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Role of Cytoskeleton in Host Cell Invasion by Intracellular Protozoa *Toxoplasma gondii*

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Abstract A microfilament-based motility in *Toxoplasma gondii* (*T. gondii*) is involved in host cell invasion, yet the exact mechanism has not yet been determined. Accordingly, the current study examined the localization of actin and tubulin in *T. gondii* using immunofluorescent (IF) and immunogold staining for electron microscopy. Indirect immunofluorescence (IF) staining using anti-actin and anti-tubulin monoclonal antibodies (mAbs) revealed localization of fluorescence on the entire surface of the tachyzoites. The actin in *T. gondii* was observed by immunogold staining, and the gold particles were seen on the surface, especially at the anterior end and in the cytoplasm of the parasite. However, there were no gold particles in the nucleus, rhoptries, and dense granules. The tubulin in *T. gondii* was located on the surface and in the cytoplasm of the tachyzoites in the extracellular parasite, compared with anterior part of tachyzoites in the intracellular parasite. The antigens of *T. gondii* recognized by anti-actin mAb were 107 kDa, 50 kDa, 48 kDa, and 40 kDa proteins, while those recognized by anti-tubulin mAb were 56 kDa, 52 kDa, and 34 kDa proteins. Tachyzoites of *T. gondii* pretreated with the actin inhibitor, cytochalasin D (20 μg/ml), and tubulin inhibitor, colchicine (2×10^{-6} M), for 30 min at 37°C were used to infect the isolated mouse macrophages (tachyzoites:macrophage=2:1). Pretreatment with the inhibitors resulted in lower multiplication of tachyzoites within the macrophages than in the untreated group 18 h post infection ($p<0.05$). Therefore, the present results suggest that actin and tubulin appear to be involved in the invasion of and multiplication in host cells.

Key words: *Toxoplasma gondii*, cytoskeleton, actin, tubulin, invasion, macrophage

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Toxoplasma gondii (*T. gondii*) is an obligate intracellular parasite that infects a wide variety of vertebrates, including humans and animals. *T. gondii* infection in immunocompetent individuals is clinically asymptomatic. However, in immunocompromised individuals, severe complications such as *Toxoplasma* encephalitis can be experienced along with abortion or neonatal malformation in pregnant women. After the ingestion of cysts or oocysts of *T. gondii*, tachyzoites or sporozoites are released within the intestinal lumen, through which they invade the host cells. Within a host cell, a tachyzoite within a parasitophorous vacuole rapidly divides by endodyogeny, eventually ruptures the host cell, and then invades new cells. *Toxoplasma* contains an apical complex with secretory organelles and a well- developed cytoskeleton. These secretory organelles are thought to contain certain components associated with enzymatic and metabolic processes involved in invasion and parasitophorous vacuole formation. The apical cytoskeleton is also suspected to play a role in the mechanical aspects of invasion [12, 22]. Nonetheless, the mechanism of the host cell entry of *T. gondii* has not yet been clarified.

The cytoskeleton of eukaryotic cells is composed of microtubules and microfilaments that are linked to motility. The microfilaments involved in cell invasion represent the true contractile machinery of the cell cytoplasm. Actin is an abundant protein in eukaryotic cells, while the dynamic assembly and disassembly of actin filaments produce cell motility [20]. Actin molecules have been found on the preconoidal rings and the polar ring in the conoid, and these organelles appear to be filamentous [25]. Monoclonal antibodies specific to mammalian β-tubulin can recognize the microtubule cytoskeleton in *T. vaginalis*, *T. gondii*, and *Leishmani donovani* [18, 19]. The presence of actin in the anterior end of *Cryptosporidium parvum* sporozoites

suggests that cytoskeletal action may provide a structural organization for the apical organelles and facilitate the host cell membrane penetration [7].

