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MINIREVIEW

Toxoplasma gondii: a Protozoan for the Nineties

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INTRODUCTION

Toxoplasma gondii is growing in popularity as a model for studying intracellular parasitism (17). The parasite has also gained more attention recently as the most common cause of focal central nervous system infections in patients with AIDS (15) and continues to cause more than 3,000 congenital infections per year in the United States alone.

T. gondii infects nearly all animals and most birds and is one of the most widely distributed of all intracellular parasites. In vitro, tachyzoites of *T. gondii* can invade and replicate within essentially all nucleated cells, an unusual feat for any intracellular organism. The intracellular tachyzoites reside within a vacuole which is incapable of acidifying or fusing with any membrane-bound organelle within the host cell endocytic system (11–13, 28) and as such is effectively hidden from the host; yet the parasite replicates rapidly. In contrast, the encysted bradyzoite stage can lie dormant within tissues for decades, apparently hidden from the host's immune system and from potentially effective antimicrobial therapy, only to emerge when host immunity wanes. What do we know about these rather prodigous accomplishments, what are we and what should we be trying to learn, and where are we likely to succeed or fail? We will attempt to answer these questions by dividing the discussion into separate areas: attachment and invasion, cytoskeletal proteins and motility, parasitophorous vacuole membrane (PVM) formation, intracellular development, and differentiation. This perspective will not attempt to cover the immunologic response to *T. gondii* or to deal with new diagnostic or therapeutic approaches to this parasite. Nonetheless, these areas interdigitate with the cell biology of *T. gondii* intracellular infection.

Several advances necessary for more rapid progress are self-evident. These will be alluded to again below but are mentioned here for emphasis. Development of a transformation system for tachyzoites (see Addendum in Proof), identifying a means to grow tachyzoites axenically, and determining methods for growing bradyzoites in vitro in high yield stand out in importance in comparison with most other considerations. Essentially every issue discussed below could be pursued more rapidly and in more depth should one or more of these fundamental advances be made.

ATTACHMENT AND INVASION

The morphological steps involved in *T. gondii* cell attachment and invasion were first described more than 30 years ago (reviewed in references 25 and 31). The processes are similar for *T. gondii* and for other members of the phylum Apicomplexa which cause human and animal diseases, including *Plasmodium*, *Eimeria*, *Cryptosporidium*, and *Sarcocystis* species. Invasion is likely to involve the surface motility of these organisms and the exocytosis of specific organelles located in a characteristic structure named the apical complex. Nonetheless, the mechanism of these processes and the molecules involved are largely unknown.

Attachment. Until recently, limited efforts were expended in identifying parasite ligands and cell surface receptors mediating attachment of *T. gondii* to host cells. Recent work has suggested that a major parasite surface protein, P30, may be involved (9), that parasites may bear surface lectin-like molecules (20), and that host cell laminin bound to the parasite participates in attachment (7, 8). In the last situation, host cell receptors for the parasite-bound laminin were identified. These conclusions have been drawn largely from antibody or peptide inhibition experiments. Until binding of a secreted or membrane-bound parasite molecule to host cells is documented, or mutant parasites or cells blocked in the attachment process are characterized, the conclusions from these experiments remain unproven.

Whether any of these approaches will lead in the near term to a fundamental understanding of the attachment process is unclear. It remains possible that a single dominant attachment mechanism is operative, but extrapolation from other systems suggests that multiple mechanisms for parasite attachment exist. In accord with this idea, a *T. gondii* mutant lacking P30 is reported to attach normally to cells. *T. gondii* is capable of attaching to all nucleated cells, and the primary attachment process probably varies depending upon host cell origin. If the ultimate goal of identifying attachment ligands is to design intervention aimed at blocking attachment, such an approach is likely to fail.

Invasion. More productive investigations will involve investigation of parasite components necessary for invasion, since these constituents will probably be common to invasion of all cells. Invasion can be envisioned as a complex cascade of individual events: attachment, protrusion of the conoid, moving junction formation, microneme exocytosis, rhoptry exocytosis, and finally, gliding in the vacuole, each event triggering the next in a sequence which is not yet clearly known. The entire process of invasion is complete in less than 10 s, an order of magnitude faster than a typical phagocytic event.

