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Immunocytochemical localization of actin in *Toxoplasma gondii*

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Abstract. The localization of actin in the trophozoites of *Toxoplasma gondii* was examined by means of immunogold staining for electron microscopy. The thin-sectioned specimens were incubated with the IgG fraction of polyclonal antibodies raised against *Ascaris* body-wall smooth-muscle actin following colloidal gold-conjugated protein A. Electron-dense gold particles were confined to the anterior polar region in the trophozoites: they were found in the conoid, preconoidal rings, possibly in the polar ring, and in the space between the anterior terminal of the inner membrane complex and conoid. The present experiments also suggest interactions of actin with subpellicular microtubules, leading to speculation that the association of actin with microtubules provides a link to myosin, a potential source of power for microtubule-dependent movements.

Toxoplasma gondii, the causative agent of worldwide human and animal toxoplasmosis, is a typical obligate intracellular protozoan parasite. In its asexual life cycle, independent movement has been reported to be transient, in most cases being observed immediately following its release from a host cell (Bommer et al. 1969; Endo et al. 1987; Jacobs 1953; Sourander et al. 1960). In our recent experiments H^+ gradients, pH gradients across the parasite membrane, proved to be prerequisite for the initiation of this motility (Endo et al. 1987). Considering the potential importance of motility in the life cycle of this parasite, a cellular mechanism of motility should be more precisely studied.

Motility in coccidian species, including *Toxoplasma,* has recently been reported to be inhibited by treatment with cytochalasin B or D, the microfilament inhibitors, but not by the microtubule inhibitors (Jensen and Edgar 1976; Russel and Sinden 1981; Ryning and Remington 1978). These facts indicate that the aspects of motility involve the actomyosin system in nonmuscle cell types (Russe and Sinden 1981), whereas the possible participation of the microtubular system is still argued (Nichols and Chiappino 1987). More recently, actin was found in the trophozoites of the organism by immunofluorescent staining (Cintra and De Souza 1985; Endo et al. 1988). We also demonstrated that actin is mainly localized in the anterior part of the organism. Interestingly, this localization of actin is consistent with that of myosin (Schwartzman and Pfefferkorn 1983).

In the present experiments, the ultrastructural localization of actin were examined by immunogold staining using rabbit anti-*Ascaris* body-wall actin antibodies; possible mechanisms of movement in *T. gondii* is discussed.

Materials and methods

Parasites

The virulent strain (RH) of *T. gondii* was maintained by serial IP passages at 3- to 4-day intervals in mice. The trophozoites of *T. gondii* were harvested from peritoneal exudates of mice on the 3rd or 4th day of infection. They were washed twice by centrifugation at 300 *g* in K_2SO_4-based isotonic solution (pH 8.2) (K-buffer, Endo et al. 1987); the sediment was resuspended in the same medium. The viability of the parasites was monitored by the trypan blue dye exclusion test.

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Preparation of anti-actin antiserum

The preparation of anti-*Ascaris* body-wall muscle actin polyclonal antibodies was carried out as previously described by Nishioka et al. (1983). The IgG fraction of anti-*Ascaris* actin antibody was prepared by actin affinity column chromatography followed by protein A affinity column chromatography. About 80% of the IgG fraction obtained showed reactivity with *Ascaris* actin alone, but the remainder showed affinity to actins of various animals, suggesting that the latter recognizes the conserved regions of the actin molecule.

Electron microscopy

Fixation and embedding: The trophozoites in K-buffer were centrifuged and fixed at room temperature for 1 h in a 0.1% glutaraldehyde-2% paraformaldehyde mixture in 100 m*M* phosphate-buffered solution (pH 7.4). The samples were soaked for 1 h in 100 m*M* NH_4Cl in 100 m*M* phosphate solution to block free aldehyde groups. After dehydration using serially graded ethanol solution, they were embedded in a resin mixture (Lowicryl K4M) and polymerized at 4° C for 24 h. For further polymerization they were stored at room temperature for 2 days.

Some parasites were extracted at 4° C for 15 min with gentle agitation against 0.3% Nonidet P-40 (NP-40) in 5 m*M* phosphate-buffered solution in the presence of 5 m*M* $MgSO_4$, 0.2 m*M* EGTA, and 1 m*M* DTT. After collection by centrifugation they were processed as described above.