Microtubules are dispersed throughout the cytoplasm and also found in cilia, flagella, and centrioles that are involved in cell motions. Although *Toxoplasma* does not have specialized organelles for motility, such as cilia or flagella, they can move along a surface by gliding [11]. *T. gondii* displays a gliding motility which relies on an actin-based contractile system in the parasite [5]. The current study was performed to identify the cytoskeleton of *T. gondii* and its influence on invasion of this parasite into host cells. The data here demonstrated that the actin and tubulin in *T. gondii* were involved in the invasion process into host cells.

MATERIALS AND METHODS

Culture of *T. gondii*

The *T. gondii* (RH strain) was maintained by serial passage in ICR mice twice a week. The trophozoites were harvested from the peritoneum of the infected mice 48 h and 96 h after infection, washed, and centrifuged in a Minimum Essential Medium (MEM, Gibco, Grand Island, NY, U.S.A.) [9]. The viability of the parasite was examined using trypan blue dye [26].

Immunoelectron Microscopy

The parasites were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in a caccodylate buffer+3% sucrose for 2 h at room temperature. After dehydration in 20%, 50%, and 70% ethanol, embedding in LR White (London Resin Co., Taab Lab., U.K.) was performed. Ultrathin sections on a nickel grid were incubated with NH_4Cl and 0.5 M Tris buffer, followed by 2 h of incubation with an anti-actin monoclonal antibody (mAb) raised against chicken gizzard actin or anti-tubulin mAb raised against *Strongylocentrotus purpuratus,* and incubation with colloidal gold (12 nm) conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, U.S.A.). After washing, the sections were stained with 4% uranyl acetate in water and observed under an electron microscope (Hitachi 600, Tokyo, Japan) [10].

Indirect Immunofluorescent (IF) Staining

After washing the *T. gondii* tachyzoites with phosphate buffered saline (PBS), the parasites were fixed with 10% paraformaldehyde for 30 min. After washing, the parasites were incubated with 1% NH_4OH for 5 min and Tween 80 for 5 min at room temperature. An emusified solution of 20 µl of *T. gondii* (1.5×10^6 cells/ml) was dropped onto a marking glass slide and dried. The trophozoites were then defatted with cold acetone for 3 min and dried at room temperature. Next, the slides were immersed in anti-actin and anti-tubulin mAb (1:40–1:1,280 dilution) and incubated at 37°C for 1 h in a wet chamber. After washing, the slides were immersed in a 1:30–1:50 dilution of FITC-conjugated goat anti-mouse IgG (Cappel, Pennsylvania, U.S.A.) and incubated at 37°C for 1 h. The slides were then observed under fluorecence microscopy (Fluoval, Jenaval Co., Germany) after staining with 0.1% Evans blue for 5 min and 90% glycine [17].

Western Blot Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed and the separated proteins were transfered onto nitrocellulose paper according to Towbin *et al.* [23]. The nitrocellulose papers were incubated with anti-actin mAb (Chemicon, Temecula, California U.S.A.) or anti-tubulin mAb (Sigma) in PBS-5% skimmed milk at 37°C for 1 h. After washing, the strips were incubated in affinipure $F(ab')_2$ fragment peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, Pennsylvania, U.S.A.) and reacted with diaminobenzidine (DAB, Sigma) solution for 5 min [4].

Pretreatment of Parasite with Actin and Tubulin Inhibitors

BALB/c mice (Korea Animal Co., Seoul, Korea) were injected peritoneally with lipopolysaccharide (LPS, 10 µg/0.1 ml, Sigma) 3 days before collecting peritoneal cells. *T. gondii* tachyzoites were harvested from ICR mice and pretreated with 20 µg/ml cytochalasin D (Sigma) in PBS and 2×10^{-6} M colchicines (Sigma) in DMSO at 37°C for 30 min. The adherent peritoneal macrophages (1×10^6) were washed with PBS and infected with the pretreated tachyzoites (tachyzoite:macrophage=2:1) in 12-well plates with a glass cover. Four and 18 h after the parasite infection, the glass covers were fixed in 100% methanol and stained with a Giemsa solution. After drying the glass covers, the number of infected macrophages and tachyzoites per 300 macrophages were counted under a light microscope.

