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Pathway of C6-NBD-Ceramide on the Host Cell Infected with *Toxoplasma gondii**

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ABSTRACT. **Fluorescence microscopy, with dyes analog of ceramide, and transmission electron microscopy, were used to analyze lipid traffic during interaction of *Toxoplasma gondii* with host cells. It is C6-NBD-Ceramide (C6-NBD-Cer), a fluorescent analog of ceramide, stained the Golgi complex where was metabolized into fluorescent shingolipid and glucosylceramide, and translocated via the Golgi complex to the plasma membrane of living cells. In uninfected cells, C6-NBD-Cer initially concentrated at the perinuclear region, and after its fluorescent products were present in the cytoplasm and the plasma membrane. In infected cells, the probe initially is stained the Golgi complex. After 4 hours incubation with C6-NBD-Cer, the parasites within the parasitophorous vacuole began to be stained, and at 5 hours incubation, the parasites are completely fluorecent. The Golgi complex, as revealed by fluorescent probe and electron microscopy, maintained its perinuclear position throughout the evolution of intracellular parasitism. These results suggested that the intracellular parasite used the lipid pathway of the host cell.**

Toxoplasma gondii is a parasitic protozoon able to infect a wide range of nucleated cells of vertebrates (18). Following penetration into the cells, tachyzoites of *T. gondii* localize within a modified endocytic vacuole, known as the parasitophorous vacuole, where they multiply until they achieve the complete destruction of the host cell. During the process of invasion and intracellular multiplication the tachyzoites secrete a large number of proteins into this vacuole (1, 6). Some of these proteins associate with the parasitophorous vacuole membrane (1, 12) while others remain free within the vacuole (1). Previous studies have shown that changes occur in the distribution of cytoplasmic structures of the host cell during the evolution of intracellular parasitism (5, 7, 16). It has been reported that host cell mitochondria accumulate around the parasitophorous vacuole (16, 28). Some elements of the endoplasmic reticulum were seen in close contact with the PV membrane (16). However, little is known about the interaction of components of the host cell Golgi complex with the parasitophorous vacuole.

In the last years new fluorescent probes have been developed which allow the analysis of the distribution and function of important cytoplasmic structures in living cells by fluorescence microscopy (10, 15, 19, 21, 24). Among these probes C6-NBD-Ceramide (C6-NBD-Cer) has been widely used, since observations show that it initially concentrates in the Golgi complex. Later on, their metabolized products, which still are fluorescent, can be incorporated in others structures, allowing the examination of lipid traffic between cell organelles. The NBD-derivated fatty acids exhibit two important properties: they are intensely fluorescent, and they are slightly water soluble and have a high hydrophobic partition coefficient that essentially allows them to be inserted into cell membranes (24). C6-NBD-Cer provided the first cytological evidence that sphingomyelin and glycosphingolipid synthesis was associated with the Golgi complex (13, 20, 21). These studies indicated that this analogue can reach the Golgi complex when exogenously added, although the relevance of this to its natural counterpart is unclear. Once reaching the Golgi, C6-NBD-Cer is converted into C6-NBD-GlcCer and C6-NBD-sphingomyelin (2).

We decided to use C6-NBD-Cer to analyze its fate in

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cells infected with *T. gondii* as an approach to study further the process of host cell-parasite relationship. The results obtained are described in this paper.

MATERIALS AND METHODS

Parasites. Tachyzoites from a virulent RH strain of *Toxoplasma gondii* were maintained by intraperitoneal passages in Swiss mice and were collected in Ringer's solution at pH 7.2, 48–72 h 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 phosphate-buffered saline solution (PBS), pH 7.2, and ressuspended to a density of 10^6 parasites/ml in 199 medium without fetal calf serum. 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 cell. Vero cells (kidney fibroblast of African green monkey) were maintained in Falcon plastic flasks using 199 medium with 4% fetal calf serum and passed 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 ($3–5 \times 10^5$/flask) and maintained at 37°C overnight in 5% CO_2.

