

## Characterization of Extreme Apical Antigens from *Toxoplasma gondii*

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MORRISSETTE, N. S., BEDIAN, V., WEBSTER, P., AND ROOS, D. S. 1994. Characterization of extreme apical antigens from *Toxoplasma gondii*. *Experimental Parasitology* 79, 445-459. We have isolated 26 monoclonal antibodies which specifically recognize the extreme apex of *Toxoplasma gondii*, a protozoan parasite which attaches to and invades host cells via its specialized apical end. The unique apical organelles which define the phylum Apicomplexa are thought to be involved in mechanical and enzymatic aspects of invasion. Immunoblots, immunofluorescent morphology, and immunogold labeling define six classes of apically localized antigens recognized by these antibodies. Three of the classes are detergent-insoluble and localize to the conoid and the cytoplasmic face of the apical membrane, suggesting that they may be part of the parasite's membrane cytoskeleton. The remaining three classes extract with detergent and are associated with internal membrane bounded vesicles (micronemes and the upper necks of rhoptries). One class of micronemal antigens appears to be cell cycle regulated. This antigen localizes to the cytoplasm, especially the perinuclear region, in thin (recently replicated) parasites, but is apical in larger parasites. © 1994 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: Apical complex; Conoid; Cytoskeleton; Rhoptries; Micronemes; *Plasmodium*; *Toxoplasma gondii*; Electron microscopy (EM); Fish skin gelatin (FSG); Human foreskin fibroblasts (HFF); Indirect immunofluorescence assay (IFA); Inner membrane complex (IMC); Phosphate-buffered saline (PBS); *N*- $\alpha$ -p-Tosyl-L-arginine methyl ester hydrochloride (TAME); Phenylmethylsulfonyl fluoride (PMSF); Sodium dodecyl sulfate (SDS).

### INTRODUCTION

*Toxoplasma gondii*, like other members of the phylum Apicomplexa, is characterized by a specialized apical end which contains unique organelles thought to be associated with the process of host invasion (see Discussion for a diagram of the *T. gondii* apical complex). Proteins associated with membrane-bounded organelles—rhoptries, micronemes, and dense granules—have been identified in *Toxoplasma* and *Plasmodium* (Achbarou *et al.* 1991a,b; Charif *et al.* 1990; de Carvalho *et al.* 1991; Foussard *et al.* 1991; Herion *et al.* 1993; Leriche and Dubremetz 1990, 1991; Mercier *et al.* 1993; Ossario *et al.* 1992; Sadak *et al.* 1988; Saffer *et al.* 1992; Sam-Yellowe 1992), but antigens of the extreme apex, in-

cluding those associated with the conoid and various other intriguing cytoskeletal structures, remain poorly understood. The study of antigens associated with the extreme apex of *T. gondii* may be expected to yield insights into the role of these cytoskeletal structures in maintaining cell polarity and in the process of cell invasion and exit.

Polarity in *Toxoplasma* is reflected in (and perhaps maintained by) a polarized membrane cytoskeleton, consisting of 22 subpellicular microtubules, the conoid, and auxiliary structures which organize these two filament systems (Chobotar and Scholtyseck 1982; Nichols and Chiapino 1987; Russell and Burns 1984). The conoid is a mysterious organelle which contains a spiral of 26- to 30-nm filaments of unknown

composition. Associated with the conoid are a variety of accessory structures of unknown composition, but which appear to organize and position the conoid in extended or retracted states relative to the corset of subpellicular microtubules. Posterior to the conoid, the 22 subpellicular microtubules extend in a gradual spiral from the apical polar ring (a microtubule organizing center) along the cytoplasmic face of the pellicle, terminating beyond the nucleus. The *Toxoplasma* pellicle is composed of three unit membranes: an outer plasma membrane and an inner membrane complex (IMC), which begins at the apical polar ring and runs in close apposition to the plasma membrane along the entire length of the parasite. The IMC consists of a patchwork of tightly fitted, flattened vesicles organized in a spiralling fashion similar to the right-handed spiral of the subpellicular microtubules which are tightly apposed to its cytoplasmic face. Electron microscopy of freeze-fractured Apicomplexan membrane complexes demonstrate a high degree of apical structural organization, suggesting coordination with the underlying cytoskeleton (Dubremetz and Torpier 1978; Porchet and Torpier 1977). Although microfilaments have not been visualized in these parasites, monoclonal antibody studies localize both actin and myosin to the parasite apex (Cintra and de Souza 1985; Endo *et al.* 1988; Schwartzman and Krug 1989; Yasuda *et al.* 1988). The posterior end of *T. gondii* does not contain any obvious cytoskeletal elements.

In order to isolate antibodies which specifically recognize the extreme apical region of *T. gondii* tachyzoites, we have screened three hybridoma libraries by immunofluorescence morphology. The classification of antigens bound by these monoclonal antibodies represents the first step in our goal to characterize proteins associated with the extreme apex, including components of the membrane-associated cytoskeleton.

## MATERIALS AND METHODS

**Culture of parasites.** *Toxoplasma* strains RH(ERP) (Sabin 1941) and P(LK) (Kasper and Ware 1985) were maintained by serial passage in confluent lines of primary human foreskin fibroblasts (HFF) in Eagle's minimal essential medium supplemented with 1% heat-inactivated fetal bovine serum and 25 µg/liter gentamycin. Large-scale culture and purification for antigen or protein preparation was carried out as previously described (Roos *et al.* 1994). *Plasmodium falciparum* merozoites (kindly supplied by Dr. Harvey Rubin and associates) were grown in human red blood cells in Hepes-buffered RPMI 1640 + 10–15% human serum and 40 mg/liter gentamycin.

**Production of hybridomas.** Three independent hybridoma fusions were generated and screened for labeling of the apical membrane region of intracellular parasites by indirect immunofluorescent staining (IFA). The immunogens for the three hybridoma libraries were (1) total RH strain protein, (2) total P strain protein, and (3) a detergent insoluble extract of RH parasites. The detergent insoluble extract was made by suspending tachyzoites in 1% Triton X-100 in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate buffered saline (PBS) followed by N<sub>2</sub> cavitation at 4°C for 20 min at 350 psi. The resulting insoluble material was pelleted at 16,000g and used as the immunogen. Balb/c mice were injected with 200 µg protein three times at 2-week intervals (alternating intraperitoneal and subcutaneous injections) and boosted intravenously with 200 µg protein 3 days before the fusion. Fusions between splenic lymphocytes and the Sp2/OAg14 myeloma line were performed using the protocol of Lane *et al.* (1986).

Hybridoma lines were grown in Kennett's HY medium (90% modified Eagle's medium, 10% NCTC-135 with 4.5 g/liter glucose) with 20% fetal bovine serum and macrophage-derived growth supplements. Lines which demonstrated apical labeling (see below) were cloned by limiting dilution and retested for apical staining. A total of 26 independent cloned lines were isolated for further analysis. The "R" fusion lines were from the total RH protein fusion: 605 samples were screened by IFA morphology and 10 lines were cloned. The "A" fusion lines were directed against P strain antigen, but were screened with RH strain parasites to ensure antibody cross-reactivity: 1470 samples were screened and 2 lines were cloned. The "C" fusion lines were directed against a detergent-insoluble fraction of RH tachyzoites: 2940 samples were screened and 14 lines were cloned.

**Immunofluorescence of *Toxoplasma*.** HFF cells on glass slides or coverslips were infected with *Toxoplasma*, fixed for 10 min in 3.7% formal saline, pH 7.4 and permeabilized for 20 min in 0.25% Triton X-100 in PBS at room temperature. After rinsing with PBS, samples were incubated in conditioned medium from the individual hybridoma lines for 1 hr at 37°C in a

hydrated chamber. The samples were rinsed for 15 min in PBS and incubated in a 1/1000 dilution of FITC-labeled rabbit anti-mouse antibody (Cappel) for 1 hr at 37°C. The secondary antibody was diluted in a solution of 5% calf serum and 1% bovine serum albumin in PBS. Following washes in PBS, the samples were briefly rinsed in water, mounted in a saturated polyvinyl alcohol/glycerol mounting medium (Harlow and Lane 1988) and viewed in a Zeiss Axiovert equipped with epifluorescence optics.

