

Microtubules, but not actin filaments, drive daughter cell budding and cell division in *Toxoplasma gondii*

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SUMMARY

We have used drugs to examine the role(s) of the actin and microtubule cytoskeletons in the intracellular growth and replication of the intracellular protozoan parasite, *Toxoplasma gondii*. By using a 5 minute infection period and adding the drugs shortly after entry we can treat parasites at the start of intracellular development and 6-8 hours prior to the onset of daughter cell budding. Using this approach we found, somewhat surprisingly, that reagents that perturb the actin cytoskeleton in different ways (cytochalasin D, latrunculin A and jasplakinolide) had little effect on parasite replication although they had the expected effects on the host cells. These actin inhibitors did, however, disrupt the orderly turnover of the mother cell organelles leading to the formation of a large residual body at the posterior end of each pair of budding parasites. Treating established parasite cultures with the actin inhibitors blocked ionophore-induced egression of tachyzoites from the host cells, demonstrating that

intracellular parasites were susceptible to the effects of these inhibitors. In contrast, the anti-microtubule drugs oryzalin and taxol, and to a much lesser extent nocodazole, which affect microtubule dynamics in different ways, blocked parasite replication by disrupting the normal assembly of the apical conoid and the microtubule inner membrane complex (IMC) in the budding daughter parasites. Centrosome replication and assembly of intranuclear spindles, however, occurred normally. Thus, daughter cell budding per se is dependent primarily on the parasite microtubule system and does not require a dynamic actin cytoskeleton, although disruption of actin dynamics causes problems in the turnover of parasite organelles.

Key words: *Toxoplasma gondii*, Cytoskeleton, Actin, Microtubule, Cell division

INTRODUCTION

In eukaryotes, cell division is a complex, highly integrated process involving chromosome replication and segregation during mitosis, as well as the partitioning of the mother cell into two daughter cells during cytokinesis. Although there are fundamental differences in the details of cell division (particularly in the process of cytokinesis) between animal and plant cells, the process involves the spatially and temporally co-ordinated participation of the actin and microtubule cytoskeletons. Thus, reagents that perturb the proper functioning of the cytoskeleton disrupt cell division. For example, drugs that disrupt actin dynamics, such as cytochalasin, prevent cytokinesis in animal cells by blocking the formation and/or functioning of the contractile ring in the cleavage furrow, whereas microtubule antagonists block the assembly of the mitotic spindle preventing chromosome separation (Rappaport, 1996).

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects a wide range of vertebrate hosts including man (Joiner and Dubremetz, 1993) and is a major cause of death in AIDS patients. Tachyzoites invade host cells and become established in a highly modified parasitophorous

vacuole (Lingelbach and Joiner, 1998; Mordue et al., 1999). After an initial period of growth, the parasite undergoes multiple rounds of replication and eventually egresses from the host cell to invade other cells, thereby initiating another round of growth and replication. Unlike most eukaryotes, cell replication in *Toxoplasma* occurs by a highly polarized internal budding process (see Chobotar and Scholtyseck, 1982). Budding is initiated close to the nucleus in association with a pair of cytoplasmic centrosomes, which form the poles of the intranuclear spindle. During each round of replication the parasite forms two new dome-shaped conoids, each with an associated inner membrane complex (IMC; a pair of closely apposed membranes that form a discontinuous set of sheets immediately beneath the outer plasma membrane) and microtubules, and a new complement of secretory organelles. The microtubule IMC extends posteriorly around each pole of the nucleus, eventually dividing the nucleus in two. While much of the mother cell cytoplasm and organelles are incorporated into the two daughter cells, other parts (the apical conoid complex and associated secretory organelles, and parts of the subpellicular cytoskeleton) disappear and are presumably broken down and recycled by the parasite.

On the basis of morphological studies, microtubules play a

central role in the biology of *Toxoplasma* providing an essential part of the subpellicular cytoskeleton (the array of 22 microtubules associated with the IMC) (Nichols and Chiappino, 1987). In addition, during cell replication microtubules form the intranuclear mitotic spindle and the formation and internal growth of the two daughter cells during the budding process is characterized by the assembly and subsequent elongation of the microtubule IMC (Chobotar and Scholtyseck, 1982). What role the actin cytoskeleton plays in the budding process is not known. In fact, little is known about the organization of the actin cytoskeleton. Biochemically tachyzoites contain a significant amount of actin, although the majority appears to be in the monomeric G-form (Dobrowolski et al., 1997) and actin filaments have only recently been observed (Shaw and Tilney, 1999).

In the present study we have used a range of cytoskeletal antagonists to investigate the role(s) of actin and microtubules in the process of daughter cell budding in *Toxoplasma* tachyzoites. A major problem when examining the effects of inhibitors on established intracellular parasites is that the parasites are generally not synchronised with regard to their cell cycle. This makes it difficult to accurately assess the effects of inhibitors on cell cycle progression and parasite replication. To overcome this problem we used a short infection period, and by adding the reagents soon after invasion, we can treat parasites at the start of intracellular development and many hours prior to the onset of daughter cell budding. Using this approach we found that, somewhat surprisingly, actin antagonists did not prevent parasite growth and replication even though these reagents had profound effects on the host cells. While actin antagonists did not directly inhibit daughter cell budding they did disrupt part of the budding process, resulting in the dumping of mother cell organelles (rhoptries, micronemes, apicoplast, part of the mitochondrion) into residual bodies at the posterior of each pair of dividing parasites. In contrast, anti-microtubule drugs (oryzalin, taxol and to a much lesser extent, nocodazole), which affect microtubule dynamics in different ways, blocked parasite replication by disrupting conoid formation and the assembly of the microtubule-IMC in the budding daughter parasites. However, centrosome replication and the assembly of intranuclear spindles occurred normally. Thus, daughter cell budding per se is dependent primarily on the parasite microtubule system and does not require a dynamic actin cytoskeleton.

MATERIALS AND METHODS

Parasites and host cells

The RH strain of *Toxoplasma gondii* was maintained by serial passage in confluent monolayers of primary human foreskin fibroblasts (HFFs) (Roos et al., 1994). Parasites were harvested shortly after complete lysis of the host cell monolayer and purified by filtration through a 3 µm pore-sized polycarbonate filter.

Reagents

Latrunculin A and jasplakinolide were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Oryzalin (3,5-dinitro- N^4,N^4 -dipropylsulfanilamide) was obtained from Drs John Keeton and Dennis Lade (Lilly Research Laboratories, Indianapolis, IN, USA). Additional samples of oryzalin were provided by Dr John Benbow

(Lehigh University). All other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless stated otherwise.

Cytochalasin D was made up as a 2 mM stock solution in methanol, while latrunculin A and jasplakinolide were prepared as 1 mM stock solutions in DMSO. Taxol and oryzalin were prepared as 1 mM stock solutions in DMSO, while nocodazole was prepared as a 10 mM stock solution in DMSO. Reagents were stored as portions at -20°C and diluted to final concentrations using culture medium.

Infection of host cells with *Toxoplasma* tachyzoites

Human foreskin fibroblasts grown in 35 mm Falcon Petri dishes (Becton-Dickinson, Oxnard, CA, USA) containing a 22 mm #1 glass coverslip (Corning, Corning, NY, USA) were infected with tachyzoites (approx. 10^5 parasites per ml). Parasites were allowed 5 minutes at 37°C to invade, after which the medium was aspirated to remove all free non-invaded parasites and replaced with fresh medium. The parasites were allowed 15-30 minutes at 37°C to become established in the host cells before reagents were added. Cultures were incubated in a humidified atmosphere containing 5% CO_2 for 24 hours at 37°C .

At the end of the incubation period the coverslips were taken and the infected cells fixed in methanol at -20°C . The remaining infected cells in the dishes were processed for electron microscopy as described below.

Parasite replication was assessed by counting the numbers of parasites per vacuole by direct visualization using a Zeiss microscope with phase objectives. To ensure random counting, fields from all regions of the coverslip were selected without prior microscopic examination and all vacuoles within each field were counted. In all experiments the number of parasites per vacuole was determined for between 500-700 vacuoles from at least three separate experiments.

To examine the longer term effects of the various actin-disrupting drugs on parasite development, monolayers of HFFs in T-25 flasks were infected with tachyzoites over a 10-15 minute period. All non-invaded parasites were removed and intracellular parasites allowed 30 minutes to become established before reagents were added and the cultures incubated at 37°C until parasites lysed-out. Since only at low drug concentrations could we obtain any viable parasites we also treated 24-hour-old infected cultures with higher concentrations of actin-disrupting drugs and collected the resulting lysed-out parasites 24-30 hours later. Tachyzoites from these cultures were harvested shortly after complete lysis of the host cell monolayer and purified by filtration through a 3 µm pore-sized polycarbonate filter. All parasites were washed by suspension and pelleting several times in culture medium before being used to infect HFFs grown in Petri dishes containing a 22 mm #1 glass coverslip as described above. The infected cells were incubated for 24 hours in the absence of reagent, after which the coverslips were taken and the infected cells fixed in methanol at -20°C . Parasite replication was assessed by counting the numbers of parasites per vacuole. The remaining infected cells in the dishes were processed for electron microscopy as described below.

