Toxoplasma Modifications Macrophage Phagosomes by Secretion of a Vesicular Network Rich in Surface Proteins

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Abstract. Modification of macrophage phagosomes begins shortly after formation as Toxoplasma cells secrete membranous vesicles that form a reticulate network within the vacuole. The Toxoplasma-modified compartments then resist normal endocytic processing and digestion. We have used the pronounced Ca++-dependent stability of the intraphagosomal membrane (IPM) network to purify and characterize the structural proteins of this assembly. In addition to the structural matrix, Toxoplasma secretes a discrete set of soluble proteins, including a newly described 22-kD calcium-binding protein.

The IPM network adheres to intact Toxoplasma cells after host cell lysis in the presence of 1 mM Ca++; however, the network readily disperses in calcium-free buffer and was purified as vesicles that sedimented at 100,000 g. Purified IPM vesicles were specifically recognized by immune sera from mice with chronic Toxoplasma infection and consisted primarily of a 30-kD protein when analyzed by SDS PAGE. IPM network proteins share a major antigenic component located on the surface of extracellular Toxoplasma cells as shown by immunoperoxidase electron microscopy using a polyclonal antibody prepared against the IPM vesicles. Moreover, in Toxoplasma-infected macrophages, anti-IMP antibody confirmed that the extensive IPM array contains proteins also found on the Toxoplasma cell surface. Our results indicate the IMP network represents a unique structural modification of the phagosome comprised in part of Toxoplasma surface proteins.

Toxoplasma exploits the endocytic process to gain entry to host cells (32), where it resides in modified vacuoles that resist microbicidal activity and normal digestion (15, 26, 33). The modified phagosome appears to act as a protective interface, supporting prolific growth and multiplication of the parasite at the expense of the host cell. The establishment of Toxoplasma within modified host cell vacuoles leads to chronic infections characterized by intracellular cysts located primarily in striated muscle and the central nervous system (5, 23). Exacerbation of latent infection due to cyst rupture and unrestricted parasite replication results in a life-threatening condition in immunocompromised patients (24). The specialized endocytic compartment in which living Toxoplasma reside contains a prominent vesicular network that forms connections with the parasite plasma membrane and the phagosome membrane (14, 21, 22). The origin and function of this assembly are unknown; however, similar networks occur in host cell phagosomes containing other protozoa that subvert host cell endocytic processing (3, 4, 13, 20, 31).

To elucidate the components of this crucial parasite–host interface, we isolated intraphagosomal membrane (IPM) vesicles from Toxoplasma–infected macrophages. We demonstrate here that the IMP network is comprised primarily of a 30-kD protein that is a major surface protein of Toxoplasma cells. Within the phagosome vacuolar space, the IMP network occupies considerable surface area that contains Toxoplasma cell surface proteins. As such, this network is ideally situated for modification of the phagocytic vacuole to evade microbicidal events during entry and to provide nutrient transport.

Materials and Methods

Parasite Culture

RH strain Toxoplasma gondii tachyzoites were harvested from ascitic fluid of CF-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) in Hanks' balanced salt solution (Gibco, Grand Island, NY) supplemented with 1 mM Ca++, 30 U/ml heparin (Sigma Chemical Co., St. Louis, MO), and 10 mM Hepes buffer (Gibco). Extracellular Toxoplasma cells were purified from remaining host cells by filtration through 30-μm polycarbonate membrane filters (Nucleopore Corp., Pleasanton, CA) (33) and washed three times by centrifugation at 250 g for 10 min.
Antisera Production

Polyclonal anti-IPM network sera were produced by intramuscular immunization of CF-1 mice with native IPM network proteins from preparations that were screened for purity by SDS PAGE. The initial injection of 10 μg protein was administered in complete Freund’s adjuvant followed by two injections of 5 μg protein in incomplete Freund’s given at 10-d intervals. Mouse anti-Toxoplasma sera were produced in CF-1 mice with chronic C-strain Toxoplasma infection followed by boosting with RH-strain Toxoplasma as previously described (27).