Extracellular parasites were isolated by filtration of freshly lysed-out tachyzoites through 3- $\mu$ m filters (Nuclepore), followed by centrifugation at 1000g for 20 min at 4°C. Parasites were resuspended in a small volume of PBS and allowed to settle onto polylysine-coated coverslips for 15 min at room temperature. Fixation and immunofluorescent staining was performed as above. Detergent extraction was carried out either by suspension of pelleted parasites in 1% Triton/PBS and incubation in suspension for 10 min prior to adhesion to polylysine coverslips or by attachment of parasites to coverslips prior to extraction.

**Immunoblotting.** *T. gondii* tachyzoites were isolated as described above. *P. falciparum* merozoites were isolated by permeabilization of parasitized red cells with 0.1% saponin in PBS on ice for 20 min, followed by centrifugation at 1000g for 20 min. Parasites and uninfected HFF control cells were lysed into approximately 10 $\times$  their pellet volume in urea sample buffer (50 mM Tris, pH 6.8, 5% SDS, 6 M urea, and 10%  $\beta$ -mercaptoethanol). A cocktail of protease inhibitors included 100  $\mu$ M Tame, 100  $\mu$ M PMSF, 2  $\mu$ M leupeptin, and 1.4  $\mu$ M pepstatin. Protein samples (10  $\mu$ l/well) were run on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose using semidry transfer with a graphite electroblotter (Millipore) in Tris-glycine transfer buffer (Harlow and Lane 1988). After Ponceau S staining, the nitrocellulose was cut into strips and blocked in 10% nonfat milk in PBS overnight. Each strip was inverted onto parafilm containing 500  $\mu$ l of conditioned medium from an individual hybridoma line and incubated for 1 hr at room temperature in a hydrated chamber. After washing with PBS for 15 min, the strips were individually incubated with a 1/5000 dilution of horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Cappel). The final wash was followed by luminol detection (Amersham or DuPont), according to the manufacturer's recommendations.

**Cryosectioning and immunoelectron microscopy.** Extracellular *Toxoplasma* tachyzoites were fixed for 1 hr at room temperature by suspension in 0.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.4), transferred to buffer alone, and stored at 4°C until use. Fixed parasites were rinsed by suspension in 10% fish skin gelatin (FSG) in PBS and pelleted in a microfuge. The FSG/PBS was replaced with 10% molten gelatin in

PBS, cells were repelleted, and the gelatin was allowed to gel on ice. The solidified pellet was transferred to 2.1 M sucrose and infiltrated for at least 1 hr on ice. After mounting, samples were vitrified in liquid N<sub>2</sub> and sectioned on an Ultracut ultramicrotome equipped with FC4 cryoattachment (Leica) and a diamond knife (Diatome). After blocking in 0.12 mM glycine (10 min) and 10% FSG (10 min), sections were stained with conditioned hybridoma medium for 30 min, bridged using a rabbit anti-mouse antibody (Cappel) for 30 min, and coupled to protein A-gold (5 nm) for 15 min. Grids were washed in six changes of PBS between each incubation. After gold labeling, the grids were briefly rinsed in water and stained in a 1:9 mixture of 3% uranyl acetate and 2% methyl cellulose in water (Griffiths 1993). Samples were viewed in a Philips 400 microscope.

## RESULTS

Twenty-six monoclonal antibodies which stain the extreme apical region of *Toxoplasma* were identified from three hybridoma screens, carried out as described under Materials and Methods. Based on their immunofluorescence morphology, immunogold localization, and immunoblot results, these antibodies define six antigen classes (summarized in Table I). The first three classes are detergent-insoluble, suggesting that they may be associated with the membrane cytoskeleton. The latter three classes are readily extracted by detergent treatment. None of the antibodies stain nonpermeabilized parasites as visualized by immunofluorescence, indicating that they react with internal antigens. *Toxoplasma* replicates by endodyogeny, in which two daughter parasites form within the mother; recently replicated parasites can be distinguished by their relatively thin morphology. All antigen classes are visible by immunofluorescence during replication; the localization of these antigens during early stages of endodyogeny can be observed in larger parasites as two subterminal spots in addition to the maternal apical staining.

Class I antibodies stain an apical region which, by immunofluorescence, appears to extend approximately one-quarter the

TABLE I  
Apical Antibody Classes and Properties

Class	Hybridoma lines <sup>a</sup>	Antigen size (kDa)		Detergent solubility	IFA morphology	Immuno-EM morphology
		<i>T. gondii</i>	<i>P. falciparum</i>			
I	A4H11 <sup>b</sup> , C12B2 <sup>b</sup> , C4F3, C21D4 <sup>b</sup>	>200, 122, 112, 104 92, 68	139, 122, 46	-	Small apical labeling; ~1/4 parasite length	Labels conoid and cytoplasmic face of the apical pellicle; ~6× length of the conoid
II	C12C2, C13D12 <sup>b</sup>	>200, 122, 112	None	-	Same as class I	Same as class I
III	R3F2	>200, 112	139, 122	-	Tiny apical labeling	Labels conoid and cytoplasmic face of the apical pellicle; ~3× length of the conoid
IV	A3H9	163, 96	None	+	Elongated, tiny apical labeling	Rhoptry necks? (inconsistent labeling)
V	C6F1 <sup>c</sup> , C16G10 <sup>c</sup> , C6G8 <sup>c</sup> , C21B9, C9B6 <sup>b</sup> , C25G3 <sup>c</sup> , C13B6, R2H1 <sup>c</sup> , C13G11 <sup>c</sup> , R10F6 <sup>c</sup>	38	None	+	Large, punctate, membrane-associated apical labeling. Cell-cycle regulated: thin parasites exhibit perinuclear/cytoplasmic pattern rather than apical morphology	Labels micronemes
VI	C29C12 <sup>c</sup> , R8D4, R2G4 <sup>c</sup> , R9C5 <sup>c</sup> , R6D2, R17E12 <sup>c</sup> , R7D2 <sup>c</sup> , R23E4 <sup>c</sup>	120	None	+	Similar to class V, but without the cell cycle aspect	Labels micronemes

<sup>a</sup> Hybridoma lines designated "A" indicate total P-strain protein; "C," detergent-insoluble RH-strain protein; "R," total RH-strain protein used as the immunogen.

<sup>b</sup> Does not bind consistently to immunoblots.

<sup>c</sup> No binding on immunoblots.

length of the parasite (Figs. 1A and 1B). The four antibodies in this class recognize six major bands on immunoblots, with approximate molecular weights of >200, 122, 112, 104, 92, and 68 kDa (Fig. 2, lane 1). Members of this class of antibodies cross-react with antigens of 139, 122, and 46 kDa on immunoblot samples from *P. falciparum* (Fig. 2, lane 2). This pattern is not affected by the presence or absence of protease inhibitors in the lysis buffer, as discussed further below. Attempts to abrogate antigenicity by treating protein samples with periodate failed, suggesting that the epitope is not a carbohydrate structure present on multiple proteins. The bands do not corre-

spond to those seen with an affinity-purified polyclonal antibody which binds to ubiquitin conjugates, suggesting that the bands are not ubiquitin-modified forms of a single antigen, as has been observed for actin in *P. falciparum* (Field *et al.* 1993) (data not shown).

When extracellular parasites are extensively extracted with 1% Triton X-100 in PBS, class I antigen remains with the insoluble cytoskeletal residue of the parasite (Fig. 3C). Immunogold EM of *Toxoplasma* with members of this antibody class shows labeling of the cytoplasmic face of the inner membrane complex in the apical region only (as illustrated for the class II antibody



C12C2 in Fig. 4). Sections through the conoid (cf. arrow in Fig. 4B) also display labeling of this organelle. In parasites where the conoid is retracted below the apical polar ring, the region between the conoid and the IMC is labeled with gold (Fig. 4B). Labeling by these antibodies appears mainly circumferential; i.e., no gold label is found within the conoid or the apical cytoplasm. Gold decoration extends down the cytoplasmic face of the apical membrane to a region approximately half-way between the apical end and the nucleus (Fig. 4A).

