexual cycle.

At some frequency within the host, there is apparently a low rate of spontaneous reactivation whereby bradyzoites differentiate back to tachyzoites. Normally, the immune response efficiently prevents the dissemination of these tachyzoites. In immunocompromised hosts, however, such reactivation may be unchecked and/or more frequent, leading to the provocative suggestion that the parasites might actively detect a lowered immunity against them (57, 60). The result, in either case, can be a massive and potentially fatal recrudescence.

Pathogenesis

In humans, clinical disease is normally limited either to immunocompromised individuals or to congenital disease resulting from an acute infection of the expectant mother. The severity of congenital infections depends on the stage of pregnancy when the acute infection occurred, and spontaneous abortions or neurological disorders such as blindness and mental retardation can result. In the past two decades, there has been a dramatic increase in the number of immunocompromised individuals and thus a concomitant increase in severe toxoplasmosis. Within this latter patient group, *Toxoplasma* is a frequent cause of intracerebral focal lesions resulting in toxoplasmic encephalitis (182). If left untreated, toxoplasmic encephalitis can be fatal, and thus this disease represents an important concern in the AIDS community (84). Although the current drug therapy (sulfonamide and pyrimethamine [171]) will effectively kill the tachyzoite stage, such treatment does not remove the chronic bradyzoite stage, and thus long-term therapy is needed. The toxic side effects of these drugs, combined with their inability to eliminate the infection, makes the need for safer and more effective treatments critical.

Genetics

***Toxoplasma* is amenable to genetic analysis.** *T. gondii* is emerging as a model system for the study of intracellular parasitism due to numerous characteristics which make it amenable to genetic analysis. The infectivity of this parasite for virtually any nucleated mammalian cell allows easy propagation in vitro using standard cell culture techniques. Classic genetic crosses can be performed in cats and used to map and quantify the number of loci involved in a specific phenotype (120). Following a cross between two distinct strains, a genetic map of the *Toxoplasma* genome has been generated using 64 restriction fragment length polymorphism markers to link drug resistance loci to specific regions within the known 11 chromosomes (153).

The nuclear genome of *Toxoplasma* is 8×10^7 bp (28) and is haploid, allowing one to easily modify or even eliminate the expression of a gene through chemical mutagenesis (118, 119). In addition, clonal isolates can be easily recovered via plaque purification due to the limited migration of the parasite (121).

Molecular tools for manipulation of the *Toxoplasma* genome. Within the past decade, studies of *Toxoplasma* have benefited greatly from the development of molecular genetics as a tool for studying intracellular parasitism. This parasite was the first obligate intracellular protozoan to be transformed with exogenous DNA (38, 75, 162). As a result, molecular tools for both forward and reverse genetics have been developed

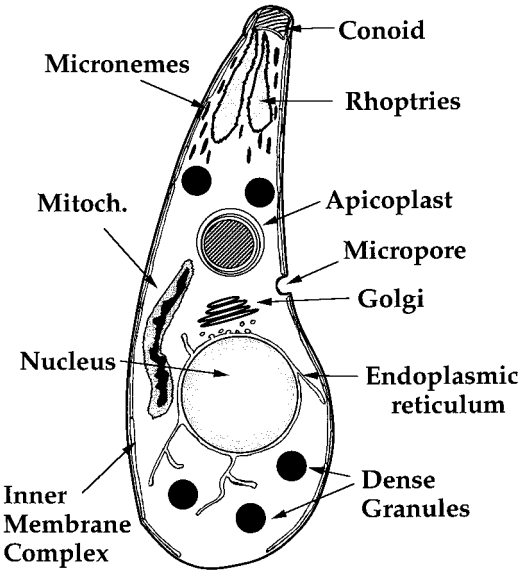

FIG. 2. *Toxoplasma* organelles. The major organelles of the asexually reproducing tachyzoite stage are shown. Mitoch., mitochondrion.

to manipulate the genome of *T. gondii* (reviewed in references 16, 133, and 160). A variety of markers have been developed to specifically select for transformed parasites without killing the host cells. These markers include chloramphenicol acetyltransferase (75), dihydrofolate reductase-thymidylate synthase (38), hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) (39), tryptophan synthase (154), and phleomycin resistance (91, 161). These markers have been used in gene replacement strategies via homologous recombination (40, 76) as well as in random insertional mutagenesis (37, 78).

The HXGPRT gene is of particular value due to its versatility for use in both positive and negative selections by treatment with mycophenolic acid or 6-thioxanthine, respectively (39). This positive and negative selection has permitted hit-and-run strategies to knock out the expression of genes (36). However, unlike the other selectable markers, HXGPRT is normally expressed by this parasite and thus its expression must be repressed to enable selection for the construct-derived copy. Since it is not an essential gene, the endogenous copy was completely removed in the RH strain, providing a clean background for selection (117, 133). Chloramphenicol acetyltransferase is unique among these selectable markers because in addition to its parasiticidal activity, it may be readily used as a reporter (162). Other reporters include the *Escherichia coli* derived β-galactosidase (146) and modified versions of the green fluorescence protein (167).

Gene discovery using expressed sequence tags. In addition to the development of molecular tools, a new database of expressed sequence tags (EST) has been generated for *T. gondii* (5, 64, 87, 178). Using cDNA libraries of both tachyzoites and bradyzoites, a single sequencing reaction from each of ~10,000 clones was entered into the database to sample the transcripts from the two stages (5). One of the applications of this database is to identify genes that are stage specific in their transcript levels (87). This database has also been used to uncover previously unidentified proteins that show homology to known attachment and motility proteins in *Plasmodium* (24, 178). By searching the EST sequences for proteins with homology to SAG1, the major surface antigen in *Toxoplasma*, a family of related proteins has been identified that coat the surface of the parasite and may be involved in attachment and/or regulation of the host immune response (17, 87).

Ultrastructure

Multiple, regulated secretory organelles. Given its obligate intracellular lifestyle, it is not surprising that *T. gondii* has a number of regulated secretory organelles (reviewed in reference 105). These are shown in Figure 2 and include the apical micronemes (from the Greek for "small threads") and rhoptries (from the Greek for "club-shaped") and the more generally localized dense granules. Each compartment has its own complement of proteins whose function is consistent with the timing of their release (24): micronemes release their contents early during the attachment-invasion process, then the rhoptries are released as invasion proceeds, and finally the dense granules discharge their contents when invasion is essentially complete. Where their functions are known or surmised, the individual protein components are described in the relevant section below.

Cytoskeletal network. The cytoskeleton of *T. gondii* is a complex orchestration of microtubules and other macromolecular structures that has apparently evolved to provide structural integrity, to direct polarized secretion, and to enable the parasite to glide across surfaces and invade host cells (53, 104, 106) (Fig. 3). At the anterior tip of the cell, two preconoidal rings surround the top of a tube-like structure called a conoid. The conoid consists of 14 elements of unknown composition that spiral counterclockwise as perceived from the posterior pole. Two microtubules approximately 400 nm in length extend from the preconoidal rings and pass through the center of the conoid, terminating within the body of the cell. These tubules are bound tightly together and are embedded in a dense matrix that is closely associated with the apically localized rhoptries and micronemes. It has been postulated that the microtubules

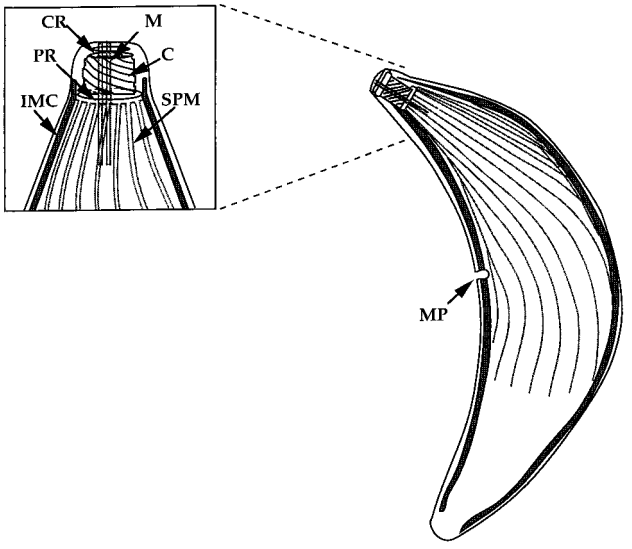

FIG. 3. The *Toxoplasma* cytoskeleton. This diagram illustrates the dominant features of the parasite's cytoskeletal arrangement. The anterior end of the parasite is enlarged in the box to the left so as to illustrate the preconoidal rings (CR), the conoid (C), the two apical microtubules (M), and the polar ring (PR) from which 22 subpellicular microtubules (SPM) emanate. The IMC is located just beneath the plasma membrane from the anterior to the posterior poles and is interrupted only by the micropore (MP) located in the middle of the parasite body. This pore is believed to be the primary portal through which endocytosis takes place.

function as a scaffold directing these organelles to pass through the conoid and secrete their contents from the apical tip (106).

