Disruption of microtubules uncouples budding and nuclear division in *Toxoplasma gondii*

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Summary

The tachyzoite stage of the protozoan parasite *Toxoplasma gondii* has two populations of microtubules: spindle microtubules and subpellicular microtubules. To determine how these two microtubule populations are regulated, we investigated microtubule behavior during the cell cycle following treatment with microtubule-disrupting drugs. Previous work had established that the microtubule populations are individually nucleated by two distinct microtubule-organizing centers (MTOCs): the apical polar ring for the subpellicular microtubules and spindle pole plaques/centrioles for the spindle microtubules. When replicating tachyzoites were treated with 0.5 μM oryzalin or 1.0 mM colchicine they retained the capacity to form a spindle and undergo nuclear division. Although these parasites could complete budding, they lost the bulk of their subpellicular microtubules and the ability to reinvade host cells. Both nascent spindle and subpellicular microtubules were disrupted in 2.5 μM oryzalin or 5.0 mM colchicine. Under these conditions, parasites grew in size and replicated their genome but were incapable of nuclear division. After removal from 0.5 μM oryzalin, *Toxoplasma* tachyzoites were able to restore normal subpellicular microtubules and a fully invasive phenotype. When oryzalin was removed from *Toxoplasma* tachyzoites treated with 2.5 μM drug, the parasites attempted to bud as crescent-shaped tachyzoites. Because the polyploid nuclear mass could not be correctly segregated, many daughter parasites lacked nuclei altogether although budding and scission from the maternal mass was able to be completed. Multiple MTOCs permit *Toxoplasma* tachyzoites to control nuclear division independently from cell polarity and cytokinesis. This unusual situation grants greater cell cycle flexibility to these parasites but abolishes the checks for coregulation of nuclear division and cytokinesis found in other eukaryotes.

Key words: Apicomplexa, Centrin, Centriole, Centrosome, Colchicine, Dinitroaniline, Endodyogeny, Oryzalin, Spindle, Subpellicular Microtubule, Tubulin

Introduction

Toxoplasma gondii and other apicomplexans (*Plasmodium*, *Cryptosporidium* and *Eimeria*) are highly polarized parasites that share distinct morphological features (Chobotar and Scholtyseck, 1982; Morrissette and Sibley, 2002). These protozoa are responsible for a range of human and animal diseases and have considerable medical and economic impact worldwide (Black and Boothroyd, 2000). The specialized apical end of the apicomplexans contains several unique organelles (rhoptries, micronemes and conoid) critical for invasion and survival within host cells (Chobotar and Scholtyseck, 1982). Apicomplexans are haploid organisms for most of their respective life cycles. They are also obligate intracellular parasites, growing and replicating only within host cells. Parasite replication occurs after invasion of a host cell, within a membrane-bound parasitophorous vacuole, and continues until the host cell is lysed by the replicating parasites. Extracellular parasites have low metabolic capacity, and without reinvasion of new host cells, they rapidly die. Apicomplexans also differentiate to gametes that undergo fusion to generate a transiently diploid zygote. The male gametes have flagella and utilize flagellar motility to reach and fertilize female gametes. All other parasite stages lack flagellar structures. In *Toxoplasma*, the invasive forms are the tachyzoite, the sporozoite and the bradyzoites. Tachyzoites are easily grown *in vitro* within monolayers of fibroblasts; therefore, we have used this stage for the current study. Invasive forms (including tachyzoites) have two groups of microtubules that mediate the critically important functions of polarity, shape and nuclear division.

The two populations of microtubules in *Toxoplasma gondii* tachyzoites are spindle microtubules and subpellicular microtubules (Fig. 1). The characteristic crescent shape of *Toxoplasma* is maintained by an interaction between the pellicle and the underlying twenty-two subpellicular microtubules (Morrissette et al., 1997; Nichols and Chiappino, 1987). The pellicle is composed of the plasma membrane and the closely apposed inner membrane complex that comprises flattened vesicles. The subpellicular microtubules (~5 μM long) have a characteristic organization and length and are nucleated from the apical polar ring, a unique microtubule-organizing center (MTOC) (Nichols and Chiappino, 1987; Russell and Burns, 1984). These microtubules are critically important for shape and apical polarity (Fig. 1A1,B1). When the subpellicular microtubules are disrupted, both cell shape and apical polarity are lost (Morrissette and Roos, 1998; Stokkermans et al., 1996). Because the subpellicular microtubules of extracellular parasites are non-dynamic, the effects of microtubule-disrupting drugs are only seen in intracellular (replicating) parasites (Stokkermans et al., 1996). The spindle microtubules (~1-2 μM long) function to form an intra-nuclear spindle (a closed mitosis) to coordinate chromosome segregation (Fig. 1A2,B2). Spindle microtubules originate in a dense plaque structure that is embedded in the nuclear membrane adjacent to cytoplasmic centrioles (Senaud, 1967). A previous study has demonstrated that disruption of microtubules with high concentrations of oryzalin prevents parasite replication, although intracellular tachyzoites continue to metabolize and grow in size (Stokkermans et al., 1996).

Parasite replication occurs by means of internal budding termed endodyogeny (Sheffield and Melton, 1968). This process preserves the capacity of tachyzoites to invade the host throughout the cell cycle by maintaining their critically important apical specialization and crescent shape. *Toxoplasma* nuclear division is followed by formation of two daughter cells within the mother parasite (Fig. 1A3,B3). These daughter cells are delimited by an inner membrane complex and associated subpellicular microtubules, and each contains a complete set of apical organelles (conoid, rhoptries and micronemes) and a nucleus, mitochondrion, Golgi and plastid. Once daughter cells are mature, the maternal apical complex is disassembled and the daughter parasites emerge from the maternal plasma membrane. Since this method of replication is non-conventional, we have illustrated it in Fig. 1 using diagrams and immunofluorescence of tubulin.

In order to define the normal activity of the subpellicular microtubule and spindle microtubule populations, we have analyzed their behavior during the tachyzoite replication cycle. Because these individual microtubule populations have independent MTOCs, we have probed the degree to which they are coordinately regulated. The microtubule-disrupting agents colchicine and oryzalin are capable of disrupting *Toxoplasma* microtubules (Morrissette et al., 1997; Morrissette and Roos, 1998; Shaw et al., 2000; Stokkermans et al., 1996). We have established that the subpellicular microtubules and the spindle microtubules are differentially sensitive to disruption with these agents. Moreover, these drugs can be used to uncouple the functions of the subpellicular microtubules from those of the spindle microtubules, showing that the checks that regulate cell division in other eukaryotes are not present in *Toxoplasma*.