The second class of antigens is represented by two antibody lines which produce an immunofluorescence pattern similar to the first class but on immunoblots appears to recognize a subset of the class I antigens at >200, 122, and 112 kDa (Fig. 2, lane 4). Neither class II antibody appears to cross-react with *Plasmodium* antigens on immunoblots. These antigens are resistant to detergent extraction, and immunogold labeling of cryosections is similar to the class I members, labeling the conoid and the cytoplasmic face of the IMC from the apex approximately half-way to the nucleus (Fig. 4).

Class III contains a single antibody which stains a tiny part of the very apical membrane by immunofluorescence (Figs. 1C and 1D); this antigen is also resistant to detergent extraction (Fig. 3D). On immunoblots, the R3F2 antibody recognizes a major band at >200 kDa and a minor band at 112 kDa in *T. gondii* (Fig. 2, lane 7) and cross-reacts with *P. falciparum* antigens of 139 and 122 kDa (lane 8). These antigens appear to be a subset of the class I antigens in both *Toxoplasma* and malaria (compare with lanes 1 and 2). Immunoprecipitation with class III antibody permits isolation of the >200 kDa antigen, which cross-reacts with class I antibodies on immunoblots (not shown). In agreement with the distinctive immunofluorescence morphology, immunogold localization labels the conoid and a

small area of the cytoplasmic face of the apical membrane (Fig. 5). The labeling of the cytoplasmic face of the IMC extends approximately three times the length of the retracted conoid (Fig. 5A), a much more restricted localization than that observed for classes I and II, which extend two to three times this distance (compare with Fig. 4A).

The class IV antibody stains a small region at the extreme apex. It can be distinguished from class III antigen by its elongate morphology and its susceptibility to detergent extraction (Fig. 1E and 1F). On immunoblots this antibody stains two bands: a minor 163-kDa antigen and a major band at 96 kDa (Fig. 2, lane 10). This antigen class is represented by a single antibody which does not demonstrate convincing immunogold localization, but its solubility in detergent argues against cytoskeletal association. The high and narrow A3H9 immunofluorescence pattern is clearly distinct from the morphology of antigens previously localized to rhoptry bodies (Sadak *et al.* 1988; Saffer *et al.* 1992), suggesting that it is restricted to the upper necks (peduncle) of rhoptries. This pattern is also distinct from that seen with dense granules (punctate) and micronemes (see below).

The last two antibody classes, V and VI, both exhibit a punctate apical staining pattern which extends over at least one-third the length of the parasite (Figs. 1G–1L). In large parasites which contain two daughter buds, it is possible to see subterminal apical staining. These antigens are sensitive to detergent extraction (Figs. 3A and 3B), and immunogold localizes both antigen classes V and VI to the micronemes—small, convoluted membranous structures in the apical region of the parasite (Fig. 6). Class V members (Figs. 1G–1J) also show an apparent cell cycle-regulated aspect: thin parasites exhibit a perinuclear/cytoplasmic IFA pattern (best seen in shorter exposures; cf. inset in Fig. 1J). In living cultures observed by time-lapse videomicroscopy, thinner

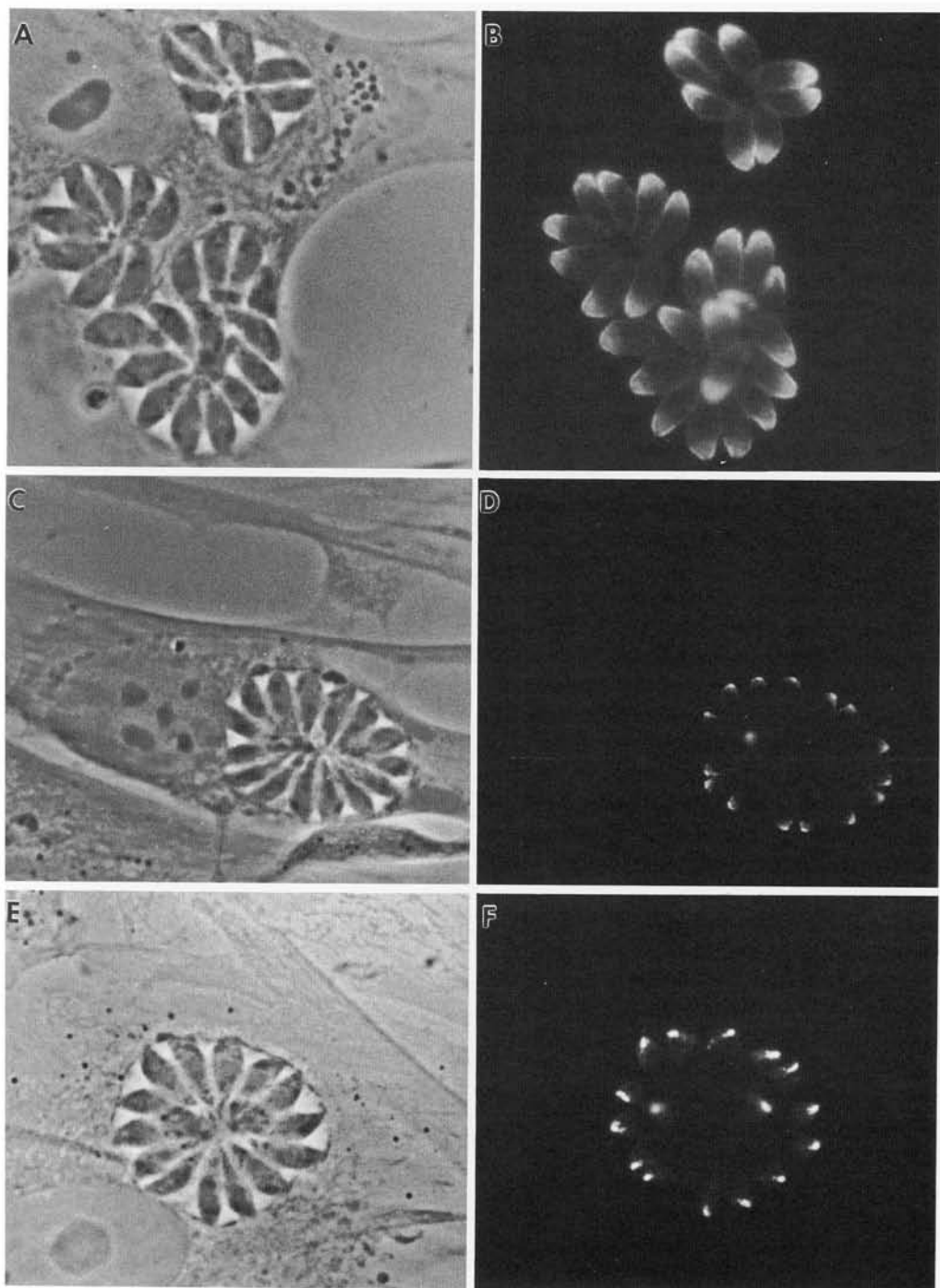


FIG. 1. Phase-contrast and immunofluorescence microscopy of *Toxoplasma* tachyzoites in HFF cells. (A and B) C4F3, representing the morphology of both the class I and class II antibodies stains the apex extending approximately one-fourth the length of the parasite. (C and D) R3F2, the single class III antibody, stains a tiny apical region. (E and F) A3H9, the only class IV monoclonal, stains a tiny elongate apex. (G, H, I, and J) C13B6, representing class V antibodies, stains a large, punctate apex in large parasites (late in the cell cycle), but stains younger (thinner) tachyzoites in a perinuclear/cytoplasmic fashion (cf. inset). (K and L) R8D4, representing class VI antibodies. This antigen is similar to class V by apical morphology but does not relocalize during different phases of the cell cycle.

