Toxoplasma gondii Exploits Host Low-Density Lipoprotein Receptor-mediated Endocytosis for Cholesterol Acquisition

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Abstract. The obligate intracellular protozoan *Toxoplasma gondii* resides within a specialized parasitophorous vacuole (PV), isolated from host vesicular traffic. In this study, the origin of parasite cholesterol was investigated. *T. gondii* cannot synthesize sterols via the mevalonate pathway. Host cholesterol biosynthesis remains unchanged after infection and a blockade in host de novo sterol biosynthesis does not affect parasite growth. However, simultaneous limitation of exogenous and endogenous sources of cholesterol from the host cell strongly reduces parasite replication and parasite growth is stimulated by exogenously supplied cholesterol. Intracellular parasites acquire host cholesterol that is endocytosed by the low-density lipoprotein (LDL) pathway, a process that is specifically increased in infected cells. Interference with LDL endocytosis, with lysosomal degradation of LDL, or with cholesterol translocation from lysosomes blocks cholesterol delivery to the PV and significantly reduces parasite replication. Similarly, incubation of *T. gondii* in mutant cells defective in mobilization of cholesterol from lysosomes leads to a decrease of parasite cholesterol content and proliferation. This cholesterol trafficking to the PV is independent of the pathways involving the host Golgi or endoplasmic reticulum. Despite being segregated from the endocytic machinery of the host cell, the *T. gondii* vacuole actively accumulates LDL-derived cholesterol that has transited through host lysosomes.

Key words: *Toxoplasma gondii* • parasitophorous vacuole • somatic cell mutant • LDL endocytic pathway • cholesterol transport

Introduction

Upon entering a host cell, many intracellular pathogens reside within membrane-bound vacuoles. Successful intracellular parasitism is dependent on the pathogen-driven control of the biogenesis and maturation of the vacuole, allowing the establishment of a replication-permissive niche. The obligate intracellular protozoan *Toxoplasma gondii* resides in a specialized parasitophorous vacuole (PV)[1] that neither acidifies nor fuses with organelles of the endocytic cascade and exocytic pathway and, as such, is totally isolated from the host cell vesicular transport system (Jones et al., 1972; Sibley et al., 1985; Joiner et al., 1990; Mordue et al., 1999). This parasite is auxotrophic for several metabolites (see review by Sinai and Joiner, 1997) and must exchange nutrients across the PV membrane (PVM), surrounding it to assure its survival and propagation. This raises the intriguing issue of how nutrients are obtained from the host cell by *T. gondii*.

Small soluble molecules of <1,400 D are able to cross the PVM through functional pores (Schwab et al., 1994). Although devoid of transmembrane transporter/receptors of host cell origin (Porchet-Hennere and Torpier, 1983), the PVM contains numerous secreted parasite proteins that might be implicated in metabolite transport (Sinai and Joiner, 1997; Lingelbach and Joiner, 1998). Within the vacuolar space is a tubulo-reticular network connected to the PVM, which likely increases the exchange surface between the host cytoplasm and intravacuolar parasites (Sibley et al., 1995).

Of importance, the PVM of *T. gondii* is tightly enshrouded by host mitochondria and endoplasmic reticulum (ER), the host cell lipid biosynthetic apparatus (Jones et al., 1972; Melo et al., 1992; Lindsay et al., 1993; Sinai et

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[1]*Abbreviations used in this paper:* CO, cholesteryl oleate; HFF, human foreskin fibroblast; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoprotein; LP, total lipoproteins; LPDS, LP-deficient serum; NBD-C, 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)23,24-bisnor-5-cholen-3β)-ol; NPC, Niemann-Pick type C; PV, parasitophorous vacuole; PVM, PV membrane; SRD cells, mutant CHO cells with a sterol regulatory defective phenotype; SSD cells, mutant CHO cells with a squalene synthase deficiency; [^{3}H-CO]-LDL, LDL that is labeled with [^{3}H-CO]; [NBD-C]-LDL, LDL that is labeled with [NBD-C].

al., 1997). This organelle association has been postulated to play a role in lipid and possibly membrane scavenging from these host organelles to the intravacuolar parasite at sites of PVM-organelle association (Sinai et al., 1997). Indeed, *T. gondii* seems to be deficient in its ability to synthesize selected phospholipids de novo (Sinai, A.P., K.A. Joiner, and D.R. Voelker, unpublished observations).

Toxoplasma membranes contain cholesterol based on both biochemical and morphological criteria (Monteiro Cintra and de Souza, 1985; Gallois et al., 1988; Foussard et al., 1991a, 1991b). Cholesterol is concentrated in rhoptries, apical secretory organelles implicated in the extension of the PVM during invasion. Indeed, these organelles have a very high cholesterol/phospholipid molar ratio of 1.5 (Foussard et al., 1991a). In higher eukaryotic cells, cholesterol homeostasis is finely regulated by transcriptional, translational, and posttranslational mechanisms (reviewed in Goldstein and Brown, 1990; Brown and Goldstein, 1999). Cells have a number of options when it comes to the use of cholesterol for membrane biogenesis or synthesis of new molecules derived from cholesterol. This latter is synthesized in the ER via the key enzyme of the mevalonate pathway, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Newly synthesized cholesterol is transported rapidly to the caveolae domains of the plasma membrane from where it constitutively cycles with the cell interior. Another important source of cholesterol is plasma low-density lipoprotein particles (LDL) that are internalized by specific receptors and delivered to late endosomes/lysosomes for hydrolysis. When cholesterol is effluxed from lysosomes, the bulk of cholesterol is transported to the plasma membrane probably by a Golgi-dependent pathway involving caveolae, while a portion is delivered to the ER by vesicular transport. Deposition of excess cellular cholesterol in the form of cholesteryl esters is catalyzed by the resident ER acyl-CoA:cholesterol acyltransferase (ACAT), leading to the biogenesis of lipid droplets (reviewed in Lange and Steck, 1996; Liscum and Munn, 1999).