A reasonable starting hypothesis is that the organelles and organellar proteins involved will be fundamentally similar in toxoplasmas and in other members of the phylum Apicomplexa. Two types of organelles at the anterior end of the parasite are likely to be involved, rhoptries and micronemes. Rhoptries are large club-shaped anterior organelles with a slender duct through which organellar contents are discharged at the time of invasion into cells. Micronemes are

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smaller, vesicular organelles which may also discharge immediately preceeding or concomitantly with invasion. Triggering mechanisms for rhoptry or microneme discharge are unknown—spontaneous discharge from extracellular parasites is not observed, and no inducing signals are known. Although cell attachment is apparently a necessary signal for organelle discharge, it is probably not sufficient.

A central question is whether rhoptry components are critical only during invasion (for example by altering the plasma membrane or cytoskeleton of the host cell) or whether these components play some more protracted role in maintaining the PVM or vacuolar space. At least 10 proteins of *T. gondii* rhoptries are identified by monoclonal antibodies (14). ROP1, a 61-kDa *T. gondii* rhoptry protein (23, 24), previously termed penetration-enhancing factor, was first identified functionally almost 30 years ago (16). The deduced amino acid sequence of ROP1 predicts an unusual charge asymmetry, with a highly acidic, proline-rich amino-terminal domain and a strongly basic carboxy-terminal domain (18). A reasonable hypothesis, based on primary structure and limited sequence homology with acidic proline-rich salivary proteins which mediate heterotypic complexing, is that ROP1 participates in attachment. ROP1 initially lines the vacuolar space but disappears as the vacuole matures, obviating a function in intracellular survival. The functions of the other *T. gondii* rhoptry proteins, several of which have been cloned, remain obscure. Lipids are also found in rhoptries, and they could participate in vacuole formation, as discussed below. Characterizing the distribution and transmembrane orientation of rhoptry components within the PVM at various times after invasion should provide initial clues as to the function of these molecules. These clues can be followed with more definitive experiments.

Microneme proteins are also likely to participate in *T. gondii* invasion, based on analogy with their involvement in *Plasmodium* invasion events (2). Homologs of another group of apical *Plasmodium* invasion proteins of uncertain organelle localization (PK66 and p83) (30) may also be found in *T. gondii*. The most problematic issue experimentally, however, will be to identify the precise timing of microneme discharge and the mechanism by which the microneme proteins facilitate invasion.

The complexity and the speed of invasion hamper investigation of the process. The inability to grow the parasite under cell-free conditions precludes any simple method to isolate invasion mutants. Hence, chemical mutagenesis will not be an easy way of investigating invasion, whereas deletion of genes encoding organelle proteins, when technically available, should allow dissection of their respective roles in the process. At present, efforts must necessarily be limited to categorizing and localizing the involved components.

CYTOSKELETAL PROTEINS AND MOTILITY

Parasite motility is required for tachyzoite invasion of cells (reviewed in reference 25). Although microtubules are the most prominent morphologic feature of the tachyzoite cytoskeleton, microfilament but not microtubule inhibitors block parasite invasion. Myosin is confined to the apical end of the parasite, and actin has either been localized to the apical end or found diffusely distributed throughout the cytoplasm. It is likely that these components are involved in protrusion of the conoid associated with initiation of invasion, while the bulk of the motility may rely on a system organized along stably polymerized microtubules not inhibited by conventional microtubule inhibitors. The corkscrew motion seen during parasite entry matches the orientation of the subpellicular microtubules, suggesting that a linkage between the parasite surface and the microtubules exists. In fact, microtubules are likely to be the frame on which a motility system is organized: the arrays of intramembranous particles found in the inner membrane complex underlying the plasmalemma may constitute the subunits of the motor along which transmembrane adhesive molecules are translocated. The connection between surface components and the cytoskeleton would necessarily be complex given the presence of three membranes overlying the microtubule complex. Identification and localization of *T. gondii* homologs of dynein and kinesin and of other components of microtubule-based motors should provide initial clues about parasite motility. The most promising approach for defining the components involved, however, is likely to be in vitro reconstruction of motility using isolated parasite cytoskeletal elements.