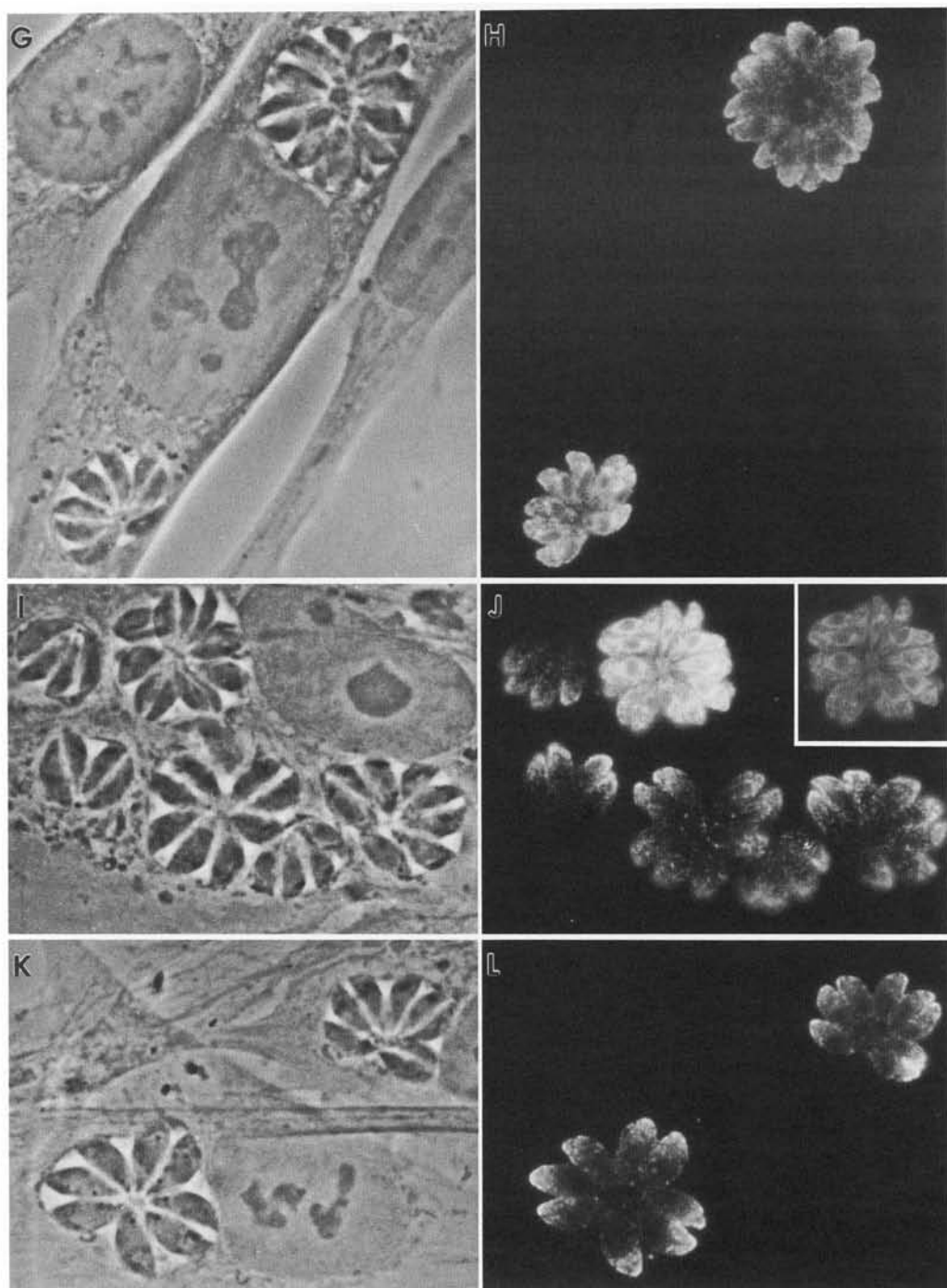


FIG. 1—Continued

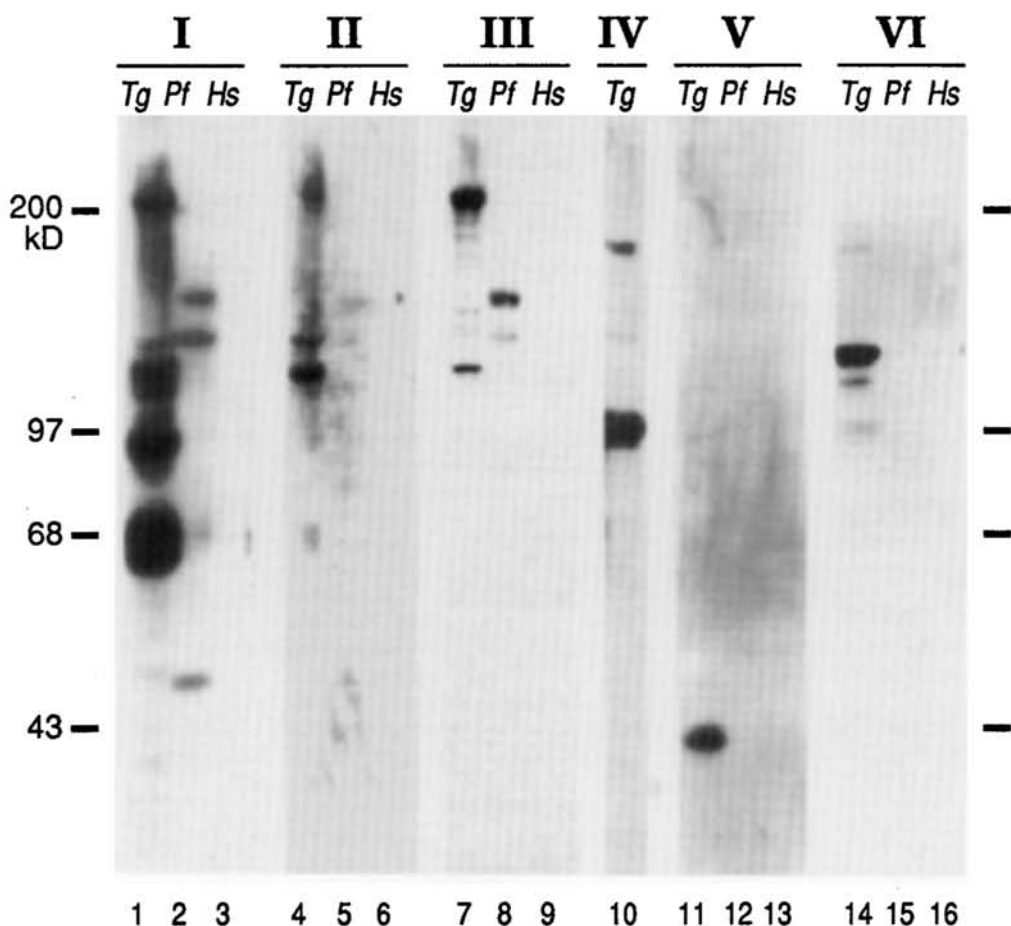


FIG. 2. Immunoblots of *Toxoplasma* apical antigen classes: Tg, *Toxoplasma gondii* protein; Pf, *Plasmodium falciparum* protein; Hs, (*Homo sapiens*) indicates protein from HFF host cells. Lane 1 (C4F3), class I members display a complex pattern of six bands at >200, 122, 112, 104, 92, and 68 kDa. The most prominent bands are at 68 and 112 kDa. Lanes 2 and 3 (C4F3), cross-reacting antigens of ~139, 122, and 47 kDa are seen in *P. falciparum*; host cell extracts do not show any cross-reacting bands. Lane 4 (C12C2), class II antigen displays three bands at >200, 122, and 112 kDa. All class II bands comigrate with bands stained with class I antibodies. Lanes 5 and 6 (C12C2), these antibodies do not bind to any cross-reacting antigens in malaria or human samples. Lanes 7-9 (R3F2), class III antibody stains a major band at >200 kDa and a minor band at 112 kDa in *T. gondii* and *P. falciparum* proteins of ~139 and 122 kDa—a subset of the class I antigens in lanes 1 and 2. Lane 10 (A3H9), class IV antibody recognizes two bands at 163 and 93 kDa; it does not cross-react with either malaria or human protein (not shown). Lanes 11-13 (C21B9), class V antibodies identify a single band at 38 kDa and do not react with either malaria or human antigens. Lanes 14-16, members of antibody class VI (R6D2) stain a single band at 120 kDa and do not react with malaria or human samples.

parasites are invariably those which have most recently divided (M. E. Modica and D. S. Roos, unpublished observations). It therefore seems likely that the perinuclear staining which is observed exclusively in thin parasites represents cell-cycle regulated expression of a micronemal antigen.

