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Redistribution of Parasite and Host Cell Membrane Components during *Toxoplasma gondii* Invasion

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ABSTRACT. **The initial association of tachyzoites of *Toxoplasma gondii* with a host cell induces an endocytic process which leads to the formation of a vacuole known as the parasitophorous vacuole (PV). We analyzed the parasite-host cell interaction process using either parasites or host cells whose membrane was previously labeled with probes specific for proteins, sialoglycoconjugates and lipids, and then allowed to interact for periods varying from 5 minutes to 24 hours. The fate of the fluorscents probes was followed by confocal laser scanning microscopy. In host cells previously labeled with PKH26, FITC-Thiosemicarbazide or DTAF, which label membrane proteins, siloglycoconjugates and lipids, respectively, a uniform labeling of the cell surface was observed before interaction. When allowed to interact with *T. gondii*, labeling for PKH26 and DTAF, but not for FITC-Thiosemicarbazide, was observed initially at the region of contact between the two cells and subsequently on the membrane lining the PV and the intravacuolar parasites. These observations show that some, but not all, membrane components contribute to the formation of the PV membrane. Previously labeled parasites attach to the host cell surface but lose the fluorescent probes during the invasion process so that no labeled parasites were seen within the PV. These observations point to the existence of a dynamic process of membrane-associated components of the parasite and host cell during the interaction process.**

Toxoplasma gondii is an important opportunistic pathogen causing congenital infections (10, 37) and severe complications in immunocompromised individuals (18). In vitro, tachyzoites of *T. gondii* can invade and replicate within essentially all nucleated cells, using at least two mechanisms: phagocytosis and active penetration (35). Once internalized the tachyzoites reside within a special cytoplasmic vacuole known as the parasitophorous vacuole (PV), which does not fuse with organelles of the host cell endocytic pathway, maintaining a neutral pH (16, 17, 35, 38). Immediately after formation, host cell mitochondria and endoplasmic reticulum (16, 21, 22, 24, 37).

It has been suggested that during formation of the PV host cell, plasma membrane components are excluded from the forming parasitophorous vacuole membrane (PVM), preventions later fusion with the host cell endocytic system (7, 35). The origin of the PVM in cells infected with *T. gondii* and other Apicomplexan parasites is not completely clarified and has been the subject of considerable controversy (14, 42). Two main models have been proposed to explain how the PVM is formed: (a) in the bilayer insertion model, the PVM is thought to be formed from lipids that the parasite secrete from apical organelles and insert into the host cell membrane during invasion: (b) an alternative model, known as induced invagination, proposes that the parasite induces the host cell membrane to invaginate to form the PVM (42).

The composition of lipids and proteins in the parasitophorous vacuole is unknown, but lipids are likely to influence the ability of this compartment to interact with other vesicles within the host cell (35). The phospholipids in the parasitophorous vacuole membrane surrounding *Plasmodium knowlesi* are primarily derived from invagination of the red cell plasma membrane (43). A similar process is involved in the formation of the PV with *T. gondii*. Electron microscopy studies indicated that the vacuole is formed by invagination of the host cell plasma membrane as soon as the parasite invades (1, 17). Specific plasma membrane proteins have been shown to be reduced or absent from

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newly formed phagosomes during engulfment of *L. pneumophila* (9), *T. cruzi* (13, 20), *L. mexicana* (26, 31) and *T. gondii* (7). These parasites have different intracellular fates, indicating that exclusion or removal of plasma-membrane proteins might interfere with the behaviour of the vacuole.

During the *T. gondii* invasion process, some host cell plasma membrane proteins are present in newly formed parasitophorous vacuoles, but are rapidly removed and are no more observed in mature vacuoles, while other membrane proteins are excluded during the formation of the vacuole (7, 34). Immunoelectron microscopy and freeze-fracture studies have shown that erythrocyte membrane proteins are essentially absent from the PVM in *Plasmodium knowlesi* (2, 4, 19) and significantly reduced in PV-containing *T. gondii* (27).

Assuming that during the process of *T. gondii*-host cell interaction, the interchange of surface components of the two cells takes place, we decided to analyze the fate of fluorescent probes for lipids, proteins and sialoglycoproteins of the interacting cells by confocal laser scanning microscopy. The results obtained are described in this paper.

MATERIALS AND METHODS

Parasites

Tachyzoites from the virulent RH strain of *T. gondii* were maintained by intraperitoneal passages in CF1 strain mice and were collected in phosphate-buffered saline (PBS) at pH 7.2, 48 to 72 hr after infection. The ascite fluid obtained from infected mice was centrifuged at 200 g for 7–10 minutes at room temperature to remove cells and debris. The supernatant, which contained the parasites, was collected and centrifuged at 1,000 g for 7–10 minutes. The pellet obtained was washed 2 or 3 times with PBS, pH 7.2, and resuspended to a density of 10^6 parasites/ml in 199 medium without fetal calf serum (FCS). The parasites were used within 30–40 minutes after removal from the mouse peritoneal cavity, and the viability was evaluated using a dye-exclusion test with trypan blue.