PVM FORMATION

One of the most enigmatic issues surrounding invasion is the origin and composition of the PVM. This specialized membrane, which lacks plasma membrane markers from the host cell, is thought to account for the irreversible fusion incompetence of the parasitophorous vacuole, presumably because it lacks any signals for fusion with other compartments. Freeze fracture analysis indicates that few if any intramembranous particles are found in the PVM immediately after invasion; hence, the newly formed PVM may represent essentially a phospholipid bilayer.

A central issue is whether the lipids necessary for initial formation of the PVM are host or parasite derived. Fluorescent lipid probes are being used to examine this problem by labeling either parasite or host cell lipids. Relatively specific labeling of rhoptries has proven difficult, lipid flip-flop between membranes complicates interpretation, and ultimate resolution of the issue will probably depend upon lipid analysis of purified PVM. The lipid content of *T. gondii* rhoptries is unusual (6) and might be reflected in a unique lipid composition of the PVM (10). At this time, purification of the PVM to the degree and in the amount required for this type of analysis is not feasible.

A *T. gondii* phospholipase has been suggested to play a part in the invasion process, possibly by altering membrane fluidity in the target cell (21, 22). This conclusion is based largely on the use of phospholipase inhibitors and will remain conjectural until mutants lacking the enzyme are constructed or until a more tractable invasion system is established.

INTRACELLULAR DEVELOPMENT

The parasitophorous vacuole space and membrane are extensively modified after *T. gondii* invasion. Parasite proteins associate with the PVM and with a tubuloreticular network within the vacuolar space (26, 27) and are mostly derived from the parasite dense granules, a third type of parasite secretory organelle (in addition to rhoptries and micronemes). To date, five dense granule proteins have been identified by monoclonal antibodies, GRA1 to -5. Partial or complete cDNA sequences are available for all five molecules and provide few clues as to function. Three of these

proteins (GRA1, -2, and -4) associate predominantly with the intravacuolar network, whereas the remaining two (GRA3 and -5) associate more specifically with the PVM (1, 3). Neither the signals for dense granule exocytosis nor the protein signals associated with GRA targeting events are understood. Although traffic of dense granule or other *T. gondii* proteins into the cytoplasm of the host cell has not been reported, such events are being actively sought by investigators studying antigen processing in *T. gondii*-infected cells.

No functions can yet be assigned to proteins present within the parasitophorous vacuole space and/or membrane. It is likely that some of these proteins are necessary for acquisition of nutrients from the host cell, by having roles in either transport or enzymatic modification of host cell constituents. Alternatively, or in addition, the dense granule proteins may play predominantly a structural role as components of a nascent cyst wall (discussed below). The best approach to define the function of the dense granule proteins is unclear. Construction of parasite mutants may ultimately provide the most broad-ranging approach but would likely require specific complementation with excess amounts of either known or suspected nutrients to link the mutation with a function (analogous to the identification of auxotrophic mutants in bacteria). The potential problems inherent in delivering these nutrients from the extracellular milieu across the cell plasmalemma, the PVM, and the parasite plasma membrane are self-evident. The observation that addition of exogenous tryptophan to gamma interferon-treated cells rescues the parasites suggests, however, that such an approach is feasible (19). Targeted searches for molecules known to be involved in salvage of needed nutrients, such as purines, may provide more information in the short run.

Although the identification of media for growth of the parasites axenically would facilitate the study of *T. gondii* biology in general by directly facilitating development of a transformation system, the most immediate benefits of such an advance would be to define the metabolic and nutritional requirements of the parasite for intracellular growth. Further elucidation of the secreted, surface, or intracellular components of the parasite needed for acquisition of these nutrients could then proceed in a more directed fashion. Although this area has been explored to only a limited extent with toxoplasma and other coccidian parasites, it is likely that *T. gondii* requires monosaccharides, lipids, and amino acids in addition to the proven requirement for purines. Extracellular cultivation may also facilitate efforts to synchronize the mitotic cycle of the parasite, permitting study of organelle biogenesis during endodyogeny.