Since the host cells contain large amounts of actin, it is possible that the host cell sequesters all available actin inhibitor, thereby reducing the level of reagent available to the parasites to below an effective concentration. To test this possibility, we examined the effect of the three actin inhibitors on the ability of the parasite to egress the host cell upon activation with the Ca^{2+} ionophore, A23187. Infected cultures at 30-34 hours post-entry were treated with inhibitor for 2 hours before 5 µM A23187 was added and the timing of parasite egression was recorded. After 15 minutes (37°C) all remaining host cells were fixed and processed for electron microscopy as described below. Control cultures were treated with appropriate amounts of DMSO or methanol for 2 hours and the parasites activated with 5 µM A23187.

Fluorescence microscopy

To assess the effects of the anti-microtubule drugs on both parasites

and host cells, infected cells grown on coverslips and treated for 24 hours with the drugs were fixed with methanol (-20°C) for 10 minutes.

After fixation, the coverslips were washed with PBS containing 1% BSA, 10 mM glycine and 0.01% sodium azide. The microtubule cytoskeletons were labeled with an anti- α -tubulin monoclonal antibody (Sigma Chemical Co., St Louis, MO, USA) used at a 1:1000 dilution. Parasite and host cell centrosomes were labeled with an anti-centrin polyclonal antibody 26-14.1 (generously provided by Dr J. Salisbury, Mayo Clinic, Rochester, MN, USA) used at a 1:500 dilution. The samples were stained with an appropriate FITC- or TRITC-conjugated secondary antibody (Sigma). To assess the effects of microtubule antagonists on the morphology of the parasite nucleus some specimens were also stained with YO-PRO 1 (Molecular Probes). Coverslips were dehydrated in ethanol and mounted in PBS-Citifluor antifadent (Ted Pella, Redding, CA, USA). Preparations were viewed on a Zeiss Universal fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY, USA) or an Olympus model BX50 confocal microscope running Fluoview software (Olympus America Inc. Melville, NY, USA). Confocal images were examined and printed using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA, USA).

Electron microscopy

For electron microscopy, infected cells were fixed in situ with a freshly prepared mixture containing 1% glutaraldehyde (made from an 8% stock; Electron Microscopy Sciences, Fort Washington, PA, USA) and 1% osmium tetroxide in 50 mM phosphate buffer (pH 6.2). The fixative was added at room temperature and then placed on ice (4°C) for 45 minutes. Samples were rinsed with distilled water to

remove excess phosphate ions and the cells released from the Petri dish by scraping gently with a bevelled cell scraper. The samples were pelleted and then en bloc stained with 0.5% aqueous uranyl acetate for 6-16 hours at 4°C . Samples were dehydrated with acetone and embedded in an Epon-Araldite mix.

Ultrathin section (50-70 nm thick) were cut and stained with uranyl acetate and lead citrate and examined using a Philips 200 electron microscope.

RESULTS

Effects of actin inhibitors

Replication of *Toxoplasma* tachyzoites is not blocked by reagents that disrupt the actin cytoskeleton

At low concentrations cytochalasin D ($1\ \mu\text{M}$) and latrunculin A ($1\ \mu\text{M}$), both of which disrupt actin filament assembly, had little effect on the initiation of parasite growth and replication as judged by the numbers of parasites per vacuole at the end of the 24 hour incubation period (Fig. 1A,B). At $10\ \mu\text{M}$, cytochalasin D caused many of the host cells to round-up, concomitant with a noticeable but variable inhibition of parasite replication as well as an increase in the numbers of abnormal parasites (Fig. 1A). Similarly, higher concentrations of latrunculin A ($5\ \mu\text{M}$) caused the majority of host cells to round-up making it difficult to count the numbers of parasites per vacuole. However, examination of these cultures at the EM

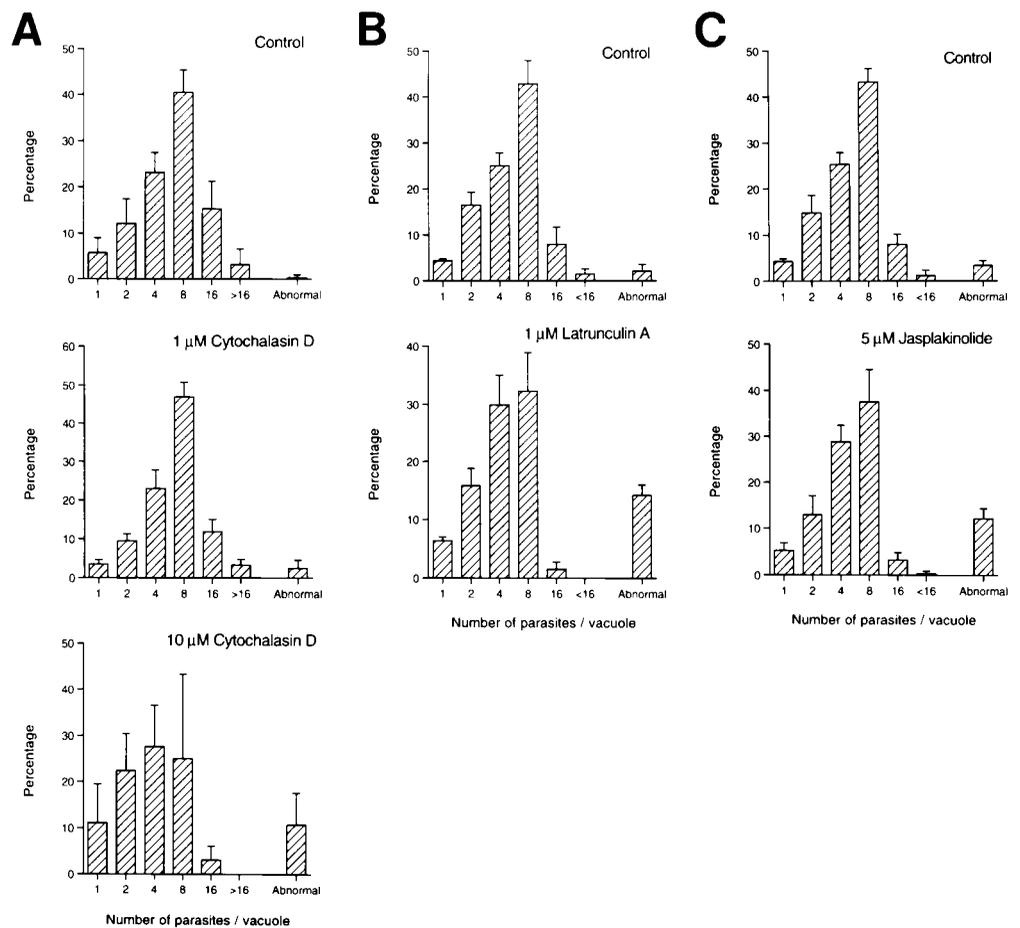
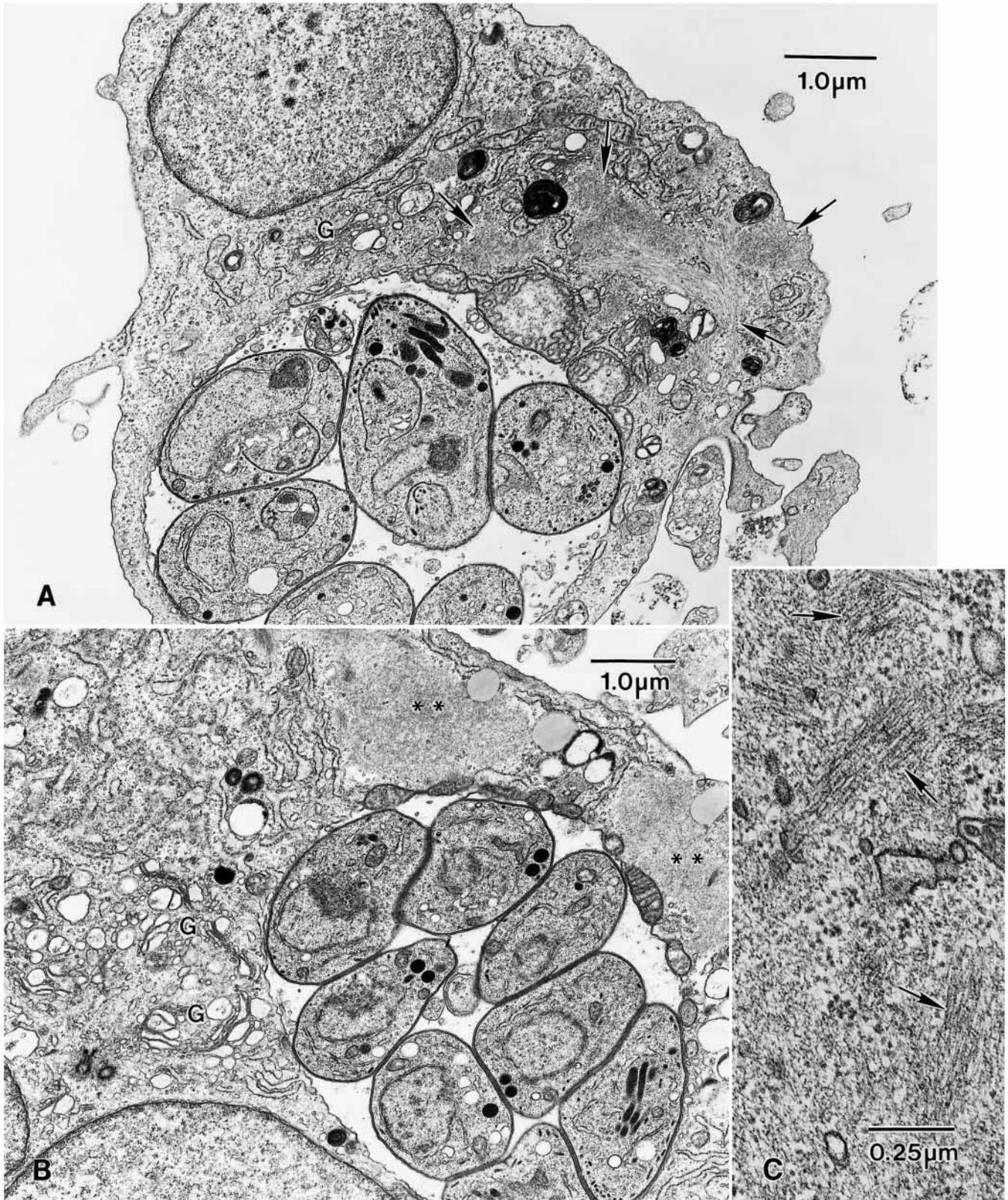