Host cell parasite interaction. Parasites suspended in 199 medium were incubated for 1 h in the presence of Vero cells using a 5 : 1 parasite-host cell ratio. After incubation, the cells were washed twice with PBS to remove extracellular parasites and incubated for periods varying from 24 to 48 hours at 37°C, and processed for fluorescence or electron microscopy, as described below.

Fluorescence Microscopy. For visualization of the Golgi complex the purified laser dye C6-NBD-Ceramide (Molecular Probes Inc., USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml and subsequently diluted to 1.4 mg/ml in 199 medium containing 1.4 mM bovine serum albumin (BSA, SIGMA). Control or infected cells grown on 12 mm-diameter glass coverslips were incubated with C6-NBD-Ceramide for 30 min to 5 hours at 37°C. Coverslips were then rinsed three to five times with 199 medium and mounted in a glass slide with 199 medium supplemented with 4% fetal calf serum, and were examined by epifluorescent illumination either in a Zeiss Axioplan microscopy equipped with a Zeiss planopochromat objective lens (63×) or a Confocal Laser Scan Microscope (CLSM), using a 488 nm argon laser. Photographs were taken on Tri-X (ASA 400 or 100) film using the automatic exposure control of the Zeiss camera to these microscopes. Tri-X film was developed for 5 min in Kodak HC 110 (dilution B).

Electron Microscopy. Vero cells were plated in culture flasks, cultivated as described above and allowed to interact with the parasites. After interaction the cultures were washed with PBS and fixed for 1 h at room temperature in a solution containing 1% glutaraldehyde, 4% paraformaldehyde, 5 mM $CaCl_2$ and 5% sucrose in 0.1 M cacodylate buffer, pH 7.2. They were then washed with cacodylate buffer with 5% sucrose and postfixed for 1 h in a solution containing 1% OsO_4, 0.8% potassium ferrocyanide and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2. The cells were rinsed with cacodylate buffer, dehydrated in acetone and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate, and observed with a Zeiss 902 Electron Microscope.

RESULTS

C6-NBD-Ceramide stain. C6-NBD-Cer was administrated to Vero cells as a complex with bovine serum albumin (C6-NBD-Cer-BSA). Examination of living cells by fluorescence microscopy after exposure to complex C6-NBD-Cer-BSA revealed intense labeling at the perinuclear region, where the Golgi complex is located, as described by Pagano et al. (1989). The Golgi complex stained with C6-NBD-Cer appeared as a bright compact structure (Fig. 1). When Vero cells were incubated for 2, 3, 4 and 5 hours at 37°C, the staining pattern was not modified (not shown), but in some cells a more diffuse labeling pattern was observed (Fig. 2). In infected cells incubated for 2 and 3 hours with C6-NBD-Cer the labeling pattern was not modified, and the Golgi complex appeared as a compact structure located at the side of the nucleus. After 3 hours incubation a diffuse fluorescence was observed thoughout the cytoplasm of the host cell (Figs. 2–3). Even after incubation for 4 hours no fluorescence was seen in the parasitophorous vacuole (PV). However, those parasites which remained outside the cells, were intensely labeled (Figs. 5, 6). After 3 hours of incubation with C6-NBD-Cer, in addition to a diffuse cytoplasm labeling, intense labeling was seen in regions close to the parasitophorous vacuole found in heavily infected cells (Figs. 5–8). In some cells, a light staining of the PV membrane and intravacuolar parasites was evident (Figs. 9, 10). Intense labeling of intravacuolar parasites was observed only after 5 hours of incubation time with C6-NBD-Cer, (Fig. 10). This labeling was more intense in the anterior, perinuclear region of the tachyzoites, although diffuse labeling throughout the cell, except the nucleus, was evident (Fig. 10).

Electron microscopy. Transmission electron microscopy of thin sections of infected cells showed the presence of stacks of Golgi complex, as well as isolated tubular structures localized near the PV (Fig. 11).