Immunogold staining for electron microscopy

Thin-sectioned specimens (approximately 50 nm thick) were mounted onto nickel grids and washed in phosphate-buffered saline containing 0.2% bovine serum albumin and 0.3% Tween 20 (PBS-BT). They were then incubated for 2 h at room temperature with the IgG fraction of rabbit antiactin polyclonal antibodies. In some experiments incubation was carried out at 4° C for 18 h. The immunoreacted specimens were washed thoroughly in PBS-BT and then reincubated with a colloidal gold-conjugated protein A solution for 45 min. They were again washed in PBS to remove nonreactive colloidal gold particles.

As a positive control, chicken-gizzard smooth muscle was stained as described above. Negative control preparations consisted of thin sections exposed to rabbit normal serum substituting for antiactin antiserum or colloidal gold-conjugated protein A only. The specimens obtained were then stained with uranyl acetate and lead citrate and examined under an electron microscope (Hitachi H-500).

Results

In this study we used the IgG fraction of rabbit polyclonal anti-*Ascaris* body-wall muscle actin antibodies, which recognizes epitopes of a large variety of actins, from human (Nishioka et al. 1983) to protozoan (Endo et al. 1988). When chicken-gizzard smooth muscle was stained with the antiactin antibody following colloidal gold-conjugated protein A as the positive control (Fig. 1), electron-dense gold particles specifically reactive to the bundles of actin filaments were seen. Gold particles were not observed in the other organelles.

In the trophozoites of *T. gondii* stained with the immunogold method, gold particles were mostly seen at the anterior pole of the parasite but not on the nucleus, mitochondria, endoplasmic reticulums, dense bodies, outer membrane, or in the cytoplasmic matrix (Fig. 2A). More precisely, the alignment of gold particles along the inner surface of the inner membrane complex was frequently seen (Fig. 2a arrows). In the semioblique section, gold particles were observed in association with the longitudinal runs of subpellicular microtubules (Fig. 2B), indicating a possible interaction of actin molecules with the microtubules. In the cross section, gold particles on the inner surface of the inner membrane complex were not evenly distributed but rather gathered as semiclusters (Fig. 2C).

The gold particles were consistently found on the preconodial rings (Fig. 3A, B) and polar ring, in the conoid (Figs. 3A–C), and in the space between the anterior terminal of the inner membrane complex and conoid (Figs. 2A, B, 3A, C). In the cross sections, the localization of gold particles was also seen along the inner surface of the conoid (Fig. 3D).

Rhoptries were also stained (Fig. 2). However, this might include a more or less nonspecific reaction of the rhoptry with immunoglobulins; gold particles were still observed in the rhoptries, although the amount of gold particles was markedly reduced even when rabbit normal serum was substituted for the antiactin antibody in the reaction (Fig. 4A). No particles were observed when the specimens were directly stained with colloidal gold-conjugated protein A alone (Fig. 4B), which may in turn indicate that rhoptries possess nonspecific reactivity with serum components, possibly immunoglobulins.

For a further experiment, we used parasites extracted with a detergent (NP-40) prior to fixation for electron microscopy. By this treatment, cytoplasmic membranes and other soluble components of the cells were extracted such that we could more precisely observe the cytoskeletal structure and microtubular arrangement of the parasite (Nichols and Chiappino 1987) (Fig. 5). When these extracted parasites were stained with the antibody, large amounts of gold particles were observed at the anterior pole of the trophozoites (Fig. 5B). In the cross section, it can clearly be seen that the inner part of the pellicle was heavily stained with the antibody, whereas the outermost pellicle was not stained (Fig. 5C). Gold particles were also found associated with the internal microtubules (Fig. 5A).