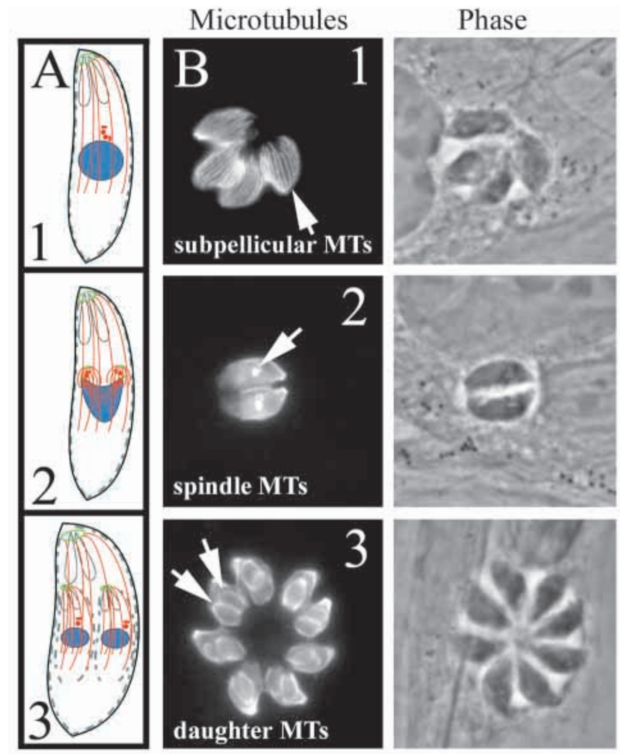

Fig. 1. *Toxoplasma* tachyzoites have two discrete populations of microtubules. (A) A diagram of the microtubules of *Toxoplasma* tachyzoites: the subpellicular microtubules and spindle microtubules. The sequence illustrates the behavior of microtubules during replication by endodyogeny. (A1) The subpellicular microtubules (red) are nucleated from the apical polar ring (green) and are tightly apposed to the inner membrane complex (gray dashed lines) that underlies the plasma membrane. (A2) The spindle microtubules (red) are nucleated from a spindle pole plaque embedded in the nuclear membrane (green). Extranuclear centrioles (red) are closely associated with the spindle pole plaques and daughter buds. (A3) Tachyzoites replicate by endodyogeny, a form of internal budding. The two daughter parasites (each containing a collection of apical organelles) are enclosed by individual sets of inner membrane complex and associated subpellicular microtubules. The mother parasite's subpellicular microtubules and apical complex are retained so that parasites are competent for invasion throughout the cell cycle. (B) Immunofluorescent labeling of the subpellicular microtubules (B1, arrow), spindle microtubules (B2, arrow) and daughter subpellicular microtubules (B3, arrows). The left-hand panels correspond to tubulin labeling and the right-hand panels are phase-contrast images of the parasites growing within primary fibroblasts.

Materials and Methods

Growth of cells

Toxoplasma tachyzoites were propagated in confluent monolayers of human foreskin fibroblast (HFF) cells in DMEM with 10% FBS. For most experiments, the RH strain (Sabin, 1941) of *Toxoplasma* was used, but for studies requiring synchronous populations, the thymidine-kinase-transfected RH strain (the CTK11 line, kindly provided by Michael White, Montana State University) was used and propagated in HFF cells in media containing dialyzed FBS (Radke and White, 1998). The colchicine-resistant (CV2-8) CHO cell line was obtained from Fernando Cabral (University of Texas, Houston) and was grown in MEM with 10% FCS. A mutation to β-tubulin underlies drug resistance in the CV2-8 line (Cabral et al., 1980).

Drug treatment

The dinitroaniline oryzalin was obtained from Riedel-deHaen (Germany), and stock solutions were made up in DMSO. Colchicine and thymidine (Sigma) stock solutions were made up in sterile DMSO and diluted into tissue culture media. For cell cycle experiments, CTK11 tachyzoites were treated with 10 μM thymidine for 4.0 hours. Although the normal doubling time of RH tachyzoites is approximately 6.5 hours, treatment with thymidine is relatively toxic to tachyzoites and the bulk of parasites are synchronous and viable at 4.0 hours. Oryzalin washout experiments were carried out by infecting and growing *Toxoplasma* tachyzoites in 0.5 or 2.5 μM oryzalin for 24 or 48 hours, after which the drug was removed, the monolayer rinsed and fresh medium without drug was added. Samples were collected at the time of drug washout, 3 hours after drug washout, and at 24 hour intervals for the next four days.

To quantify microtubule behavior, 30 random microscope fields were scored from two coverslips within an experiment. Independent washout experiments were quantified. Values representing the sum of the 30 fields from a coverslip were normalized to fractions of a total of 100 parasites. The average value of the samples within an experiment was averaged between experiments and the standard error of the mean was calculated. The resulting values were plotted in Excel. To document nuclear division in 0.5 μM oryzalin, parasites were scored as (1) carrying out nuclear division and segregation correctly, (2) carrying out nuclear division correctly but showing a defect in nuclear segregation to daughter buds or (3) showing unequal nuclear division or arrested without nuclear division. To quantify recovery from treatment with 0.5 or 2.5 μM oryzalin, parasites were scored as (1) first generation replicating parasites (equal or greater than 8 parasites per parasitophorous vacuole), (2) first generation aberrant vacuoles, (3) second generation non-replicating parasites (single crescent-shaped parasites) or (4) second generation replicating parasites (2 or 4 parasites per parasitophorous vacuole).

Immunofluorescence

Intracellular parasites on 12 mm circular glass coverslips were fixed, permeabilized and stained as previously described (Morrissette et al., 1994). They were mounted in Vectashield Mounting Media with DAPI (Vector). Phase contrast and immunofluorescence images were collected on a Zeiss Axioskop using the Axiovision camera and software. Images were exported as tif files and manipulated in Photoshop 5.5. Confocal images were collected on a Leica TCS SP2 Confocal microscope. Half-micron step optical sections were converted into parallel projections using the Leica software, and these images were overlaid and modified using Adobe Photoshop 5.5.

Antibodies

A *Toxoplasma*-specific rabbit anti-tubulin polyclonal antiserum was raised against the peptide KGEMGAEEGA conjugated to hen egg albumen (Cocalico). Mouse antiserum generated against this peptide was kindly provided by John Boothroyd (Stanford University). Anti-centrin monoclonal (20H5) and polyclonal antibodies were kindly provided by Jeffrey Salisbury (Mayo Clinic). The monoclonal antibody 45.15 against the subpellicular network component IMC-1/net-1 was provided by Gary Ward (University of Vermont). Secondary antibodies conjugated to Alexa 568 and Oregon Green were obtained from Molecular Probes as was the ToPro3 DNA stain used in the confocal samples.