DISCUSSION

Previous studies have shown that C6-NBD-Cer clear-

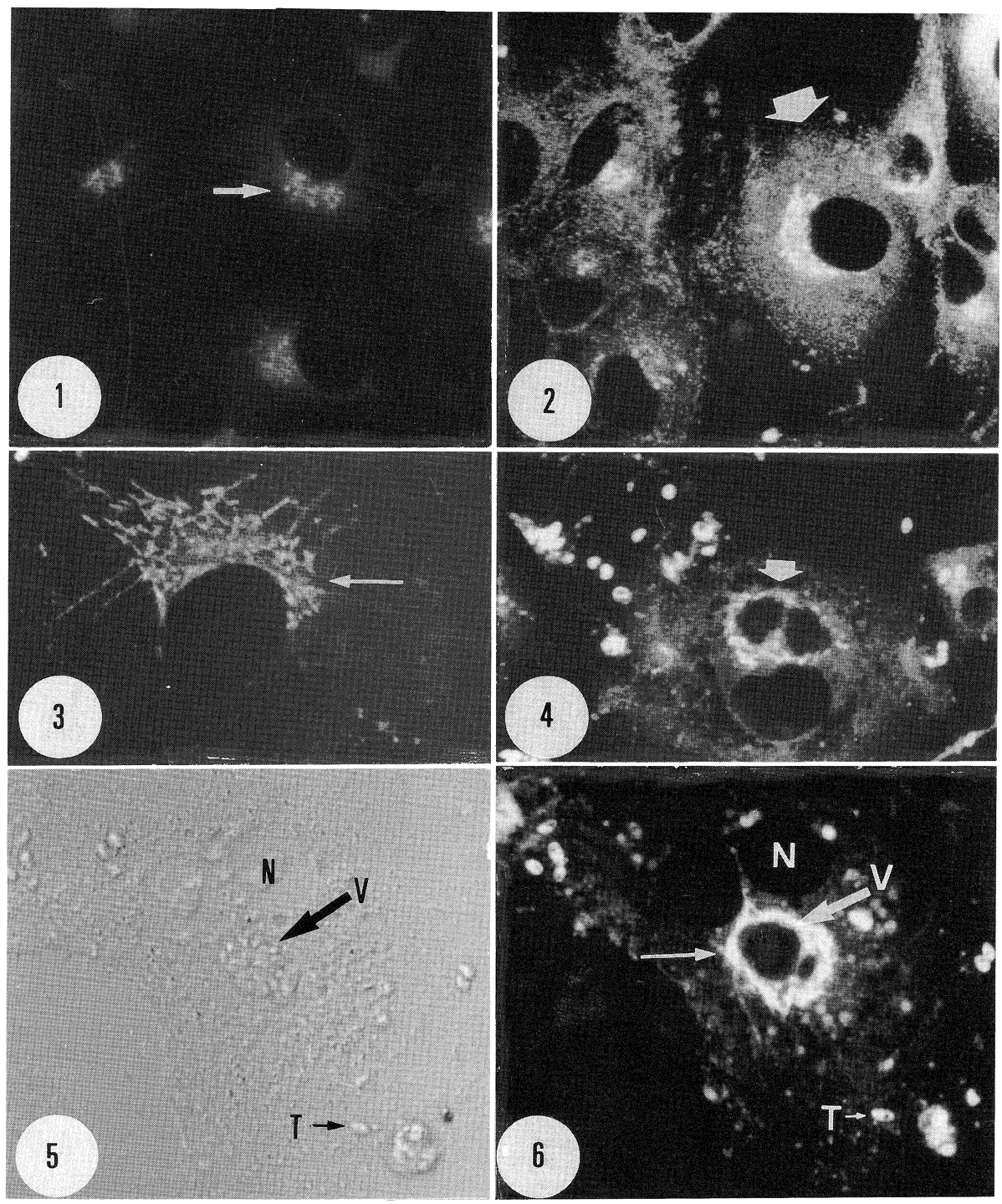

Fig. 1–6. Living Vero cells labeled with C6-NBD-Cer and examined by fluorescence microscopy. Fig. 1, The Golgi complex stained with C6-NBD-Cer appears as a bright compact structure (arrow). Fig. 2 and 3, Uninfected cells show a diffuse fluorescence from C6-NBD-Cer incubated for 3–4 hours (arrows). Infected cells incubated for 3 (Fig. 4) and 4 hours (Fig. 6) with C6-NBD-Cer. Fig. 5 and 6 are interferential and fluorescence microscopy, respectively, of the same cell. Fig. 1: ×370; Fig. 2: ×450; Fig. 3: ×340; Fig. 4: ×350; Figs. 5, 6: ×400. N=nucleus; T=*Toxoplasma gondii*; V=parasitophorous vacuole.