Fig. 1. Effects of actin inhibitors on parasite growth and replication. (A) Cytochalasin D; (B) latrunculin A; (C) jasplakinolide. Tachyzoites were allowed 5 minutes (at 37°C) to invade host cells and were then treated with various concentrations of actin inhibitors for 24 hours at 37°C , after which the cultures were processed as outlined in Materials and Methods. The data shows counts (means \pm s.d. from a minimum of five separate experiments) of the numbers of parasites per vacuole.



level revealed that multiple rounds of parasite replication had occurred (see below).

Treatment of newly infected cells with jasplakinolide (up to 5 μM), a membrane-permeable actin polymerizing and

filament stabilizing drug (Bubb et al., 1994), did not inhibit parasite growth and replication (Fig. 1C). Again, however, the drug did have increasingly severe effects on the host cells with many cells becoming rounded up. Invariably, with all three

Fig. 2. Electron micrographs of infected host cells treated with (A) 10 μM cytochalasin D, and (B,C) 1 μM jasplakinolide for 24 hours at 37°C. (A) Treatment with cytochalasin D caused the host cells to become rounded with the cytoskeleton showing signs of disruption (arrows) although the host cell Golgi (G) is not disrupted. (B) Jasplakinolide treatment caused the accumulation of snarls of randomly orientated actin filaments in parts of the host cell (**). However, despite the disruption to the host cell cytoskeleton the Golgi (G) and much of the cytoplasm are not disrupted. (C) High magnification of an area of randomly orientated actin filaments in a jasplakinolide-treated host cell. Arrows highlight groups of actin filaments cut in a longitudinal section. Note that with both drugs the parasites were still able to undergo several rounds of replication.

drugs, parasite replication was more limited in the most severely affected host cells, although in all cases some rounds of replication occurred.

Actin inhibitors disrupt the orderly turnover of the mother cell organelles

Examination of treated cultures by electron microscopy showed that both cytochalasin D (Fig. 2A) and latrunculin A treatment led to the disruption of the host cell cytoskeleton, causing many of the cells to round up. By contrast, jasplakinolide treatment produced extensive polymerization of actin filaments throughout the host cell cytoplasm (Fig. 2B,C). In general, despite the often extensive disruption to the host cell cytoskeleton, the Golgi complex and much of the ER in the host cells was not disrupted. Nevertheless, at the highest concentrations tested, all three drugs did cause a general breakdown in cytoplasmic organization in the host cells leading ultimately to host cell death.

Although each actin inhibitor caused significant disruption to the host cytoskeleton, the majority of intracellular parasites appeared normal and all stages in the budding process were observed (Figs 3, 4). In general, the ability of the parasites to undergo one or more rounds of replication correlated with the degree to which the host cells were affected. The majority of abnormal parasites were found in those host cells that showed the most disruption to their cytoskeleton. The most conspicuous abnormality seen with all three inhibitors was the presence of large residual bodies at the posterior end of dividing or recently divided parasites (Figs 3, 4). These residual bodies were surrounded by only the parasite plasma membrane with no IMC or microtubules present (Figs 3D, 4B,C). The residual bodies varied in size and could contain rooptries, micronemes, parts of the mitochondrion, the occasional apicoplast, many free ribosomes and profiles of ER. In addition, treatment with 5 μM jasplakinolide also caused the formation of accumulations of actin filaments within the parasite. In general, this filamentous actin formed in the apical region around the conoid, but was most conspicuous towards the posterior regions and within the residual bodies (Fig. 4B). A few tachyzoites also had actin-containing apical extensions resembling acrosomal process similar to those induced in free, motile tachyzoites by jasplakinolide (Fig. 4C; see Shaw and Tilney, 1999). The numbers of tachyzoites having jasplakinolide-induced apical protrusions increased both with time post-entry and when the intracellular parasites were activated by the addition of Ca^{2+} ionophore (see below).

In contrast, in 24-hour-old control cultures, parasite budding

invariably results in small volumes of parasite cytoplasm containing dense granules and parts of the mitochondrion being released into the parasitophorous vacuole. In no case was rooptry or microneme material seen in these small volumes of residual cytoplasm. In the control cultures, these small amounts of residual material never accumulated and appeared to be degraded within the vacuole. Only at later times post-entry (>36 hours) and/or in vacuoles containing large numbers of dividing parasites (>64 parasites/vacuole) were accumulations of residual material seen, although these were never large and the surrounding single plasma membrane was often ruptured.

To examine whether similar effects occurred in established, dividing parasites, 24-hour-old infected cultures were treated with 10 μM CD or 5 μM jasplakinolide for 8 hours and then fixed and processed for electron microscopy. As expected, both drugs induced disruption of the host cell cytoskeleton leading to severe abnormalities in many host cells. By contrast, the only observable effect of either drug on parasite replication was to cause the formation of residual bodies at the posterior end of divided parasites and abnormalities in the recycling of mother cell organelles during daughter cell budding. These residual bodies contained a similar array of organelles (rooptries, micronemes, parts of the mitochondrion, the occasional apicoplast and profiles of ER) to those seen in parasites treated immediately following invasion. In addition, the residual bodies in jasplakinolide-treated parasites also contained accumulations of actin filaments and some parasites possessed actin-filled protrusions from the conoid.

We attempted to examine the long-term effects of disrupting the parasite actin cytoskeleton on parasite survival. Only at low drug concentrations (<1 μM) did the host cells survive long enough for the parasites to grow and lyse-out. These parasites were able to invade host cells and develop in an identical manner to control cultures. At these low drug levels, however, the intracellular parasites showed little or no morphological damage. At higher concentrations the disruptive effects of the drugs led to host cell death before the parasites could develop and replicate to high enough numbers to naturally egress from the host cells. Only when we incubated established, 24-hour-old infected cultures with high concentrations of inhibitor did we obtain viable parasites released from the host cells 24–30 hours later. Thus, under the conditions tested, all three actin inhibitors appeared not to block parasite growth and replication, and the failure of the parasites to survive these drugs correlated with the deleterious effects of the drugs on the host cells.