Just posterior to the conoid, a polar ring functions as a microtubule-organizing center from which 22 microtubules emanate and spiral down two-thirds of the body (~4 to 5 μm) aligned in the same counterclockwise orientation as the conoid subunits (134). Although microfilaments have not been observed in this organism (27), actin has been detected in the conoid, preconoidal rings, and subpellicular microtubules by immunoelectron microscopy (183). Immunofluorescence of intracellular tachyzoites demonstrates that actin resides predominantly in the anterior portion of 75% of extracellular parasites (the remaining 25% demonstrated staining throughout the cell) (50).

Actin is encoded by a single gene in *Toxoplasma* and is generally found in a monomeric, soluble form within the cytoplasm (34). *Toxoplasma* also has a novel actin-binding protein (dubbed "toxofilin"), which sequesters G-actin and may play a key role in the creation and function of actin filaments (124). Myosin, a mechanoprotein that interacts with actin, colocalizes with actin in the anterior portion of the parasite as well as along the inner membrane complex (33, 143). Three unconventional myosins have been cloned from *Toxoplasma* by a PCR screen using generic myosin head primers (65). Two clones (TgM-B and TgM-C) appear to be products of differential RNA splicing with estimated protein sizes of ~114 and 125 kDa, respectively. Both TgM-B and TgM-C contain a single IQ motif (consensus, IQXXXRGXXXRK) in the neck region, which is normally important in light-chain binding and regulation of activity (89, 99). The third clone (TgM-A) defines a novel class of myosins since it is the only myosin identified to date that lacks a definable neck domain or IQ motif. This protein is one of the smallest myosins known, with an expected size of 93 kDa. The tail regions of all three proteins have no homology to other known myosins apart from a highly basic charge (65). Their size resembles that of the small myosins of *Acanthamoeba* and *Dictyostelium* (79), which were determined to be membrane associated and which have been implicated in cell translocation (96). There are currently no data on the function or regulation of the *Toxoplasma* myosins.

Trimembrane pellicle. *T. gondii* is a member of the Alveolates because it possesses an inner membrane complex (IMC) consisting of flattened membrane vesicles that lie just beneath the plasma membrane (110). In the Apicomplexa, this trimembranous structure (two membranes from the IMC and one from the plasmalemma) is called the pellicle; it runs from the anterior preconoidal rings to the posterior end of the cell (47). The only apparent interruption in this complex is at the micropore, which is positioned in the middle of the parasite body (Fig. 3). This pore is believed to be the active site of endocytosis, and vesicles have been observed in this region that appear to have clathrin-like coats (108).

The vesicles that make up the IMC measure ~20 to 100 nm in diameter (128) and are "sutured" together in a spiral with the same pitch as that observed for the subpellicular microtubules (104). The function of the pellicle has not been determined, although its close association with the microtubules suggest that it may be involved in structural integrity and motility of the cell (see below). Fourier analysis of electron micrographs demonstrates that microtubule-associated proteins (MAPs) and intramembranous particles (IMPs) found within the IMC both exhibit a 32-nm periodicity longitudinally (104). These structures appear to overlap and are further ordered by a second dimension of IMPs that are found in rows between the microtubules. The interdigitating rows of IMPs are oriented at an angle of ~75° relative to the vertical MAP-associated rows and are constrained to a uniform lateral spacing of 30 nm and the 32-nm longitudinal repeat observed in the MAPs. This ordered arrangement of IMPs in the IMC is neither actin based nor dependent on the microtubules, since it extends beyond the localization of these proteins and is not lost on exposure to drugs which disrupt their polymerization (104). Since there are large open spaces of membrane between the IMPs, the distribution is thought to be maintained by something other than nearest-neighbor contacts and may require a second set of filaments. In related apicomplexans, a filamentous lattice of unknown composition extends throughout the length of the cell both parallel and perpendicular to the microtubules (32). This framework has not been observed in *Toxoplasma* but is likely to be responsible for the ordered orientation of these IMPs.

In addition to the interaction with microtubules, the pellicle is connected to the plasma membrane by bridges of unknown composition (127). The function of these filamentous networks linking the plasma membrane, IMC, and subpellicular microtubules has not been determined but is most probably required, at least in part, for directing parasite motility.

HOST CELL ATTACHMENT

Surface of *Toxoplasma*

For an intracellular pathogen to gain entry into a cell, it first must make intimate contact with the surface of the cell. Since the lipid membranes of the host and pathogen normally possess a negative net charge, receptor-ligand interactions are required to overcome this repulsive force and to attain the firm attachment required for both motility and invasion (described below). *T. gondii* is unusual among the known intracellular pathogens in being quite promiscuous in its ability to invade a wide variety of host cells. When cultured in vitro, this parasite is capable of invading almost any mammalian cell type and even insect and fish cell lines (20, 179). This broad range of possible host cells would require *Toxoplasma* to express either multiple receptors for a variety of ligands or a few receptors that bind to ligands common to numerous cell types.

To discriminate between these two scenarios, the surface of *Toxoplasma* has been extensively characterized to identify the components involved in this process (17). The plasma membrane of this parasite appears to consist predominantly of a variety of proteins that are linked to the membrane by a glycosylphosphatidylinositol (GPI) moiety. Upon identifying the genes for these proteins, it has been determined that most of the surface consists of a family of proteins related to the surface antigen SAG1 (87). SAG1 (21) is the most abundant of these surface proteins (72, 73) and has been implicated, at least in part, in the initial events of attachment to the host membrane (59, 92, 93, 131). This protein is clearly not the only parasite molecule involved in attachment since Sag1$^-$ mutants are still infectious although with altered properties: they attach less well (92) but take less time on average to enter a host cell: 1 h after adding syringe-released tachyzoites to a fresh host cell monolayer, about twice as many Sag1$^-$ mutants had invaded compared to wild-type parasites (M. Grigg and J. C. Boothroyd, unpublished results).

The interaction between SAG1 and the host cell can be partially blocked using the neoglycoprotein bovine serum albumin glucosamide as a competitive inhibitor (74, 94, 131). There is also evidence demonstrating the ability of the parasites to bind the extracellular matrix protein laminin, and this may be used as a bridge for the ubiquitous laminin receptors found on the host cell (54, 55). While antibodies against lami-

nin blocked attachment at a similar level to that seen with anti-SAG1 antibodies (54, 92), the laminin receptor on *Toxoplasma* has not been identified.

Host Ligand

The contribution of the host cell surface to attachment has been less well characterized. The expression of the as yet unidentified attachment ligand(s) on the surface of the host cell appears to be cell cycle dependent in at least some of the lines tested in vitro (48, 58). In synchronized populations of Chinese hamster ovary (CHO) and bovine kidney (MDBK) cells, parasite attachment increased threefold as the cells proceeded from the G_1 phase to the mid-S phase and decreased back to baseline as the cells entered G_2/M (58). This association between attachment and cell cycle may explain the observation that within a population of fibroblasts, there is significant, nonrandom variation in the number of vacuoles within individual host cells (although this cannot be the entire explanation because most of these host cells grown to near confluency would probably be in G_0). Antibodies raised against synchronized MDBK cells that were harvested at different points during the cell cycle showed that polyclonal serum raised against cells taken at mid-S phase was almost threefold more effective at blocking attachment than was serum raised against cells in the G_1 phase (58). This block was not specific to the MDBK cell line, since a similar effect was noted using CHO cells as the host. These data would suggest that a similar antigenic ligand in both cell lines is utilized in attachment by *Toxoplasma*, although it does not exclude the possibility of a shared, cell cycle-dependent epitope that blocks the interaction through steric hindrance.

Some clues to the nature of the interaction between host and parasite have come from studies that use polysaccharides as inhibitors and probes (111). These experiments show that there is a sugar-lectin type of interaction involved in the attachment of the parasite because certain polysaccharides (heparin, fucoidan, and dextran sulfate) can facilitate or block parasite attachment to host cells depending on the concentration of the polysaccharide used. Cell lines deficient in proteoglycan synthesis also show a decreased ability to bind the parasites, again implicating a parasite lectin-like activity in the attachment phenomenon. The identity of these lectins is not yet known.

PARASITE INVASION

The entry of *Toxoplasma* into nonphagocytic host cells is an active process that apparently involves actin/myosin motors inside the parasite and certain transmembrane proteins that link these motors to the ligands outside. This conclusion is based on data summarized in the following sections.

Toxoplasma Demonstrates Substrate-Dependent Gliding Motility

Unlike ameboid movement, gliding motility does not involve obvious conformational changes in the cell but instead appears as a sliding motion across the surface by a mechanism that has not been clearly elucidated. It is an energy-dependent process that will move a cell at rates varying from 1 to 10 μm/s, considerably faster than the movement of cells which demonstrate amoeboid motility (77). The parasitic sporozoa (including *Toxoplasma*, *Eimeria*, *Gregarina*, and *Plasmodium*) and certain green algae (desmids, diatoms, and *Chlamydomonas*) are among the protists which demonstrate the gliding form of cellular translocation (14).