Equally perplexing as those events associated with entry into the cell are the processes involved in liberation of parasites from infected cells. The observation that the calcium ionophore A23187 induces rapid release of parasites from infected cells (5) implicates calcium in release, but whether this calcium is derived from cellular or parasite stores has not been determined. One approach which should allow a clearer elucidation of host factors involved in the process is the use of permeabilized cells. We have found that permeabilizing the plasma membrane of infected cells at 15°C with streptolysin O results in immediate release of parasites when the cells are warmed to greater than 18°C in typical intracellular buffer. Manipulation of buffer conditions to prevent release should be possible.

DIFFERENTIATION

All apicomplexan parasites undergo several rounds of differentiation during their life cycle. Nonetheless, the key events driving differentiation and control of gene expression during the process are almost completely uninvestigated. The bradyzoite-tachyzoite interconversion in *T. gondii* is an ideal model for study, given the limited morphological differences between the stages and the likelihood that the process can be manipulated in vitro. Tachyzoite-bradyzoite interconversion is also becoming a major clinical concern because of its involvement in toxoplasmic reactivation in patients with AIDS. Although the immune response has classically been considered as driving formation of bradyzoites in vivo, several recent reports indicate that bradyzoites form in vitro in the absence of any effector of immunity (4). Stage-specific probes are now available to distinguish between tachyzoites and bradyzoites (29). These probes will facilitate analysis of factors such as host cell type, culture medium composition, and culture conditions which induce in vitro bradyzoite formation. The development of an in vitro system for bradyzoite formation constitutes one of the most important goals in studying *T. gondii* biology.

Important cell biological questions which can be approached by using an in vitro system for bradyzoite formation include the following. (i) Is there a connection between in vitro cyst formation and in vivo virulence? (ii) Are the signals for bradyzoite formation derived from the parasite or the host cell? (iii) Do parasite strains which vary in the propensity to form cysts respond differently to the same host cell signals? (iv) What is the composition of the cyst wall and how do these components participate in nutrient exchange (of note, recent results suggest that dense granule proteins are major components of the cyst wall)? (v) Is the difference between vacuole and cyst initiated upon invasion or at some point thereafter; i.e., are two types of vacuoles formed at the time of invasion or is a single vacuole type formed which can later transform into a cyst upon metabolic changes in the host cell or in the host cell environment? (vi) What factors control breakdown of cysts and liberation of bradyzoites? Equally important questions involving bradyzoite-specific gene expression, host immunity to bradyzoites/cysts, and chemotherapeutic eradication of bradyzoites/cysts will also be resolved naturally from success in generating in vitro cysts.

CONCLUSION

Among apicomplexan parasites, *T. gondii* provides many advantages for investigation of common invasion mechanisms, processes of parasite motility, mechanisms for formation and modification of the PVM, and shared features of stage-specific differentiation. However, as described above, major technical limitations impede rapid progress in these areas. Increased research initiatives with *T. gondii* and the resultant methodologic advances will provide basic knowledge relevant to all members of the Apicomplexa and consequently to the array of diseases caused by these organisms.

ADDENDUM IN PROOF

Since this paper was submitted, a system for DNA transformation of *T. gondii* has been developed in the laboratory of John Boothroyd at Stanford University. Chloramphenicol acetyltransferase, as well as the major surface protein SAG1

(P30), has been transiently expressed under the control of either *T. gondii* or *Trypanosoma brucei* promoters (D. Soldati and J. Boothroyd, Science, in press). Stable transfection of parasites has also been obtained by two alternative strategies: (i) chloramphenicol acetyltransferase has been used as a selectable marker, and (ii) a SAG1-minus mutant has been rescued by introduction of the SAG1 gene (K. Kim and J. Boothroyd, personal communication).

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