The immunoblot results for class V identify a band at 38 kDa; those for class VI stain a 120-kDa antigen (Fig. 2, lanes 11 and 14).

#### DISCUSSION

Previous work on apical structures has focused on antigens associated with mem-



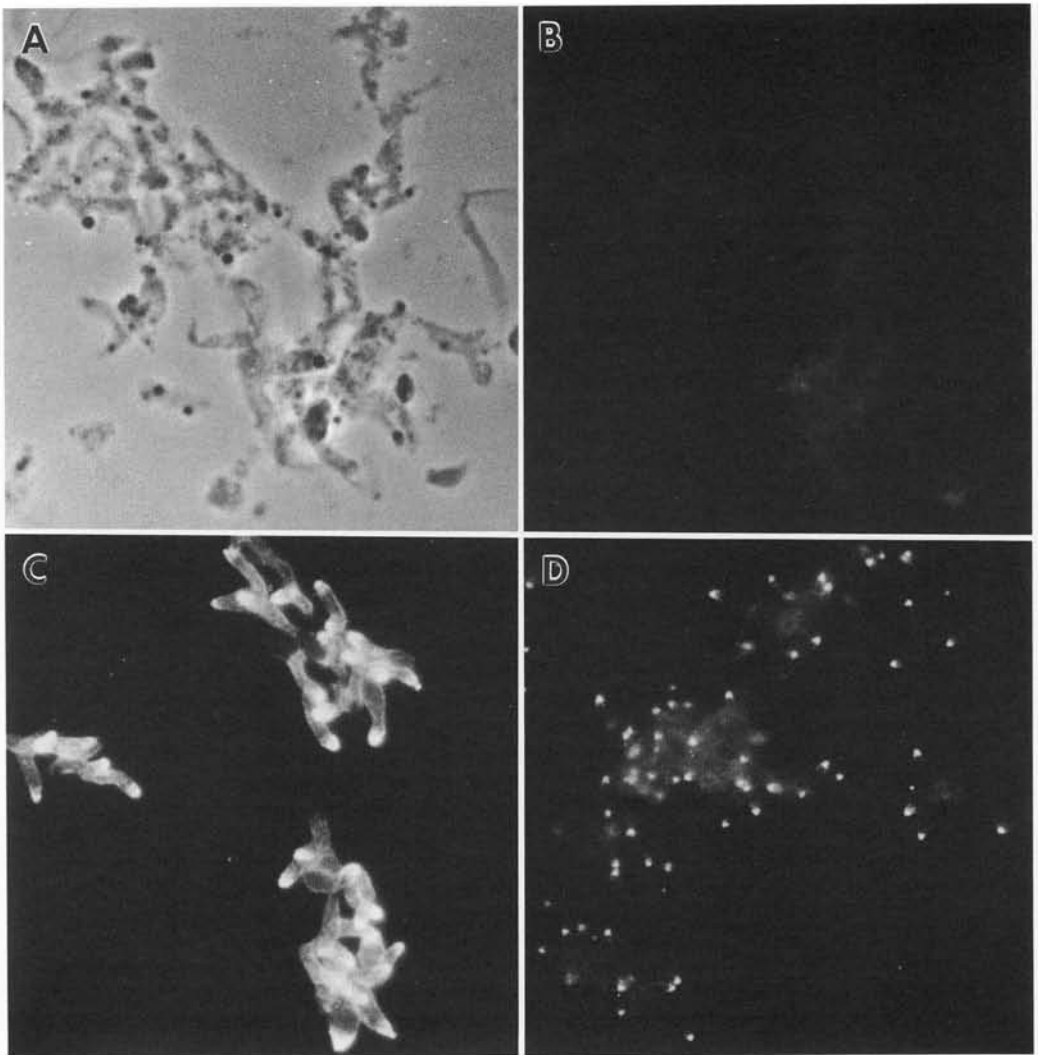


FIG. 3. Detergent solubility and insolubility of the antigen classes. (A) Phase-contrast micrograph of 1% Triton-extracted extracellular parasites. (B) IFA of the same field shown in panel A, using antibody R8D4. The class VI antigen is completely extracted by detergent. (C) Members of classes I and II are detergent-insoluble and remain apical (antibody C4F3 shown). (D) R3F2, the class III antigen, is also detergent-insoluble.

brane-bound organelles: the rhoptries, micronemes, and dense granules (Achbarou *et al.* 1991a,b; Charif *et al.* 1990; de Carvalho *et al.* 1991; Foussard *et al.* 1991; Herion *et al.* 1993; Leriche and Dubremetz 1990, 1991; Mercier *et al.* 1993; Ossario *et al.* 1992; Sadak *et al.* 1988; Saffer *et al.* 1992; Sam-Yellowe 1992). These secretory organelles are thought to contain components

associated with enzymatic and metabolic processes during invasion and parasitophorous vacuole formation. In contrast, the apical cytoskeleton has long been suspected to play a role in the mechanical aspects of invasion. Motility is essential for parasite invasion, (Endo *et al.* 1982, 1987, 1990) and while the nature of motility in *Toxoplasma* is not yet understood, it is