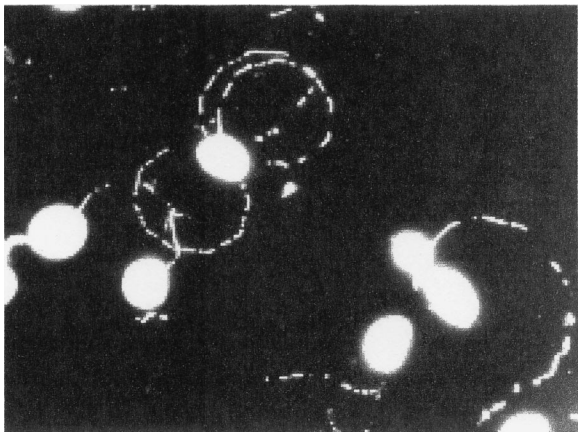

FIG. 4. Trajectory of *Toxoplasma* gliding motility. As parasites glide across a surface, they leave behind a trail of surface proteins. These trails were detected using immunofluorescence directed against the major surface protein SAG1. The circular trajectory is a result of the crescent shape of the parasite and its forward corkscrew motility, which is believed to be dictated, at least in part, by the subpellicular microtubules (106).

Toxoplasma gliding motility can be observed in vitro by phase-contrast microscopy or indirectly by staining for surface molecules that are deposited on the substrate, forming trails (Fig. 4) (35, 135, 165). Whole-cell locomotion occurs by moving the position of contact between the cell surface and the substrate along the body of the parasite in the same orientation as the subpellicular microtubules. Due to the parasite's crescent shape and the spiral of its cytoskeleton, this corkscrew type of gliding across a surface results in a trajectory of helical turns revolving around the longitudinal axis of the cell (62, 77, 135, 165).

Toxoplasma Invasion Requires Parasite Motility

The first step in the invasion process (in vitro, at least) is for the parasite to glide across the surface of the host cell, just as it does on a plastic substrate (62). Next, the parasite enters the host cell. Studies on nonphagocytic cells, such as fibroblast lines, have addressed which partner (host or parasite) provides the motive force for entry. While *Toxoplasma* invasion resembles phagocytosis in that invagination of the host plasma membrane is followed by residence within a cytoplasmic vacuole, several aspects clearly distinguish it from this process. Early reports using both cinematography (15, 66) and electron microscopy (3, 70, 109) demonstrated that invasion is polarized, with the apical end of the parasite leading. Entry is rapid (15 to 30 s), with a constriction on the parasite body as it passes into the host cell (Fig. 5). This event is quite different from that observed during the phagocytosis of *Toxoplasma* by professional phagocytes (e.g., macrophages); the parasite does not reorient, and the process is much slower (2 to 4 min). Phagocytosis is also normally associated with a marked rearrangement of the cytoskeleton in mammalian cells, and no such rearrangement is seen during normal invasion by *Toxoplasma* (3, 70). These observations, plus the unusual deformation of the parasite as it passes into the cell, suggest that *Toxoplasma* invasion is an active process distinct from currently known host endocytic events.

The requirement for parasite motility in host invasion was first implicated when it was observed that cytochalasin D, a potent inhibitor of *Toxoplasma* gliding, would block the infection of both phagocytic and nonphagocytic host cells (136,

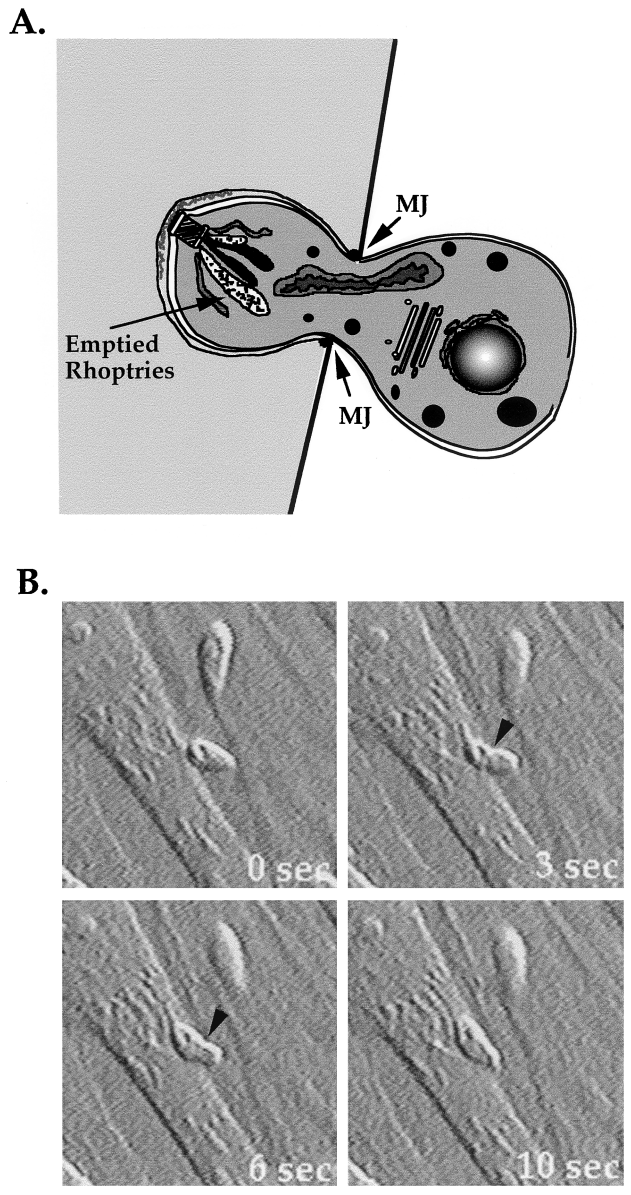

FIG. 5. *Toxoplasma* invasion. (A) Illustration of the critical events associated with host cell invasion. Secretion from the rhoptries is represented by the emptied vesicles and release of membranous material into the forming PV. As the parasite squeezes into the cell (as indicated by the constriction on the parasite body), a moving junction (MJ) forms between the parasite surface and host plasmalemma. (B) Still frames from a video of *Toxoplasma* invading a human fibroblast. The timing of the event is indicated in the lower right corner of each frame and was virtually complete 10 s after attachment. As in the cartoon, the constriction is apparent as the parasite crosses through the host cytoskeleton (indicated by arrowheads).

143). Since host microfilaments are also sensitive to cytochalasins and are required for the endocytic uptake of bacterial pathogens (52), further testing was required to discriminate between the two actin-based processes (i.e., host versus parasite). It was not until combinations of cytochalasin-resistant host and parasite mutants were tested against their sensitive counterparts that parasite motility was shown to be absolutely required for invasion (35). These tests demonstrated that actin mutants of *Toxoplasma* that are resistant to cytochalasin could invade sensitive cell lines in the presence of the drug while the opposite scenario completely blocked cell entry. Similarly, inhibitors of myosin activity will also block motility and invasion, indicating an essential role of the actomyosin motor in cell penetration.

Gliding Motility Is Dependent on an Actomyosin Motor

The active involvement of microtubules in motility has been tested using inhibitors that prevent polymerization of the tubulin subunits. The inhibitors griseofulvin, colchicine, vinblastine sulfate, and nocodazole did not appear to inhibit gliding or to affect the existing microtubules (135). This observation suggests that motility does not require a dynamic polymerizing-depolymerizing microtubular activity and that the subpellicular microtubules are not in flux but, rather, serve a structural function.

The polymerization of actin microfilaments is instrumental in the formation and extension of pseudopods during amoeboid "crawling" (56). In *T. gondii*, actin is found predominantly in a monomeric form (34); however, chemical agents that disrupt its polymerization into microfilaments, such as cytochalasins and latrunculins, or its depolymerization, such as jasplakinolide, paralyze the motility of the parasite and block invasion (125, 136, 150). While filament assembly is necessary, it is unlikely to be sufficient to generate the force required to move the cell along a substrate. Instead, the colocalization of actin and myosin suggests that the microfilaments of actin function as a scaffold for a myosin motor. This is further supported by the observation that inhibitors of the myosin ATPase (butanedione monoxime) and light-chain kinase (KT5926) are also effective at blocking motility and invasion (33, 62). These data all strongly support the notion that gliding motility is an essential aspect of active invasion of nonphagocytic host cells by *Toxoplasma*.