Host cells

Vero cells were maintained in Falcon plastic flasks using 199 medium with 5% FCS and subcultured by trypsinization when the cell density approached a confluent monolayer. One day before use in the experiments, approximately 2×10^5 Vero cells were placed on Linbro tissue plates that contained a round sterile coverslip, or were plated into 25 cm^2 flasks (3–5×10^5/flask) and maintained at 37°C overnight in 5% CO_2.

PKH26 labeling

Vero cells were washed three times in glucose isotonic solution (5.4%) and incubated with PKH26 (100 (μg/ml)) in a glucose isotonic solution for 30 seconds at 4°C. Then 500 μl of FCS was added and the cells washed twice with glucose isotonic solution. After that the parasites were incubated with labeled cells (50:1 parasite-host cell ratio) for 15 and 30, 60 minutes and 24 hours at 37°C in a 5% CO_2 atmosphere. The cells were washed twice with PBS to remove extracellular parasites and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and observed in a Zeiss 410 confocal laser scanning microscope (CLSM).

DTAF labeling

The cells were washed once in PBS, pH 7.2, and with PBS/200 mM borate, pH 8.5, in a 1:1 proportion. The cells were incubated with DTAF {5-(4,6-diclorotriazinyl)aminofluorescein} solution in the dark for 30 minutes at 4°C. The cells were washed in PBS+20% FCS once and incubated in PBS +20% FCS for 5 minutes a 4°C, washed in PBS once and incubated in 199 medium. Subsequently the parasites were incubated with labeled cells (50:1 parasite-host cell ratio) for 15 and 30, 60 minutes and 24 hours at 37°C in a 5% CO_2 atmosphere. The cells were then washed twice with PBS to remove extracellular parasites and processed for microscopy as above described.

Fluorescein-5-thiosemicarbazide labeling

The cells were washed in PBS twice, and incubated in a freshly prepared sodium m-periodate (5 mM in PBS) at a concentration 0.5 mM for 30 minutes at 4°C. The cells were then washed three times with PBS at 4°C and incubated in PBS containing 0.66 mg/ml fluorescein-5-thiosemicarbazide for 30 minutes at 4°C. Subsequently the cells were washed in 199 medium twice and incubated with parasites (50:1 parasite-host cell ratio) for 15, 30, 60 minutes and 24 hours at 37°C in a 5% CO_2 atmosphere. The cells were then washed twice with PBS to remove extracellular parasites and processed for microscopy as above described.

Parasite labeling

The ascite fluid obtained from infected mice was centrifuged at 200 g for 7–10 minutes at room temperature to remove cells and debris. The supernatant, which contained the parasites, was collected and centrifuged at 1,000 g for 7–10 minutes. The pellet obtained was washed 2 or 3 times with PBS, pH 7.2 and resuspended to a density of 10^6 parasites/ml in 199 medium without fetal calf serum (FCS). The parasites were adhered for 10 minutes to coverslips previously coated with 0.1% poly-L-lysin. The coverslips were washed in PBS and incubated with PKH26, DTAF and fluorescein thiosemicarbazide, separately, for 5 minutes at 4°C, in the dark, washed and processed for microscopy as above described. The parasites were also stained with 4′,6′-diamino-2-phenylindole (DAPI).

Vero cells-parasite interaction

When used for interaction with host cells, the parasites were labeled in suspension with PKH26, DTAF and fluorescein thiosemicarbazide, separately, for 5 minutes at 4°C, in the dark, and washed as described previously, annd incubated

with the Vero cells for 15, 30 and 60 minutes at 37°C in a 5% CO_2 atmosphere. The cells were then washed twice with PBS to remove extracellular parasites and processed for microscopy as above described.

RESULTS

PKH26

Vero cells were labeled with the fluorescent lipophilic probe PKH26, washed in buffer to remove unbounded label, and incubated with a *T. gondii* suspension for 5, 15, 30 and 60 minutes and 24 hours. Non infected Vero cells labeled only with PKH26, for 30 minutes at 4°C, were used as control. After the fixation, the cells were observed in a confocal laser scanning microscope (CLSM). The observation of the untreated cells incubated with PKH26 at low temperature and then fixed, showed labeling of the cell surface (Figs. 1a–b). No significant labeling of the surface region corresponding to the nuclear region was observed. The observation of cultures after short incubation times with the parasites (about 5 minutes) showed intense labeling of areas where parasites attached to the host cell surface and where recently internalized parasites were seen (Figs. 1c–d). After 15 and 30 minutes of interaction the PVM presented a light staining and showed an intravacuolar parasite intense labeling (Figs. 1e–f), mainly at the posterior portion of the parasite. The host cell perinuclear region showed intense labeled. After 60 minutes and 24 hours of interaction the intravacuolar parasites present the membrane labeled (Figs. 1g–j).