Upon infection with *T. gondii*, the mammalian cell acquires a novel dynamic compartment, the PV, which contains live, dividing microorganisms. We have addressed the following questions, relating to the origin of cholesterol for the parasite: Can *T. gondii* synthesize its own cholesterol via the classical mevalonate pathway? Is the PV accessible to host cell cholesterol? If accessible, is it the cholesterol synthesized by the host cell or the exogenous cholesterol delivered by LDL endocytosis that can be transported into the parasite? If acquired exogenously from LDL, is cholesterol transported from lysosomes to the PV by a direct transfer, a Golgi-, or an ER-dependent pathway? Is the host cell altered in its cholesterol biosynthesis or LDL uptake in response to parasitization? Is the parasite capable of replication in host cells unable either to synthesize cholesterol de novo, or to use LDL-delivered cholesterol, or both?

Although the *Toxoplasma* PV remains segregated from vesicular trafficking through the endo- and exocytotic pathways in the host cell, the results presented here demonstrate that this parasite can actively intercept host LDL-derived cholesterol, in transit from the lysosomes to other cellular compartments.

Materials and Methods

Chemicals and Antibody

All chemicals were obtained from either Sigma Chemical Co. or Boehringer Mannheim Biochemicals, unless indicated otherwise. Analytical grade solvents were used for lipid analysis. The nitrobenzoxadiazole-cholesterol (NBD-C) was obtained from Molecular Probes, Inc. and stored at −20°C as a 10-mg/100-μl solution in dimethylformamide. U18666A was from Biomol. Compactin was kindly provided by Dr. Akira Endo (Noko University, Tokyo, Japan). The SDZ 215-918 cyclosporin A analogue was provided by Sandoz Pharma, Ltd. Radiolabeling reagents, purchased from Amersham Corp., included [1α,2α(n)-^{3}H]cholesteryl oleate (sp act, 43 Ci/mmol), 3-hydroxy-3-methyl[3-^{14}C]glutaryl coenzyme A (sp act, 61 mCi/mmol), RS-[2-^{14}C]mevalonic acid lactone (sp act, 58 mCi/mmol), [5,6-^{3}H]uracil (sp act, 45 Ci/mmol), and [methyl-^{3}H]thymidine (sp act, 6.7 Ci/mmol). Human [^{125}I]-diferric transferrin was from DuPont NEN Research Products. The monoclonal anti–low-density lipoprotein receptor antibody was obtained from Amersham Corp.

Preparation of LDL, Lipoprotein-deficient Serum, LDL Labeled with NBD-C, or with [^{3}H]cholesteryl oleate–LDL

Human LDL (density 1.019–1.063 g/ml) was isolated from fresh plasma by zonal density gradient ultracentrifugation as described (Poumay and Ronveaux-Dupal, 1984). Lipoprotein-deficient serum (LPDS) was prepared by ultracentrifugation of fetal bovine serum (Gemini Bio-Products, Inc.) after the density was increased to 1.215 g/ml with KBr (Havel et al., 1955). Total lipoproteins (LP) contained in the supernatant were used to reconstitute LPDS. The fluorescent lipid NBD-C was incorporated into LDL by mixing 50 μl of the lipid stock solution with 20 ml of filtered fresh human plasma. After incubation for 16 h at 37°C, LDL was isolated as described above. The association of the NBD-C to the LDL fraction was estimated by the specific uptake of the fluorescent LDL by microscopy. The fluorescence observed after uptake of labeled LDL in the presence of a 40-fold excess of nonlabeled LDL was negligible. Radiolabeling of LDL with [^{3}H]cholesteryl oleate ([^{3}H-CO]) was previously described (Coppens et al., 1995).

Cell Lines and Culture Conditions

The cell lines used in this study included: Chinese hamster ovary cells, primary human foreskin fibroblasts (HFF), and African green monkey (Vero) cells. Somatic mutants of CHO cells were generous gifts from Drs. M. Brown, J. Goldstein, and A. Nohturftt of University of Texas S.W. Medical Center (Dallas, TX): CHO-7 cells (Metherall et al., 1989), UT-1 cells (Luskey et al., 1983), and a sterol regulatory-defective (SRD) mutant, SRD-1 cells (Metherall et al., 1989). Dr. L. Liscum (Tufts University School of Medicine, Medford, MA) kindly provided the 2-2 mutant (Dahl et al., 1992, 1993) and Dr. R. Simoni (Stanford University, Stanford, CA) generously gave a squalene synthase-deficient (SSD) mutant (Bradfute et al., 1992). All cell lines were grown as monolayers at 37°C in an atmosphere of 5% CO_2 in α-minimum essential medium (prepared at the Media Core Facility of the Department of Cell Biology, Yale University), supplemented with 2 mM L-glutamine and penicillin/streptomycin (100 U/ml per 100 μg/ml). This culture medium also contained variable % (vol/vol) of either FBS or LPDS that was reconstituted (or not) with LP, as indicated in each experiment. Specific requirements added in media for the mutants of CHO cells were: 40 μM compactin for the UT-1 cells, 1 μg/ml of 25-hydroxycholesterol for the SRD-1 cells, 0.2 mg/ml of geneticin for the SSD cells.