Electron microscopy

Cells in 60 mm dishes were infected with tachyzoites and treated with drugs as above. These samples were fixed at 4° for 30 minutes in ‘double fix’ containing 1% glutaraldehyde and 1% osmium tetroxide in 25 mM phosphate buffer, pH 6.2. After three washes in cold deionized water, the samples were postfixed in 1% aqueous uranyl acetate for 3 hours at 4°. These samples were embedded in Epon and processed for electron microscopy.

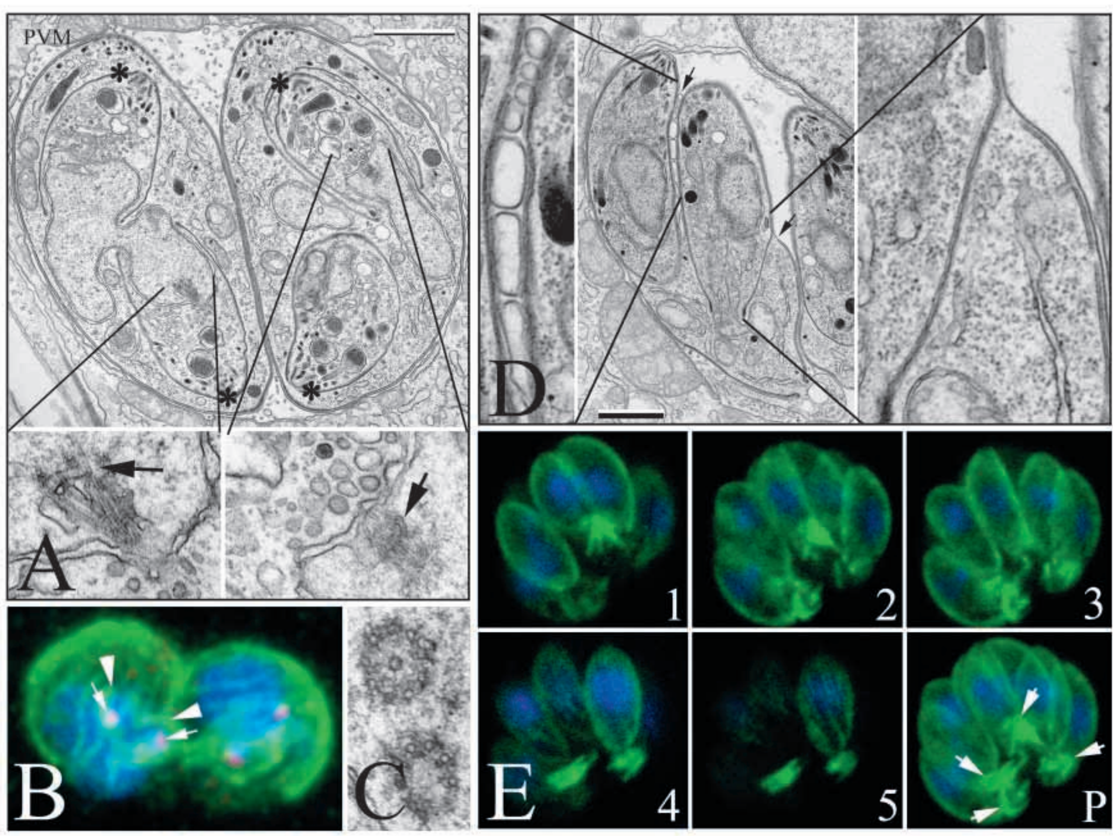

Fig. 2. (A) A thin section of a parasitophorous vacuole (PVM) enclosing two replicating parasites, each tachyzoite contains two daughter buds (marked with *). Half of the spindle is visible in each of the mature parasites and terminates in an invagination of the nuclear membrane (enlarged insets). Scale bar=1 μm. (B) Parallel projection of optical sections obtained with a confocal microscope showing tubulin (green), centrin (red) and DNA (blue) in replicating parasites. As replication proceeds, the dividing nucleus is V-shaped, as is the spindle (arrows), and it is surmounted by the subpellicular microtubules of the forming daughter buds (arrowheads). (C) A thin section of centrioles; the centriolar microtubules are singlet microtubules rather than the triplet microtubules observed in other centrioles. (D) A thin section of daughter parasites emerging from the maternal cell. In the outward-facing areas of the daughter cells, escape involves the coordinated dissociation of the maternal inner membrane complex from the plasma membrane and association of the daughter inner membrane complex onto the plasma membrane (arrow and enlarged inset). Between the two daughter cells, scission involves membrane fusion events to create new plasma membrane (arrow and enlarged inset). Scale bar=1 μm. (E) Optical sections (1-5) and parallel projection of optical sections (P) obtained with a confocal microscope showing tubulin (green) and DNA (blue) in eight tachyzoites that have just completed budding from four maternal cells. The fully intact maternal subpellicular microtubules, apical polar ring and conoid are located at the posterior of the daughter parasites (arrows).