DTAF

The fluorescent amino-reactive DTAF was used to label Vero cells in culture. After labeling, Vero cells were incubated with *T. gondii* for 5, 15, 30 and 60 minutes and 24 hours. Control Vero cells labeled with DTAF for 30 minutes at 4°C showed labeling of the whole cell surface, including the nuclear region (Figs. 2a–b). After interaction with the parasites for different periods of time, images of tachyzoites attached to as well as internalized were observed. In those attached to the cell surface, the labeling of the host cell surface and the parasite surface was evident (Figs. 2c–d). After 30 minutes of interaction tachyzoites were seen within recently formed PV. Labeling of the PVM as well as of the intravacuolar tachyzoites was noticed (Figs. 2e–f). After prolonged incubation times (60 minutes and 24 hours) no labeling of the PVM and intravacuolar tachyzoites was observed. However, labeled structures dispersed throughout the host cell cytoplasm were observed (Figs. 2g–h).

Fluorescein-5-thiosemicarbazide

Vero cells were labeled with fluorescein-5-thiosemicarbazide under conditions in which glycol groups of sialic acid residues formed following oxidation by m-periodate, and then incubated with *T. gondii* for 5, 15, 30 and 60 minutes and 24 hours. Control cells showed a punctate labeling of the cell surface (not shown). Following incubation with the tachyzoites, intense labeling of the parasites attached to the host cell surface was observed (Figs. 3a–b). After internalization no labeling of the PVM or the intravacuolar parasites was seen (Figs. 3c–d). However, a punctate labeling of some cytoplasmic structures of the host cell was observed (Figs. 3e–f).

Parasite Labeling

The parasite suspension was incubated with PKH26, fluorescein-5-thiosemicarbazide, or DTAF, fixed with formaldehyde, labeled with DAPI and observed with the confocal laser scanning microscope. Labeling of the whole surface of tachyzoites was observed when they were incubated in the presence of DTAF (Figs. 4a–c) and PKH26 (Figs. 4d–f). The surface of cells from the peritoneal fluid was intensely labeled. No significant labeling was observed with fluorescein-5-thiosemicarbazide (Figs. 4g–i).

Incubation of labeled parasites with Vero cells

The parasites were labeled with PKH26 (Figs. 5a–b) fluorescein-5-thiosemicarbazide (Figs. 5c–d) or nd DTAF (Figs. 5e–f), washed and then incubated in the presence of unlabeled Vero cells, fixed and analyzed in CLSM. Labeling was observed only at the sites of contact of the parasites with the host cell and initial internalization points (Figs. 5a–f). No labeling of intracellular parasites or of structures of the host cell was observed. It is important to point out that the parasites were viable and able to infect the cells.

DISCUSSION

One of the most enigmatic issues related with host cell invasion by *T. gondii* is the origin and composition of the PVM. In general, endocytic vacuoles are formed by one of three processes: (a) receptor-mediated endocytosis, involving clustering of ligand-receptor complexes in clathrin coated pits (30); (b) pinocytosis of fluid into small vesicles and macropinocytosis, in association with extensive membrane ruffling that is stimulated by growth hormones (29); (c) phagocytosis, through the action of multivalent receptors, and organization of the cytoskeleton (12). In some situations *T. gondii* invasion involves a phagocytic activity by the host cell (40). In most of the cases, however, invasion does not appear to be similar to any of these three processes refered to above.