RESULTS

Immunoelectron Microscopy

The localization of actin and tubulin in the *T. gondii* tachyzoites was examined with each mAb under an electron microscope. The immunogold staining of the tachyzoites with anti-actin mAb revealed specific labeling of gold particles on the surface, especially at the anterior end and in the cytoplasm of the parasite. The tubulin of *T. gondii* was located on the surface and in the cytoplasm of the tachyzoites in the extracellular parasite, and in the anterior part of the tachyzoites in the intracellular parasite. However, there were no gold particles in the nucleus,

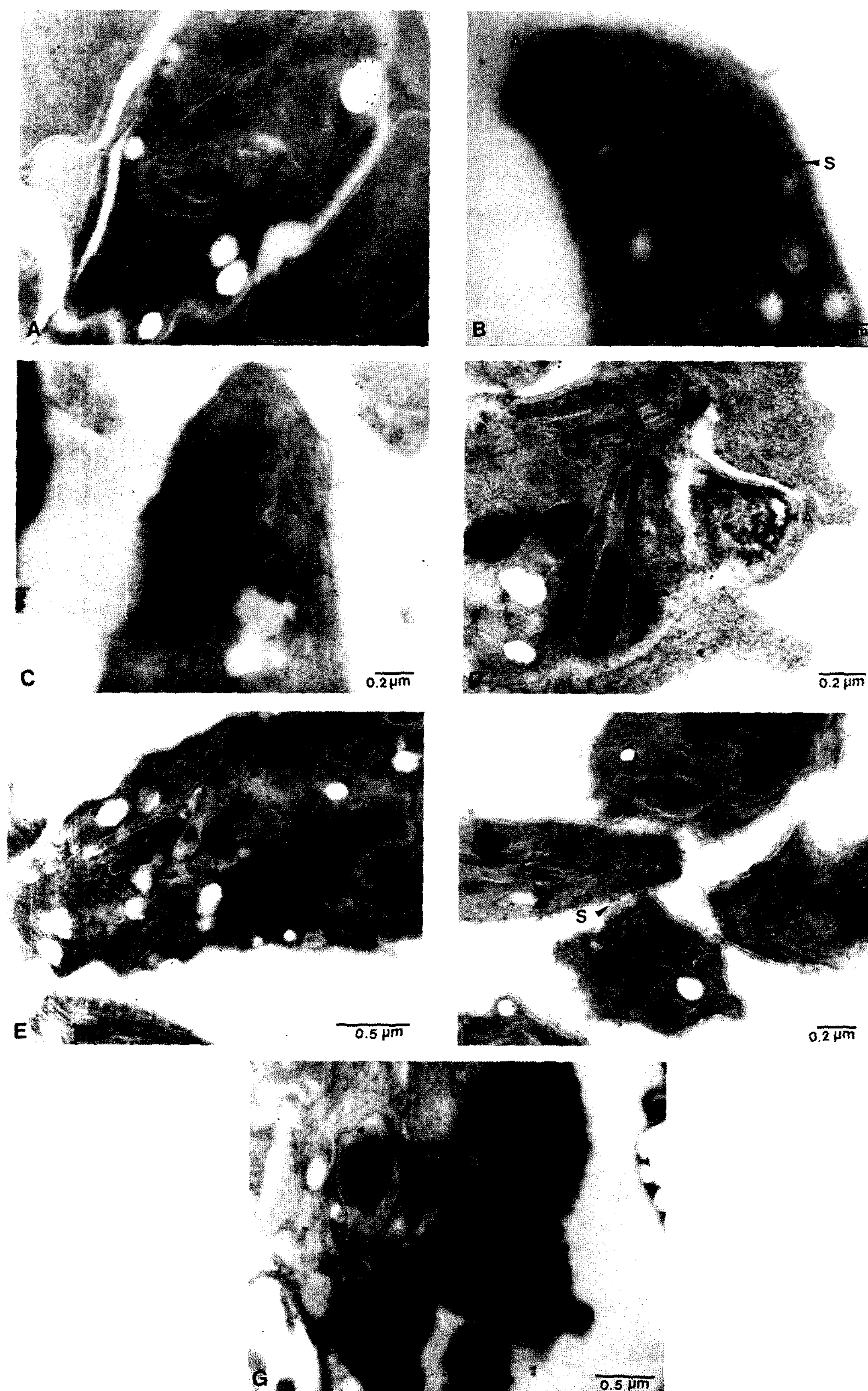

Fig. 1. Electron micrographs of trophozoite of *T. gondii* using the immunogold method.
Gold particles with anti-actin (A-C) and anti-tubulin (D-F) mAbs are seen at the anterior pole of the parasite and along the surface membrane. G, normal serum; A, anterior part of parasite; S, surface membrane of tachyzoite.

rhoptries, or dense granules (Fig. 1A-F). In addition, no particles were observed when the specimens were directly stained with colloidal gold conjugated goat anti-mouse IgG as the control (Fig. 1G).

Immunofluorescence Microscopy

The patterns of the fluorescence in the formaldehyde-fixed extracellular parasites were examined by staining with anti-actin or anti-tubulin mAbs to determine whether the actin and tubulin antigens were on the surface of the parasite. The *T. gondii* tachyzoites exhibited intense fluorescence labels on the entire surface with both anti-actin mAb (1:40–1:160) and anti-tubulin mAb (1:40–1:1,280) (Figs. 2C and 2D). There was no difference