Actin inhibitors block ionophore-induced egression of tachyzoites from host cells

One possible reason for the failure of the actin inhibitors to block parasite replication is that the host cells sequestered most of the available drug so that the concentration around the parasites was significantly less than in the medium and/or host cell cytoplasm. To examine whether the actin inhibitors were available to the parasite at concentrations able to block actin-dependent processes, we treated 30–34-hour-old infected cultures with each drug (10 μM cytochalasin D; 1 μM latrunculin A, 5 μM jasplakinolide) for 2 hours before inducing parasite egression by the addition of the Ca^{2+} ionophore A21387 (5 μM). In control cultures, the tachyzoites became active and started to escape from the host cells within 30 seconds following the addition of A21387. By 5 minutes >70% of the parasites had egressed from

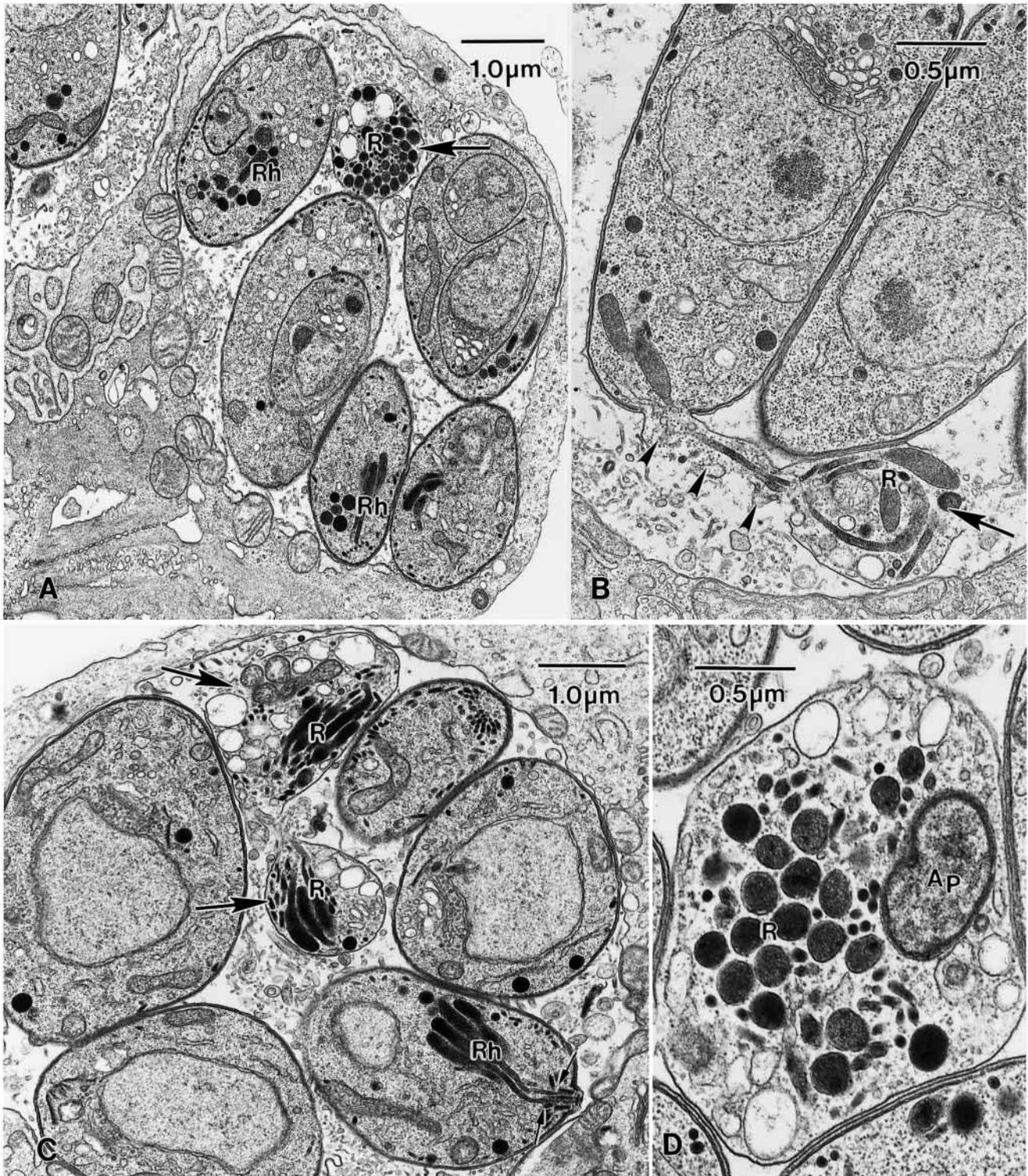


Fig. 3. Electron micrographs of infected host cells treated with (A,B) 10 μ M cytochalasin D, and (C,D) 5 μ M latrunculin A for 24 hours at 37°C. Treatment with either reagent did not block parasite division but did result in the presence of residual bodies (arrows) which were formed at the posterior of the parasites (arrowheads in B). These residual bodies contained rhoptries (R), micronemes, ribosomes and other materials, and occasionally an apicoplast (Ap in D). Note that the divided parasites still contain rhoptries (Rh) associated with the apical complex (small arrows in C).

the host cells. In contrast, in cultures treated with the actin inhibitors, the host cells had become rounded-up and very few (<5%) parasites escaped into the medium upon the addition of

A23187. Even after 15 minutes (37°C) there were very few extracellular tachyzoites present and the majority of parasites remained intracellular (data not shown). With all three inhibitors,