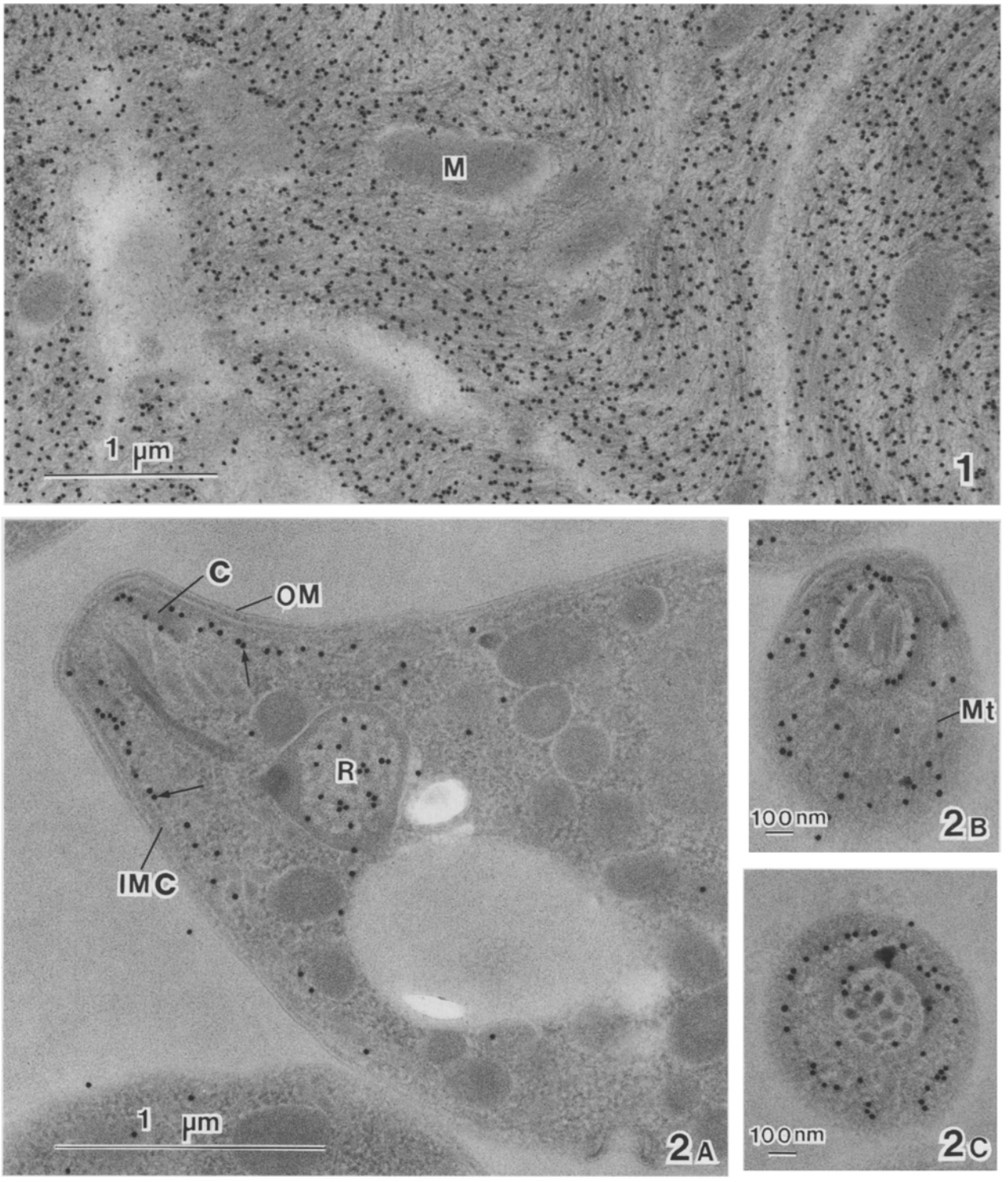

Fig. 1. Electron micrograph of a chicken-gizzard smooth muscle stained with anti-*Ascaris* actin antibody following colloidal gold-conjugated protein A gold. Gold particles were localized on the bundles of actin fibers in the muscle. *M*, mitochondria. **Fig. 2A–C.** Electron micrographs of the trophozoite of *T. gondii* stained with the immunogold method. **A** The gold particles were localized mainly at the anterior pole of the parasite. The alignment of gold particles (*arrows*) was frequently seen along the inner surface of the inner membrane complex (cf. Fig. 4). Gold particles were also seen in the space between the inner membrane complex and conoid, around the conoid, and in the rhoptries. **B** In the semioblique section, gold particles were observed in association with the longitudinal runs of subpellicular microtubules, indicating a possible interaction of actin molecules with the microtubules. **C** In the cross section, gold particles at the inner surface of the inner membrane complex were not evenly distributed but rather gathered as semiclusters. *C*, conoid; *OM*, outer membrane; *Mt*, microtubules; *M*, mitochondria; *R* rhoptry