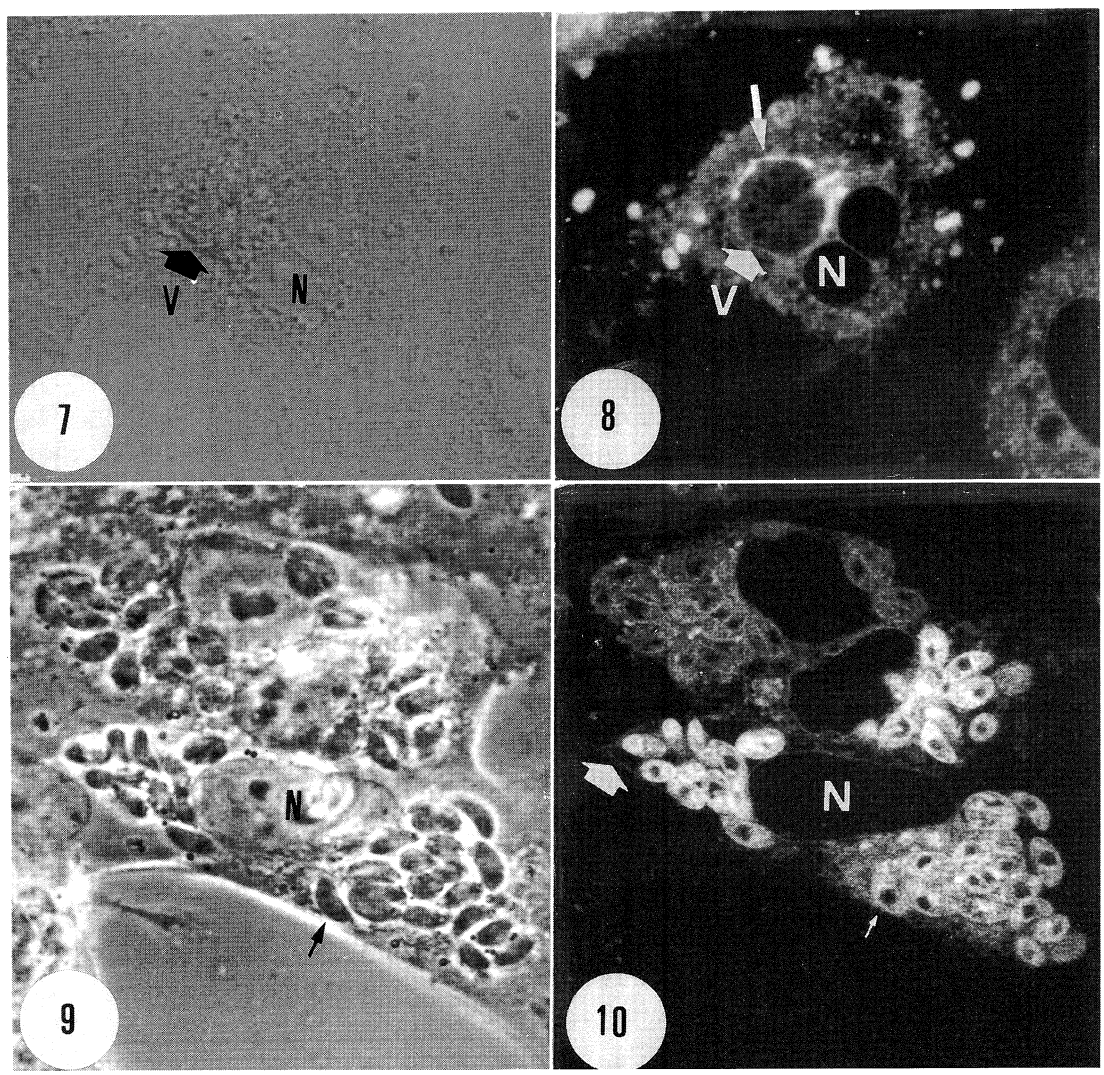

Fig. 7–10. Vero cells infected by tachyzoites of *T. gondii*, and labeled for 4 (Fig. 8) and 5 (Fig. 10) hours with C6-NBD-Cer. Fluorescence is initially concentrated around the parasitophorous vacuole (Fig. 8), and later on the parasitophorous vacuole (Fig. 10). Tachyzoites within parasitophorous vacuole are stained after 5 hours of incubation from C6-NBD-Cer. Fig. 7 and 8 show the same field of infected cells by interferencial and fluorescence microscopy. Fig. 9 and 10 also show the same field of infected cells by contrast and fluorescence microscopy. Figs. 7, 8: ×300; Figs. 9, 10: ×370

ly concentrates in the perinuclear region, where the elements which form the Golgi complex are localized. Rosenwald and Pagano (1993) suggested that this labeling distribution pattern is the result of a series of steps: (1) monomer transfer of the fluorescent lipid from an appropriate donor (Cer-BSA complex) to the plasma membrane of the recipient cell; (2) transbilayer movement from the outer to the inner leaflet to the plasma membrane; (3) spontaneous transfer of the fluorescent lipid to intracellular membranes, and (4) concentration of the lipid at the Golgi complex. C6-NBD-Cer is metabolized to both C6-NBD-Glyceroceramide and C6-NBD-Sphingomyelin at the Golgi complex in cultured cells (2, 24). Studies at the electron microscopic (EM) level using C6-NBD-Cer and C5-DMB-Cer have shown that these lipids preferentially label a subset of Golgi compartments, most likely corresponding to the trans-Golgi stacks (14). Lipids may move between intracellular membranes by one of a variety of mechanisms: diffusion of lipid monomers, either freely in solution or mediated by transport proteins; vesicular movement between compartments; or lateral diffusion between membranes through intermembrane bridges (14).

Toxoplasma gondii belongs to a group of virulent intracellular parasites that reside and multiply within a host cell cytoplasm vacuole which does not fuse with lysosomes. The membrane which lines the parasitophorous vacuole is formed during host cell invasion by compo-