The fluorescent lipophilic probe PKH26, that binds

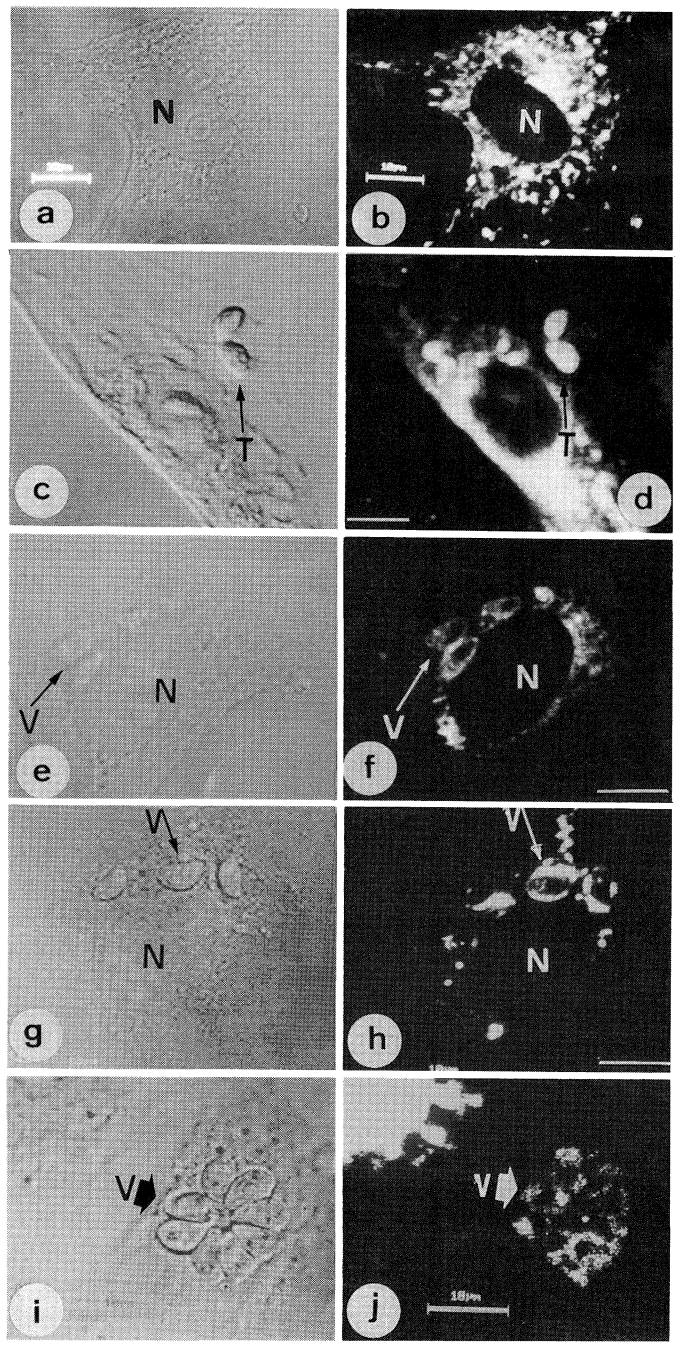

Fig. 1. Cells incubated with PKH26 only, demonstrated the cell surface labeled as shown in a and b. Analyses of interaction Vero cells-*T. gondii* after 5 minutes, demonstrated in the parasites bind on the host cell surface and labeled (c–d). After 15 and 30 minutes the PVM showed a light staining and intravacuolar parasite intense labeling (e–f), mainly at the posterior portion of the parasite. The host cell perinuclear region showed intense labeled. After 60 minutes (g–h) and 24 hours of interaction the intravacuolar parasites showed membrane labeled (i–j). N=nucleus; T=*Toxoplasma gondii*; V=vacuole. bar=10 μm.

irreversibly within cell membranes (11), was used to label the host-cell membrane and to follow the fate of lipids during PV formation in cells allowed to interact with *T. gondii*. Our present observations show clearly that during the internalization process labeled portions of the host cell plasma membrane are internalized and be come part of the PVM. In addition, later on, the intravacuolar parasites release proteins located within