Candidate Bridge Molecules between the Exterior and the Actomyosin Motors

Since the actomyosin motor that drives motility is intracellular, at least one transmembrane bridge is required for *Toxoplasma* to link it to an extracellular ligand for cell locomotion. As described in "Surface of *Toxoplasma*" (above), current data suggest that most of the abundant proteins normally found on the surface of tachyzoites are linked to the membrane via a GPI moiety. Since this linkage only connects the proteins to a lipid of the membrane and does not actually cross the bilayer, other transmembrane proteins must either exist at levels below detection or be regulated in their transport to the surface. By using the EST database, a protein called MIC2 (microneme protein 2) was recently identified in *Toxoplasma* that is homologous to secreted transmembrane attachment proteins in *Plasmodium* (178). It contains six thrombospondin-like domains that are found on several plasmodial attachment proteins including the *P. falciparum* thrombospondin-related anonymous protein (TRAP) (132). TRAP was shown to be involved in motility in *Plasmodium*, since it was shed in slime trails of sporozoites (the stage transmitted from the mosquito vector) and antibodies against this protein significantly blocked movement (137). The mature polypeptide of *Toxoplasma* MIC2 is expected to have 743 residues with a hydrophobic stretch comprising the transmembrane domain from amino acids 698 to 718. In addition to the thrombospondin-like motifs, MIC2 contains a region at its N terminus that is highly homologous to the I domain found in many integrins, including several leukocyte adhesion receptors (178).

Immunoelectron microscopy of intracellular parasites localized MIC2 to the lumen of the microneme, with none being

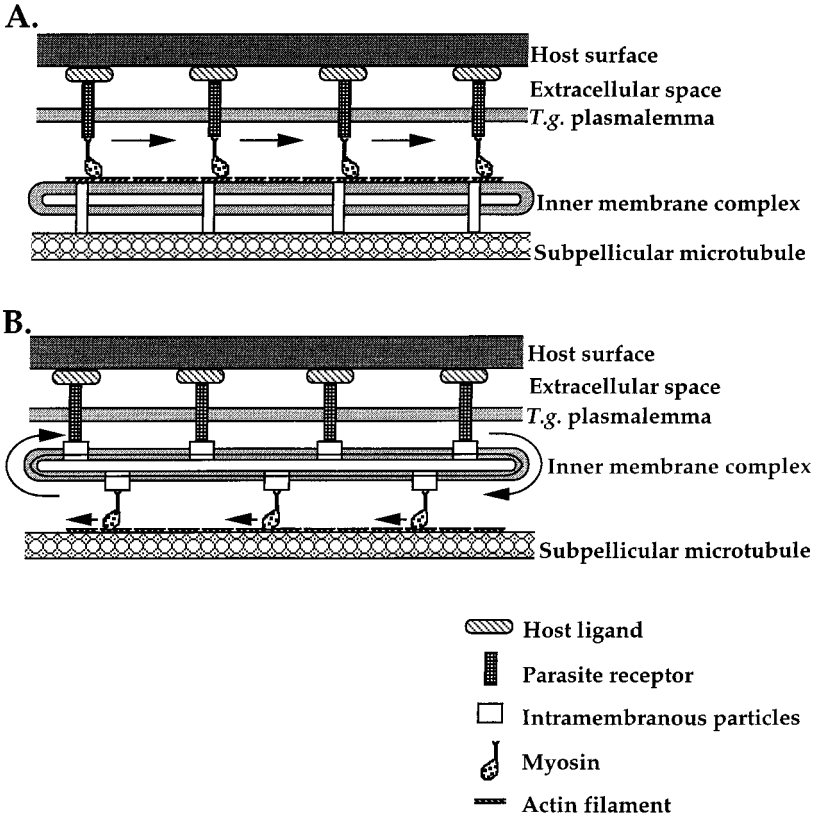

FIG. 6. Current models for the gliding motility exhibited by *T. gondii*. (A) Graphic illustration of the linear motion model (77). In this model, a transmembrane attachment protein(s) binds to an extracellular ligand and associates with myosin via its cytoplasmic tail. Myosin binds to the actin microfilaments located just beneath the plasmalemma and ratchets the transmembrane protein down the length of the parasite. The microfilaments are aligned to the orientation of the subpellicular microtubules via trans-IMC linkages, which results in the characteristic forward corkscrew locomotion. (B) The second model suggests that the IMC plays a more active role in motility (104). This model localizes both actin and myosin beneath the IMC in direct contact with the subpellicular microtubules. Instead of myosin, the IMPs associated with the IMC bind to the cytoplasmic tail of the attachment protein(s). These IMPs are connected to a secondary network of filaments that encapsulates the individual flattened vesicles. Myosin binds to either the same IMPs or distinct particles that are on the inner face of the IMC and move along microfilaments associated with the subpellicular microtubules. As these myosin-associated IMPs move along the actin tracks, they pull the secondary network like a conveyor belt. Since the outer surface IMPs are also connected to this filamentous network, they transduce this force to the attachment protein and move it down the length of the vesicle.

detectable on the surface of the parasite or within the parasitophorous vacuole (PV) (2, 178). In extracellular parasites, however, MIC2 is secreted onto the apical surface of *Toxoplasma* but only when there is contact with the host cell (24). This protein can also be seen in gliding trails (61), and it appears to be capped to the posterior end during invasion (24). A smaller, soluble form of MIC2 can be recovered from medium containing extracellular parasites and is believed to be proteolytically cleaved from the surface, leaving the transmembrane portion in the membrane (24, 178). Micronemal proteins sharing this TRAP-like conservation have also been identified in other Apicomplexans (e.g., *Eimeria* [173] and *Cryptosporidium* [163]), suggesting a family of proteins that may have a common function in attachment and motility.

The intimate association between TRAP-dependent motility and host cell entry was observed by using knockout mutants that were engineered in the erythrocytic stage of *Plasmodium berghei* (a stage that does not express this gene). After differentiation to the sporozoite stage, this mutant not only failed to glide but also was unable to invade either mosquito salivary glands or human hepatocytes (168). Similar studies have yet to be conducted in *Toxoplasma*.

Another *Plasmodium* protein that may serve as a bridge between extracellular ligands and the intracellular milieu is the apical membrane antigen AMA1, found in the rhoptries (115). *Toxoplasma* has a similarly apical homologue of this protein (albeit in the *Toxoplasma* micronemes instead of the rhoptries), complete with putative extracellular, transmembrane and intracellular domains (A. Hehl, C. Lekutis, E. Ortega, J.-F. Dubremetz, P. Bradley, and J. C. Boothroyd, unpublished results). However, the molecules inside and outside the parasite that are interacting with *Toxoplasma* AMA1 and MIC2 have yet to be identified.

Current Models for *Toxoplasma* Motility

Two models of how *Toxoplasma* is able to glide across a substrate have been suggested. The first scenario (Fig. 6A) is called linear motion (77) and requires that short actin filaments associate with the outer face of the IMC. Myosin would then interact directly, or via linking proteins, with the cytoplasmic tail of a transmembrane attachment protein (e.g., MIC2 or AMA1) and the microfilament scaffold. The path of these actin filaments would be guided by the subpellicular microtubules through IMPs that cross both membranes of the pellicle to mimic the helical trajectory set by the microtubular network. As the myosin contracts down the spiraling actin filaments, it pulls along the attachment protein toward the posterior end, where cleavage would occur to release the external portion of the protein. Since the subpellicular microtubules are found

only in the anterior two-thirds of the parasite, they may serve only to guide the second network of unknown filaments that run the full length of the parasite and are apparently used to order the interdigitating IMPs (see above). This model of *Toxoplasma* motility closely resembles that of capping ligands (often seen in the removal of antigen-antibody complexes), which also use an actin-myosin motor (51).

The second model (Fig. 6B) suggests that the inner membrane complex may not act as a stationary support matrix but instead moves like a conveyor belt driven by a microtubule-associated motor (104). The interaction between short actin microfilaments and microtubules has been documented in numerous events including axonal transport and mitotic spindle formation (123). In *T. gondii* motility, myosin may transiently bind to the tethered IMPs found associated with the pellicle and move these particles along microtubule-associated actin filaments. As the IMPs are transported along the actin, the entire surface of the vesicle would move as a result of the lattice-like connections between these particles. The cytoplasmic tails of transmembrane attachment proteins at the surface may bind to the IMPs on the outer surface of the pellicle and be pulled along by this moving belt with the same spiral as directed by the microtubules. At the end of each vesicle, the attachment protein may either be passed on to the next conveyor belt or cleaved to maintain uninterrupted movement along the length of the cell.

Two major assumptions are required by this second model that present formidable obstacles and may weaken its plausibility. The first is the supposition that the filamentous network used to order the IMPs is set up individually for each of the vesicular subunits of the pellicle. Since the pellicle is compartmentalized with the components sutured together, it would be difficult to envision the pellicle with a continuous belt of IMPs moving across these junctions and traversing the length of the parasite. The second assumption is the requirement for a direct association between the subpellicular microtubules and this secondary IMP network. In this model, vesicles that are posterior to the microtubules must be influenced by those in the anterior two-thirds of the cell. If, as mentioned above, the vesicles function as independent units with each demonstrating the same direction of movement, the force would be in opposite directions at the points of contact between the anterior and posterior vesicles. Although there are insufficient data to formally confirm or reject either of the two scenarios, the linear-motion model lacks the mechanistic problems presented by the pellicular-belt proposal.