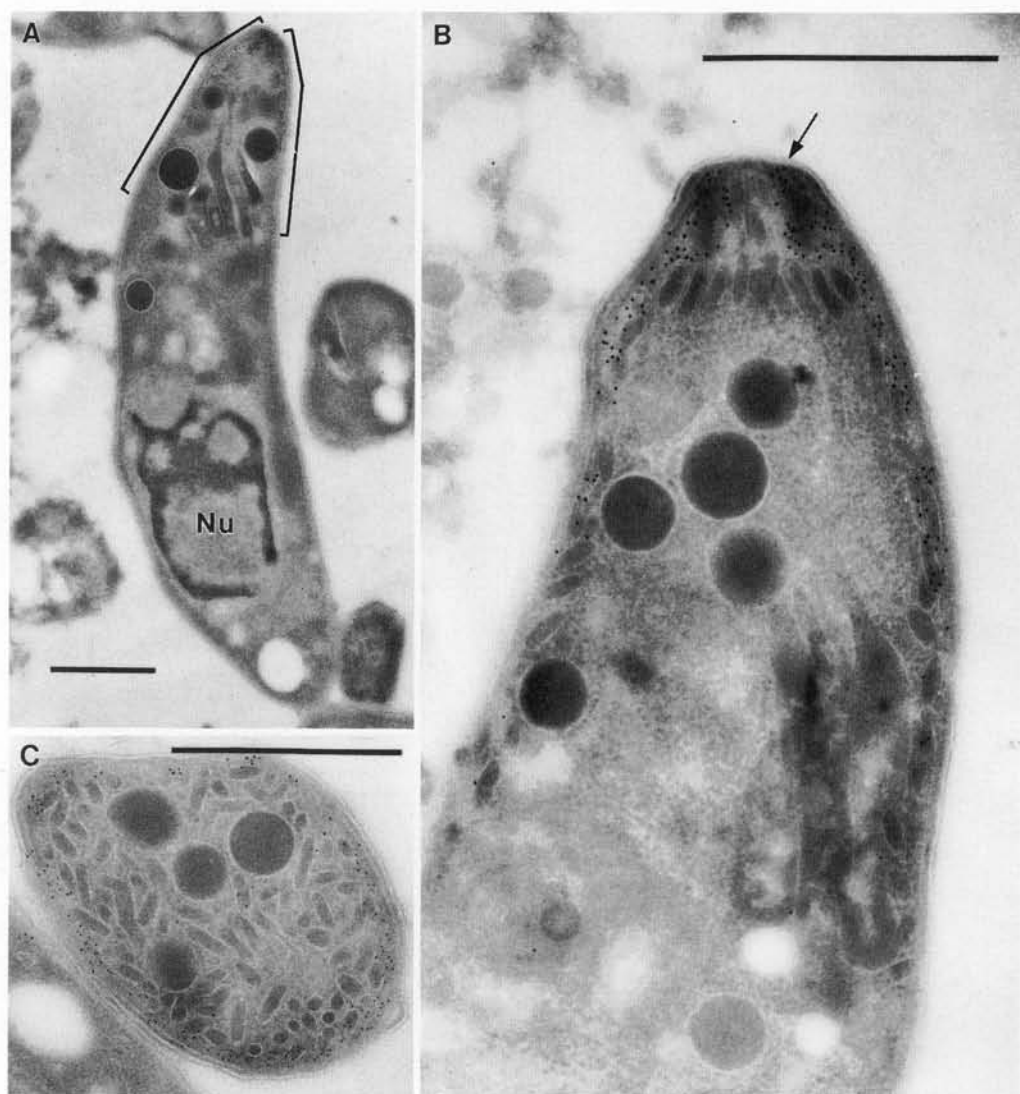


FIG. 4. Immunogold labeling of class I and II antibodies (C12C2 shown). Gold labels the conoid and along the cytoplasmic face of the IMC apical domain. Labeling extends downward along the inner face of the IMC and appears to terminate half-way between the apex and the nucleus. (A) A full-length aspect shows that labeling is confined to the conoid and the apical IMC. Intense gold labeling extends approximately half-way to the nucleus (brackets). Gold particles occasionally observed further along the pellicle may represent either nonspecific background or low concentrations of this antigen. (B) In addition to labeling the cytoplasmic face of the IMC, class I and II antibodies label longitudinal sections of the conoid (arrow) and, in retracted conoids, the space between the conoid and the lateral pellicle. (C) A cross section through the apical region confirms that staining is confined to the cytoplasmic face of the IMC and is absent from dense granules, rhostry necks, or micronemes. Scale bar is 1  $\mu$ M in all panels.

likely that cytoskeletal elements are involved. The cytoskeleton may also be associated with the establishment and maintenance of parasite polarity. For reference,

a diagram of the parasite apex, including antibody staining patterns described in this report, is shown in Fig. 7.

Three classes of extreme apical antigens

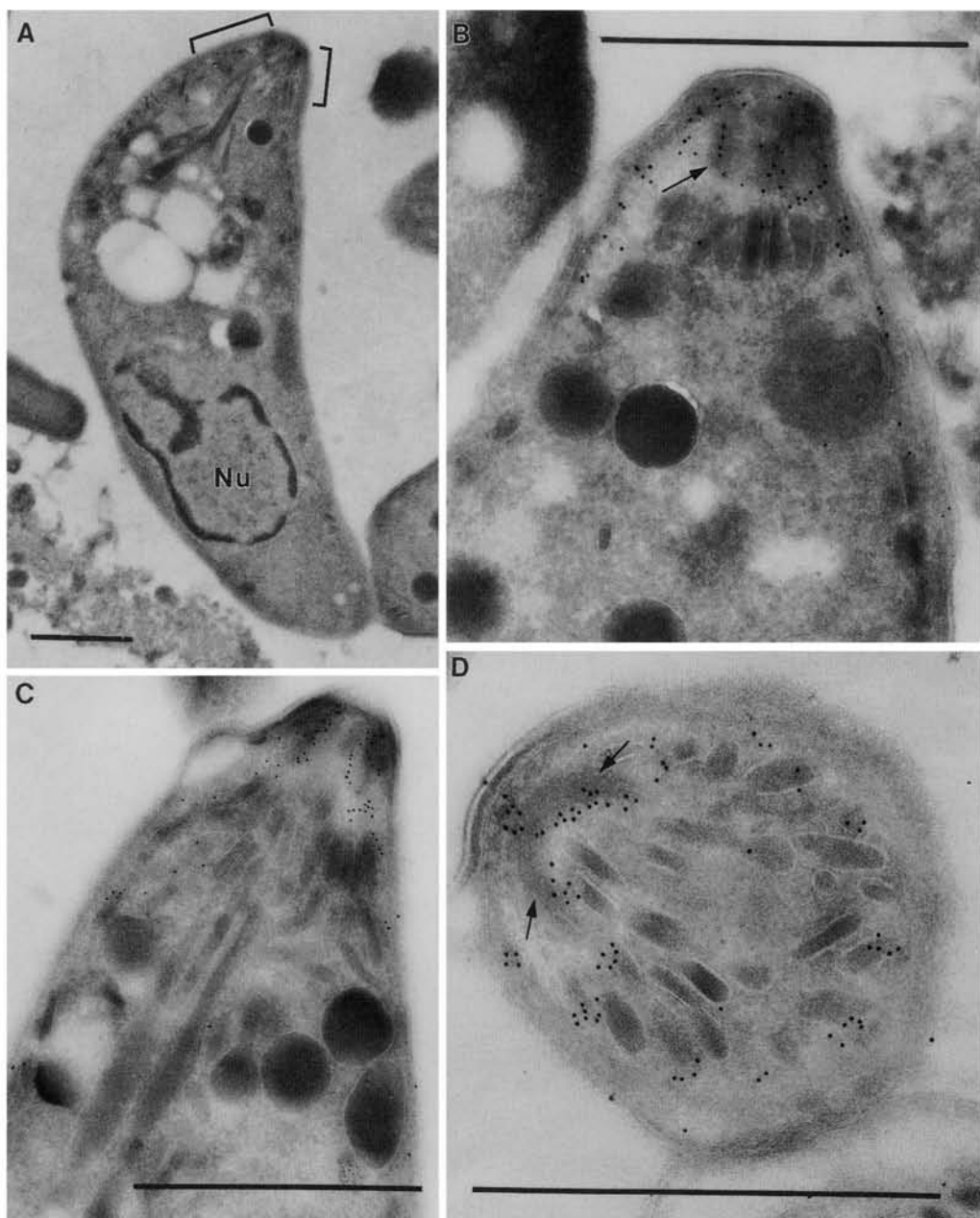


FIG. 5. Immunogold labeling of class III antigen. Consistent with the IFA results, the area labeled by this class is significantly smaller than that labeled by classes I and II. (A) Labeling of the cytoplasmic face of the IMC extends approximately three times the length of the conoid (brackets) and is also observed in the space between the apical pellicle and retracted conoid. (B) Labeling of tangential sections across the conoid filaments (arrow) and (C) longitudinal sections through the conoid. (D) Although well separated from the membrane in this micrograph, conoid filaments (arrows) are clearly labeled. Pellicle-associated label sometimes displays an apparent periodicity, suggesting that this antigen may be organized by or associated with the subpellicular microtubules. Scale bar is 1  $\mu$ M in all panels.