in the localization of the tachyzoites with the two mAbs, although the fluorescence of the anti-tubulin mAb was more intense than that of the anti-actin mAb. The controls consisted of polyclonal anti-*Toxoplasma* serum, which exhibited brighter staining than both mAbs (Fig. 2B).

Western Blot

The immunoblot patterns of *T. gondii* against anti-actin and anti-tubulin mAbs revealed 107 kDa, 50 kDa, 48 kDa, and 40 kDa for actin and 56 kDa, 52 kDa, and 34 kDa for tubulin. Similar patterns for actin and tubulin were observed in the crude lysate and soluble antigens of *T. gondii* (Figs. 3A and 3B).

Infectivity of *T. gondii* after Treatment with Inhibitors

To determine whether microfilament inhibitors can influence the invasion process of *T. gondii* into host cells, *T. gondii* invasion into mouse peritoneal macrophages was examined by microscopic observation after Giemsa staining. The motility in *T. gondii* was inhibited by pretreatment with microfilament inhibitors, such as cytochalasin D and colchicine. The percentage of macrophages infected with cytochalasin D-treated *T. gondii* was significantly lower than in the untreated control cultures. The number of macrophages infected with cytochalasin D-treated *T. gondii* was significantly reduced to 33.7±8.17 (number of infected cells in PBS treated control was 55.6±4.53), while the infected number with colchicine-treated tachyzoite was reduced to 31.2±5.32, 18 h PI ($p<0.05$). The number of tachyzoites per macrophage was reduced from 3.7±5.32 to 2.6±0.65 after treatment with cytochalasin D and 2.9±0.50 after treatment with colchicine, 18 h PI (Table 1).