While other forms of movement, such as the anterior-posterior flexing and forward protrusion of the conoid, have also been observed in *Toxoplasma*, they do not appear to be coordinated with gliding motility (26, 106, 145). The localization of actin in the space between the anterior end of the inner membrane complex and the conoid suggests that extrusion of the conoid may be powered by a similar actomyosin motor (183). The involvement of actin microfilaments in conoid motility is further supported by the observation that cytochalasin D blocks conoid protrusion (97). The regulation of this activity, as well as that demonstrated in gliding motility, is unknown.

Secretory Events during Invasion

It has long been suggested that the organelles within the apical complex, namely, the rhoptries and micronemes, are involved in invasion. The micronemes are thin, possibly vesicular organelles that are adjacent to the rhoptries at the apical end of the parasite. These organelles appear to secrete their contents just after making contact with the host cell (24). The possible role of micronemal proteins in the invasion process is discussed above.

There are generally 8 to 16 rhoptries per parasite that are identified by electron microscopy as club-shaped, electron dense organelles (149). These highly acidic organelles (148) are thought to be derived from Golgi secretory vesicles and are faithfully reproduced in each daughter cell during endodyogeny (69, 95). During invasion, rhoptries secrete their contents into the nascent PV through the narrow duct formed at the conoid by fusion of the rhoptry membrane with the apical tip (107, 127). Rhoptries in *T. gondii* contain at least eight known proteins, ROP1 to ROP8 (12, 82, 113), and while some become associated with the PV membrane (11, 139), their role in cell penetration and vacuole formation remains largely unknown. The basic pI of several of the major rhoptry proteins (ROP2 to ROP5) has led to the proposal of a role for polycationic polypeptides in host penetration (180). This is based on the observation that a histidine-rich protein isolated from *Plasmodium* was shown to induce the invagination of the erythrocytic plasma membrane (10). The mode of activity of polycationic polypeptides is suspected to be through energy-dependent alterations of membrane proteins or altered membrane microviscosity through intercalation with the lipid bilayer (180).

A penetration enhancement factor (PEF) (86) ascribed to these organelles will cause morphological degeneration of host cell membranes at high concentrations (85). In *Toxoplasma*, PEF activity is dependent on temperature, Ca^{2+} and Mg^{2+} concentrations, and pH (139, 172). The effect of PEF on host membranes and its dependence on calcium suggested that a parasite-derived phospholipase may be responsible for this activity. Phospholipase A2 (PLA_2) activity is commonly used among pathogens for the penetration of host cells (e.g., rickettsiae [181]) and cytolysis (e.g., *Entamoeba histolytica* [130]). When host cells are treated with exogenous PLA_2 from snake venom (*Naja naja*), a morphological disruption of their membranes is observed concurrent with an augmented invasion frequency by *Toxoplasma* (138). Similarly, treating parasites with the irreversible PLA_2 inhibitor *p*-bromophenacyl bromide or the $cPLA_2$-specific inhibitor LY311727 or $AACOCF_3$ reduces penetration without metabolically disabling the parasites (46, 116). This inhibition is specific to the parasite-derived enzyme(s), since pretreatment of host cells does not alter parasite invasion. A soluble phospholipase has been identified in crude lysates of *Toxoplasma* (140), and it is proposed that this enzyme may play a role in the initial events of invasion either through augmenting rhoptry exocytosis or by increasing membrane fluidity in the host plasmalemma during vacuole formation (140).

VACUOLE FORMATION

The Parasitophorous Vacuole Is Distinct from Phagosomes

After intracellular pathogens enter a cell, they must confront or avoid the endocytic apparatus which has evolved to degrade ingested material (including a phagocytosed microbe). When a vacuole is formed by receptor-mediated endocytosis, the lumen of the compartment is rapidly acidified by ATP-driven proton pumps derived from the plasma membrane (88). This acidic environment plays a crucial role in the preparation of the vacuole for fusion with lysosomes bearing hydrolytic enzymes. Since the host cell repertoire of *Toxoplasma* includes professional phagocytes, the parasite must circumvent a variety of additional antimicrobial effectors including reactive oxygen and nitrogen intermediates (31).

The vacuole generated during *Toxoplasma* invasion (83) is

unusual in that it does not acidify (156) or fuse with any cytoplasmic vesicle, even those which are taken up at the time of parasite entry (70, 152). This difference distinguishes the two types of vacuoles that can be observed within professional phagocytes: the phagosome and the PV. When opsonized parasites are phagocytosed, a vacuole is formed that is spacious and noticeably distinct from the more confined PV. This phagosome will rapidly acidify and follow the fate of the endocytic pathway, resulting in the digestion of the internalized parasite (101, 103). To prevent acidification and endocytic processing, the PV formed by parasite invasion must either exclude or rapidly eliminate the proton pumps and surface proteins that are used for vesicular fusion. This proposed activity is supported by the observation that a newly formed PV is smooth and almost completely free of intramembranous particles (44).

The site where protein exclusion is most likely to occur is at the moving junction (Fig. 5), which is observed only during invasion, not in host-mediated phagocytosis. This structure was best defined in malaria during the erythrocytic invasion (4) and represents a bridge between the parasite and host membranes. In freeze-fracture electron microscopy, the junction appears very tight with a crystalline array of lipids in the host plasmalemma that may be responsible for preventing the diffusion of proteins into the PV membrane (43). The exclusive nature of this structure has been demonstrated in two ways. First, antigen-antibody complexes, but not uncoupled antigen, are shed from the parasite surface as it passes through the moving junction (45). Second, host plasmalemma proteins are selectively excluded depending on the nature of their membrane association: GPI-anchored proteins pass through the moving junction and associate with the nascent vacuole, whereas transmembrane-anchored proteins are excluded from it (100). The actual structure and composition of the moving junction are poorly understood due to its transient appearance in both *Plasmodium* and *Toxoplasma* (for which invasion is complete within 15 to 30 s).

An alternative explanation for the absence of host proteins in the PV is that the parasite provides much of the membranous material for this vacuole after punching a hole in the host plasmalemma. Electron micrographs of early invasion events show rhoptries releasing membranous whirls which may be responsible for extending this membrane into the cytoplasm (107). Additional evidence for this model comes from observations of aborted invasion events by parasites which have been paralyzed with cytochalasin D (24). Although these parasites were prevented from penetrating the host, small vacuoles were still formed at the point of contact between the apical end of the parasite and its host. Immunoelectron microscopy demonstrated that these empty vacuoles contain proteins secreted from the rhoptries.

To determine the relative contribution of these two potential sources of the newly formed PV, invagination of host plasmalemma versus parasite-derived, time-resolved capacitance measurements were used to measure the host cell surface area during parasite invasion (169). Since the nascent PV appears to be continuous with the host membrane during the invasion event (43), extension of this membrane by adding parasite-released lipids would be expected to increase the host surface area. Conversely, if the vacuole was derived from host plasmalemma, the surface area of the host cell should be measurably reduced once invasion is complete and the vacuole has budded off. The result was that no significant change in capacitance was seen during invasion but a marked decrease occurred after the vacuole had budded off. These results indicate that while up to 20% of the vacuole may be provided by the parasite during penetration, the majority of the initial membrane is derived from the host cell. These electrophysiology experiments also indicated that one of the earliest events in the invasion process is a brief spike of conductance between the inside and outside of the host cell. This may represent a transient permeabilization of the host plasma membrane, perhaps involving the PLA_2 activity discussed above, although no data to suggest a role for this enzyme have yet been obtained.

Postinvasion Secretory Events Further Modify the Parasitophorous Vacuole

After the vacuole has formed, a third set of organelles, called dense granules, release their contents from both the anterior and posterior poles of the parasite (24). Multilamellar vesicles, similar to those secreted from the rhoptries during invasion, are secreted from a specialized invagination at the posterior end 10 to 20 min postinvasion (155). These long tubules appear to be responsible for the construction of the spaghetti-like, tubulovesicular network that extends from the PV membrane into the lumen of the vacuole (151). The function of this network is unclear but appears to dramatically increase the surface area of the PV and is associated with at least four dense-granule proteins (GRA1, GRA2, GRA4, and GRA6) (1, 80). Three other dense-granule proteins, GRA3, GRA5, and GRA8, localize with the vacuolar membrane and remain segregated from those within the tubular network (22, 112). Although the two membranous structures (vacuole and network) appear to be contiguous in some regions, this apparent partition suggests that they are maintained as separate domains.

The mechanism by which dense-granule proteins go from being soluble within the secretory organelles to having a transmembrane location in the PV membrane or the tubulovesicular network is poorly understood but presumably involves a profound change in their topology. Studies of selected members of this group of proteins show a dependence on certain elements within their sequence, especially within the eventual cytoplasmic domain of GRA4 (71), the transmembrane domain of GRA5 (81), and the amphipathic helices of GRA2 (90).