Parasite Culture and Purification

The RH strain tachyzoite of *Toxoplasma gondii* was used throughout this study, and was maintained by passage in the peritoneum of Swiss-Webster mice or by in vitro culture in Vero cells or HFF, as previously described (Roos et al., 1994). A purification scheme of intracellular parasites based on density gradient separation using Nycodenz and isopycnic centrifugation was developed. Confluent HFF or CHO cell monolayers were infected with *T. gondii*, which were further harvested from the culture supernatants. After two passages through a 27-gauge needle to disrupt any contaminating host cells, parasites were washed three times in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, adjusted to

pH 7.4) by centrifugation at 1,000 *g* in a GRP tabletop centrifuge (Beckman Instruments, Inc.) for 10 min. Parasites were then resuspended in 10% Nycodenz before application at the top of a linear Nycodenz gradient from 30 to 10%. After centrifugation in a swing-out rotor at 2,000 *g* for 30 min at room temperature, fractions were collected and analyzed for density and protein content. Fractions containing parasites were equilibrated at a density of 1.09–1.11 g/ml of Nycodenz. They were then diluted 2.5-fold with PBS, centrifuged at 5,000 *g* for 30 min, and washed three times in PBS before use for lipid extraction. The purity of the parasite preparation was monitored by the comparison of the protein content in the 1.09–1.11-g/ml fractions after inoculation of the same number of parasites in either one or three dishes of cell monolayers. When parasites were purified from three dishes, no increase in protein concentration was observed in the parasite fractions, as compared with parasites from one dish.

Determination of Parasite Concentration, Replication, and Viability

Parasite concentration was determined by numeration using a Hausser counting chamber at 400× magnification. The analysis of the number of parasites per vacuole to estimate the parasite replication and the measurement of [^{3}H]uracil incorporated into the parasites to evaluate their viability were determined as previously described (Nakaar et al., 1999). Parasite cultures were synchronized by removal of parasites that had not yet invaded 4 h after their inoculation into confluent cells.

Ligand Radiolabeling and Uptake Experiments

LDL was radiolabeled with ^{125}I by means of ICl (McFarlane, 1958) and specific radioactivity of ^{125}I-LDL was 300–550 cpm/ng protein. ^{125}I-transferrin was used at a specific radioactivity of 100–200 cpm/ng protein. For uptake experiments, confluent cultures of CHO cells in a six-well plate were inoculated with 10^6 freshly lysed-out parasites and incubated for 4 h at 37°C before washing and resuspension in culture medium containing either 10% LPDS (for LDL uptake experiments) or 10% FBS (for transferrin and horseradish peroxidase uptake experiments). The presence or absence of LP during the 24-h incubation is without influence on the subsequent transferrin or HRP uptake. 24 h post-infection, cultures were washed twice with PBS, incubated at 37°C for 2 h in culture medium (devoid of serum) containing 1% of serum albumin and different concentrations of ^{125}I-LDL, ^{125}I-transferrin, or HRP, and then chilled to 4°C and washed twice in PBS plus 5% FBS. Cell monolayers were further incubated for 60 min at 4°C in the presence of 0.1% pronase (wt/vol) to remove cell surface–bound ligand, washed twice in PBS and lysed in 0.01% Triton X-100. The pronase-resistant fraction was considered as internalized ligand. For comparison, noninfected CHO cells were used as controls, and processed identically. Cell-internalized radioiodinated ligand in the lysate was evaluated in a liquid scintillation system (LS 6000SC; Beckman Instruments, Inc.) and cell-internalized HRP was determined by measurement of the peroxidase activity in the lysate by the stopped colorimetric assay using ortho-phenylenediamine as a substrate, according to Steinman et al. (1976). Values of cell-internalized ligand in infected and uninfected cells were normalized to total cell protein.

Incubation of Infected Cells with [^{3}H-CO]-LDL and Lipid Analysis

Synchronized infected cells were incubated in culture medium containing 10% FBS or 10% LPDS. After 24 h, cells were treated with the indicated inhibitor, and then pulse-labeled with 5 mg of [^{3}H-CO]-LDL. After washing, parasites were isolated, their protein concentration determined, and lipids extracted to quantify the amount of [^{3}H-C] associated with the sterol fraction, as described (Coppens et al., 1995). Results were calculated as counts per minute per milligram cell protein, and then expressed in percent control values corresponding to the amount of [^{3}H-C] associated with the sterol fraction of infected cells that were incubated in the absence of any inhibitor.

Incubation of Infected Cells with LDL Labeled with NBD-C, Filipin, Lipid Droplet Dyes, and Fluorescence Microscopy

To visualize fluorescent cholesterol associated with *T. gondii*, synchronized infected cells were seeded on coverslips in a 24-well plate and incubated in culture medium containing 10% LPDS. After 24 h, cells were treated with the indicated inhibitor, and then labeled with 0.1 mg of [NBC-C]-LDL for various pulse-chase times. Coverslips with live cells were directly observed with an epifluorescence microscope (Microphot FXA; Nikon Inc.). Images were captured with a CCD camera (Photometrics), processed with Image-Pro Plus (Media Cybernetics), and their contrast was enhanced with Adobe Photoshop 5.0 (Adobe Systems Inc.). For cytochemical staining of β-hydroxysterols with filipin, infected cells seeded on coverslips were incubated in culture medium containing 10% FBS, washed in PBS, and then fixed in 3% paraformaldehyde for 30 min at room temperature and washed again with PBS. Coverslips with fixed cells were incubated with 25 μg/ml of filipin for 15 min, washed in PBS, and mounted with glycerol to be viewed by a fluorescence microscope using an excitation filter of 350–410 nm. For detection of parasite lipid droplets, extracellular *T. gondii* from cell cultures incubated in culture medium plus 10% FBS or 10% LPDS were fixed in paraformaldehyde and stained with either Oil Red O or Nile Red before observation by phase-contrast and fluorescence microscopy, as described (Greenspan et al., 1985; El-Jack et al., 1999).

Assay for HMG-CoA Reductase Activity

Confluent cultures of CHO cells in 10-cm plates were inoculated with 10^7 freshly lysed-out parasites and incubated for 4 h at 37°C before washing and resuspension in culture medium containing either 10% FBS or 10% LPDS. After 24 h of infection, the activity of HMG-CoA reductase was measured in situ on digitonin-permeabilized cells using [^{14}C]HMG-CoA as substrate, as described (Leonard and Chen, 1987; Geelen et al., 1991). In parallel, noninfected CHO cells were used as controls and similarly processed. Reductase activity was expressed as picomoles of radioactive HMG-CoA converted to radioactive mevalonate per minute per milligram detergent-solubilized protein.