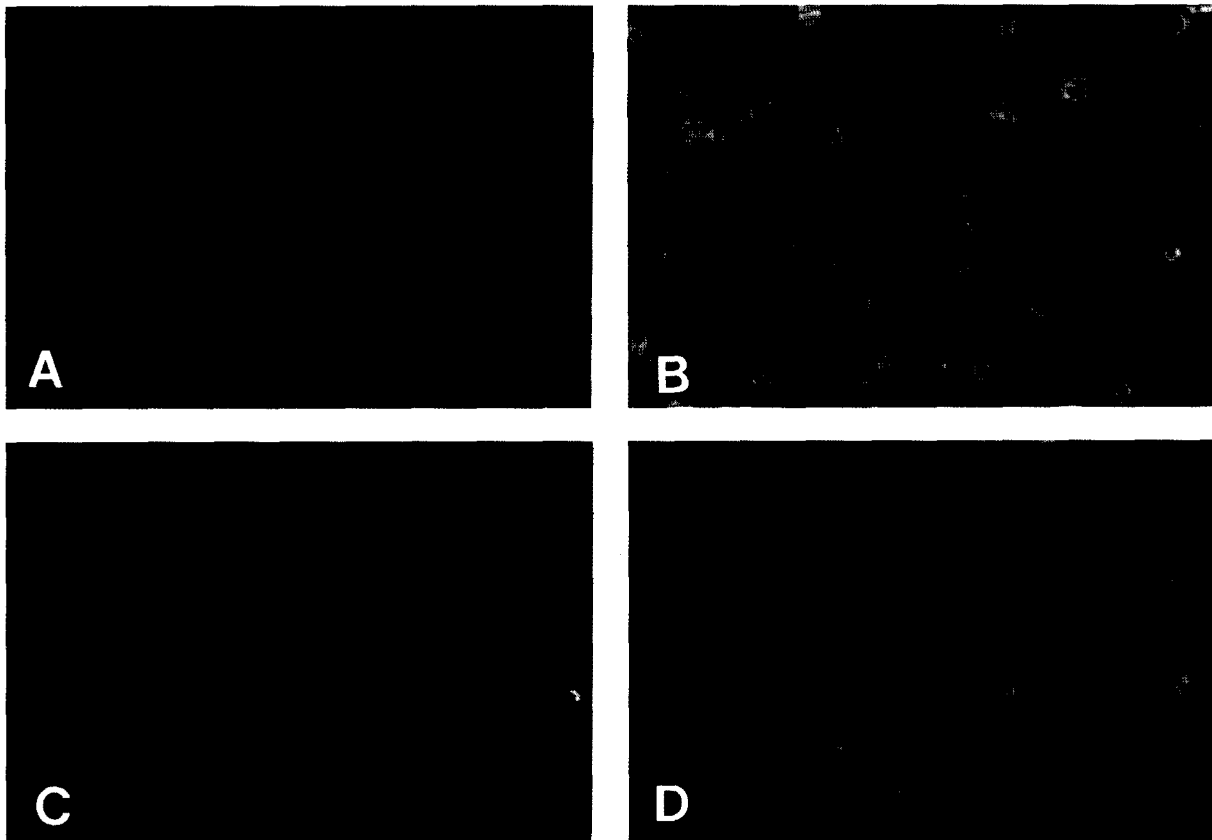

Fig. 2. *T. gondii* tachyzoites after reacting with anti-actin and anti-tubulin mAbs using IFAT.
In the parasite, fluorescein is localized on the surface and faintly localized in the cytoplasm. A, negative control; B, positive control; C, anti-actin mAb (1:160); D, anti-tubulin mAb (1:640).