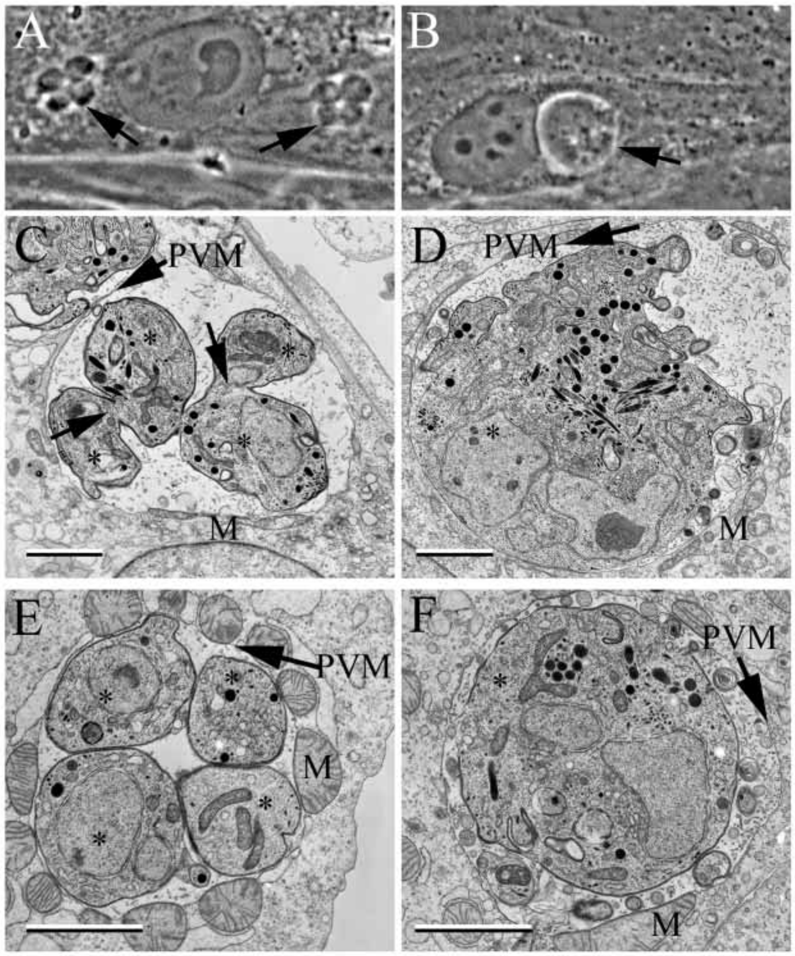

Fig. 3. *Toxoplasma* subpellicular microtubules and spindle microtubules are differentially sensitive to disruption by oryzalin or colchicine. Phase contrast of *Toxoplasma* tachyzoites (arrows) grown in HFF fibroblasts in 0.5 μM oryzalin (A) or 2.5 μM oryzalin (B). Electron microscopy of *Toxoplasma* grown in HFF fibroblasts in 0.5 μM oryzalin (C) or 2.5 μM oryzalin (D). Arrows in (C) indicate incomplete scission between daughter parasites in 0.5 μM oryzalin. Thin sections of tachyzoites in CV2-8 CHO cells in 1.0 mM colchicine (E) or 5.0 mM colchicine (F). In all electron micrographs, the scale bar represents 2 μM. The parasitophorous vacuole membrane is designated PVM and the host cell mitochondria that surround it are labeled M. Parasites are marked *.

Results

Toxoplasma tachyzoites must regulate three populations of microtubules (spindle microtubules, maternal subpellicular microtubules and daughter subpellicular microtubules) throughout endodyogeny (Fig. 1). Spindle microtubules were associated with spindle pole plaques and adjacent centrioles. Each spindle pole terminated in a plaque located within an invagination of the nuclear membrane (Fig. 2A). *Toxoplasma* centrioles were separated within G_1/S phase transition (at the time of thymidine release, not shown). In replicating parasites (from 1 to 4 hours after release), the spindle microtubules (labeled with tubulin) are found in close proximity to the centrioles (labeled with centrin; Fig. 2B). The dividing nucleus assumed a horseshoe shape, and spindle microtubules inserted into it at an acute angle (arrows, Fig. 2B). *Toxoplasma* centrioles were composed of nine singlet microtubules and a central singlet microtubule (Fig. 2C). At the completion of endodyogeny (5 to 7 hours after removal of the thymidine block), daughter parasites emerged from the mother parasite (Fig. 2D). In the outward-facing areas of the daughter cells, escape involved the coordinated release of the maternal inner membrane complex from the plasma membrane and association of the daughter inner membrane complex onto the plasma membrane (Fig. 2D, arrow and enlarged inset). Between the two daughter cells, scission involved membrane fusion events to create new plasma membrane (Fig. 2D, arrow and enlarged inset). At the completion of budding, the intact complex of maternal subpellicular microtubules, the apical polar ring and conoid were translocated to the posterior end of the parasite and discarded in the residual body (Fig. 2E). When synchronized parasites were followed through a replication cycle, ~70% showed obvious maternal microtubule complexes in the residual body, suggesting that this structure was a normal step in endodyogeny. Since residual body maternal microtubule complexes were not present in later samples, these structures must be resolved by disassembly or degradation. Electron microscopy also provided evidence of microtubules in the residual body (not shown), but the fully intact nature of the subpellicular microtubules, apical polar ring and conoid were best appreciated by immunofluorescence. It was unclear whether the subpellicular microtubules were disassembled (to recycle tubulin dimers) or degraded (to eliminate extra tubulin).

When replicating *Toxoplasma* were treated with serial dilutions of the microtubule disrupting drug oryzalin, nascent subpellicular microtubules were more sensitive to disruption than spindle microtubules were. Phase contrast microscopy of *Toxoplasma* tachyzoites grown in HFF fibroblasts showed the continued budding of tachyzoites in 0.5 μM oryzalin, although daughter parasites were round rather than crescent-shaped (Fig. 3A). At 2.5 μM oryzalin, both spindle and subpellicular microtubules were disrupted and tachyzoites grew as enormous intracellular inclusions, incapable of cell division (Fig. 3B). Electron microscopy of *Toxoplasma* grown in 0.5 μM oryzalin showed the segregation of daughter nuclei and organelles into daughter buds (Fig. 3C). Fig. 3D illustrates the non-dividing, non-polarized nature of the intracellular tachyzoite in 2.5 μM oryzalin. Both the round dividing and the round, non-dividing oryzalin-treated tachyzoites are incapable of reinvasion after lysing out of host cells (N.M., unpublished).

In colchicine-sensitive host cells (such as HFF cells), parasites arrested after host cell invasion in the presence of relatively low concentrations of colchicine. In colchicine-resistant CV2-8 cells, parasites in colchicine entered aberrant replication cycles that parallel behavior in oryzalin. This suggests that parasites do not proceed with intracellular replication in HFF cells because host microtubules are required to set up a functional parasitophorous vacuole following invasion (data not shown). This notion is supported by observations of the 'evacuoles' that arise when *Toxoplasma* entry is arrested but rhoptry secretion continues; these evacuoles move along host microtubules (Hakansson et al., 2001; Sinai et al., 1997). Tachyzoites grown in colchicine-resistant CV2-8 CHO cells displayed differential microtubule stability. Tachyzoites in 1.0 mM colchicine continue budding but were round rather than crescent-shaped; 5.0 mM colchicine disrupted both spindle and subpellicular microtubules and tachyzoites grew but were incapable of cell division (phase contrast data not shown). Electron microscopy of tachyzoites grown in CV2-8 CHO cells in 1.0 mM colchicine showed the round, budding phenotype observed with tachyzoites in 0.5 μM oryzalin (Fig. 3E) and in 5.0 mM colchicine the round, non-budding appearance akin to the shape of tachyzoites in 2.5 μM oryzalin (Fig. 3F).