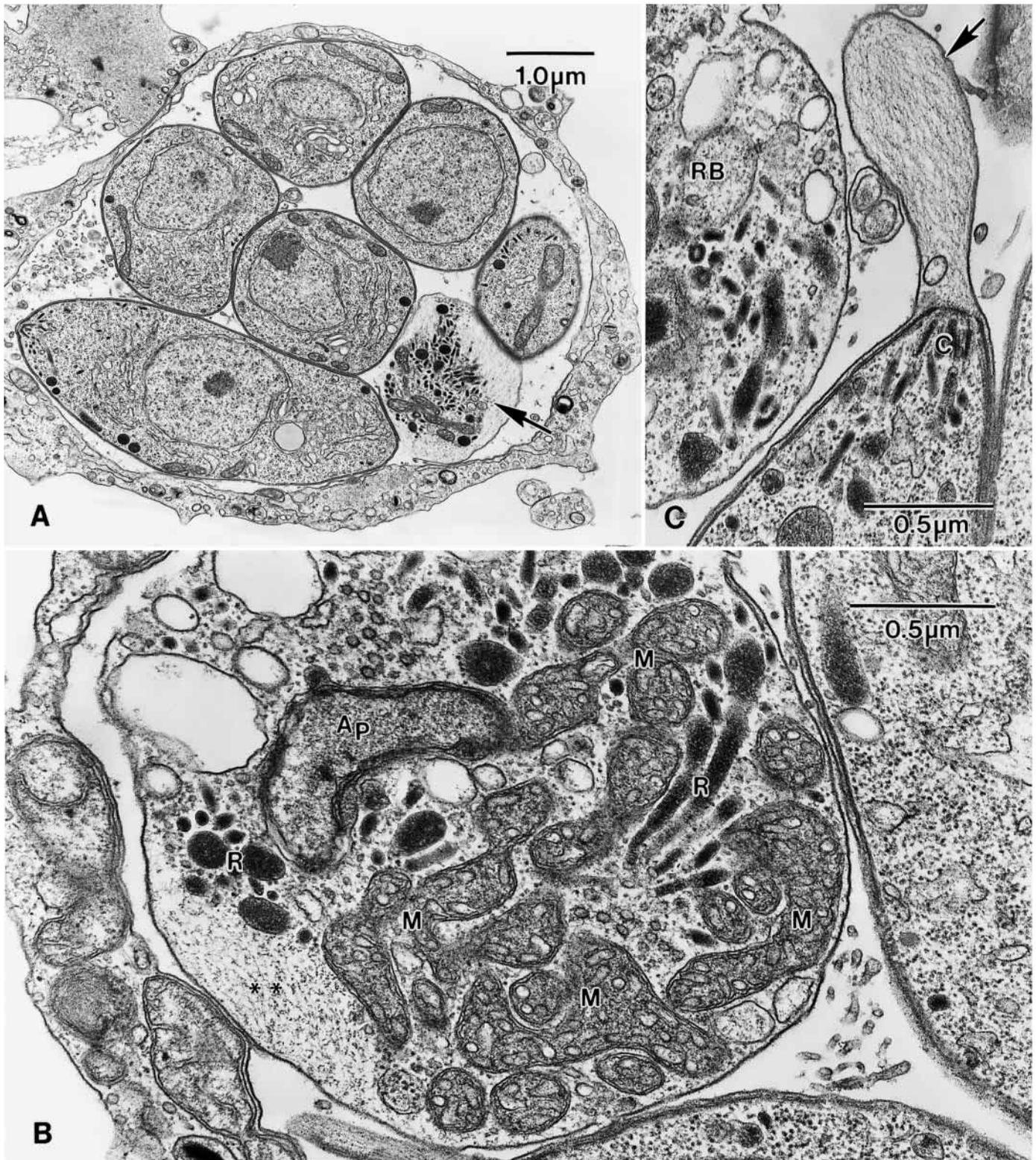
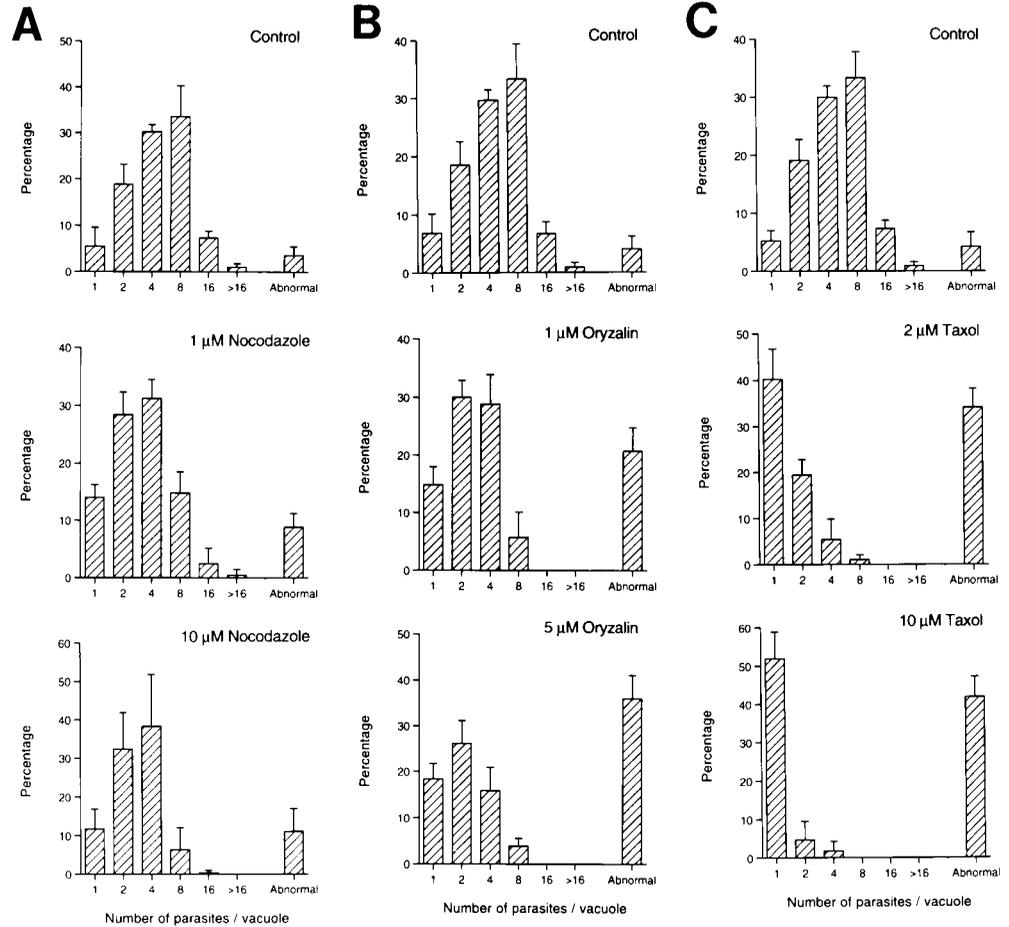


Fig. 4. Electron micrographs infected host cells treated with 5 μM jasplakinolide for 24 hours at 37°C. (A) Low magnification micrograph showing a vacuole containing tachyzoites and the presence of a large residual body (arrow) containing rhoptries, micronemes and fibrous material. (B) High magnification of one of these residual bodies showing the presence of numerous profiles of mitochondria (M), some rhoptries (R), part of an apicoplast (Ap), various vacuoles and some filamentous material (**). Note that the residual body is surrounded by only a single plasma membrane and no IMC or microtubules can be seen. (C) High magnification of the apical end of an intracellular tachyzoite showing the presence of an apical protrusion containing filamentous material (arrow). Note also the presence of part of another residual body (RB) enclosed within a single plasma membrane containing an array of organelles. C, conoid.

Fig. 5. Effects of the anti-microtubule agents (A) nocodazole, (B) oryzalin and (C) taxol, on parasite growth and replication. Tachyzoites were allowed 5 minutes (at 37°C) to invade host cells and were then treated with various concentrations of microtubule inhibitors for 24 hours at 37°C, after which the cultures were processed as outlined in Materials and Methods. The data shows counts (means \pm s.d. from a minimum of five separate experiments) of the numbers of parasites per vacuole.



after only 2 hours treatment, the host cells and tachyzoites showed morphological abnormalities identical to those described above.

A similar inhibition of Ca^{2+} -induced parasite escape was also seen when we added the actin inhibitors 5 minutes prior to the addition of A23187. Interestingly, adding jasplakinolide to 30-34-hour-old intracellular parasites did not on its own lead to parasite escape from the host cell. This is despite the fact that jasplakinolide causes free tachyzoites to twist, flex and move faster than normal as well as inducing the polymerization and stabilization of actin filaments (Shaw and Tilney, 1999). Thus, the induction of actin filament polymerization per se in intracellular tachyzoites is not sufficient by itself to cause parasite egression.

Effects of anti-microtubule drugs

Parasite replication is blocked and the proper assembly of the daughter conoids disrupted

Treating newly invaded parasites with the microtubule-depolymerizing drugs, nocodazole or oryzalin, or with taxol, which induces microtubule polymerization and stabilizes pre-existing microtubules, blocked tachyzoite replication and led to the formation of highly abnormal parasites (Fig. 5). Nocodazole (10 μ M) was the least effective at inhibiting parasite replication (Fig. 5A) and naturally lysed-out parasites were recovered from treated cultures 40-50 hours post-entry. These recovered parasites were viable and were able to enter new host cells and replicate in a manner similar to untreated controls (data not shown). In contrast, oryzalin and taxol both caused significant reductions in parasite replication and the formation of large numbers of abnormal parasite masses (Fig. 5B,C). Nevertheless, with both oryzalin and taxol, while some parasites were able to divide successfully over the initial 24 hours post entry even at the highest drug concentrations used, no viable parasites were recovered from oryzalin- and taxol-treated cultures.

To assess the effects of each reagent on host cell microtubules, we stained 24 hour-treated infected cells with

anti- α -tubulin antibody. As expected nocodazole caused the complete breakdown of the host cell microtubules, while oryzalin had only a marginal effect on the microtubule staining pattern. Taxol treatment led to an increase in the numbers of microtubules throughout the host cell cytoplasm but did not greatly disrupt the normal orderly microtubule arrays seen in control cells (data not shown).

Examination of treated cultures by electron microscopy showed that while nocodazole caused the breakdown of the host cell microtubules, parasites were able to grow and replicate normally. In contrast, both oryzalin and taxol blocked parasite replication and led to the formation of large abnormal parasite masses within each parasitophorous vacuole (Figs 6, 7). Since each drug was added to newly established intracellular parasites well before they committed to division (approximately 6-8 hours post-invasion), it is clear that neither drug totally blocked microtubule polymerization and parasites were able to initiate daughter cell budding. In particular, parasites formed intranuclear microtubule spindles and were able to manufacture and assemble, with varying degrees of success (see below), new daughter conoids with the accompanying IMC and associated microtubules. Even at 5 μ M oryzalin parasites were still able to form microtubular spindles and to manufacture and assemble new daughter conoids with the accompanying IMC and associated microtubules (Fig. 6A,B). In addition, the elongation of the daughter parasites could proceed to a point where new rhoptries and micronemes were produced and the apicoplast was partitioned into the