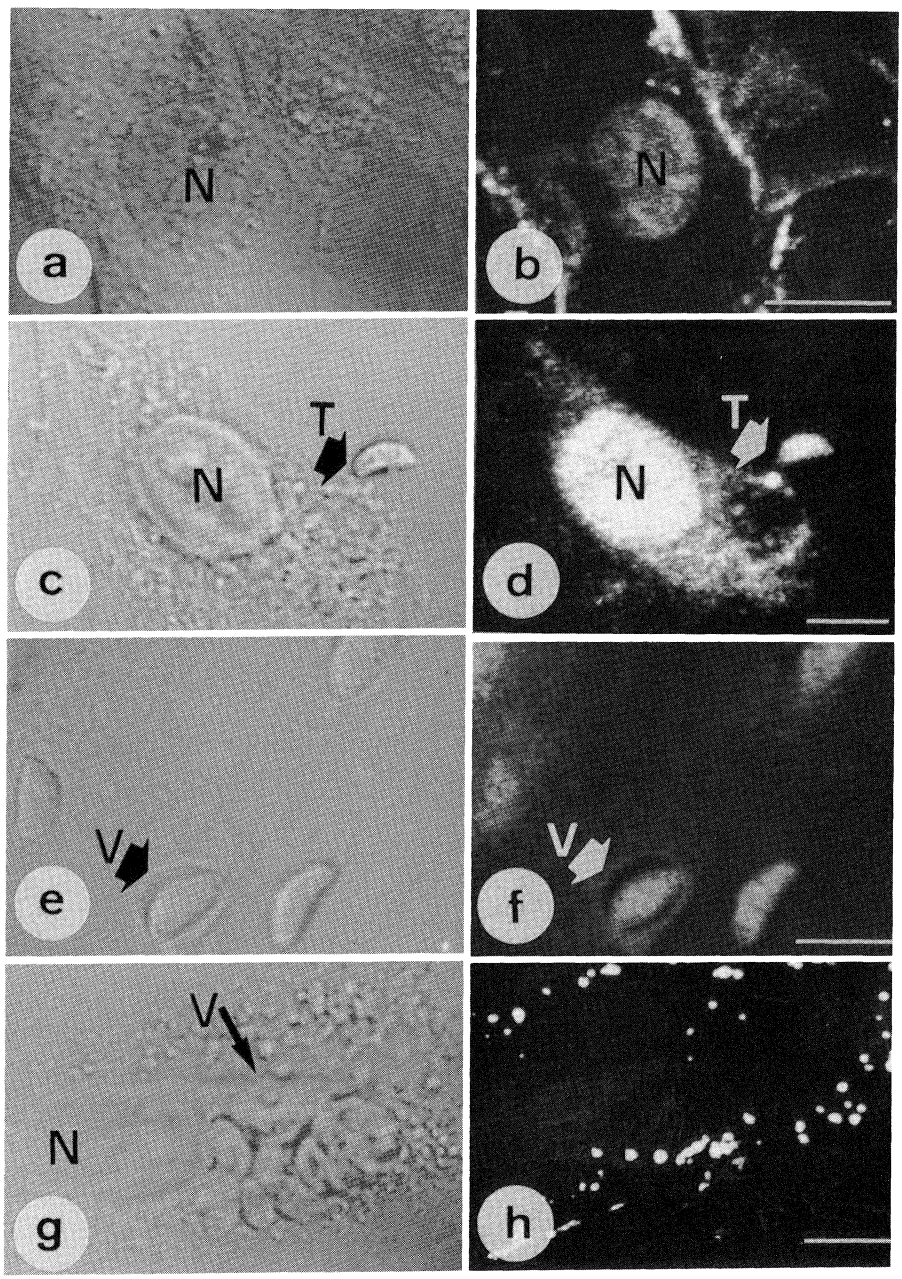

Fig. 2. Control cells showed surface labeled (a–b). After 5 minutes the interaction the parasites bind at the cellular surface present labeled (c–d). In the of the 15 and 30 minutes the PVM was slightly labeled and the surface of intravacuolar parasites intensely labeled (e–f). Host cell perinuclear region was intensely labeled with DTAF. After 60 minutes (data not show) and 24 hours (g–h) of interaction the intravacuolar parasites were not labeled and the host cell showed labeling dispersed throughout the cytoplasm. N=nucleus; T=*Toxoplasma gondii*; V=vacuole. Figs. a–b, bar=25 μm. Figs. c–h, bar=10 μm.

the dense granules, such as GRA1, GRA2 and GRA4, which remain in the vacuole (6) and are also incorporated into the intravacuolar membranous network and the PVM (25). As intravacuolar parasitism evolves the intensity of labeling decreases, possibly due to dilution of the label among the new parasites formed after division (3). Observation of malaria invasion in red blood cells labeled with fluorescent lipids clearly showed that the vacuole originated by invagination of the red cell membrane (43). Joiner and Puhreneto (1991) suggested the insertion of lipids of the parasite on PVM. Suss-Toby *et al.* (1996) reported that a small amount of par-