Isolation of IPM Networks

Freshly isolated, washed Toxoplasma cells were incubated for 30 min at 4°C in dissociation buffer consisting of calcium-magnesium-free (CMF) phosphate-buffered saline (PBS) (Irvine Scientific, Santa Ana, CA) pH 7.2 containing 10 U/ml heparin, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml phenylmethylsulfonyl fluoride, and 0.1 mM dithiothreitol (Sigma Chemical Co.). Incubation in isotonic dissociation buffer did not affect the viability of Toxoplasma cells as shown by mouse inoculation and the ability of treated Toxoplasma to infect host cells in vitro as previously described (27). The protocol used to isolate IPM network and characterize secretion products of Toxoplasma under Ca++ free conditions is illustrated in Fig. 1. After incubation in dissociation buffer, intact Toxoplasma cells were pelleted and the supernatant clarified by five spins at 1,000 g for 10 min each. The clarified supernatant was fractionated by centrifugation first at 10,000 g for 60 min then at 100,000 g for 120 min. The 100,000 g pellet was washed twice in dissociation buffer using an air ultramicrofuge (Beckman Instruments, Inc., Palo Alto, CA) at 100,000 g for 60 min. The 100,000 g supernatant, referred to as the soluble protein fraction, was concentrated with Centricon-10 columns (Amicon, Danvers, MA) and stored at -20°C.

Electron Microscopy

Transmission EM. Cells were fixed with 2% glutaraldehyde (GA) in 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in LR White resin (London Resin Co. Ltd., Hampshire, England). To enhance membrane staining, cells were postfixed in 1% osmium tetroxide plus 0.5% potassium ferricyanide (9) or fixed with GA plus 1% tannic acid and postfixed with osmium plus 1% tannic acid (28). Thin sections were stained with uranyl acetate and lead citrate and viewed with a JOEL 100CX EM.

Serologically Specific Electron Microscopy (SSEM). For SSEM (7), carbon-formvar-coated grids were incubated 30 min in 1:100 dilution of mouse anti-Toxoplasma sera, or normal mouse sera, washed three times in PBS, and incubated 1 h with suspensions of the isolation fractions. SSEM grids were then washed three times and stained with either 1% uranyl acetate or 2% phosphotungstic acid, air dried, and viewed with a JOEL 100CX EM.

Immunoperoxidase Localization. Mouse peritoneal macrophages cultured on Lab-Tek chamber slides (Miles Scientific Div., Naperville, IL) in RPMI-1640 (Gibco) media containing 20% heat-inactivated fetal calf serum (Sterile Systems Inc., Logan, UT) were infected with freshly harvested Toxoplasma at a ratio of 2:1. At 1 h after infection, monolayers were fixed in cold 2% formaldehyde, 0.1% GA in 0.1 M cacodylate buffer for 30 min, 4°C. Monolayers were washed three times and incubated 10 min in PBS containing 10% glycerol, then frozen in liquid nitrogen and rapidly thawed to 37°C. Monolayers were successively incubated in antisera at 37°C and washed with PBS at 4°C as follows: (a) normal rabbit sera diluted 1:20 in PBS; (b) primary antibody consisting of normal mouse sera or polyclonal mouse anti-IPM sera diluted 1:20 in PBS; (c) peroxidase-conjugated goat (IgG-F(ab’)2) anti-mouse IgG (Tago Inc., Burlingame, CA) diluted 1:20 in PBS. Monolayers were rinsed in 2.5% GA in 0.1 M cacodylate buffer for 1 h (4°C). Monolayers were rinsed in Tris-HCl, developed for 3 min with diaminobenzidine (Polysciences, Inc., Warrington, PA), and fixed in 1% osmium tetroxide containing 0.5% potassium ferricyanide. Thin sections of LR White resin embedded monolayers were examined without further staining using a Philips EM 410.