Replicating tachyzoites discard excess organelles and cytoplasm in a posteriorly located structure termed the residual body. This structure can resemble the rounded daughter buds observed in oryzalin-treated parasites. In order to distinguish daughter parasites from the residual body, we used an antibody (45.15, anti-IMC-1) that recognizes an intermediate-filament-like component of a network that is associated with the inner membrane complex (IMC). In untreated tachyzoites, IMC-1 labeling was localized directly below the plasma membrane and ran from a region directly under the extreme apex of the parasite to a region close to the posterior of the tachyzoite (Fig. 4A, top row). It began in a region coincident with the subpellicular microtubules but extended significantly beyond them. The residual body was not labeled with the IMC-1 antibody. Immunofluorescence with the *Toxoplasma*-specific tubulin antibody demonstrated that at 0.5 μM oryzalin, the subpellicular microtubules were shortened or absent (Fig. 4A middle row), but nuclear division proceeded with correct segregation of the centrioles (Fig. 4B middle row). In contrast, in 2.5 μM oryzalin, both spindle and subpellicular microtubules were disrupted (Fig. 4A bottom row), nuclear division and budding ceased and centrioles continued to duplicate unchecked (Fig. 4B bottom row). Equivalent results are obtained with colchicine-treated *Toxoplasma* in CV2-8 cells (not shown). Fig. 4C shows two daughter parasites (demarcated by the IMC-1 antibody) that have completed nuclear division (DAPI) and budded (tubulin and IMC-1 labeling). One nucleus was correctly segregated to a daughter bud (DB), but the other bud failed to capture a nucleus, which was retained within the residual body (RB). Quantification of nuclear division and budding behavior in 0.5 μM oryzalin demonstrated that the majority of replicating parasites (~60%) correctly divided and segregated their nuclei (Fig. 4D). A smaller set of parasites underwent nuclear division but retained one or both nuclei in the residual body (~20%). A similar number of parasites (~20%) either underwent aberrant nuclear division (producing unequally sized nuclei) or arrested prior to nuclear division although daughter buds were formed. The innate failure rate of division in untreated cells is >1% as judged by DAPI staining. The important conclusion of this quantification is that nuclear division was unaffected in 80% of replicating parasites, and the bulk of these parasites correctly segregated their nuclei to daughter cells under conditions that disrupted the subpellicular microtubules.

To assess the recovery of microtubule function in parasites treated with oryzalin, we treated tachyzoites with drug for 24-48 hours, removed the oryzalin and followed recovery (Fig. 5). We observed that parasites treated with 0.5 μM oryzalin for 48 hours (Fig. 5A) recovered the ability to form subpellicular microtubules (Fig. 5B). Invasiveness was restored in these parasites, suggesting that the subpellicular microtubules played a role in tachyzoite invasion. These parasites continued to invade and replicate correctly, indicating that their chromosomes were correctly segregated during the 48 hour exposure to oryzalin (six to seven doublings). When oryzalin was washed out from *Toxoplasma* tachyzoites treated with 2.5 μM oryzalin for 48 hours (Fig. 5C), the polyploid nuclear mass was not correctly segregated (Fig. 5D). Daughter parasites were made that contained large aggregates of DNA, contained only an apicoplast genome or lacked DNA altogether. Astoundingly, parasites without nuclei completed budding and escaped from the parasitophorous vacuole (Fig. 5E1-4).

Washout experiments consisting of a 48 hour oryzalin treatment followed by a 48 hour recovery were quantified to assess the subsequent recovery of tachyzoites. Parasitophorous vacuoles containing equal to or greater than eight parasites per parasitophorous vacuole were assumed to be vacuoles that were existent during the oryzalin treatment and washout/recovery (primary parasitophorous vacuoles). Vacuoles containing large aberrant masses were considered to be irretrievably altered parasites in primary parasitophorous vacuoles. Parasitophorous vacuoles containing 1-4 parasites were considered to be parasites that lysed from the original parasitophorous vacuoles and invaded new host cells during the washout/recovery phase, creating 'secondary' parasitophorous vacuoles. Parasites were scored as (1) primary vacuoles with replicating parasites, (2) primary vacuoles with aberrant parasites, (3) secondary vacuoles with non-replicating parasites (single crescent-shaped parasites) or (4) secondary vacuoles with replicating parasites (2 or 4 parasites per parasitophorous vacuole). Parasites treated with 0.5 μM oryzalin retained the capacity to undergo correct nuclear division and scission despite lacking the bulk of their subpellicular microtubules. After oryzalin was removed, the tachyzoites recovered their subpellicular microtubules and lysed out of host cells, reinvaded and continued to replicate as second generation parasites (Fig. 6, left panel). Slightly more abnormal versus normal primary vacuoles were observed in these samples because many of the normal first generation vacuoles have lysed and the parasites have gone on to make secondary vacuoles. When oryzalin was removed from parasites treated with 2.5 μM of the drug, an increased number of aberrant masses in primary vacuoles was observed. Second-generation parasites bud off of these masses and escape from the parasitophorous vacuole but they contained irregular nuclei or lacked nuclei altogether. The majority of these second generation parasites could not initiate growth and replication because they lacked adequate genetic material (Fig. 6, right panel).

Discussion

The model of microtubule organization illustrated by most vertebrate cells or by yeast is characterized by two microtubule populations organized by a single MTOC, the centrosome or spindle pole body (Kuntziger and Bornens, 2000; Palazzo et al., 2000). In interphase cells, this juxtanuclear MTOC