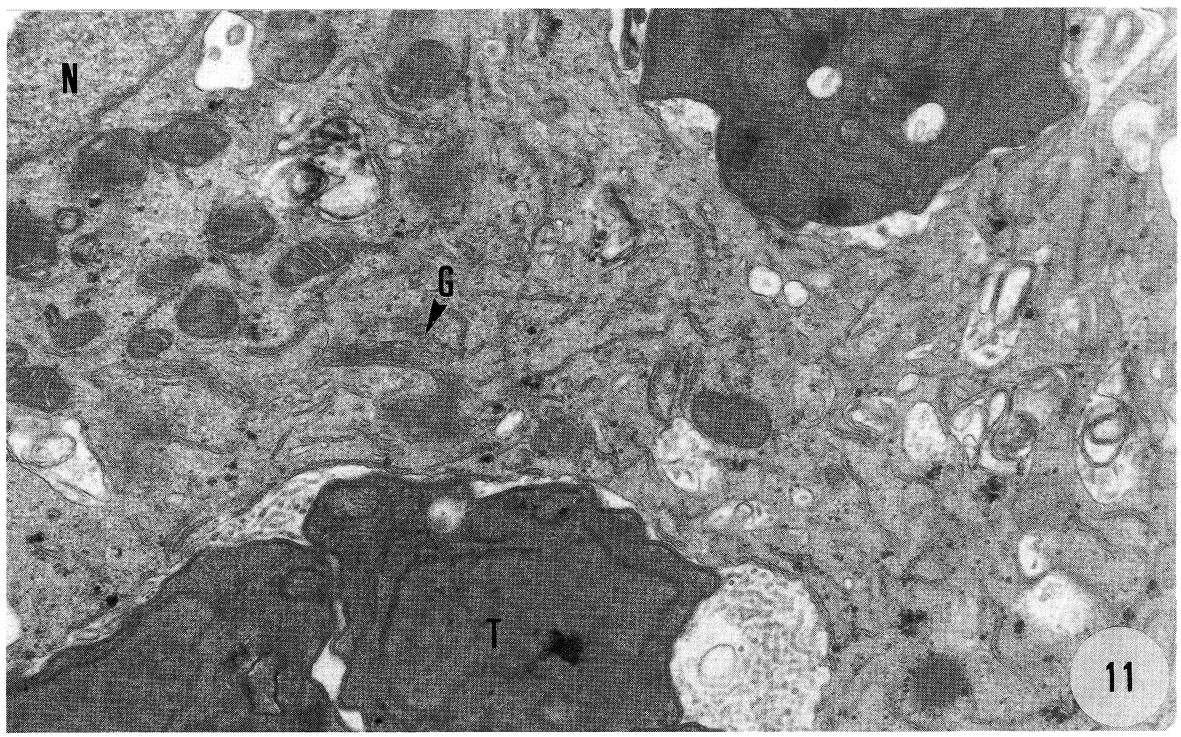

Fig. 11. Transmission electron microscopy of thin sections of Vero cells infected by *T. gondii*. After 24 hours of interaction time, the Golgi complex (G) is located near the parasitophorous vacuole and the nucleus (N). ×21,000

nents originated from both the parasites and the host cell membrane (3, 4, 23, 26). Concomitant with parasite division there is a clear increase in the size of the parasitophorous vacuole and the surface area of the membrane lining it. Nothing is known about the mechanism(s) involved in this process. Previous studies have provided evidence that interchange of metabolic products takes place between the parasite and the host cell, with the parasitophorous vacuole membrane acting as interplayer. For instance Pfefferkorn and Schwartzmann (1981) demonstrated that *T. gondii* incorporated pyrimidines and purines from the host cell. It has also been shown that the mitochondria from the host cell concentrated around the parasitophorous vacuole. This fact has been interpreted as an indication of metabolic cooperation between the two cells (16, 28).

Our present observations following the fate of C6-NBD-Cer by fluorescence microscopy show that as occurs in normal mammalian cells it initially concentrates in the Golgi complex of *T. gondii*-infected cells. Later on, however, the labeled metabolic products follow a distinct route. In untreated cells labelling of the cell surface is observed (21). In infected cells, however, we could initially observe labelling of structures near and in between the various parasitophorous vacuoles and later on (after 5 hours), labeling of the membrane lining the vacuoles and the intravacuolar parasites. These observations suggest that tachyzoites of *T. gondii* incorporate lipids transfered from the host cell. At present we do not known how the fluorescent products reach the parasitophorous vacuole. Recent studies have shown that the membrane lining the vacuole act as a molecular sieve and that molecules up to 1900 kDa can cross it from the host cell cytoplasm to the vacuole (8, 27).

The observation of labeling of the membrane lining the parasitophorous vacuole is interesting and may explain the process of increase on the size of the vacuole during the evolution of the intracellular parasitism. The growth of parasitophorous vacuole containing parasites such as *Leishmania* has been explained by the incorporation of new membrane components during fusion of lysosomes with the vacuole (8, 11). In the case of *T. gondii*, however, lysosomes do not fuse with the vacuoles (9, 24, 26).

It is important to point out that tachyzoites of *T. gondii* also incorporate C6-NBD-Cer. In the case of free extracellular parasites, labeling was intense and observed even with short incubation time. In the case of intracellular parasites labeling was observed only after 5 hours, which is probably the time required for the intracellular degradation of C6-NBD-Cer in to its products, and distribution of these products into the vacuole.

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