Electrophoresis

SDS PAGE. Toxoplasma cells and fractions isolated as described above were boiled 2 min in 1% SDS, 2% 2-mercaptoethanol, 1% Triton X-100, 10 mM Tris-HCl containing 10% glycerol, then frozen in liquid nitrogen and rapidly thawed. Monolayers were successively incubated in antisera at 37°C and washed with PBS at 4°C as follows: (a) normal rabbit sera diluted 1:20 in PBS; (b) primary antibody consisting of normal mouse sera or polyclonal mouse anti-IPM sera diluted 1:20 in PBS; (c) peroxidase-conjugated goat (IgG-F(ab’)2) anti-mouse IgG (Tago Inc., Burlingame, CA) diluted 1:20 in PBS. Monolayers were refixed in 2.5% GA in 0.1 M cacodylate buffer for 1 h (4°C). Monolayers were rinsed in Tris-HCl, developed for 3 min with diaminobenzidine (Polysciences, Inc., Warrington, PA), and fixed in 1% osmium tetroxide containing 0.5% potassium ferricyanide. Thin sections of LR White resin embedded monolayers were examined without further staining using a Philips EM 410.

Results

Development of Intraphagosomal Networks

The expression of Toxoplasma networks within macrophage phagosomes was examined by electron microscopy at time intervals of 5, 10, 30, and 60 min after infection. Membranous profiles were observed within the newly formed phagocytic vacuole, typically near the apical end of the organism.

MOUSE ASCITES, 2 × 10^6 TOXOPLASMA

![Figure 1. Purification of IPM networks that adhere to Toxoplasma cells released from mouse macrophages. Toxoplasma cells were incubated in CMF PBS dissociation buffer to disperse IPM networks. The network assembly and soluble products were then separated by differential centrifugation.](image-url)
Figures 2–4. (Fig. 2) Electron micrograph of *Toxoplasma* cell within newly formed macrophage phagocytic vacuole. Several membrane vesicles (V) are evident within the vacuole near the apical end of the parasite where the rhoptries (R) connect. M, mitochondria. Bar, 1.0 μm. (Fig. 3) Electron micrograph of modified phagocytic vacuole at 10 min after *Toxoplasma* infection. The prominent IPM network consists of tubular elements (T) and membranous vesicles (V) which occupy the lumen of the modified phagocytic vacuole. Bar, 1.0 μm. (Fig. 4) Electron micrograph of modified phagocytic vacuole at 30 min after *Toxoplasma* infection. The expanded lumen of the modified vacuole contains a fully developed reticular network. Connections with the parasite surface and vacuole membrane (arrowheads) are observed and the unit membrane profile of the tubular elements is evident (*). The close alignment of host rough endoplasmic reticulum (RER) gives the modified vacuole its characteristic triple membrane appearance (arrows). P, pellicle. PM, plasma membrane. M, mitochondria. Bar, 0.5 μm.
Figures 5-9. Electron micrograph of *Toxoplasma* cells with adherent clusters of IPM networks (IPN). The networks are readily purified from host cell components due to their adhesion to *Toxoplasma* cells pelleted at low speed (200 g) in the presence of calcium. Bar, 1.0 μm. (Fig. 6) Electron micrograph of *Toxoplasma* cell (TC) incubated in CMF PBS demonstrating protrusions of the plasma membrane. The membrane extensions formed in vitro resembled the association of IPM networks with intracellular *Toxoplasma* cells. Membrane-bound vesicles (V) which contain an electron-dense core may form by budding of surface membrane protrusions. P, pellicle. PM, plasma membrane. Bar, 0.1 μm. (Fig. 7) Electron micrograph of *Toxoplasma* cell incubated in CMF PBS and fixed in the presence of tannic acid. The membrane protrusions are comprised of multiple lamellar whorls that resemble stacked membranes with spacing of 5-7 nm. P, pellicle. PM, plasma membrane. Bar, 0.1 μm. (Fig. 8) Electron micrograph of purified network vesicles that were thin sectioned and stained with uranyl acetate and lead citrate. Vesicles contained in the 100,000 g pellet demonstrate unit membrane profile similar to IPM networks within modified phagosomes (compare Fig. 4). Bar, 0.1 μm. (Fig. 9) Serologically specific electron micrograph of purified network vesicles negatively stained with phosphotungstic acid. IPM network vesicles were specifically absorbed by mouse anti-*Toxoplasma* antibody, coated grids (a) but not by normal mouse sera-coated grids. (b). Bar, 0.1 μm.

within 5 min after invasion (Fig. 2). The phagosome membrane was continuous in all cases indicating the initial vacuole is formed by invagination of the host cell plasma membrane. Strands of host cell rough endoplasmic reticulum were closely adjacent to the limiting vacuole membrane, but were not connected during the early stages of infection.