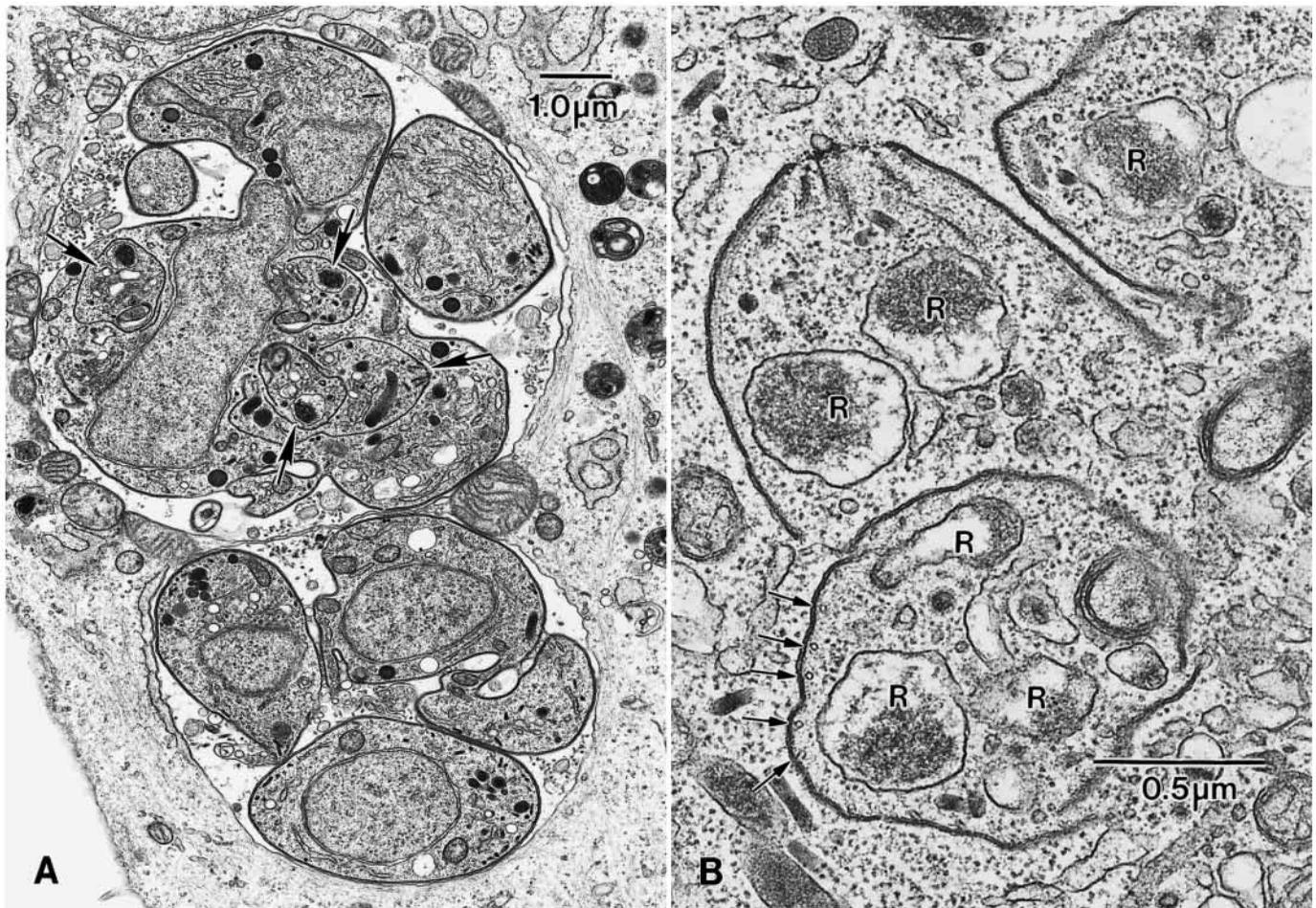


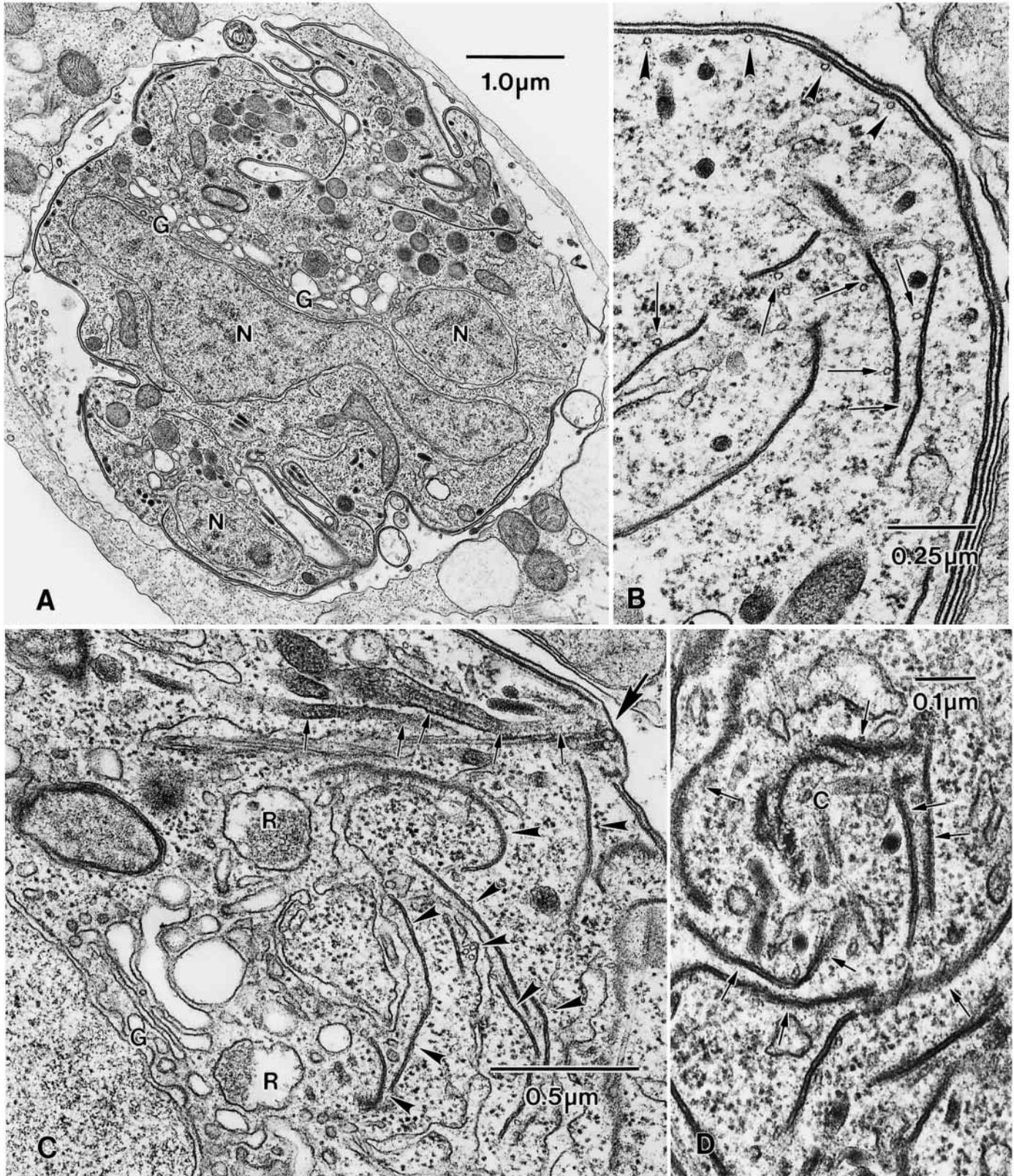
Fig. 6. Electron micrographs showing the effects of treating newly infected cultures with 5 μM oryzalin for 24 hours at 37°C. (A) Low magnification showing two vacuoles containing abnormal parasite masses. Note that the parasite mass in the upper vacuole contains a number of daughter conoids (arrows). (B) Higher magnification image of part of an abnormal parasite mass showing sections of different orientations through the apical ends of several developing daughter conoids. Note that each daughter conoid contains forming rhoptries (R) and that the IMC has microtubules associated with it (small arrows).

daughters. However, the parasite nucleus never became anchored into the developing daughters. In all but in the most severely affected parasites, the subpellicular microtubule IMC complex of the mother cell was still present, indicating that these microtubules do not readily turn over and that oryzalin does not induce microtubule depolymerization.

Similarly with taxol, while a small proportion of parasites are able to divide, the majority of vacuoles contained large, misshapen parasite masses (Fig. 7A). Nevertheless, these abnormal parasites were still able to form intranuclear microtubule spindles and could manufacture large amounts of IMC, often with associated microtubules (Fig. 7B,C). Taxol also caused an increase in the incidence of microtubules within the parasite cytoplasm above those seen associated with the newly formed sections of IMC. In most parasites, assembly of new daughter conoids was much less successful than that seen in oryzalin-treated parasites. In fact, even in cultures treated with 2 μM taxol, few complete daughter conoids were seen and the assembly of the apical pole (the polar rings and conoid) and the organization of the IMC-microtubule units around the conoid was significantly disrupted (compare Figs 7D and 6B).

In these cases, the parasite nucleus failed to become incorporated into the developing daughters and no new parasites were formed.