Mevalonate and HMG-CoA Incorporation into Sterols

To study the biosynthesis of sterols in *T. gondii*, 500 nmol of [^{14}C]mevalonic acid or 300 nmol [^{14}C]HMG-CoA was added for 3 h at 37°C to confluent cultures of CHO cells in 10-cm plates infected with 10^7 freshly lysed-out parasites for 24 h in medium containing 10% LPDS. For labeling with HMG-CoA, infected cells were previously permeabilized with digitonin (Leonard and Chen, 1987). After washing, parasites were isolated, their protein concentration determined, and lipids extracted to quantify the amount of [^{14}C]cholesterol associated with the sterol fraction, as described (Coppens et al., 1995). An assay of radioactive mevalonate or HMG-CoA incorporation was performed on extracellular *T. gondii* in conditions where parasites are metabolically active, as described previously (Coppens et al., 1999).

Protein Determination

Protein content was determined by the bicinchoninic acid assay (Smith et al., 1985), using serum albumin as standard.

Statistical Analysis

For comparison of means, *P* was determined by analysis of variance against control (ANOVA 2).

Results

Cholesterol Is Not Uniformly Distributed within T. gondii

The polyene antibiotic filipin was used to visualize the distribution of cholesterol or other β-hydroxy-sterols by light fluorescence microscopy. In the presence of extracellular lipoproteins, uninfected fibroblasts showed a filipin-cholesterol staining in the plasma membrane and in intracellular perinuclear organelles (Fig. 1 A) that have been identified as lysosomes, endocytic recycling compartments, and Golgi structures (Butler et al., 1992; Neufeld et al., 1996; Mukherjee et al., 1998). Cholesterol distribution in parasites inside fibroblasts was observed as intense and pre-

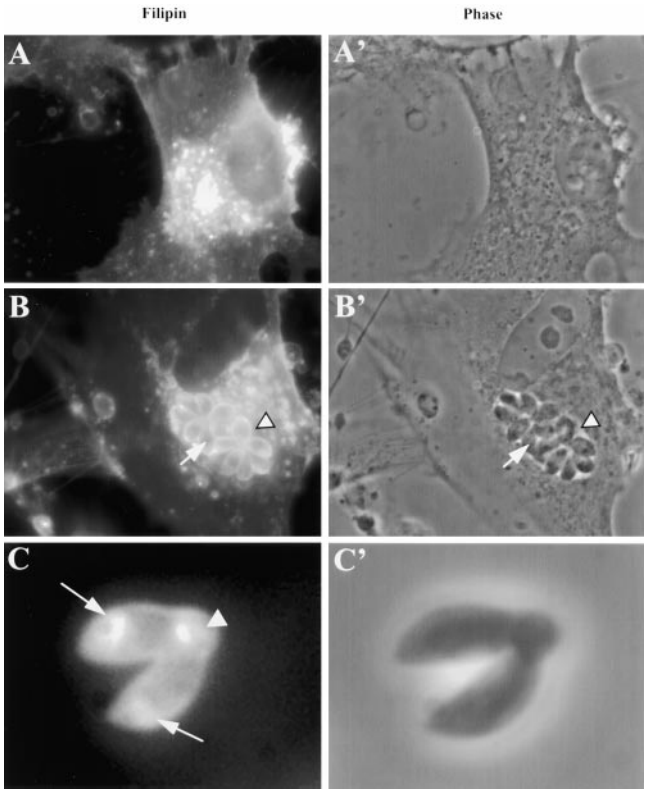

Figure 1. Intracellular distribution of cholesterol in uninfected and *T. gondii*–infected fibroblasts. Uninfected fibroblasts (A) or infected with *T. gondii* (B, arrow) were cultivated for 24 h in medium containing 10% FCS, fixed, cytochemically stained with filipin for cholesterol detection, and observed by fluorescence microscopy. In B, the arrowhead shows the location of the PVM. Alternatively, freshly lysed-out and dividing parasites (C) were similarly processed for filipin-cholesterol labeling. In C, the arrows indicate the nascent apical complexes of the two progenies and the arrowhead, the residual body of the parent cell.

dominantly located in the plasma membrane and the apical region, including the specialized secretory rhoptries (Fig. 1 B). This is in accordance with the unusually high cholesterol/phospholipid molar ratio of these organelles (Foussard et al., 1991a). No prominent filipin staining was noted in the PVM, leading to the suggestion that this membrane, if accessible to filipin, is low in cholesterol compared with plasma membranes. In parasites dividing by endodyogeny, filipin stained the nascent apical complexes of the two progenies as well as the residual body, which is the site of accumulation of structures remaining from the parent cell (Fig. 1 C).

Parasites Are Deficient in their Ability to Synthesize Sterols via the Mevalonate Pathway

To determine whether intracellular parasites are able to synthesize cholesterol, infected cells were incubated with radioactive precursors of the mevalonate pathway. Very low amounts of radioactivity were detected in the parasite sterol faction compared with CHO cells after incubation either in the presence of [^{14}C]mevalonate or [^{14}C]HMG-CoA (Table I). To circumvent a problem of substrate accessibility, parasite sterols were isolated from extracellular *T. gondii* incubated in the presence of the same radioactive precursors and no radioactivity was found after sterol isolation (not shown).

Table I. Absence of Incorporation of Mevalonate and HMG-CoA in the Sterol Fraction of T. gondii

	Incorporation into the sterol fraction of:	
	Mevalonate	HMG-CoA
	cpm/mg cell protein	
T. gondii	130 ± 35	88 ± 7
CHO cells	455,150 ± 49,800	3,670 ± 230

Infected or uninfected CHO cells grown for 24 h in medium containing 10% LPDS were incubated with [^{14}C]mevalonate or [^{14}C]HMG-CoA. After washing, the sterol fractions of parasites or CHO cells were isolated to determine the radioactivity content. Values are means ± SD of three separate experiments.