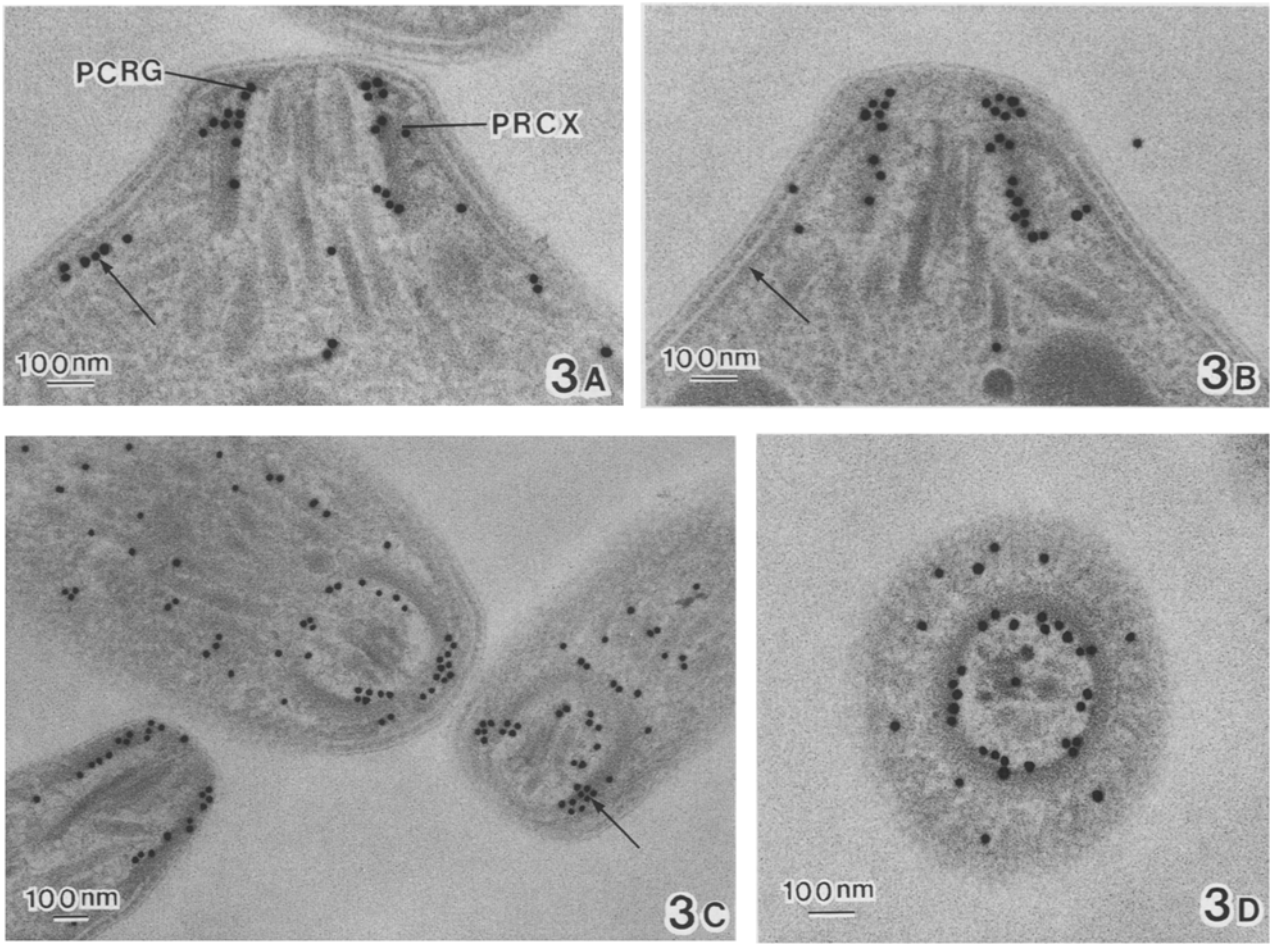

Fig. 3A–D. Electron micrographs of *T. gondii* stained with the immunogold method. **A** Gold particles were seen on the preconoidal rings (*PCRG*) and polar ring complex (*PRCX*) and in the conoid. In this section, the alignment of gold particles along the pellicle was seen (*arrow*). **B** Gold particles were clearly seen in the preconoidal rings and in the conoid. Note that in this section, alignment of the gold particles along the pellicle was not seen (*arrow*). **C** In the semioblique sections, gold particles were clearly demonstrated in the space between the polar ring and conoid (*arrow*). **D** Gold particles were also localized along the inner surface of the conoid

Discussion

In our previous report (Endo et al. 1987), the presence and localization of actin in the anterior part of the trophozoite of *T. gondii* was demonstrated by means of SDS-PAGE and immunofluorescence. This localization of actin was further confirmed in the present experiments. In *Toxoplasma,* actin molecules are localized as follows:

1. The frequently observed alignment of the actin along the inner surface of the inner membrane complex in the anterior part of the parasites may exlain the interactions of actin with subpellicular microtubules (Figs. 2A, 5B). Nevertheless, ultrastructural studies have not yet found a corresponding distribution of actin filaments in *T. gondii* (Sheffield and Melton 1968; Cintra and De Souza 1985; Nichols and Chiappino 1987; present experiment), indicating that the actin molecules are monomeric or form only very short filaments, if any. We feel it important to consider whether such interactions are mechanisms of structure or motility.

2. We cannot yet explain why much larger amounts of gold particles were observed in the detergent-extracted parasites than in intact ones. This might be due to the extraction of soluble materials from subpellicular structures, which resulted in the revelation of buried actin networks.

3. Actin molecules were consistently found on the preconoidal rings (Fig. 3A, B) and polar ring and in the conoid (Figs. 3A–C). Nichols and Chiappino (1987) have recently reported that these organelles were filamentous.