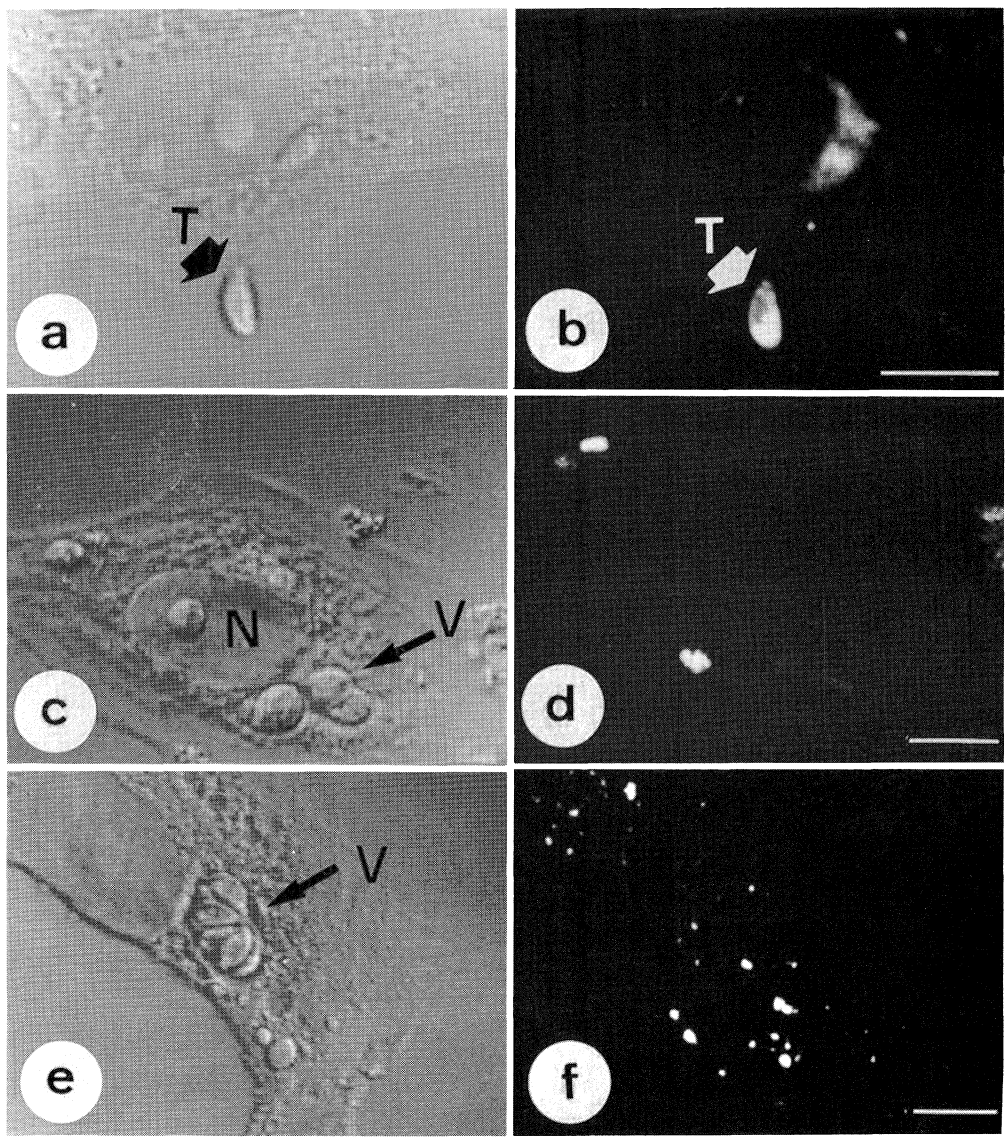

Fig. 3. Cells incubated with FITC-Thiosemicarbazide only demonstrated the surface cell labeling (data not show). In the interaction Vero cells-*T. gondii* after 15 and 30 minute (a–b), slight labeling was observed in surrounding PVM and some of the intravacuolar parasites were intensely labeled. After 60 minutes (c–d) and 24 hours of interaction (e–f) the parasites were not labeled and the host cell demonstrated slightly labeling dispersed throughout the cytoplasm. N=nucleus; T=*Toxoplasma gondii*; V=vacuole. bar=10 μm.

asite-derived material (0–18.5% of the total surface area of the PVM) may be inserted into the host cell plasma membrane. Our present observations cannot definitively rule out bulk insertion of lipids as another possible mechanism of PVM formation as previously suggested (14).

We used DTAF, which reacts with primary and secondary amines groups (39), to label membrane proteins of the Vero cell before interaction with tachyzoites in order to follow the fate of the labeled proteins during the parasite-host cell interaction process. As expected, only the Vero cells surface was labeled when incubation was carried out at 4°C. However, when these labeled cells were incubated in the presence of parasites at 37°C the labeling of the surface of attached parasites was evident. This is an interesting observation which suggests a process of transference of components of the host cell surface to the tachyzoites surface during the early steps of the interaction process.

Previous studies have shown that, following parasite attachment secretion of microneme components, the secretion of rhoptry components takes place (15, 41). Our present observations suggest that components of the