The PV membrane is a porous structure with a size exclusion limit of ~1,300 Da (142). It has been proposed that GRA3, a soluble protein that oligomerizes and inserts into the PV membrane, may function as the pore-forming complex (112). Since *Toxoplasma* is auxotrophic for purine biosynthesis (114, 144), these pores may be required for the acquisition of purines in the form of host cytosolic ATP. Using mutant host cells that are incapable of purine synthesis, it was determined that parasites do not require or directly utilize the host ATP as an energy source but, instead, hydrolyze the molecule to adenosine for purine salvage (144). An NTP hydrolase (NTPase) has been identified in the lumen of the PV and may be partly responsible for this salvage process (8).

In addition to purine salvage, the PV membrane is responsible for recruiting host mitochondria and endoplasmic reticulum (ER) immediately following invasion (127, 158). These organelles will form an intimate interaction with the PV membrane, covering almost 75% of the vacuolar membrane by 4 h postinfection (158). The PV-associated mitochondria may be physiologically distinct since they label more intensely when stained with rhodamine 123 than do those which are not so associated (170). A similar recruitment of host mitochondria occurs in the vacuole formed during *Legionella pneumophila* invasion of monocytes (67). While this interaction is essential

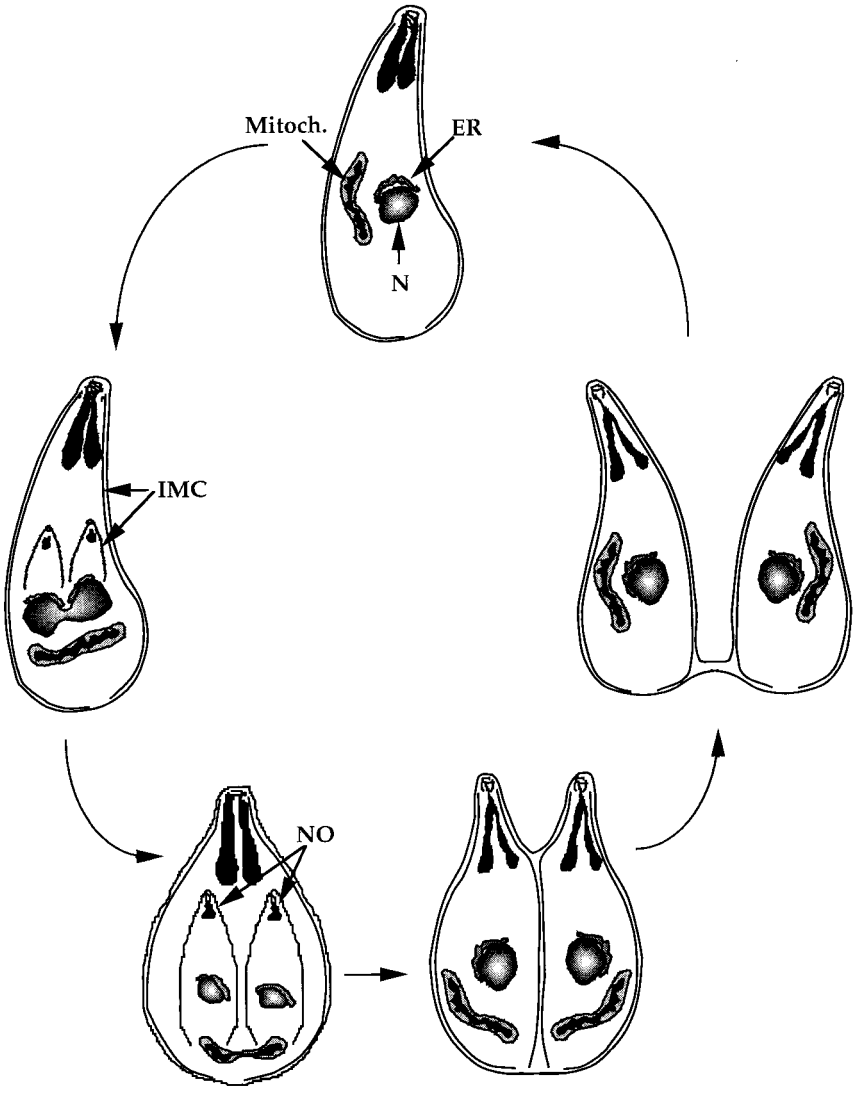

FIG. 7. Diagram of endodyogeny. As a parasite begins to divide, two IMCs begin to develop in the middle of the cell from what appears to be a rudimentary conoid and microtubule-organizing center (polar ring). As the IMC extends from these structures, the nucleus (N) and mitochondrion (Mitoch.) divide into these membranous outlines. Nascent apical organelles (NO) develop within the anterior poles as the daughter cells grow. Eventually, the entire cytoplasm is divided between the daughters and the IMC of the mother dissociates. A cleavage furrow divides the cells from the anterior pole. This division continues down the length of the cells until it reaches the posterior end, where it can leave a residual body connecting the two daughters.

for the intracellular growth of this bacterial pathogen (13), its role in intracellular survival is not known. For *Toxoplasma*, it has been proposed that recruitment of host ER and mitochondria may serve to provide lipids for the PV (174). Similar regions of membrane apposition between the mitochondria and ER have been observed in a variety of cell types and are thought to be sites of lipid trafficking (174, 175). Since preliminary data suggest that *Toxoplasma* may be incapable of de novo fatty acid synthesis (158), this interaction may provide the means for lipid transport to intravacuolar parasites through the tubulovesicular network.

PARASITE REPLICATION

Morphological Examination of Endodyogeny

Once *Toxoplasma* enters a cell, it begins replication by a process called endodyogeny (Fig. 7). This has been defined morphologically by electron microscopy (176, 177), but the cellular signals and machinery responsible for division are relatively unknown except that it is known that endodyogeny is dependent on proper functioning of the microtubules of the parasite much more than on the actin filaments (147).

The onset of endodyogeny is marked by the appearance of two pellicular membranes that begin to form from the middle of the cell. These membranous structures begin to define the daughters within the cytoplasm of the mother cell at points that resemble a rudimentary conoid and microtubule-organizing center (see "Ultrastructure" above). As the inner membrane complex extends from these regions, the mother cell becomes more spherical and apical organelles (i.e., nascent rhoptries and micronemes) are formed at the two anterior poles of the growing daughters. The nuclear envelope remains intact as the nucleus, apicoplast (the plastid-like anterior organelle), and single mitochondrion divide between the daughters. Each cell continues to mature until the cytoplasm and all of its contents are divided between the two progeny; thus, for a time each