4. The presence of actin was also demonstrated in the space between the anterior terminal of the

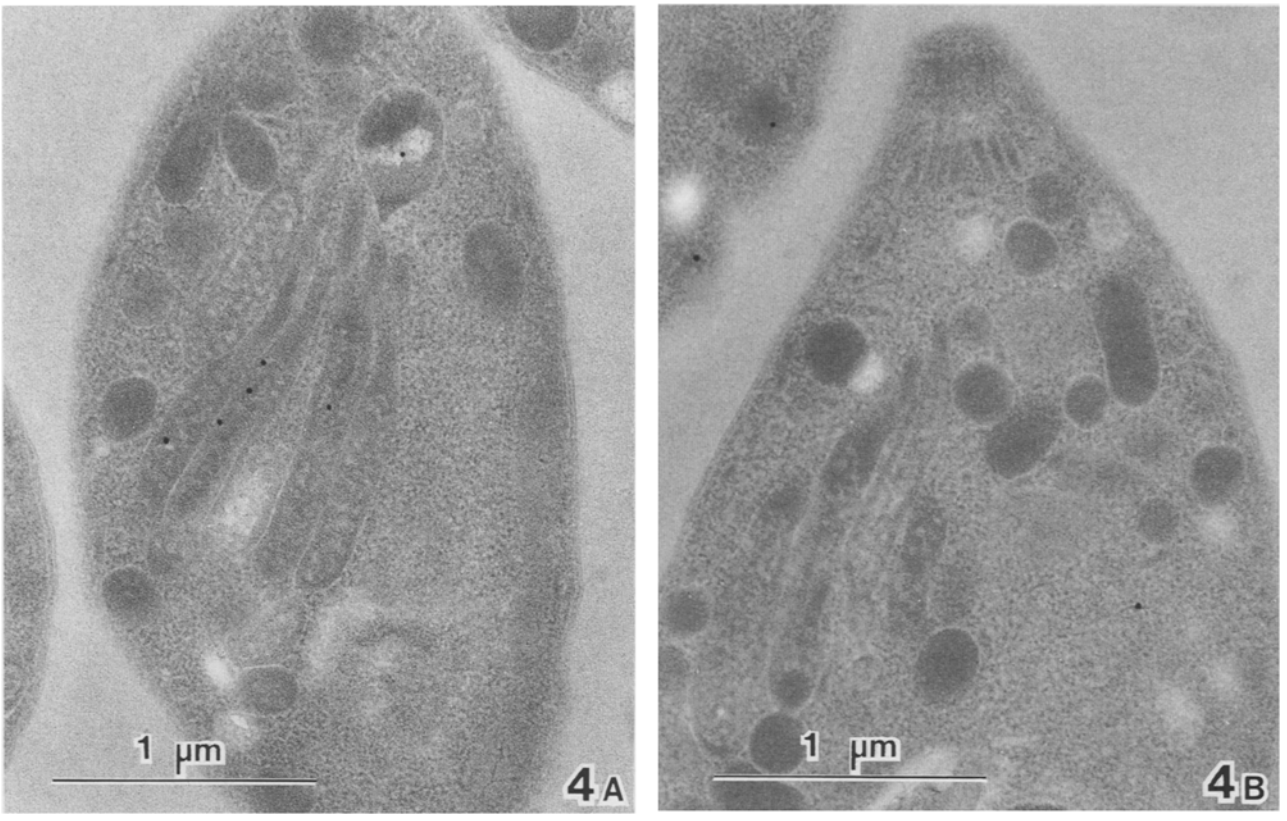

Fig. 4A, B. An electron micrograph of the trophozoite of *T. gondii* treated either with rabbit normal serum followed by colloidal gold-conjugated protein A or with colloidal gold-conjugated protein A alone. **A** As the negative control, the parasite was stained with rabbit normal serum followed by colloidal gold-conjugated protein A alone. No organelle other than the rhoptries was stained with gold particles in this reaction. A possible nonspecific reaction of the rhoptries with immunoglobulins was observed, although the amount of gold particles was markedly reduced. **B** The nonspecific reaction could not be detected in the specimen treated with colloidal gold-conjugated protein A alone

inner membrane complex and conoid (Figs. 2A, B, 3A, C), were curved arms bridge the gap between them (Nichols and Chiappino 1987).

Concerning mechanisms of movement in coccidian species, Russel and Sinden (1981) have noted that the parasites adhere to the substratum by surface ligands. This ligand/substratum complex is then capped along the fixed spiral of the parasite body by a microfilament-based contractile system. However, considering that the anterior polar region is the motility-generating center (Chiappino et al. 1984; Endo et al. 1987; Jacobs 1953; Muhlpfordt 1952; Pulvertaft et al. 1954; for a review see Doran 1972), this system may not apply to the *Toxoplasma* locomotion system. Apart from this, Nichols and Chiappino (1987) have postulated that the polar ring complex moves diagonally along the conoid subunits during conoid extension and retraction. This imparts a twist to the polar ring complex with its attached microtubules, resulting in torsion of the body. In this system, they presumed the possible presence of dynein as an energy-transducing enzyme associated with microtubules that powers the microtubule-dependent movements (Gibbons and Fronk 1979; Porter and Johnson 1983). However, it is also possible, that the association of actin with microtubules provides a link to myosin, another possible potential source of power for microtubule-dependent movements (Pollard et al. 1984). Interestingly, Pollard et al. (1984) have reported that short actin filaments seen in the mitotic spindle can interact with microtubules via microtubule-associated proteins, whereas long filaments in the cell cortex are structural, contributing to the integrity of the cytoplasmic matrix. The results obtained in the present experiments, together with the fact that studies localizing myosin have found a corresponding distribution (Schwartzman and Pfefferkorn 1983), support the latter idea. The fact that experiments with cytochalasin B (Jensen and Edgar 1976) and D (Ryning and Remington 1978; Schwartzman and Pfeffer-