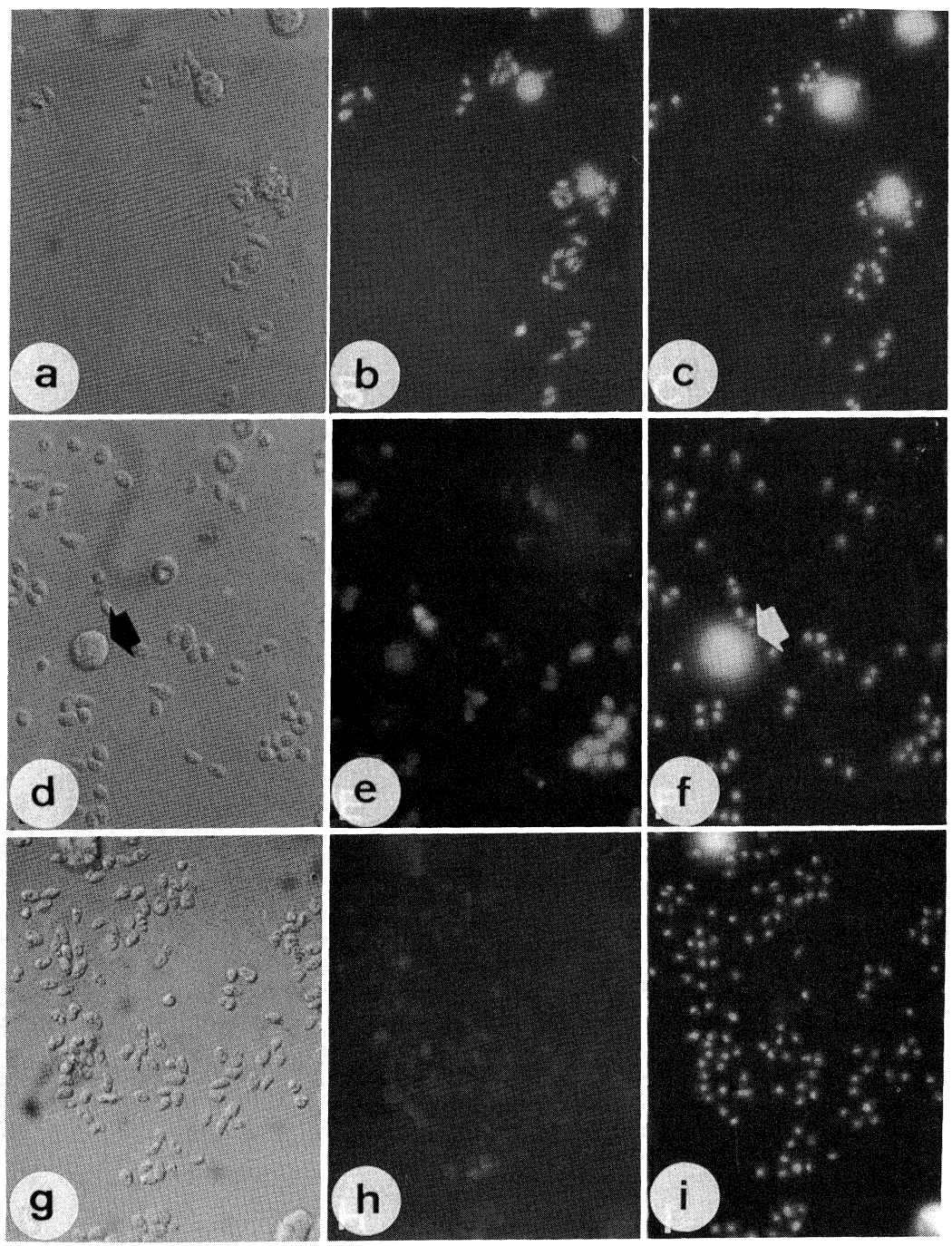

Fig. 4. The parasite suspension was incubated with PKH26, fluorescein-thiosemicarbazide, or DTAF. Labeling of the whole surface of tachyzoites was observed when they were incubated in the presence of DTAF. (a–b) interferential and fluorescence microscopy; (c) DAPI fluorescence microscopy; and PKH26; (d–e) interferential and fluorescence microscopy; (f) DAPI fluorescence microscopy. The surface of cells from the peritoneal fluid was intensely labeled (arrow). No significant labeling was observed with fluorescein-thiosemicarbazide (g–h) interferential and fluorescence microscopy and (i) DAPI fluorescence microscopy. ×1,000.

host cell may also be transferred to the parasite, indicating the complexity of the interaction process. Once the parasites were internalized labeling of the PVM and of the intravacuolar parasites was evident, indicating that at least some plasma membrane components of the host cell are internalized to be come part of the PVM.