While daughter cell budding was dramatically reduced by both oryzalin and taxol, the parasite nucleus increased dramatically in size (with an accompanying increase in the parasite Golgi, see Fig. 7A) and became highly lobed. Nevertheless, the parasite nucleus appeared to remain as a single entity and did not become divided. It should be emphasized that, despite the profound effects on parasite replication and morphology, neither drug caused the parasite Golgi to become disassembled. In fact, Golgi function was maintained in the presence of these drugs as measured by the continued formation of new rhoptries and micronemes. In oryzalin-treated parasites the newly formed rhoptries and micronemes were localized within the developing daughter conoids (Fig. 6A,B), whereas in taxol-treated parasites the majority of newly synthesized secretory organelles were found scattered throughout the parasite cytoplasm (Fig. 7A,C). In comparison, both nocodazole and, to a lesser extent, oryzalin caused a loss of host cell microtubules and the disassembly of



the host cell Golgi and a disruption of cellular architecture. Taxol produced an increase in host cell microtubules throughout the cytoplasm but no significant disruption to the host cell Golgi.

While oryzalin and taxol severely disrupted parasite replication, neither drug totally blocked the formation of intranuclear spindles. We therefore examined the effects of all three anti-microtubule drugs on centrosome replication by

Fig. 7. Electron micrographs of infected host cells treated with 2 μM taxol for 24 hours at 37°C. (A) Low magnification showing a vacuole containing an abnormal parasite mass. Note that the parasite nucleus is greatly enlarged and lobated so that there are several profiles of parasite nucleus (N) in this section. With the increase in the size of the nucleus, the parasite Golgi (G) has also become enlarged but still maintains its normal morphology. (B) High magnification image of part of an abnormal parasite mass showing the presence of several sections of IMC with associated microtubules (arrows). Note also that the subpellicular cytoskeleton of the parasite is still intact and consists of the outer plasma membrane, IMC and microtubules (arrowheads). (C) High magnification image of part of an abnormal parasite mass showing sections of IMC-microtubules (arrowheads), the Golgi region of the parasite nucleus (G) with forming rophtries (R), and a partially broken-down apical complex at the plasma membrane (large arrow). Note that several mature rophtries are still attached to the partially broken-down apical complex (small arrows). (D) High magnification image of an assembling daughter conoid (C) with a number of sections of IMC-microtubules in the vicinity (arrows). The nascent conoid has a polar ring and the beginnings of the conoid but the overall organization and the positioning of the IMC-microtubules is significantly different from the situation in control parasites.

staining 24-hour-treated infected cells with an antibody against centrin, an intracentriolar protein (Paoletti et al., 1996), which labels the parasite centrosome (unpublished observation).

In 24-hour-old control cultures, the host cells contained a single or two closely associated spots corresponding to the centrosomes, while each parasite contained one or two bright spots closely associated with the nucleus (Fig. 8A). In cultures treated with nocodazole, the majority of vacuoles contained rosettes of normal looking parasites each of which had one or two bright spots closely associated with the nucleus (Fig. 8B). Cultures treated with oryzalin and taxol contained significant numbers of vacuoles with abnormal parasite masses, which contained multiple centrin-labeled structures (Fig. 8C,D), indicating that centrosome replication proceeded despite the failure of the parasite to successfully complete cell division.

DISCUSSION

In the present study, we used a number of drugs to examine the role(s) of the actin and microtubule cytoskeletons on parasite growth and replication. To be effective the actin and microtubule inhibitors must not only bind to their target protein(s), but in many cases also require that these cytoskeletal polymers be in a dynamic state during the treatment period. By using a short infection time (5 minutes at 37°C) and by adding the reagents soon after invasion, we were able to examine the effects of these drugs on populations of parasites treated at the start of intracellular development and many hours prior to the onset of daughter cell budding.

Actin inhibitors do not block parasite growth and replication but do disrupt the orderly turnover of mother cell organelles during daughter cell budding

Somewhat unexpectedly, we found that treating newly infected cells with three actin inhibitors (cytochalasin D, latrunculin A and jasplakinolide), each of which has a different effect on actin filament dynamics, had little or no effect on parasite

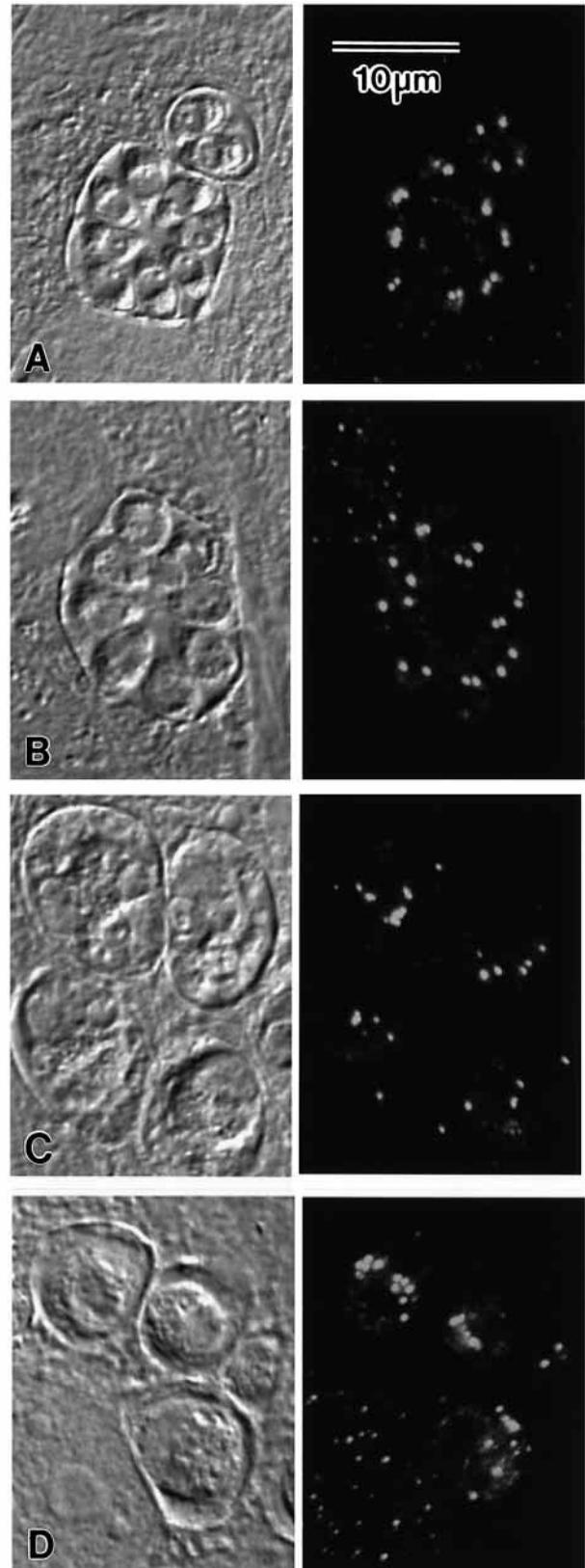


Fig. 8. DIC (left) and corresponding confocal fluorescence images (right) of newly infected cultures incubated for 24 hours with microtubule inhibitors and then stained with an antibody against centrin to localize the centrosomes. (A) control; (B) 10 μM nocodazole; (C) 5 μM oryzalin; (D) 2 μM taxol.

replication although the host cells underwent profound structural changes. We know that the parasite actin cytoskeleton is susceptible to the action of these drugs since each one blocks tachyzoite entry into host cells (Morisaki et al., 1995; Dobrowolski and Sibley, 1996; Shaw and Tilney, 1999; unpublished observations). Since we used concentrations well above those needed to block parasite entry, two possible explanations for the failure of these drugs to block parasite replication are that the drugs were not gaining access to the intracellular parasite and/or the host cells were sequestering all available reagent. However, each drug was clearly entering the host cell and, since the parasite resides in a vacuole surrounded by a 'leaky' membrane (Schwab et al., 1994), it seems unlikely that they would not reach the parasite.

The problem of the host cells sequestering all available drug is more difficult to discount. *Toxoplasma* is an obligate intracellular parasite and therefore any treatment(s) are likely to affect the host cells and produce effects, either directly or indirectly, on the parasite. All three actin inhibitors had profound effects on the host cells and caused them to die before the parasites could replicate and develop sufficiently to egress from the host cells. Only when we incubated established, 24-hour-old infected cultures with each inhibitor did we obtain viable infective parasites released from the host cells 24–30 hours later. Thus, provided the host cells survive the effects of the actin inhibitors long enough, the parasites can grow, replicate and eventually escape. Nevertheless, all three actin inhibitors blocked ionophore-induced egression of tachyzoites from the host cell, demonstrating that each reagent was available to the intracellular parasites at sufficiently high concentrations to block an actin-dependent physiological process.