Well developed structural networks, consisting of 200-nm diam vesicles and elongate tubules, were apparent within modified phagocytic vacuoles as early as 10 min (Fig. 3). The tubules measured 40-60-nm diam by 200-500 nm in convoluted length and appeared bilaminar in cross-section. A dramatic enlargement of the modified vacuole occurs between 30 and 60 min and connections are evident between the vacuole membrane and the parasite surface membrane via an extensive IPM network (Fig. 4). A closely apposed layer of host rough endoplasmic reticulum and mitochondria gives the modified vacuole a double or triple membrane profile not seen in normal phagosomes (Fig. 4, arrows).

**Calcium Responsiveness and Purification of Extracellular IPM Networks**

*Toxoplasma* cells liberated from lysed host cells were sepa-
rated from host cell components by filtration and repeated washing. Extensive IPM networks adhered to *Toxoplasma* cells pelleted at low speeds (i.e., 100–500 g) in the presence of 1 mM Ca \(^{++}\) (Fig. 5); however, in the absence of Ca \(^{++}\), IPM networks dispersed and are not found associated with *Toxoplasma* cells pelleted at low speed (not shown). We have used this Ca \(^{++}\) sensitivity to purify dispersed IPM network components from intact *Toxoplasma* cells under isotonic conditions (Fig. 1).

Washed *Toxoplasma* cells were agitated for 30 min in CMF PBS to disperse the IPM network allowing intact organisms to be removed by low speed centrifugation. *Toxoplasma* cells incubated in CMF PBS showed numerous extensions of the plasma membrane although the internal cell structure remained normal (Fig. 6). These plasma membrane extensions did not form when *Toxoplasma* cells were fixed in the presence of calcium. Bilaminar extensions of the parasite membrane formed during CMF PBS incubation were similar to tubular components of the IPM network associated with *Toxoplasma* cells within macrophage vacuoles (compare Figs. 4 and 6). Tannic acid staining demonstrated these membrane extensions consisted of lamellar whirls with 5–7-nm spacing that resembled concentric membrane layers (Fig. 7).

### Immune Recognition of IPM Networks

Serologically specific EM (7) was used to identify conditions necessary to purify IPM networks after disaggregation in CMF PBS. Fractions of the isolation protocol were screened using mouse anti-*Toxoplasma* antibody coated grids to specifically detect components of the IPM network. Membrane-delineated vesicles of similar size and appearance to the IPM network tubules were observed in thin sections of the 100,000 g pellet (Fig. 8). The vesicles were specifically recognized by mouse anti-*Toxoplasma* sera coated grids incubated in the 100,000 g pellet, but were not detected in the 10,000 g pellet by SSEM (Fig. 9).

### Protein Analysis

SDS PAGE was used to characterize secretion products of *Toxoplasma* cells including the structural components of IPM networks. *Toxoplasma* cells secreted a number of soluble proteins, detected in the CMF PBS 100,000 g supernatant (Fig. 10, lane 3) which were significantly different from proteins detected by deliberate lysis of whole *Toxoplasma* cells (Fig. 10, lane 2). The 100,000 g pellet, which contained membrane vesicles recognized by anti-*Toxoplasma* sera, was comprised primarily of a 30-kD protein with minor components of 35 and 20 kD (Fig. 10, lane 4). The lack of detectable protein in the 10,000 g pellet, which would normally contain organelles from disrupted cells, confirmed that *Toxoplasma* cells were not lysed by CMF PBS incubation (Fig. 10, lane 5).