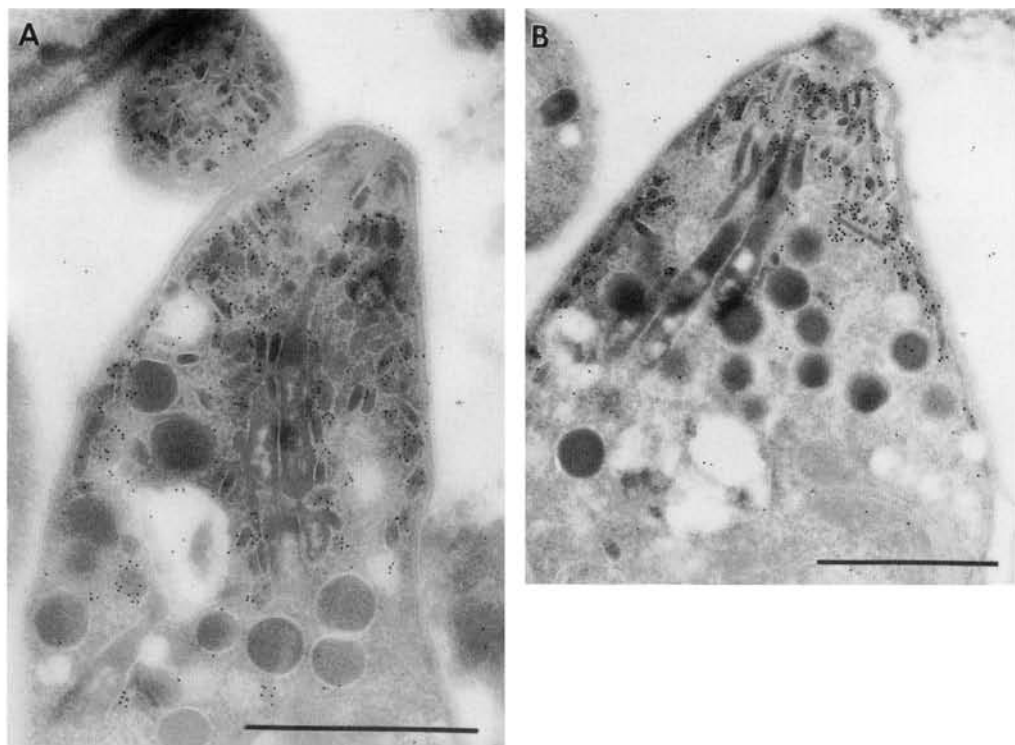


FIG. 6. Immunogold labeling of classes V and VI (antibody RIOF6 shown). Members of these classes label the micronemes. (A) Longitudinal and apical and apical cross section (upper left) both demonstrate labeling of the micronemes. (B) Gold particles are confined to the micronemes and do not label dense granules or rhoptries. Scale bar is 1  $\mu$ M in both panels.

(I, II, III) are detergent-insoluble and localize to the cytoplasmic face of the inner membrane complex. All three classes label the conoid (Figs. 4 and 5), but labeling extends beyond this organelle, particularly in the case of classes I and II. Labeling also extends into the space between retracted conoids and the laterally adjacent pellicle. In some cases, the circumferential labeling through cross sections of the parasite displays an apparent periodicity, suggesting that this antigen may be organized by or associated with the subpellicular microtubules (Fig. 5D). None of these antigens is found along the entire pellicle length occupied by subpellicular microtubules, however, and the labeling pattern is clearly distinct from that seen with an anti-tubulin antibody (not shown).

Antigen classes I–III also show similarities on immunoblots. All three classes appear to recognize a >200-kDa antigen and a band at 112-kDa; classes I and II both recognize bands at 122 kDa. The larger (>200 kDa) antigen can be immunoprecipitated by class III antibody, and this band cross-reacts with class I antibodies on immunoblots. Class I members also identify unique bands of 104, 92, and 68 kDa. This pattern of *T. gondii* bands is reminiscent of an antibody against a *Plasmodium yoelii* apical antigen which cross-reacts with a number of Apicomplexan parasites (Taylor *et al.* 1990) (confirmed using antibody kindly provided by Dr. D. Taylor). As reported by Taylor *et al.*, *T. gondii* is unique in showing multiple bands on immunoblots using this antibody. In contrast to this previous re-

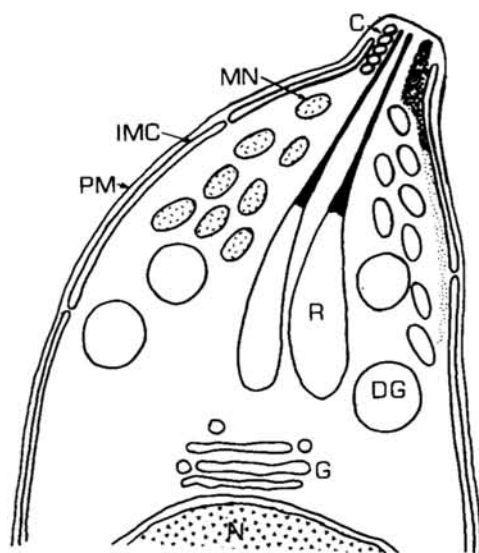


FIG. 7. Diagram of the *T. gondii* apical complex. Superior to the nucleus (N) lie the Golgi apparatus (G) and specialized secretory organelles: the dense granules (DG), rhoptries (R), and micronemes (MN). The conoid (C; shown in cross section) is a spiraling cytoskeletal structure found at the extreme apex and is linked to additional cytoskeletal elements including the pre-conoidal rings and apical polar ring (not shown). Subpellicular microtubules are nucleated by the apical polar ring, and are closely apposed to the inner membrane complex (IMC), extending distally past the parasite nucleus. Antibodies characterized in this report recognize the conoid and internal face of the IMC [classes I and II, light stippling; class III, dark stippling] (shown on the right side only). Class IV antibodies may recognize the upper necks of rhoptries (black). Class V and VI recognize micronemal antigens (shaded on left side only). PM, plasma membrane. See text for further discussion.

port, our most avid class I antibody (C4F3) recognizes three bands in *P. falciparum* and at least six in *Toxoplasma* (Fig. 2, lanes 1 and 2) and labels both the apical pellicle and the conoid (of *T. gondii*) by immunofluorescence.

There are several possible explanations for the large number of bands observed on immunoblots with class I antibodies and the cross-reactivity between antibody classes I, II, and III. It appears unlikely that the multiplicity of bands observed results from degradation alone, since the same pattern

of bands is seen in multiple protein preparations, and no evidence of degradation has been observed using the same preparations for reaction with other antibodies. The immunoblot pattern is unaffected by the presence or absence of protease inhibitors (TAME, PMSF, leupeptin, and pepstatin). We cannot, however, rule out the possibility that the multiple bands result from proteolytic processing or degradation *in vivo*, perhaps associated with disassembly of the maternal architecture during the later stages of endodyogeny. Alternatively, classes I, II, and III may recognize shared epitopes on multiple proteins, or the antibodies may recognize a highly modified class of proteins in such a way that their reactivity is differentially affected by the modification. The cytoplasmic localization of these antigens argues against the notion of an N-linked carbohydrate epitope, however, as does the apparent cross-reactivity of the class I and III antibodies with *P. falciparum*, which lacks N-linked glycosylation (Dieckmann-Schuppert *et al.* 1992, 1994). Carbohydrate epitopes remain a formal possibility for these antibodies, but we have been unable to alter antigenicity using periodate treatment, (Woodward *et al.* 1985).

Dubremetz *et al.* have reported a 220-kDa antigen restricted to the upper necks of rhoptries (Dubremetz and Schwartzman 1993)—a morphology similar to that seen with class IV antibody A3H9 (which binds to a major band at 93 kDa and a minor 163-kDa band). Class VI antigens are localized to the micronemes and are probably identical to the 120-kDa antigen MIC2 (Achbarou *et al.* 1991a). Antibodies in Class V bind to a cell-cycle-regulated micronemal antigen in immunofluorescence assays and a 38-kDa antigen on immunoblots. The members of this class probably recognize MIC3, a ~90 kDa micronemal antigen which is a heterodimer of two 38-kDa polypeptides resolvable under reducing conditions (Achbarou *et al.* 1991a). The cell-cycle aspect of



this antigen may prove of interest. Although apical at other times, recently replicated parasites show distinctive perinuclear staining (Fig. 1J, inset). This pattern suggests association with the parasite's secretory apparatus, which is probably synthesizing proteins for deposition in specialized apical organelles at this time.

The isolation and initial characterization of 26 monoclonal antibodies to the extreme apex of *T. gondii* provides tools for the study of proteins associated with this region. The intricate organization of the apical cytoskeleton and the maintenance of apical polarity in these parasites suggests that characterization of extreme apical antigens will be an intriguing story.

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*Note added in proof.* The suspected identity of class V and VI antigens with (respectively) the MIC3 and MIC2 antigens of Achbarov *et al.* (1991a) is supported by comigration on immunoblots (antibodies kindly provided by Dr. J. F. Dubremetz). The class V/MIC3 cell-cycle-associated perinuclear staining observed in intracellular parasites (Fig. 1G-1J) localizes to the Golgi apparatus by Immuno EM.

#### REFERENCES

ACHBAROU, A., MERCEREAU-PUJALON, O., AUTHEMAN, J., FORTIER, B., CAMUS, D., AND DUBREMETZ, J. 1991a. Characterization of microneme proteins of *Toxoplasma gondii*. *Molecular and Biochemical Parasitology* 47, 223-234.