Using as host cells the UT-1 mutant, a clone of CHO cells resistant to the HMG-CoA reductase inhibitor compactin (mol wt 390.5), we demonstrated that parasite replication was not affected in the presence of 40 μM compactin (not shown), suggesting that this enzyme, if present in the parasite, is not necessary for parasite growth. These data indicate that intra- or extracellular parasites do not synthesize and are not dependent upon de novo sterols via the classical mevalonate pathway.

Host Cholesterol Biosynthesis Remains Unchanged in Infected Cells

To assess the influence of parasites on host cholesterol production, we monitored the formation of mevalonate from [^{14}C]HMG-CoA in parasitized or uninfected cells after permeabilization with digitonin. As illustrated in Table II, a similar activity of HMG-CoA reductase was observed in control cells or in cells containing tachyzoites. The main contribution of the mevalonate production is from the host cells since parasites fail to use precursors from the mevalonate pathway, as demonstrated above in Table I. The removal of LP from the culture medium induces an increase of the enzyme activity to the same extent in both conditions (Table II).

Deficiency in Host Cell De Novo Biosynthesis Does Not Limit Parasite Growth

We have further explored whether parasite replication is totally independent of host cholesterol biosynthesis, using as host cells mutants that are defective for cholesterol bio-

Table II. Comparison of HMG-CoA Reductase Activity in Untreated or Infected Cells by T. gondii

	Infected CHO cells	Uninfected CHO cells
	pmol mevalonate/min per mg cell protein	
3.5% LPDS + LP	13.5 ± 2.6	15.9 ± 4.1
3.5% LPDS	29 ± 7.4	27.1 ± 6.3

CHO cells were grown for 24 h in medium containing LP (or not) in the presence or absence of parasites. After washing, cells were permeabilized and incubated in the assay medium containing [^{14}C]HMG-CoA for 30 min at 37°C. The reductase activity was determined by measurement of the production of radioactive mevalonate. Values are means ± SD of three separate experiments.

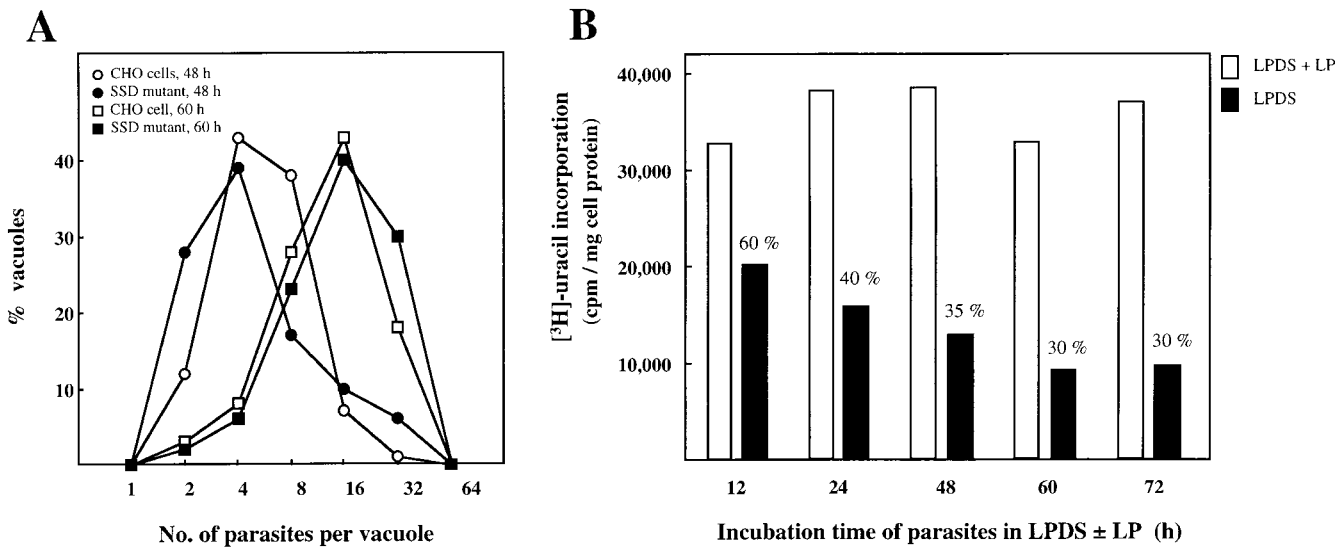

Figure 2. Parasite replication in the SSD mutant in the presence or absence of LP. (A) The distribution of PV sizes expressed in percent was determined at 48 and 60 h after infection of the SSD mutant or CHO cells with parasites in the presence 10% FBS in the culture medium. Data represent the averages for at least 50 randomly selected vacuoles in two different experiments. (B) At 12, 24, 48, 60, and 72 h, corresponding to incubation times of intracellular parasites in the presence or absence of LP, uracil incorporation was assayed using the SSD mutant infected every 12 h, as described in Materials and Methods. Results are expressed in counts per minute of [^{3}H]uracil incorporated into parasites per milligram cell protein and are means of two separate experiments done in duplicate.

synthesis and, therefore, auxotrophic for LDL-derived cholesterol.

The SSD mutant has a metabolic block at squalene synthase, converting farnesylpyrophosphate to squalene, the first enzymatic step committed solely to the biosynthesis of sterols, and is therefore incapable of endogenous cholesterol biosynthesis (Bradfute et al., 1992). As determined by enumeration of parasites per vacuole, *T. gondii* developed normally in SSD cells compared with CHO cells in medium containing 10% FBS (Fig. 2 A). The levels of uracil incorporation into newly synthesized parasite nucleic acids (99 ± 4% as control of uracil incorporated in parasites) as well as the filipin-cholesterol staining of these parasites maintained in these two types of cells were also similar (not shown). This suggests that host cholesterol production has no significant effect on parasite replication and that the bulk of the parasite cholesterol requirement can be satisfied by exogenous cholesterol.