### Calcium Binding

Calcium binding activity of proteins immobilized on NCP was detected by autoradiography after labeling with \(^{45}\)Ca \(^{++}\) in the presence of excess Mg \(^{++}\). Dot blots of native proteins contained in whole *Toxoplasma* cell lysates and in purified IPM network vesicles did not bind significant amounts of \(^{45}\)Ca \(^{++}\) (Fig. 11, lanes 2 and 4). However, *Toxoplasma* protein(s) secreted during CMF PBS incubation and concentrated in the 100,000 g supernatant showed a high affinity for \(^{45}\)Ca \(^{++}\) (Fig. 11, lane 3). To identify the component responsible for this calcium-binding activity, proteins were separated by SDS PAGE and transferred to NCP for autoradiography after \(^{45}\)Ca \(^{++}\) labeling. Bovine brain calmodulin, which retains calcium-binding activity after SDS treatment (19), was used as a positive control (Fig. 12, lane 3). A 22-kD pro-
protein contained in 100,000 g CMF PBS supernatant demonstrated a high affinity for $^{45}$Ca$^{++}$ (Fig. 12, lane 2). This 22-kD protein was not evident in an equal amount of total protein from lysates of whole Toxoplasma cells (Fig. 12, lane 1), indicating it is specifically secreted in a soluble form by Toxoplasma cells during incubation in CMF PBS.

**Immunoperoxidase Localization of IPM Network Proteins**

Polyclonal mouse anti-IPM antibody was produced in mice by immunization with IPM networks which consist predominately of a 30-kD protein. The anti-IPM antibody recognized primarily a similar 30-kD protein in extracts of whole Toxoplasma cells by Western blotting analysis (Fig. 13). Normal mouse sera did not label any Toxoplasma proteins under the conditions shown in Fig. 13.

Immunoperoxidase EM was used to localize in situ the IMP network proteins recognized by anti-IMP antibody (predominately 30 kD). Toxoplasma cells treated with normal mouse sera lacked peroxidase reaction product (Fig. 14). Anti-IMP antibody recognized determinants on the surface of extracellular Toxoplasma cells (Fig. 15) and on the tubular extensions of the cell surface characteristic of incubation in CMF PBS (Fig. 16). In Toxoplasma-infected macrophages, the anti-IMP antibody recognized proteins on the surface of intracellular Toxoplasma and IPM networks (Fig. 17). Moreover, peroxidase reaction product was evident in the region of the phagosome vacuole membrane but not on the macrophage plasma membrane (Fig. 17), indicating that the IMP Toxoplasma proteins may be associated with the modified phagosome membrane. Although this later reaction product may be the result of nonspecific deposits of diaminobenzidine, we have minimized the potential for such artifacts by using short development times as previously described (6).

**Discussion**

Toxoplasma secretes a protein-rich network within host cell phagosomes that is evident within 5 min of entry. The rapid expression of the parasite-derived network suggests it may function in evasion of microbicidal events initiated as the phagosome forms (2, 10, 17). Consistent with a role in intracellular survival, the IMP network is produced by live Toxoplasma entering macrophages but not when heat killed or specific antibody-coated parasites are engulfed and digested by macrophages (15, 21). The exceeding stability of this
modified compartment is demonstrated by ultraviolet-irradiated *Toxoplasma* cells, which fail to divide intracellularly, but secrete a characteristic network within phagosomes that resist lysosome fusion (8). The elaborate IPM network is not restricted to infections in macrophages but also forms when *Toxoplasma* cells enter kidney cell lines (25) and fibroblasts (Sibley, L. D., unpublished observations).

Calcium binding is evidently important in regulation of *Toxoplasma* surface interactions involved in host cell entry since IPM networks and *Toxoplasma* cell surfaces display pronounced calcium-dependent adhesion. Furthermore, *Toxoplasma* cells produce a 22-kD soluble protein that shows high calcium-binding affinity in both native state and after SDS treatment. This 22-kD calcium-binding protein appears to be secreted by intact *Toxoplasma* cells and is therefore unlike other known calcium-binding proteins that regulate intracellular events (11). Notedly, *Toxoplasma* releases this 22-kD protein under the same conditions that favor in vitro formation of membrane protrusions resembling the IPM network. Whether the 22-kD calcium-binding protein is present within the phagosome or if it plays a role in regulating IMP development in situ is presently unknown.