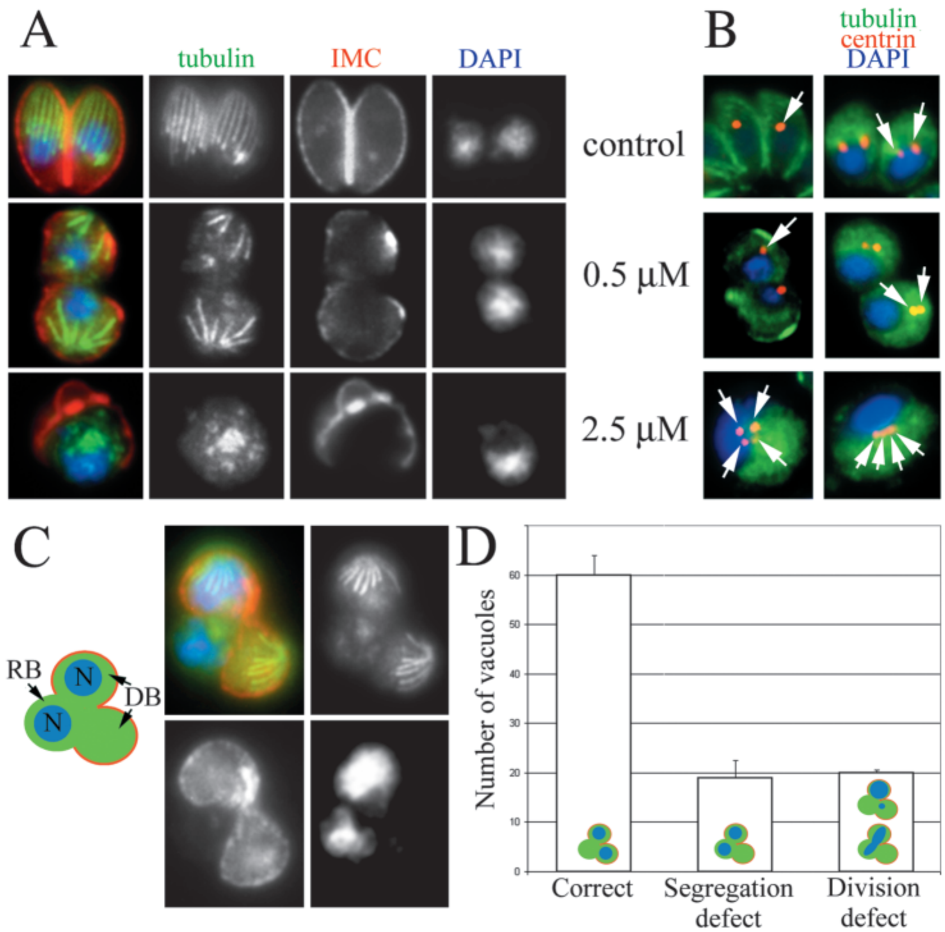

Fig. 4. In 0.5 µM oryzalin, the subpellicular microtubules are shortened or absent but nuclear division proceeds with correct segregation of the centrioles; in 2.5 µM oryzalin, both spindle and subpellicular microtubules are disrupted, nuclear division and budding cease and centrioles continue to duplicate unchecked. (A) Triple immunofluorescence of microtubules (green), IMC-1 (red) and DNA (blue) in control cells (top row), samples treated with 0.5 µM oryzalin (middle row) or samples treated with 2.5 µM oryzalin (bottom row). Without a drug, the subpellicular microtubules extend along two-thirds of the length of crescent-shaped tachyzoites. In 0.5 µM oryzalin, the subpellicular microtubules are greatly shortened or absent, although nuclear division continues with nuclear segregation. Daughter parasites are round rather than crescent shaped. At 2.5 µM oryzalin, all microtubules are disrupted and the parasite grows as a large round blob. (B) Triple immunofluorescence of tubulin (green), centrin (red) and DNA (blue) in control cells (top row), samples treated with 0.5 µM oryzalin (middle row) or samples treated with 2.5 µM oryzalin (bottom row). Centrioles appear as a single (non-replicated) spot or two (replicated) spots in untreated parasites. Centrioles continue to segregate correctly in 0.5 µM oryzalin, but in 2.5 µM oryzalin they continue to replicate although nuclear division is inhibited. (C) Triple immunofluorescence of microtubules (green), inner membrane complex (red) and DNA (blue) in cells treated with 0.5 µM oryzalin illustrates that although nuclear division occurs in 0.5 µM oryzalin, a subset of the nuclei fail to segregate correctly to the daughter buds and are retained in the residual body. (D) Quantification of nuclear division and segregation in 0.5 µM oryzalin demonstrates that the majority of replicating parasites (~60%) correctly divides and segregates their nuclei. A smaller set of parasites undergoes nuclear division but retains one or both nuclei in the residual body (~20%). A similar number of parasites either undergo aberrant nuclear division (producing unequally sized nuclei) or arrest prior to nuclear division although daughter buds are formed (~20%).

organizes the bulk of cytosolic microtubules. During the course of replication, the cytosolic microtubules are reorganized into a bipolar spindle following MTOC duplication. In contrast, during endodyogeny, *Toxoplasma* tachyzoites do not dedifferentiate, therefore their subpellicular microtubules are not disassembled or reorganized prior to nuclear division (Sheffield and Melton, 1968). Because cell shape and apical specialization are essential to invasion, this permits tachyzoites to remain invasive throughout their cell cycle. Perhaps by necessity, each of the tachyzoite microtubule populations has an individual MTOC. The subpellicular microtubules are nucleated from and organized by the apical polar ring (Nichols and Chiappino, 1987; Russell and Burns, 1984). The spindle microtubules are nucleated from two spindle pole plaques (Chobotar and Scholtyseck, 1982; Senaud, 1967). Both daughter subpellicular microtubules and spindle microtubules are adjacent to cytoplasmic centrioles during replication (Fig. 2). *Toxoplasma* centrioles contain nine singlet microtubules and a central singlet microtubule (Fig. 2C). Although centrioles typically contain a '9 + 0' organization of triplet microtubule blades, centrioles containing singlet microtubules are found in *Caenorhabditis* testes and *Drosphila* embryos; however, neither of these examples contains a central microtubule (Preble et al., 2000).

Nuclear division in the Apicomplexa proceeds without nuclear membrane breakdown. Spindle pole plaques (variously termed centrocones, centriolar equivalents or centriolar plaques) organize the spindle microtubules of apicomplexans (Chobotar and Scholtyseck, 1982; Senaud, 1967). The Apicomplexan spindles terminate in poorly defined regions of electron density located within invaginations of the nuclear membrane; these regions (the spindle pole plaques) are found in close proximity to extranuclear centrioles (Fig. 2). In turn, the centrioles are intimately associated with the apicoplast (Striepen et al., 2000) and are also close to the forming daughter buds. The processes of nuclear division and budding in *Toxoplasma* are somewhat akin to these behaviors in *Chlamydomonas*. The biflagellated alga *Chlamydomonas* also has a closed nuclear division (Preble et al., 2000). Membrane-associated rootlet microtubules originate from the basal bodies, and these microtubules enfold the daughter nuclei during replication, similar to the behavior of the daughter subpellicular microtubules in *Toxoplasma*. *Chlamydomonas* has prominent fibers formed from centrin that link the axoneme, basal bodies/centrioles and nucleus (Baron et al., 1995; Salisbury et al., 1988; Taillon et al., 1992; Wright et al., 1989). The *Chlamydomonas vfl2* mutant has a point mutation in centrin and a defect in organelle segregation, indicating that centrin fibers play a role in correct segregation during replication (Taillon et al., 1992). Since the *Toxoplasma* centrioles do not appear to be nucleating microtubules directly, we suggest that they may function to organize centrin fibers that in turn link the subpellicular microtubules to the apicoplast and nucleus.