Blockade of both Exogenous and Endogenous Sources of Host Cholesterol Strongly Reduces Parasite Replication

To determine whether the parasites are still capable of surviving and multiplying in the absence of any sources of cholesterol originating from the host cell, we infected the SSD mutant in medium containing 10% LPDS and monitored parasite viability over a period of 72 h. Assayed by thymidine incorporation, the SSD mutant can be maintained in a medium devoid of extracellular LP for a maximal time of 12 h (not shown). To avoid effects of host cell viability loss on parasite replication, we used a serial passage protocol. Each 12 h after infection, intracellular parasites were mechanically harvested from the SSD cells, purified, and a portion was used for uracil incorporation. Another portion was used to infect fresh SSD cells (mechanically released parasites are fully invasive) at the beginning of their starvation cycle in LPDS. Prolonged time in the absence of host exogenous and endogenous sources of cholesterol, achieved by this serial passage protocol, led to a progressive reduction of parasite replication (Fig. 2 B). Parasite growth was not totally abrogated after 60 h of LP starvation and beyond, since 30% of the parasite population was still detectable. This decrease in replication rate was reversible since the addition of extracellular LP restored the parasite growth to normal values (not shown).

Extracellular LDL and Exogenous Free Cholesterol Stimulate Parasite Growth

We next asked whether parasite growth can be directly dependent upon exogenously supplied cholesterol. The replication rate of intracellular parasites incubated in culture medium devoid of LP or with an excess of exogenous cholesterol, either freely administrated or associated with LDL, was analyzed. Removal of any lipoproteins from the incubation medium resulted in a reduced incorporation of [^{3}H]uracil into parasites by 10 and 16%, after 24 and 48 h, respectively, as compared with incubation in the presence of 10% FBS (Fig. 3 A). These lower values of radioactive uracil incorporation were coincident with the presence of smaller vacuoles containing fewer parasites when parasites were maintained without LP (Fig. 3 B). This slowing in parasite multiplication also caused a delay in the parasite-induced lysis of the monolayer of host cells as monitored by an increase in the parasite doubling time (10–13 h in LPDS vs. 6–8 h in FBS).

The cell line CHO-7, a derivative clone of CHO cells selected for growth in lipoprotein-deficient serum (Metherall et al., 1989), was used as the host cell for *T. gondii*.

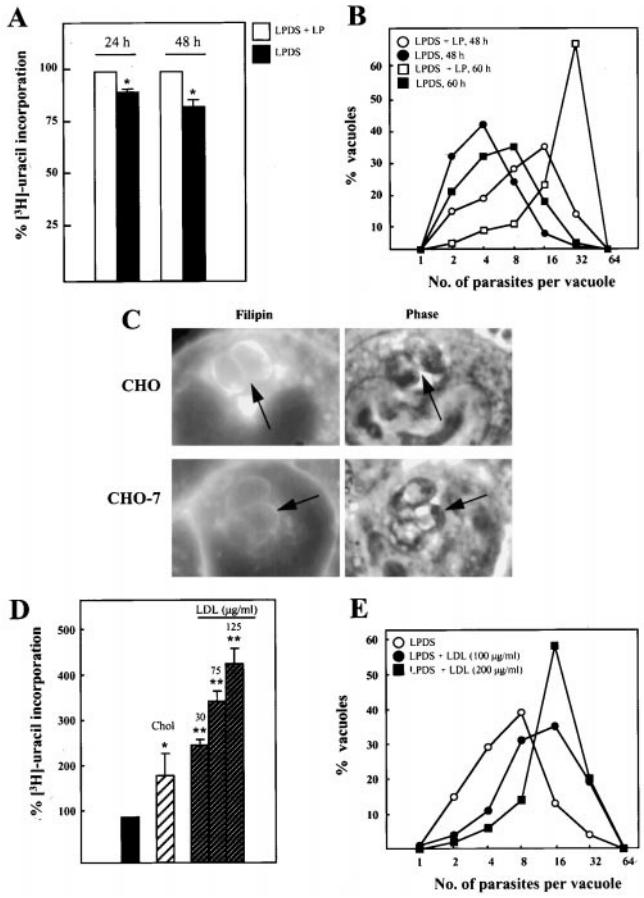

Figure 3. Influence of exogenous cholesterol and lipoproteins on parasite proliferation. (A) Uracil incorporation was assayed using CHO cells infected for 24 or 48 h in medium containing 10% LPDS, supplemented (or not) with LP. Data in percent are expressed relative to control (incubation in the presence of LP) taken as 100% ± SEM from four separate experiments done in triplicate. Differences between values of control and experimental groups were statistically significant (*$P < 0.01$). (B) The distribution of PV sizes expressed in percent was determined at 48 and 60 h after infection of HFF with parasites in 10% LPDS in the presence or absence of LP. Data represent the averages for at least 50 randomly selected vacuoles in four different experiments. (C) The CHO and CHO-7 cells infected with parasites (arrows) were cultivated for 24 h in medium containing 10% FCS or 10% LPDS, respectively, fixed, cytochemically stained with filipin for cholesterol detection, and observed by fluorescence microscopy. (D) Uracil incorporation was assayed using CHO cells infected for 24 h in medium containing 10% LPDS (solid bars), or supplemented with 10 μg/ml of water-soluble cholesterol (chol), or with LDL at the indicated concentrations. Data in percent are expressed relative to control (incubation in the presence of LPDS) taken as 100% ± SEM from three separate experiments done in duplicate. Differences between values of control and experimental groups were statistically significant (*$P < 0.01$; **$P < 0.005$). (E) The percent distribution of PV sizes was determined at 24 h after infection of HFF with parasites in medium containing 10% LPDS, supplemented or not with LDL at the indicated concentrations. Data represent the averages for at least 50 randomly selected vacuoles in two different experiments.