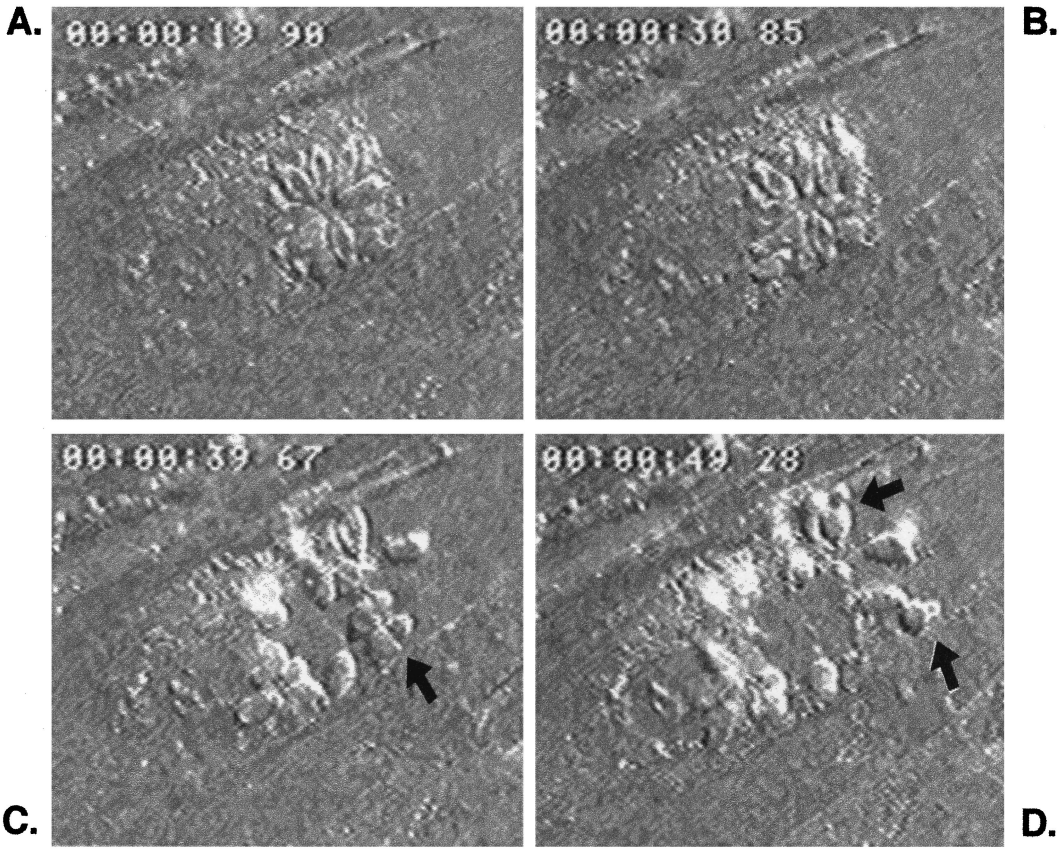

FIG. 8. Ionophore-induced egress. Four video frames of human fibroblasts infected with *Toxoplasma* are shown starting about 30 h postinfection. The first frame (A) shows a typical rosette of 16 parasites that are positioned with their anterior poles pointing away from the center. The clock in the upper left corner of each frame was activated at the time of replacing the medium with Hanks balanced salt solution containing 1 μM A23187 (a calcium ionophore) to induce egress. The frames were obtained at ~10-s intervals (starting at ~20 s postinduction in frame A) to illustrate the speed of the response to A23187. By ~31 s (B), the parasites are beginning to move within the PV, and at ~40 s (C) they are leaving the PV (see arrow) and beginning to cross through the host cytoplasm. The last frame (D), taken at ~49 s postinduction, shows two parasites apparently passing through the cytoskeleton of the host as suggested by the severe constriction on the bodies of the parasites (arrows).

daughter has five membranes between the bulk of its cytoplasm and the lumen of the PV. Eventually, the mother's inner membrane complex disappears and the original plasmalemma is used to envelope the two new cells. A cleavage furrow is initiated at the anterior pole and extends between the daughters throughout most of the cell, leaving only a residual body at the posterior end. This posterior attachment results in the formation of a rosette as the parasites continue to replicate with the separate anterior ends pointing away from the center. The fact that *Toxoplasma* maintains an intact cytoskeletal and organellar network throughout most of endodyogeny could provide it with the ability to immediately exit and invade at virtually any point during intracellular growth. Indeed, ionophore-induced egress will cause >99% of parasites in asynchronous culture to exit the host cell, and most appear well able to immediately reinvade (see below) (49).

Toxoplasma Cell Cycle

Examination of *Toxoplasma* endodyogeny, beyond the level of morphological observations, requires the synchronization of the cell cycle. A strategy that is routinely used for synchronizing mammalian cells is based on the accumulation of dTTP following thymidine treatment in cells that express thymidine kinase (164). This increase leads to a dTTP-specific inhibition of ribonucleotide reductase activity and a concurrent depletion of dCTP during DNA synthesis, leading to a reversible cell cycle arrest at the onset of the S phase. Since *Toxoplasma* does not carry the thymidine kinase gene, a strain that expresses the herpes simplex virus thymidine kinase has recently been engineered specifically for the purpose of synchronization (129). This recombinant strain demonstrates the expected late-G_1-, early-S-phase arrest when thymidine (10 μM) is added to the medium. If thymidine is removed after a 4-h block, the arrest is reversed and the newly synchronized population immediately enters the S phase. By harvesting this population at different time points after removing the thymidine block, each stage of the cell cycle can be determined by relative fluorescence using propidium iodide stain and fluorescence-activated cell sorter analysis (30). It was estimated that the length of S is ≤2 h, that of G_2/M is <1 h, and that of G_1 is ~4 h, for a total cell cycle of >7 h (129). Although the process of endodyogeny is clearly distinct from mitosis of other eukaryotes, the relative timing of the major phases in *Toxoplasma* appears similar to that observed in other organisms (18).

PARASITE EGRESS

Egress Is a Rapid, Cytolytic Event

As with the process of invasion, intracellular organisms have evolved a variety of mechanisms for egress and transmission to neighboring cells. For *Toxoplasma* grown in vitro at least, egress is a rapid event that results in the lysis of the host cell

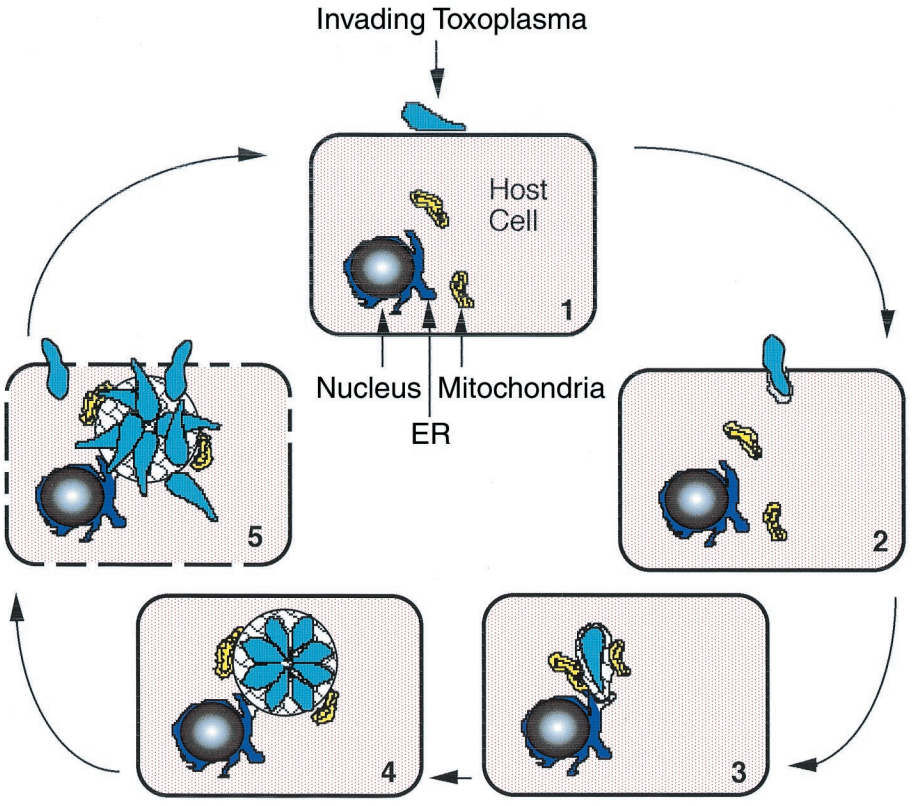

FIG. 9. The *Toxoplasma* lytic cycle. This diagram depicts the five events that are discussed in the text which are responsible for the acute phase of toxoplasmosis. The first event is attachment of *Toxoplasma* to its host cell (step 1). This step may involve two forms of contact: an initial transient interaction that signals microneme secretion followed by a firm adherence that utilizes the transmembrane micronemal proteins (e.g., MIC2). As the parasite glides across the surface, it is reoriented to make contact with the host surface at its apical end and to initiate invasion (step 2). During the invasion event, the PV is formed and modified by secretion from both the rhoptries and, later, the dense granules. After the parasite enters its host, it stops moving and the vacuole closes at its posterior end by pinching off via a fission pore. The newly formed PV immediately recruits host mitochondria and portions of the host ER (step 3). This close association between host organelles and the PV is likely to play a role in acquiring essential nutrients (possibly lipids) for intracellular growth. The parasites undergo several rounds of replication (step 4) until they receive a signal from either the parasite itself or the host cell, resulting in egress (step 5). Host cell egress is typically a destructive process that lyses the host cell and releases motile parasites. These parasites quickly invade neighboring cells to complete the cycle.

and release of very motile parasites. By video microscopy, a synchronized mass exodus of all the intracellular parasites within a single host can be seen passing through the PV membrane, the host cytoplasm, and finally the plasmalemma. In Fig. 8, video frames of ionophore-induced egress are shown (described further below). As the parasites pass through each of the membranes, an obvious constriction runs down the body of the parasite, resembling the junction ring observed during invasion. Although there have been no reports of secretory events associated with *Toxoplasma* egress, the merozoite stage of *Plasmodium* appears to secrete from their rhoptries just prior to erythrocyte exit (29, 115). If micronemal proteins are indeed required for *Toxoplasma* motility (see "Candidate bridge molecules between the exterior and the actomyosin motors" above), it would be expected that these organelles (and possibly rhoptries) are induced to exocytose during egress. Once outside the confines of the lysed host cell, the parasites will quickly invade neighboring cells and halt after they are enveloped by their new vacuole, thus completing the lytic cycle (Fig. 9).