Cytokinesis in higher eukaryotes is regulated by the position of the spindle poles (Rappaport, 1961; Wheatley, 1999). The cleavage furrow forms perpendicular to the plane of the spindle, thus ensuring that each daughter cell will inherit a nucleus. The apical polar ring MTOC drives *Toxoplasma* budding, whereas nuclear division is controlled by the spindle pole plaque structure. This arrangement permits *Toxoplasma* to replicate while maintaining a fully differentiated phenotype. In the related parasite *Plasmodium*, this arrangement permits schizogeny, the accumulation of multiple nuclei prior to a synchronous budding of ~64 daughter parasites. One consequence of having multiple MTOC is that it is possible to disconnect nuclear division and budding to create anucleate daughter parasites. We have generated anucleate parasites by disrupting the synchrony of nuclear division and parasite budding (Fig. 5). Anucleate 'zoids' were previously identified in the unrelated protozoan parasite *Trypanosoma brucei* after treatment with drugs (such as rhizoxin) that disrupt microtubules (Ploubidou et al., 1999; Robinson et al., 1995). In this case, the spindle microtubules appear to be more susceptible to disruption than the subpellicular microtubules. When *T. brucei* is treated with rhizoxin, the nuclear genome is replicated but spindle function and nuclear segregation is inhibited. These parasites divide, creating a diploid daughter cell and an anucleate zoid, both bounded by subpellicular microtubules and containing a functional flagellum. Trypanosome zoids contain kinetoplast DNA, reminiscent of the *Toxoplasma* zoids described here, which generally contain the apicoplast genome (Fig. 5E).

Shaw and Tilney have previously studied the effect of 1-5 μM oryzalin on cell division in *Toxoplasma*. These authors used morphological observations to conclude that oryzalin prevented parasite budding (cytokinesis) but did not block centriole replication or mitotic spindle formation. In the present study, we have used functional assays to quantify the effects of low (0.5 μM) and high (2.5 μM) levels of oryzalin on nuclear and cell division in *Toxoplasma*. Our studies reveal that the spindle and subpellicular microtubules have different sensitivity to oryzalin. Thus, while we would agree with Shaw and Tilney that spindle microtubules are resistant to low levels of oryzalin (which disrupt subpellicular microtubule function), we observed disruption of both subpellicular and spindle microtubule populations at high levels of drug. This was most dramatically shown by the reversibility experiments. Removal of 0.5 μM oryzalin permitted daughter cells to establish functional subpellicular microtubules during cytokinesis. In turn, these restored subpellicular microtubules re-establish infectivity in daughter parasites. A dramatically different outcome was observed when parasites were removed from 2.5 μM oryzalin. These parasites failed to correctly segregate nuclei or centrioles into daughter cells, although cytokinesis generated anucleate progeny. We interpret this to mean that in 2.5 μM oryzalin, nuclear spindles were disrupted. Since DNA replication continued unchecked, upon oryzalin removal

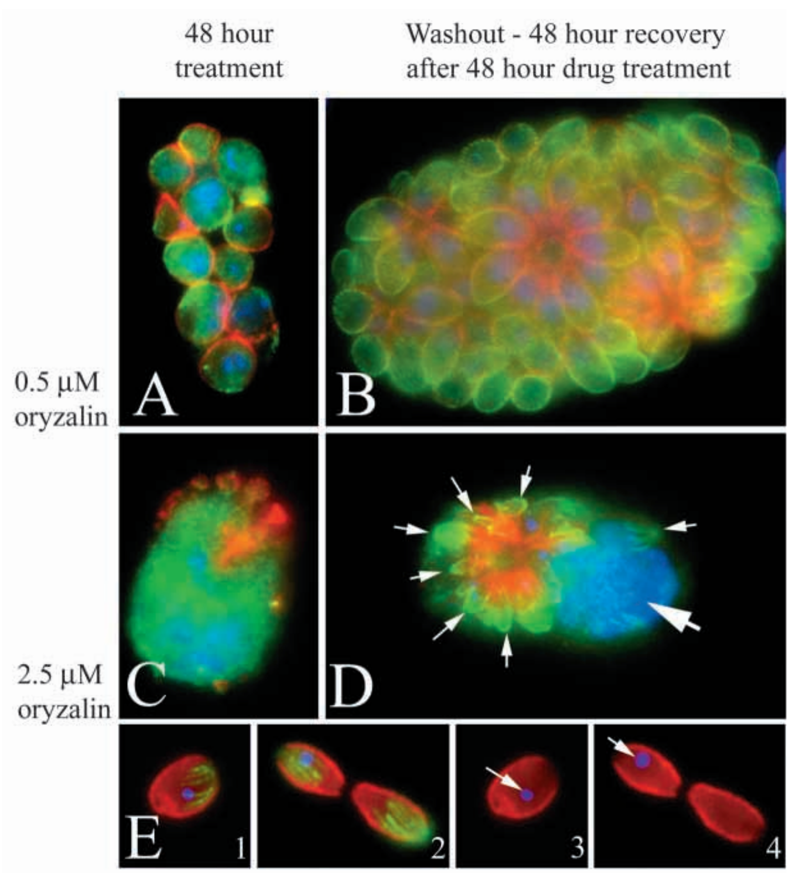

Fig. 5. Triple immunofluorescence of microtubules (green), IMC-1 (red) and DNA (blue) in samples treated for 48 hours with 0.5 μM oryzalin (A,B) or 2.5 μM oryzalin (C-E). Tachyzoites treated with 0.5 μM oryzalin undergo nuclear division and bud as round parasites (A). 48 hours after drug removal, these parasites continue replicating as crescent-shaped parasites with correctly sized and segregating nuclei and fully restored subpellicular microtubules (B). Tachyzoites treated with 2.5 μM oryzalin continue to grow (this is a single parasite) but fail to undergo nuclear division or bud off daughter parasites (C). When these samples are removed from the drug for 48 hours, they attempt to bud as multiple daughter parasites in a process reminiscent of schizogeny (D). The large, polyploid nuclear mass (large arrow) cannot be segregated correctly, so daughter buds (small arrows) are completely devoid of nuclear material or contain the apicoplast. Anucleate zoids (E1-4) can complete budding, escape from the vacuole and in some cases reinvade new cells but are incapable of growth or replication. E1 and 2 show triple-labeled zoids and E3 and 4 show the same cells with the tubulin staining omitted for clarity. The zoid in E1/3 has an apicoplast but lacks a nucleus, as does the left-hand zoid in E2/4. The right-hand zoid in E2/4 lacks both nucleus and apicoplast.