When *T. gondii* infected CHO-7 cells in medium containing 10% LPDS, a decrease of parasite growth was observed (85 ± 5% of uracil incorporation after 24 h), as compared with infection of CHO-7 cells in medium containing LP. In addition, filipin staining of the parasite plasma membrane inside CHO-7 cells was notably less than when incubated with LP and was not concentrated at the apical complex as compared with parasites grown in CHO cells in the presence of 10% FBS (Fig. 3 C). By comparison, the intensity of the plasma membrane labeling of the CHO-7 cells was unchanged in the absence of extracellular LP.

The addition of exogenous cholesterol, particularly when incorporated into LDL in the incubation medium, has a stimulatory effect on *T. gondii* replication, measured by uracil incorporation into parasites (Fig. 3 D), as well as enumeration of parasites per PV in infected cells (Fig. 3 E). This growth stimulation was proportional to the concentration of LDL in the medium.

Altogether, these data indicate that an exogenous source of cholesterol, mainly provided by LDL, is required for optimal intracellular replication of *T. gondii*, although it is not essential for growth.

Receptor-mediated Endocytosis of LDL Is Specifically Increased in Infected Cells

To assess whether the presence of replicating tachyzoites in CHO cells resulted from an increased uptake of extracellular LDL as a source of cholesterol for the parasites, infected and noninfected cells were incubated in parallel with ^{125}I-LDL, and the extent of radioactive ligand uptake was determined. Internalization of LDL was stimulated by two- to threefold in a ligand dose-dependent manner in infected CHO cells (Fig. 4). This uptake was specific for LDL as internalization of this ligand was inhibitable by an excess concentration of nonlabeled LDL, indicating that the enhanced endocytosis of LDL in infected cells did not result from receptor-independent fluid-phase endocytosis (not shown). Furthermore, the increased LDL uptake in infected cells was not a consequence of a global stimulation of endocytosis since uptake of transferrin as another receptor-internalized ligand, or the fluid-phase tracer HRP was unchanged after infection (Fig. 4).

LDL-derived Cholesterol Is Rapidly Delivered to the PV and Is Concentrated in the Parasite

To determine whether intravacuolar parasites can take up sterols directly from the external medium, we incubated infected and uninfected fibroblasts with fluorescent NBD-C incorporated into LDL at 37°C. Noninfected cells exposed to [NBD-C]-LDL exhibited shortly after endocytosis a pattern of punctate fluorescence consistent with endocytic structures. After a lag time of ~10 min, [NBD-C]-LDL is degraded to free NBD-C in lysosomal compartments, which subsequently is transferred to the plasma membrane via the Golgi apparatus (Craig et al., 1981; Ohashi et al., 1992; Fig. 5 D). Cholesterol that has reached the plasma membrane is then slowly transferred to cholesterol acceptors in the surrounding medium (serum proteins), contributing to a gradual loss of NBD-C, as shown by a de-

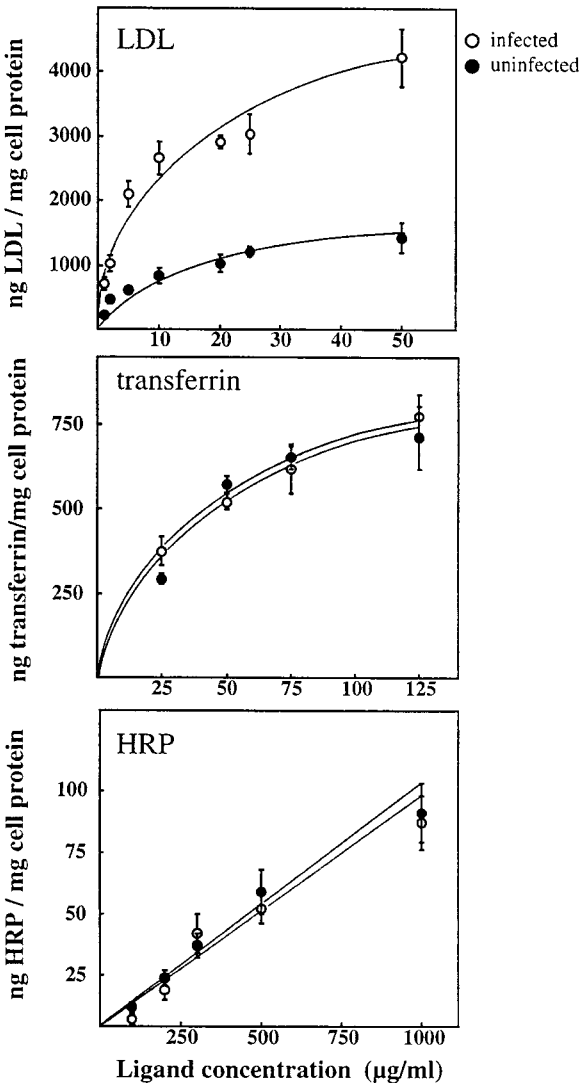

Figure 4. Comparison of receptor-mediated endocytosis and pinocytosis in uninfected and *T. gondii*–infected fibroblasts. Uninfected CHO cells (●) or infected with *T. gondii* (○) for 24 h were then incubated at 37°C in the presence of the indicated concentration of ^{125}I-LDL, ^{125}I-transferrin, or HRP for 2 h. After washing, the amount of cell-associated ligand was determined by radioactivity or enzymatic activity measurement and expressed as nanograms of ligand per milligram cell protein. Values are means ± SD of three separate experiments.