In the presence of calcium, parasites released from host cells aggregate with IPM networks. A marked calcium-responsive stability was used to disperse this association without lysing *Toxoplasma* cells and purify IPM network vesicles by differential centrifugation. Purified IPM vesicles were comprised primarily of a 30-kD protein. Immunoperoxidase EM confirmed that the IMP proteins, including the 30-kD protein, are located on the *Toxoplasma* cell surface as well as in the tubular extensions which form the IPM network. In addition, anti-IMP peroxidase reaction product was evident along the phagosome membrane of *Toxoplasma*-containing vacuoles. However, the potential limitations of diaminobenzidine in resolving the precise localization of bound immunoglobulin (6) preclude us from establishing whether IMP proteins are in fact intimately associated with the phagosome membrane.

We have recently confirmed that the 30-kD protein isolated from IPM networks reacts with a monoclonal antibody (1EU, reference 12) to the 27-30-kD major surface protein of *Toxoplasma* (manuscript in preparation). The 30-kD surface protein is a major antigenic component of *Toxoplasma* recognized serologically by both chronically infected mice and humans (12). In the present study, chronic mouse sera specifically absorbed the IPM vesicles from solution, indicating that antigenic proteins are exposed on the surface of the vesicles. The 30-kD *Toxoplasma* surface protein contains an extensive hydrophobic region (16) that may serve to anchor this protein within the IMP vesicles.

The distribution of the IMP proteins (predominately 30 kD) revealed by immunoperoxidase localization confirms initial EM observations that IPM networks connect the *Toxoplasma* cell surface to the phagosome vacuole membrane. Formation of the IPM network may involve secretion of vesicles from the parasite surface as suggested by: (a) apparent
unit membrane profile of the IPN network, (b) connections between IPN networks and Toxoplasma surface membranes, and (c) in vitro formation of membrane protrusions by extracellular Toxoplasma which resemble a whorled stack of membranes when stained with tannic acid. Similar membranous whorls are observed within paired apical organelles known as rhoptries of Plasmodium and when extruded show an affinity for the parasite cell surface (29). Although we have not observed any involvement of the rhoptries, previous reports have implicated the rhoptries as storage organelles for vesicular material extruded by Toxoplasma (22).

The Toxoplasma-containing vacuole forms by invagination of the macrophage plasma membrane (1, 32) and thus may also contain proteins normally distributed on the host cell surface. However, the uniqueness of the Toxoplasma-modified vacuoles is evident by their failure to trigger host cell oxygen intermediate formation (33), phagosome acidification (26), and avoidance lysosome fusion (15). The elaborate structural modification of macrophage phagosomes by Toxoplasma surface proteins may account for this resistance to digestion. Alternatively, the modified vacuole may be important in maximizing surface area for nutrient transport needed to support the prolific growth of the parasite (4). The absence of host cell plasma membrane transport systems for phagosomal excretion of nutrients may be compensated for by the Toxoplasma-derived IMP network within this compartment.

We report here that Toxoplasma modifies macrophage phagosomes by elaborating a vesicular network comprised primarily of a 30-kD protein also found on the Toxoplasma cell surface. To what extent this elaborate structural modification alters physiological events within the phagosome is intriguing given the propensity for Toxoplasma to evade microbicidal activity. Provided with a method for purifying this interface as an intact unit, it should now be possible to examine the effect of the IMP network on macrophage endocytic processing.

We thank Dr. Jack Remington (Palo Alto Medical Research Foundation, Palo Alto) for generously providing monoclonal antibodies to Toxoplasma, Dr. Larry Rubin (Epidemiology and Public Health, Yale University) for advice on Ca2+ labeling of proteins and for generously providing bovine brain calmodulin and Michaela Mooney (Pathology, Louisiana State University) for helpful technical advice and the suggestion to use SSEM.

Received for publication 12 March 1986, and in revised form 3 June 1986.

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