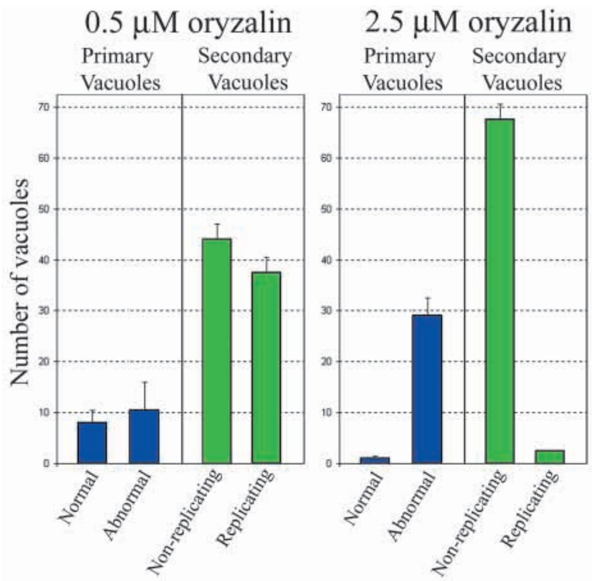

Fig. 6. Drug washout experiments demonstrate that treatment with 0.5 μM oryzalin is fully reversible, but treatment with 2.5 μM oryzalin causes irreparable damage. Quantification of 48-hour oryzalin treatment followed by a 48-hour recovery in the absence of the drug demonstrates that *Toxoplasma* nuclear division and segregation occur in 0.5 μM oryzalin (left panel). The blue columns enumerate the numbers of normal and abnormal parasitophorous vacuoles that were present at the time of drug treatment (primary vacuoles). The green columns quantify the numbers of secondary vacuoles that were made by parasites that lysed from primary vacuoles and invaded new host cells during the washout and recovery phase. Parasites treated with 0.5 μM oryzalin can recover and go on to make invasive parasites that are capable of replication after drug removal. However, in 2.5 μM oryzalin (right panel), the continued replication of DNA is uncoupled from spindle microtubule-mediated chromosome segregation and produces both aberrant primary vacuoles and second generation parasites that have unbalanced nuclei or lack nuclei and are incapable of continued growth.

parasites could not segregate chromosomes correctly and were unable to resolve this defect in nuclear division.

Observations of nuclear division in 0.5 μM oryzalin also suggest that the subpellicular microtubules are not required for scission of the horseshoe-shaped nucleus during mitosis. Both the washout studies and staining with DAPI indicate that nuclear division was accomplished in 0.5 μM oryzalin, where the subpellicular microtubules are greatly shortened or absent. It is, however, clear that the subpellicular microtubules play an important role in segregation of organelles to daughter buds. Although daughter buds (with correctly assembled inner membrane complex) form in 0.5 μM oryzalin, in approximately 20% of the population the divided nuclei do not segregate into daughter cells but are retained in the residual body. In 0.5 μM oryzalin, unequal segregation can also be observed for the apical complex organelles such as the rhoptries and micronemes (N.M., unpublished).

Previous work has shown that the subpellicular microtubules are extraordinarily stable during isolation and are heavily decorated with associated proteins (Morrissette et al., 1997; Nichols and Chiappino, 1987; Russell and Burns, 1984; Russell and Sinden, 1982). Drugs such as colchicines or the dinitroanilines that disrupt dynamic microtubules are completely ineffective against the subpellicular microtubules of extracellular apicomplexan parasites, indicating that these microtubules are not dynamic (Russell, 1983; Stokkermans et al., 1996). Once parasites are intracellular and replicating, colchicine or the dinitroanilines disrupt the nascent (dynamic) microtubules of daughter parasites. Consistent with this extraordinary stability, at the completion of endodyogeny, the maternal subpellicular microtubules, apical polar ring and conoid are removed to the residual body at the posterior of budding cells (Fig. 2E). This relocation may be a prelude to microtubule disassembly and recycling or to degradation of the complex. In either case, maternal microtubule disassembly is not coincident with daughter cell budding.

It is intriguing that the subpellicular microtubules that are highly stable in vitro should be more susceptible to disruption by drugs such as oryzalin or colchicine. The differential stability of the microtubule populations may be explained by the influence of an associated protein or proteins that specifically interact with only one of the microtubule populations. Alternatively, the intranuclear nature of the *Toxoplasma* spindle may afford some protection from the destabilizing drugs. However, we favor the hypothesis that the tachyzoites can still build short microtubules in lower concentrations of oryzalin or colchicine. These shorter microtubules may be adequate to make a functional spindle but are incapable of providing sufficient scaffolding to generate crescent-shaped rather than spherical tachyzoites. As would be predicted, centriole segregation (as assessed by centrin staining) occurs correctly as long as the spindle microtubules are intact. In the presence of lower concentrations of the microtubule-disrupting drugs colchicine or oryzalin, the centrioles segregate as long as spindle formation is unimpaired. In contrast, centriole segregation fails, although duplication remains unchecked, in concentrations of the drugs that disrupt both spindle and subpellicular microtubule populations (Fig. 4B).

Although *Toxoplasma* microtubules are disrupted by colchicine, compared with oryzalin, they are relatively insensitive to it (0.5-2.5 μM oryzalin versus 1.0 to 10.0 mM colchicine is required). This is consistent with the ‘plant-like’ nature of apicomplexan tubulin revealed by phylogenetic analysis (Stokkermans et al., 1996). Plant tubulins are exquisitely sensitive to oryzalin (a commercial herbicide) and relatively insensitive to colchicine (a plant product). The related apicomplexan *Plasmodium* is also susceptible to similarly high concentrations of colchicine that inhibit nuclear division and re-invasion. A previous study of the behavior of the *Plasmodium* erythrocytic stage in colchicine established that there were concentrations (10 μM-1mM) where schizont nuclear division and budding continued but invasion was inhibited, and higher concentrations (>10 mM) where nuclear division and schizogeny were affected (Bejon et al., 1997). Owing to the much smaller size of *Plasmodium* merozoites, the appearance of microtubules was not directly examined; however, they produced remarkably similar results to the data presented here, which suggests that *Plasmodium* also has differential stability for spindle and subpellicular microtubules.

In this paper we have investigated the dynamics of spindle microtubules and subpellicular microtubules. These two populations of microtubules are each organized by a different

MTOC and are differentially sensitive to disruption by drugs. These observations have permitted us to isolate the essential functions of these microtubules. The presence of subpellicular microtubules is necessary for host cell invasion. Parasites lacking intact subpellicular microtubules are incapable of invading host cells. Parasites containing subpellicular microtubules but lacking a nucleus are capable of completing scission from the maternal cell and are capable of invasion. Conversely, spindle microtubules are necessary and sufficient for chromosome segregation and nuclear scission. Apicomplexans use multiple MTOCs to independently control nuclear division and cell polarity/cytokinesis. Having multiple MTOCs permit greater flexibility but eliminates the opportunity for checks for accurate nuclear division and correct cytokinesis found in other eukaryotes.

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