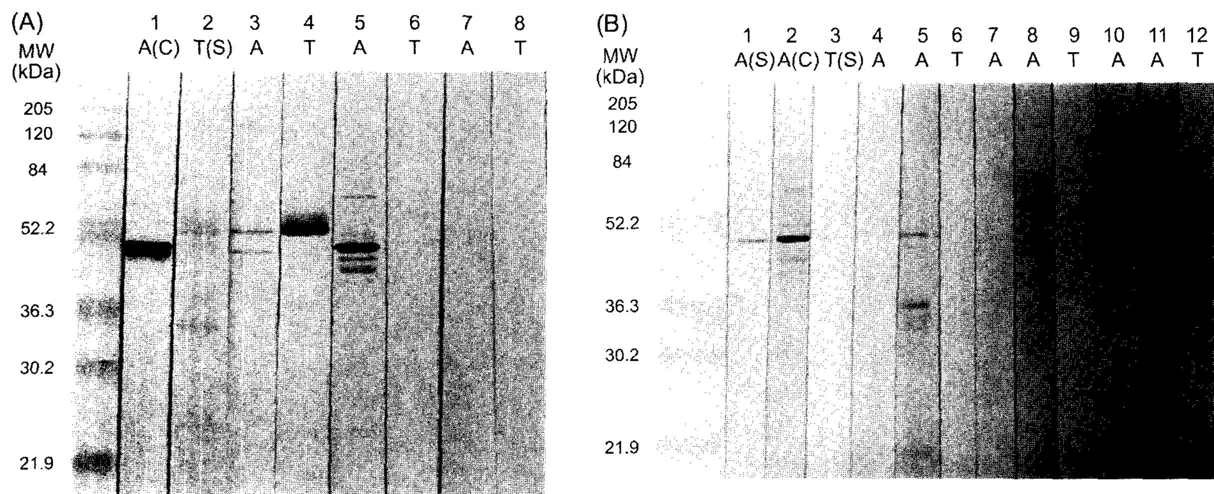

Fig. 3. Western blot analysis of *T. gondii* with anti-actin and anti-tubulin mAbs.
The anti-actin mAb reacted with 50 kDa and 46 kDa proteins, while the anti-tubulin mAb reacted with proteins of 56 kDa, 54 kDa, and 34 kDa. (A) Lanes 1–2, *T. gondii* lysate; lanes 3–4, whole *T. gondii*; lanes 5–6, *Anisakis* sp.; lanes 7–8, *T. vaginalis*. (B) Lanes 1–3, *Anisakis* sp.; lanes 4–6, *P. westermani*; lanes 7–9, *T. vaginalis*; lanes 10–12, *T. gondii* lysate. A, actin; T, tubulin; S, Sigma; C, Chemicon.

DISCUSSION

Apicomplexan parasites, including *Toxoplasma*, *Plasmodium*, *Cryptosporidium*, and *Eimeria*, have a common mechanism for motility and cell invasion [7, 15], including attachment to a host cell, parasite orientation prior to invasion, protrusion of the conoid, formation of a moving junction, exocytosis of micronemes and rhoptries, and membrane penetration and formation of intracellular vacuoles following invasion [8, 11, 12]. The current study demonstrated the localization of actin and tubulin in *T. gondii* and their influence on the invasion of the parasite into host cells where actin and tubulin were distributed. Indirect IF staining of the tachyzoites then showed across the entire surface of *T. gondii*. The actin was mainly localized in the anterior part of the *Toxoplasma*, based on immunofluolescent staining. Several previous studies have shown, through indirect IF staining, that the *T. gondii* actin is intensely localized in one end of the tachyzoites and faintly throughout the cytoplasm [6, 21]. The sporozoites of *C. parvum* typically exhibit a diffuse and moderately intense fluorescence labeling following indirect IFA with anti-actin mAb [7]. In order to analyze the presence of actin and tubulin molecules in *T. gondii*, immunolocalization was carried out using colloidal gold. The result showed that immunogold particles of actin and tubulin were found on the surface membrane and in the cytoplasm, especially in the anterior portion of the parasite. In *Toxoplasma*, actin molecules are localized along the inner surface of the inner membrane complex in the anterior part of the parasite, the preconoidal rings, polar ring, in the conoid, and in the space between the anterior terminal of the inner membrane complex and the conoid [25]. The immunoblot pattern of the crude extract from *T. gondii* revealed several actin and tubulin protein bands. However, there was no difference between the bands of the crude and soluble extracts of *T. gondii*. Since some different bands for actin and tubulin in *Anisakis* sp. larva have been reported and the mAb against *Amoeba proteus* actin cross-reacts with actins of both vertebrate and invertetrate origin, including *Acanthamoeb*

Table 1. Invasion of macrophages and multiplication of *T. gondii* (RH) tachyzoites pretreated with actin and tubulin inhibitors.