Calcium Acts as a Signal for Egress

While the actual stimulus that signals the timing of natural egress is unknown, Ca^{2+} has been shown to play a major role in the activation of this process (49, 142). The relationship between calcium and egress was first demonstrated using the calcium ionophore A23187 on infected murine macrophages (49). This drug will increase the permeability of membranes for the passive diffusion of divalent cations (preference for Ca^{2+}) along a concentration gradient (126). It was observed that intracellular parasites which are exposed to this drug would rapidly leave their host cells in a manner resembling that of natural egress. This ionophore-induced egress appears to be a destructive event for the host cell, resulting in the formation of membranous blebs and lysis (observed even in cells infected by single parasites [49]). This cytolysis is dependent on viable intracellular parasites, since uninfected macrophages and those that had phagocytosed dead parasites were not affected by the ionophore.

Upon closer examination, it appears that during egress, the parasite pushes the host plasmalemma out from the surface, much like "invading" out of the cell (49). Eventually this membrane is ruptured as the parasite escapes, resulting in host cell lysis. Nonspecific effects of A23187 (e.g., pH shifts resulting from the exchange of protons for Ca^{2+}) do not appear to be responsible for ionophore-induced egress, because it is specifically blocked by chelation of intracellular Ca^{2+} with BAPTA-AM (a cell-permeable Ca^{2+} chelator [M. W. Black and J. C. Boothroyd, unpublished results]) and can also be induced by the direct microinjection of Ca^{2+} into infected cells (142).

Investigation of the influence of elevated extracellular [Ca^{2+}] on *Toxoplasma* has uncovered a putative polyvalent cation receptor similar to those observed in kidney cells (9). In mammalian cells, receptors such as these function via coupling to heterotrimeric GTP-binding proteins (G-proteins) which influence the regulation of Ca^{2+} channels by the generation of inositol 1,4,5-trisphosphate (19). Although there is evidence for such G-proteins in *Toxoplasma* (63), there is no information about their function or association with such a receptor.

Dithiothreitol Acts as a Signal for Egress

In addition to directly elevating host cytosolic [Ca^{2+}], *Toxoplasma* egress has been induced by treating infected monolayers with dithiothreitol (DTT) (157, 166). Infected cells treated with this reducing agent demonstrated a 15 to 50% increase in the level of cytoplasmic Ca^{2+} immediately before the activation of parasite motility and egress (166). This peculiar response to DTT appears to be dependent on both the host and parasite, since DTT will not increase the cytoplasmic [Ca^{2+}] of uninfected cells or the motility of extracellular parasites. Although the calcium flux and parasite egress could be induced using other dithiol reducing agents, including dithioerythritol and to a lesser extent dimercaptopropanol, there was no response to monothiols such as 2-mercaptoethanol (166).

This preference for dithiols directly correlates with data on the in vitro activation of parasite vacuolar NTPases (7, 157). In the virulent RH strain, there are two isoforms of this enzyme called NTPase 1 (equally active against NTP and NDP) and NTPase 3 (specific for NTP) (7). They function as an apyrase, degrading ATP to ADP and AMP, and are expected to be involved in purine salvage of host cytosolic ATP that diffuses into the vacuole (see above). An unusual feature of *Toxoplasma* NTPases is that they are each equipped with 15 cysteine residues that require dithiol reducing agents for in vitro activation. This DTT-dependent form of NTPase is unique to *Toxoplasma* among the Apicomplexans, and its role in intracellular parasitism is not understood (6). It is estimated that less than 5% of the enzyme expressed within the PV is functional during intracellular growth. A plausible compensation for this inactivity is the gross excess of NTPase that is secreted into the vacuolar space; a sufficient amount is secreted from a single parasite to deplete the entire pool of host ATP in a matter of minutes if activated (157). Infected host cells treated with DTT demonstrated a dramatic increase in ATP degradation that correlated with the induction of parasite egress (157). As seen in the DTT-induced calcium flux, degradation of ATP was observed only in parasite-infected cells.

While the connection between NTPase activity and cytoplasmic [Ca^{2+}] is unclear, a candidate may be the Ca^{2+}-ATPase channels found within host ER and mitochondria. When ATP is depleted, these cation pumps would not be expected to prevent the release of Ca^{2+} into the cytosol. Interestingly, activation of *Toxoplasma* NTPases also appears to be triggered by protein-protein interactions, as determined by their spontaneous activation during immunoprecipitation (157). It has been postulated that NTPases may use this trait as a novel timing mechanism for the induction of a burst of activity upon reaching a critical threshold. As observed with DTT, this enzymatic activation could result in (i) the rapid degradation of host ATP, (ii) a flux in cytosolic [Ca^{2+}], and (iii) parasite egress (157, 166).

INFLUENCE OF CA^{2+} ON OTHER ASPECTS OF *TOXOPLASMA* GROWTH

Rearrangement of the *Toxoplasma* Cytoskeleton

Although Ca^{2+} homeostasis in *T. gondii* has not been well characterized, it is clear that its regulation is key for several cellular activities. Three distinct intracellular Ca^{2+} stores have been identified in this parasite including the ER, mitochondria, and an unusual acidic compartment called the acidocalcisome (102). Using the calcium ionophores A23187 and ionomycin, it was determined that Ca^{2+} release from one or more of these organelles results in the forward protrusion of the conoid (97). Although the presence or absence of extracellular Ca^{2+} in the medium had little influence on protrusion, when parasites were pretreated with BAPTA-AM to chelate intracellular Ca^{2+}, ionophore induction of conoid extrusion was almost completely abolished. This BAPTA-AM paralysis was reversed by adding excess (1 mM) $CaCl_2$ to the medium containing the ionophore, indicating that elevation of cytosolic [Ca^{2+}] is directly responsible for conoid extrusion. As predicted by these results, Ca^{2+} fluxes also stimulate the discharge of micronemes, an event closely tied to conoid protrusion (23).

Parasite Motility and Invasion

The gliding motility of *Toxoplasma* appears to be activated by elevated cytosolic [Ca^{2+}] levels. When extracellular parasites are exposed to calcium ionophores (97) or treated with low concentrations of trypsin in the presence of calcium and ATP (98), they begin to exhibit the characteristic gliding motility. Trypsinization of these parasites appears to briefly permeabilize their plasma membrane, resulting in the diffusion of Ca^{2+} and ATP into (and presumably out of) the cytosol. This is supported by the fact that parasites trypsinized in Ca^{2+}- or ATP-free medium did not become motile. The requirement for intracellular Ca^{2+} was demonstrated by the BAPTA-AM inhibition of gliding in both the trypsinization induction and ionophore induction procedures. As one might expect, pretreatment of extracellular parasites with BAPTA-AM also resulted in a >90% reduction in host cell invasion compared to untreated controls (97). Interestingly, the same study revealed that invasion was inhibited almost fourfold when parasites were briefly treated with ionomycin compared to untreated controls. Although it is possible that this is a consequence of exhausting the resources required for motility and invasion, little is known about the overall effect of calcium ionophores on *Toxoplasma*. The unconventional myosins found in this parasite (see "Ultrastructure" above) represent possible targets for the Ca^{2+}-dependent control of conoid extrusion and gliding, since both these processes involve actomyosin motors.

Intracellular Survival and Replication

While the influence of [Ca^{2+}] on intracellular growth has not been directly examined in *Toxoplasma*, a synthesized drug that blocks calcium import across the host plasmalemma (L-651,582) has been found to effectively inhibit intracellular growth (68). This dependence on host [Ca^{2+}] or Ca^{2+}-dependent pathways is consistent with data obtained concerning the antiparasitic effects of Ca^{2+} and calmodulin antagonists in *P. falciparum* (141). An investigation of host cytosolic [Ca^{2+}] in *Toxoplasma*-infected cells demonstrates that the PV imports Ca^{2+} from the host cytoplasm (122). After 48 h of intracellular replication, a significant decrease in the level of host cytosolic Ca^{2+} was observed compared to that in uninfected cells. Since the porous PV would allow free diffusion of Ca^{2+} across the

membrane, these data suggest that products secreted or imported into the vacuole bind Ca^{2+}. A candidate for this activity is the 23-kDa dense-granule protein GRA1 (25). This protein has a high affinity for Ca^{2+} and is closely associated with the tubulovesicular network of the PV. The stability of this network appears to be calcium dependent and can be recovered from intracellular parasites only in the presence of 1 mM Ca^{2+} (151). Other than this apparent stabilization, no functional roles have been ascribed to vacuolar sequestration of host Ca^{2+}.

CONCLUSION

In this review, we have described the current knowledge about the lytic cycle of the asexual, tachyzoite form of *T. gondii*. While many of the basic phenomena are beginning to be well described, relatively few of the molecules involved have been identified. Many of the most important questions remain unanswered. For example, which specific receptor-ligand interactions mediate the attachment and invasion processes? What is the mechanism by which gliding motility is used to actively penetrate the host cell? What functions do the many novel proteins found within the various secretory compartments of the parasite (micronemes, rhoptries and dense granules) serve in this cycle? What is the role of the spaghetti-like network elaborated inside the parasitophorous vacuole? Which critical molecules enter (or exit?) via the micropore? And, last but not least, which signals lead to egress? This is no simple agenda, but the intricacies are likely to be as interesting as they are important.

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