ACHBAROU, A., MERCEREAU-PUJALON, O., SADAQ, A., FORTIER, B., LERICHE, M., CAMUS, D., AND DUBREMETZ, J. 1991b. Differential targeting of dense granule proteins in the parasitophorous vacu-

ole of *Toxoplasma gondii*. *Parasitology* 103, 321-329.

CHARIF, H., DARCY, F., TORPIER, G., CESBRON-DELAUW, M., AND CAPRON, A. 1990. *Toxoplasma gondii*: Characterization and localization of antigens secreted from tachyzoites. *Experimental Parasitology* 71, 114-124.

CHOBOTAR, B., AND CHOLTSECK, E. 1982. Ultrastructure. In "The Biology of the Coccidia" (P. Long, Ed.), pp. 101-165. Univ. Park Press, Baltimore.

CINTRA, W., AND DE SOUZA, W. 1985. Immunocytochemical localization of cytoskeletal proteins and electron microscopy of detergent extracted tachyzoites of *Toxoplasma gondii*. *Journal of Submicroscopic Cytology* 17, 503-508.

DE CARVALHO, L., SOUTO-PADRON, T., AND DE SOUZA, W. 1991. Localization of lectin-binding sites and sugar-binding proteins in tachyzoites of *Toxoplasma gondii*. *Journal of Parasitology* 77, 156-161.

DIECKMANN-SCHUPPERT, A., BAUSE, E., AND SCHWARTZ, R. 1994. Glycosylation reactions in *Plasmodium falciparum*, *Toxoplasma gondii*, and *Trypanosoma brucei brucei* probed by the use of synthetic peptides. *Biochimica et Biophysica Acta* 1199, 37-44.

DIECKMANN-SCHUPPERT, A., BENDER, S., ODENTHAL-SCHNITTLER, M., BAUSE, E., AND SCHWARTZ, R. 1992. Apparent lack of N-glycosylation in the asexual intraerythrocytic stage of *Plasmodium falciparum*. *European Journal of Biochemistry* 205, 815-825.

DUBREMETZ, J., AND SCHWARTZMAN, J. 1993. Subcellular organelles of *Toxoplasma gondii* and host cell invasion. *Research in Immunology* 144, 31-33.

DUBREMETZ, J., AND TORPIER, G. 1978. Freeze-fracture study of the pellicle of an Eimerian sporozoite (Protozoa, Coccidia). *Journal of Ultrastructure Research* 62, 94-109.

ENDO, T., SETHI, K., AND PIEKARSKI, G. 1982. *Toxoplasma gondii*: Calcium ionophore A23187-mediated exit of trophozoites from infected murine macrophages. *Experimental Parasitology* 53, 179-188.

ENDO, T., TOKUDA, H., YAGITA, K., AND KOYAMA, T. 1987. Effect of extracellular potassium on acid release and motility initiation in *Toxoplasma gondii*. *Parasitology Research* 75, 102-106.

ENDO, T., AND YAGITA, K. 1990. Effect of extracellular ions on motility and cell entry in *Toxoplasma gondii*. *Journal of Protozoology* 37, 133-138.

ENDO, T., YAGITA, K., YASUDA, T., AND NAKAMURA, T. 1988. Detection and localization of actin in *Toxoplasma gondii*. *Parasitology Research* 75, 102-106.

FIELD, S., PINDER, J., CLOUGH, B., DLUZEWski, A., WILSON, R., AND GRATZER, W. 1993. Actin in the

- merozoite of the malaria parasite, *Plasmodium falciparum*. *Cell Motility and the Cytoskeleton* 25, 43-48.
- FOUSSARD, F., LERICHE, M., AND DUBREMETZ, J. 1991. Characterization of the lipid content of *Toxoplasma gondii* rhoptries. *Parasitology* 102, 367-370.
- GRIFFITHS, G. 1993. "Fine Structure and Immunocytochemistry," pp. 137-197, Springer-Verlag, New York.
- HARLOW, E., AND LANE, R. 1988. "Antibodies: A Laboratory Manual," Cold Spring Harbor Press, Cold Spring Harbor, NY.
- HERION, P., HERNANDEZ-PANDO, R., DUBREMETZ, J., AND SAVEDRA, R. 1993. Subcellular localization of the 54 kDa antigen of *Toxoplasma gondii*. *Journal of Parasitology* 79, 216-222.
- KASPER, L., AND WARE, P. 1985. Recognition and characterization of stage-specific oocyst sporozoite antigens of *Toxoplasma gondii* by human antisera. *Journal of Clinical Investigation* 75, 1570-1577.
- LANE, R., CRISSMAN, R., AND GINN, S. 1986. High efficiency fusion procedure for producing monoclonal antibodies against weak immunogens. *Methods in Enzymology* 121, 183-192.
- LERICHE, M., AND DUBREMETZ, J. 1990. Extocytosis of *Toxoplasma gondii* dense granules into the parasitophorous vacuole after host cell invasion. *Parasitology Research* 76, 559-562.
- LERICHE, M., AND DUBREMETZ, J. 1991. Characterization of the protein contents of rhoptries and dense granules of *Toxoplasma gondii* tachyzoites by subcellular fractionation and monoclonal antibodies. *Molecular and Biochemical Parasitology* 45, 249-260.
- MERCIER, C., LECORDIER, L., DARCY, F., DESLEE, D., MURRAY, A., TOURVIELLE, B., MAES, P., CAPRON, A., AND CESBRON-DELAUW, M. 1993. Molecular characterization of a dense granule antigen (Gra 2) associated with the network of the parasitophorous vacuole in *Toxoplasma gondii*. *Molecular and Biochemical Parasitology* 58, 71-82.
- NICHOLS, B., AND CHIAPPINO, M. 1987. Cytoskeleton of *Toxoplasma gondii*. *Journal of Protozoology* 34, 217-226.
- OSSORIO, P., SCHWARTZMAN, J., AND BOOTHROYD, J. 1992. A *Toxoplasma gondii* rhoptry protein associated with host cell penetration has unusual charge asymmetry. *Molecular and Biochemical Parasitology* 50, 1-16.
- PORCHET, E., AND TORPIER, G. 1977. Etude du germe infectieux de *Sarcocystis tenella* et *Toxoplasma gondii* par la technique du cryodécapage. *Zeitschrift für Parasitenkunde (Berlin)* 54, 101-127.
- ROOS, D., DONALD, R., MORRISSETTE, N., AND MOULTON, L. 1994. Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Methods in Cell Biology*, in press.
- RUSSELL, D., AND BURNS, R. 1984. The polar ring of Coccidian parasites: A unique microtubule organizing centre. *Journal of Cell Science* 50, 345-359.
- SABIN, A. 1941. Toxoplasmic encephalitis in children. *Journal of the American Medical Association* 116, 801-807.
- SADAK, A., TAGHY, Z., FORTIER, B., AND DUBREMETZ, J. 1988. Characterization of a family of rhoptry proteins of *Toxoplasma gondii*. *Molecular and Biochemical Parasitology* 29, 203-211.
- SAFFER, L., MERCEREAU-PUJALON, O., DUBREMETZ, J., AND SCHWARTZMAN, J. 1992. Localization of a *Toxoplasma gondii* rhoptry protein by immunoelectron microscopy during and after host cell penetration. *Journal of Protozoology* 39, 526-530.
- SAM-YELLOWE, T. 1992. Molecular factors responsible for host cell recognition and invasion in *Plasmodium falciparum*. *Journal of Protozoology* 39, 181-189.
- SCHWARTZMAN, J., AND KRUG, E. 1989. Immunofluorescent localization of myosin at the anterior pole of the Coccidian, *Toxoplasma gondii*. *Journal of Protozoology* 30, 657-661.
- TAYLOR, D., EVANS, C., ALEY, S., BARTA, J., AND DANFORTH, H. 1990. Identification of an apically-located antigen that is conserved in protozoan parasites. *Journal of Protozoology* 37, 540-545.
- YASUDA, T., YAGITA, K., NAKAMURA, T., AND ENDO, T. 1988. Immunocytochemical localization of actin in *Toxoplasma gondii*. *Parasitology Research* 75, 107-113.
- WOODWARD, M., YOUNG, W., AND BLOODGOOD, R. 1985. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *Journal of Immunological Methods* 78, 143-153.

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