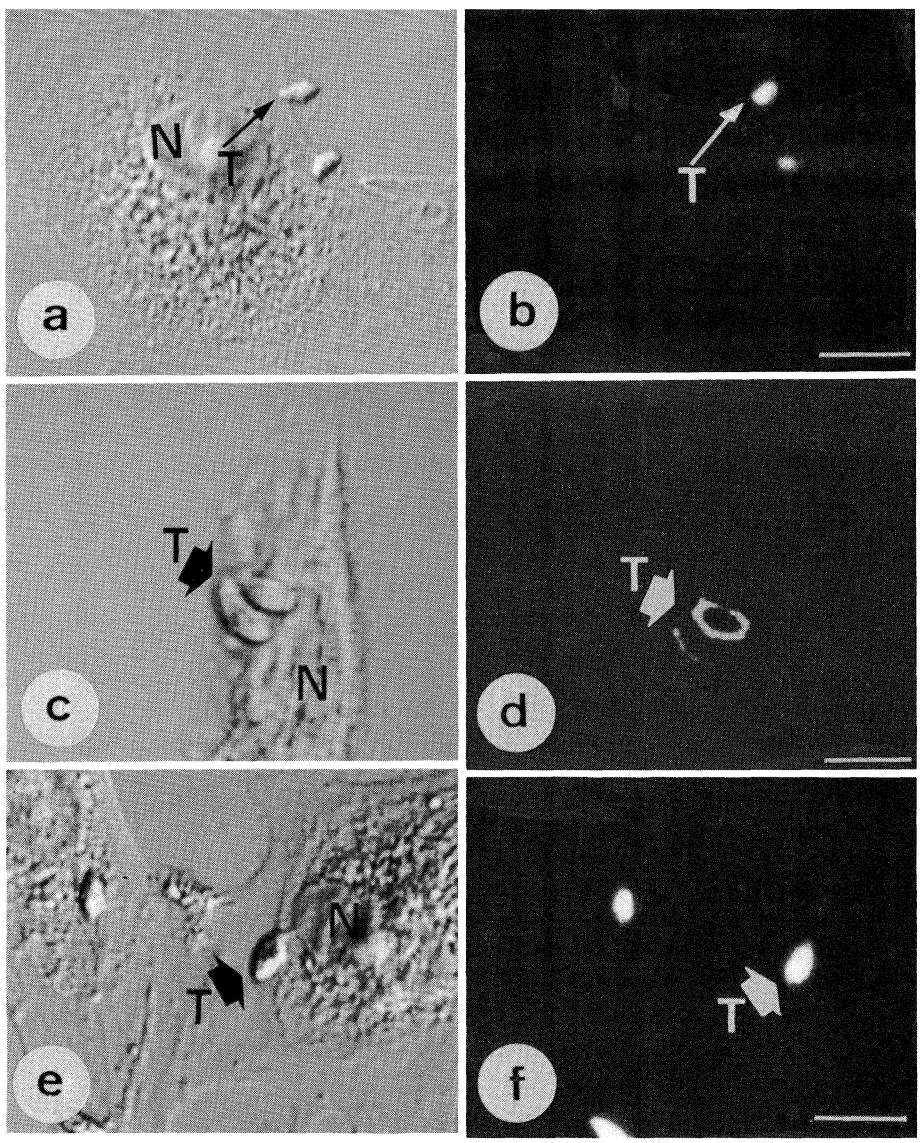

Fig. 5. Interaction after 5 minutes demonstrated only the parasites (arrow) were labeled and intracellular parasites were not labeled, with the probes: (a–b) PKH26; (c–d) thiosemicarbazide the parasites slightly labeled (arrow) and (e–f) DTAF. N=nucleus; T=*Toxoplasma gondii*. Bar=10 μm.

Previous studies using freeze-fracture have shown that the PVM of *T. gondii*-containing PV presents a lower density of intramembranous particles as compared with the host cell plasma membrane (28).

Our observations using host cell previously labeled with fluorescein-thiosemicarbazide showed intense labeling of the surface of parasites attached to the cell surface, suggesting transference of labeled molecules from the host cell to the parasite surface. However, in contrast to what was observed with the labels for proteins and lipids, no labeling of the PVM and intravacuolar parasites was observed. This observation suggests that sialoglycoconjugates exposed on the host cell surface are not internalized during the process of internalization of tachyzoites of *T. gondii*, being excluded in a not yet defined step of the process. This result was unexpected in view of previous studies that showed surface anionic sites, detected using cationizad ferritin particles, were internalized together with untreated tachyzoites and excluded when antibody-coated parasites were used (8). The observation that attached, but not internalized parasites were labeled also suggests removal of surface components of the parasite during the internalization process.

We observed that incubation of tachyzoites in the presence of PKH26, DTAF, and to a lesser extent,

FITC-thiosemicarbazide, labeled their surface. When labeled parasites were allowed to interact with host cells, intense labeling of the areas of contact between the parasite and the host cell was observed. However, neither the PVM nor the intravacuolar parasites were labeled, indicating that the surface molecules were released into the extracellular medium before the interiorization process. Previous studies have shown that malaria merozoites labeled with fluorescent fatty acids transfer fluorescent material to the parasitophorous vacuole during invasion (22). Specific proteins (5, 31) and lipids (5) are thought to be transferred from the apical organelles during invagination contributing to the formation of the PVM.

Taken together the available data suggest that following the initial contact of tachyzoites of *T. gondii* with the surface of the host cell interchange of surface components of the two interaction cells take place. This phenomenon, in association with the release of macromolecules found in the micronemes and rhoptries and the invagination of the host cell plasma membrane led to the formation of the parasitophorous vacuole membrane.

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