Pretreatment of *T. gondii*	Infected cells/100 Mac.		No. of tachyzoites/cell		No. of tachyzoites/infected cell	
	4 h	18 h	4 h	18 h	4 h	18 h
No serum	12.65±9.09	55.59±4.53	0.25±0.25	2.04±1.29	1.75±0.41	3.68±2.34
NI serum	2.64±0.59*	32.68±4.52*	0.06±0.02*	1.21±0.42*	2.37±0.75	3.73±1.30
Anti-actin	3.78±1.1*	23.48±11.18*	0.08±0.04	0.53±0.19*	2.1 ±0.9	2.41±0.53
Anti-tubulin	5.28±1.2*	28.50±7.67*	0.07±0.03*	0.85±0.26*	1.48±0.84	2.97±0.22
Cytochalasin D	6.97±1.29	33.73±8.17*	0.13±0.05	0.84±0.06*	1.92±0.72	2.61±0.65
Colchicine	6.39±0.99*	31.24±5.32*	0.13±0.06	0.88±0.1*	2.04±1.05	2.86±0.5
DMSO	13.22±7.84	47.32±12.93*	0.18±0.11	1.54±0.36	1.42±0.38	3.28±0.29

*$P<0.05$ (Mann-Whitney U Test).

[3], further analysis of antigens for the actin and tubulin of *T. gondii* is needed to elucidate the invasion process into host cells.

In an assay of the invasion of the parasite into host cells, after treating the *T. gondii* tachyzoites with cytochalasin D, the invasion process was partially inhibited, while cytochalasin B totally inhibited the invasion of the parasite into the host cells [14]. In the current experiment, the treatment of the tachyzoites with cytochalasin D or colchicine inhibited the number of parasites 18 h postinfection, compared with the untreated control. These results indicated that the *T. gondii* tachyzoite motility appeared to be dependent on the action of both the actin and the tubulin in the parasite. The inhibited entry of *T. gondii* into peritoneal macrophages due to cytochalasin D was dose dependent and comparable to the inhibition of phagocytosis [16]. Accordingly, since the invasion mechanism depended on actin polymerization, and the effect of the drug was to destabilize this polymerization [5], the capability of the invasiveness of the sporozoite of *C. parvum* was not greatly influenced by the pretreatment of the parasite with cytochalasin D [27]. The decreased number of parasites in the cells was related to the treatment with colchicines, which interfer with motility, cell invasion, or replication of the parasite due to the microtubules [2]. There is a previous report that colchicine failed to modify the clinical outcome of acute toxoplasmosis in mice [1], although the addition of colchicine to a culture of glial cells infected with *T. gondii* decreased the number of parasites in comparison with the controls. However, when considering the current results and the earlier report that colchicine reduced the infectivity of *C. parvum* oocysts in cells *in vitro* [24], it is highly possible that the polymerization of the microtubules in *T. gondii* might be involved in the pathogenesis of the parasite.

Thus, the cytoskeleton would appear to be involved in diverse cellular functions, such as maintenance of cell shape, in cell motility, intracellular transport, and mitosis [21]. The detergent-extracted parasite exhibits a spiral arrangement of 22 pellicular microtubules and these microtubules have been shown to be free posterior ends [12, 13]. In the current experiment, the monoclonal antibodies of the microfilaments and microtubules were found to be localized in the apical region and on the surface of the parasite. In conclusion, the actin and tubulin in *T. gondii* were found to be involved in host cell invasion, thus providing targets for preventing intracellular parasite infection.

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