Thus, from the available evidence it seems reasonable to conclude that a dynamic actin cytoskeleton in the parasite is not essential for either parasite growth or the maintenance of cell polarity, or in the process of daughter cell budding. This would imply that the events underlying cell replication in *Toxoplasma* are mechanistically distinct from cytokinesis in both animal and plant cells, in which the actin cytoskeleton plays a vital role (Rappaport, 1996). In addition, the fact that a dynamic actin cytoskeleton is not essential for daughter cell budding in this highly polarized parasite cell suggests that the process differs from polarized budding in other systems. For example, in budding yeast, the actin cytoskeleton plays a critical role in the establishment and maintenance of polarized growth and is involved in bud site selection and the polarized secretion of components, as well as the movement and segregation of organelles (the mitochondrion and vacuole) to the daughter cell (Bretscher et al., 1994; Drubin and Nelson, 1996; Yang et al., 1997). However, in *Toxoplasma* the initiation of daughter cell budding occurs internally in association with the centrosomes that form the pole of the intranuclear spindle. In contrast, in other apicomplexan parasites the budding of new cells occurs from the surface of the parasite or a syncytial parasite mass (see Shaw and Tilney, 1992 and references therein). Whether the actin cytoskeleton is necessary for determining the sites on the outer plasma membrane from which new cell polarized cells bud in these parasites remains to be examined.

While the actin inhibitors tested did not block parasite growth and replication per se, they did cause subtle changes in the ability of the parasite to recycle the mother cell organelles.

Toxoplasma tachyzoites replicate by a process of internal budding that is fundamentally different from cell division in most other eukaryotes. During daughter cell budding, much of the mother cell cytoplasm and organelles becomes incorporated into the two daughters although other parts (the apical conoid complex and associated secretory organelles, and parts of the subpellicular cytoskeleton) disappear and are presumably broken down and recycled by the parasite. Treatment with all three actin inhibitors blocked the recycling of some of these mother cell organelles and led to their accumulation into a residual body formed at the posterior pole of each budding parasite. Interestingly, although treating 24-hour-old infected host cells resulted in the disruption of the orderly turnover of the mother cell organelles, the parasites were able to continue to replicate and produce viable, invasive tachyzoite. Thus, under the culture conditions used, the recycling of the mother cell organelles during daughter cell budding was not crucial to parasite survival.

At present little is known about how or where within the cell the parasite recycles its organelles. While tachyzoites contain a range of proteolytic enzymes (Manafi et al., 1993) their biological roles and precise cellular locations within the parasite are unknown. Moreover, substantive evidence for a lysosomal-autophagic system in the tachyzoite remains elusive. The only acid compartments so far described within the tachyzoite are the mature and forming rhoptries (Shaw et al., 1998). Until more is known about the proteolytic system(s) in the tachyzoite, it is difficult to comment on the significance of or link between inhibition of actin filament dynamics and organelle recycling in the parasite.

Disruption of microtubule dynamics leads to a reduction in parasite replication by preventing the normal assembly and/or elongation of the microtubule IMC cytoskeleton in the budding daughter parasites

While daughter cell budding in *Toxoplasma* tachyzoites was not dependent on the actin cytoskeleton, the process clearly required the polymerization of microtubules. Nocodazole (10 μ M) had little effect, consistent with the unusual nature of the microtubules in *Toxoplasma* (see Stokkermans et al., 1996). In contrast, both oryzalin and taxol caused dramatic reductions in parasite replication and resulted in the development of large abnormal parasite masses. In general, despite the often extensive morphological damage, the parasite subpellicular cytoskeleton was not disrupted and the associated microtubules did not depolymerize. Moreover, tachyzoites were able to undergo multiple rounds of centrosome replication, form mitotic spindles and initiate daughter conoid assembly in the presence of the drugs. These observations are consistent with the idea that the general mode of action of most microtubule inhibitors involves a suppression of microtubule dynamics, either by preventing the assembly of new microtubules or by stabilizing pre-existing ones, rather than directly depolymerizing pre-existing microtubules.

The initiation of daughter cell budding in *Toxoplasma* requires the synthesis and assembly of a new conoid and associated IMC-microtubule array inside the parasite in response to positional information provided by the pair of cytoplasmic centrosomes that form the poles of the intranuclear spindle. Both the mitotic spindle and each new array of 22

microtubules associated with the developing daughter cells are formed de novo since the subpellicular microtubules in the mother cell do not disappear until near the end of the budding process. Thus, the behaviour of microtubules in *Toxoplasma* differs from what occurs in higher eukaryotes where the mitotic spindle is assembled by the reorganization of the radial interphase microtubule network following nuclear envelope breakdown.

While both oryzalin and taxol prevented parasite replication, neither blocked centrosome replication or mitotic spindle formation, and the primary target of both drugs was the actual budding process. In particular taxol, by inducing microtubule polymerization, disrupted the initial assembly of the conoid and polar ring complex, whereas oryzalin mainly blocked the incorporation of the parasite nucleus into the developing daughters and the continued elongation of the IMC-microtubule assembly, thereby preventing the completion of the budding process. It should, however, be emphasized that with both drugs, although mitotic spindles were formed and the nucleus became enlarged and highly lobed, complete nuclear division did not occur and each abnormal parasite mass contained only a single, albeit unusually enlarged, nucleus. Interestingly, when we blocked DNA synthesis in newly invaded parasites, the parasite was still able to initiate multiple rounds of daughter cell budding even though nuclear division was blocked and no new parasites were formed (Shaw et al., 1997). Together these results suggest that the events of karyokinesis and cytokinesis are separate and independent of each other and imply that cell cycle checkpoints, as defined in higher eukaryotes, are absent or not functional in *Toxoplasma* tachyzoites. Such a separation is not altogether surprising since it is clear that many apicomplexan parasites can replicate their nuclei without an accompanying cytokinesis, thereby forming multinucleate syncytial cells (a process termed schizogony). Nevertheless, in *Toxoplasma* tachyzoites, which divide by an indefinite series of binary divisions, one might expect nuclear division, and conoid formation and subsequent daughter cell budding, to be tightly linked. Recent work on *Plasmodium falciparum* (Sinou et al., 1998) and on trypanosomes (e.g. Robinson et al., 1995; Grellier et al., 1999) have demonstrated a similar separation of the nuclear and cytoplasmic events of cell division, suggesting that cell cycle progression in protozoan parasites may not operate through the same pathways as in higher eukaryotic cells.

During parasite budding the parasite centrosomes act as the microtubule-organizing centre (MTOC) for the spindle microtubules, while in mature parasite the polar ring associated with the conoid complex is thought to act as the MTOC and site of origin for the 22 subpellicular microtubules associated with the IMC. However, the presence of many individual microtubule IMC units in the cytoplasm of parasites treated with both taxol and oryzalin implies that the target for these drugs may not be the polar ring and/or microtubule polymerization per se. Rather, individual microtubule IMC units may be formed at the polar ring, but in the presence of taxol and, to a lesser extent, oryzalin, they do not join together properly to form the elongating microtubule IMC cytoskeleton that drives daughter cell budding. Alternatively, the microtubule IMC units may be manufactured elsewhere in the cell and moved to the elongating daughters as preformed units. In higher eukaryotic cells microtubule polymerization can

occur at non-centriolar sites (e.g. Yvon and Wadsworth, 1997; Vorobjev et al., 1997), while in trypanosomes, which possess an elaborate microtubule-based plasma membrane-associated cytoskeleton, the distribution of gamma tubulin indicates that multiple microtubule-polymerization sites exist (Scott et al., 1997).

Lastly, while many details of the mechanism(s) controlling cell division (daughter cell budding) in *Toxoplasma* remain obscure, the results of this study highlight two important features that differentiate this parasite (and possibly apicomplexan parasites in general) from higher eukaryotes. Firstly, the process of parasite replication does not require a functionally dynamic actin cytoskeleton (but see the caveat above) and is not driven by the polymerization of actin filaments. Secondly, daughter cell budding requires and is driven by microtubule polymerization. Together these findings suggest that cell division in this protozoan parasite differs from other eukaryotes where the actin and microtubule cytoskeletons play critical interdependent roles (Gavin, 1997). The fact that a dynamic actin cytoskeleton is not essential to parasite division implies that the mechanism of force generation is different from a number of other types of cellular movement including the protrusion of lamellipodia (Cramer et al., 1994) and the propulsion of intracellular bacteria (Tilney and Tilney, 1996) and viruses (Cudmore et al., 1997); all of which involve actin filament polymerization. Nevertheless, preliminary studies using myosin inhibitors indicate that the assembly and elongation of the IMC-microtubule complex in the budding daughter parasites involves myosin-based motor(s).

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