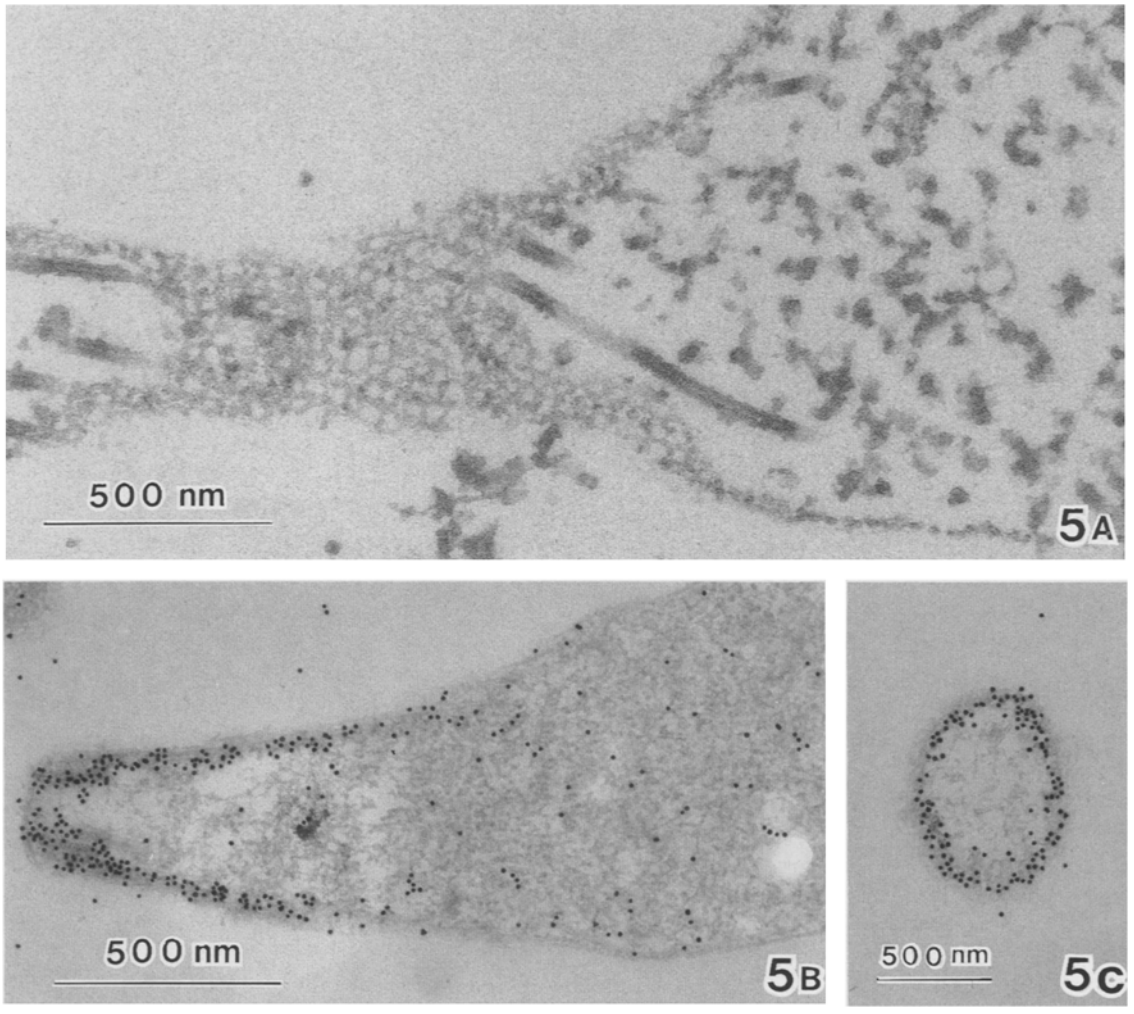

Fig. 5A–C. Electron micrographs of trophozoites of *T. gondii* treated with NP-40 prior to fixation. **A** Subpellicular meshwork was seen in the parasite treated with 0.3% Nonidet P-40; subpellicular microtubules also remained in the sample. **B** After immunogold staining, large amounts of gold particles were seen on the anterior part of the pellicle. **C** In the cross section, the inner part of the pellicle was heavily stained with gold particles but the outermost pellicle was not stained

korn 1983) have reversibly inhibited parasite motility can also be circumstantial evidence supporting this idea.

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