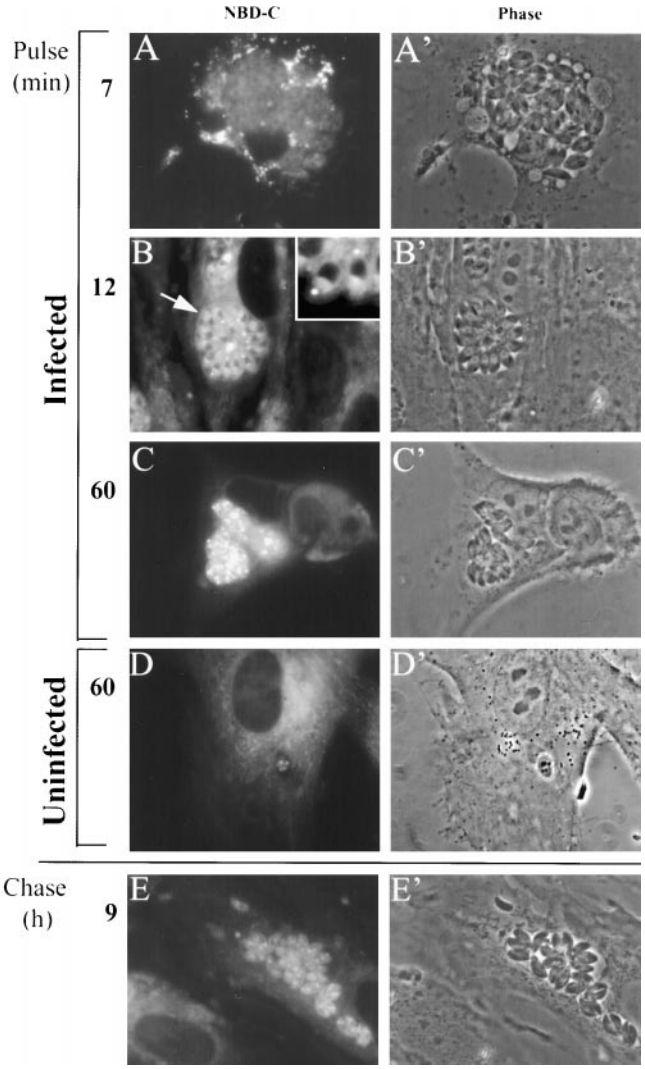

Figure 5. Kinetics of [NBD-C]-LDL acquisition in uninfected or *T. gondii*–infected cells. 24 h after infection in medium containing 10% LPDS, infected fibroblasts were pulse-labeled at 37°C with 0.1 mg/ml of [NBD-C]-LDL for 7 min (A), 12 min (B; fluorescent PVM is indicated by the arrow), or 60 min (C). Cells were washed and processed for fluorescence observation. In parallel, noninfected fibroblasts were labeled for 60 min with the fluorescent lipoproteins and treated under the same conditions (D). In one experiment, infected fibroblasts were pulse-labeled at 37°C with 0.1 mg/ml of [NBD-C]-LDL for 60 min, and then chased for 9 h in medium containing 10% LPDS (E).

crease of the plasma membrane signal (Ohashi et al., 1992).

Short pulses of infected cells with fluorescent LDL led to a gradual labeling of the PVM, followed by labeling of intravacuolar parasites (Fig. 5, A–C). Within the parasite, the labeling corresponding presumably to NBD-C was not evenly distributed, but was concentrated in the plasma membrane, the anterior region, and in compact spherical structures (one to four per parasite) throughout the cytoplasm. These latter were identified as lipid droplets (Speer et al., 1998), based on their specific accumulation of Oil Red O or Nile Red dyes (not shown). Interestingly, the number of lipid droplets was reduced in tachyzoites grown in LPDS-cultured cells as compared with parasites originating from cells cultivated with extracellular excess LDL (not shown).

A pulse (1 h)–chase (9 h) experiment performed on infected cells revealed a maintenance of fluorescence associated with the parasite plasma membrane, the apical structures, and the lipid droplets (Fig. 5 E); the intensity was slightly decreased due to a dilution of the NBD-C, concomitant with parasite division, while a diffuse labeling was observed throughout the host cell. The fluorescence initially associated with the PVM gradually disappeared with the chase time.

No specific fluorescence was detectable in infected cells

or in parasites when incubated in medium containing 10% LPDS and NBD-C administrated as lipid alone, indicating a requirement for LDL in the acquisition of NBD-C by intracellular *T. gondii* (not shown).

LDL-derived Cholesterol Acquisition Requires Live Intracellular Parasites

The above data demonstrated that the PVs containing *T. gondii* gain access to exogenous NBD-C associated with LDL. To investigate whether this cholesterol uptake is induced by the parasite itself or resulted from a passive transfer to the PV, we infected cells for 24 h, treated for the next 20 h with the anti–*Toxoplasma* drug pyrimethamine, and then incubated with [NBD-C]-LDL. The reduction of parasite viability was confirmed by measurement of uracil incorporation, revealing <10 and <1% incorporation, compared with control without pyrimethamine, after exposure to 1 and 10 μM of drug, respectively. The fluorescent labeling in *T. gondii* was abrogated by pyrimethamine treatment in a dose-dependent manner.

The SDZ 215-918 cyclosporin A derivative is a particularly potent and rapid inhibitor of *T. gondii* invasion and replication (Silverman et al., 1997). Incubation of infected cells with SDZ 215-918 in conditions that inhibit the metabolic activity of intracellular parasites, before exposure to [NBD-C]-LDL, led to a decrease of fluorescence associated with the PV (not shown). This indicates that active parasite metabolism is necessary for cholesterol uptake (Fig. 6, A–C).

No parasite-associated fluorescence was observed in preparations of live extracellular tachyzoites exposed to [NBD-C]-LDL (Fig. 6 D), indicating that parasites are unable to internalize these lipoproteins when outside the host cell and that they acquire cholesterol only after it is liberated from LDL by host lysosomes. Parasite sequestration of NBD-C is therefore specific for intracellular *T. gondii*.

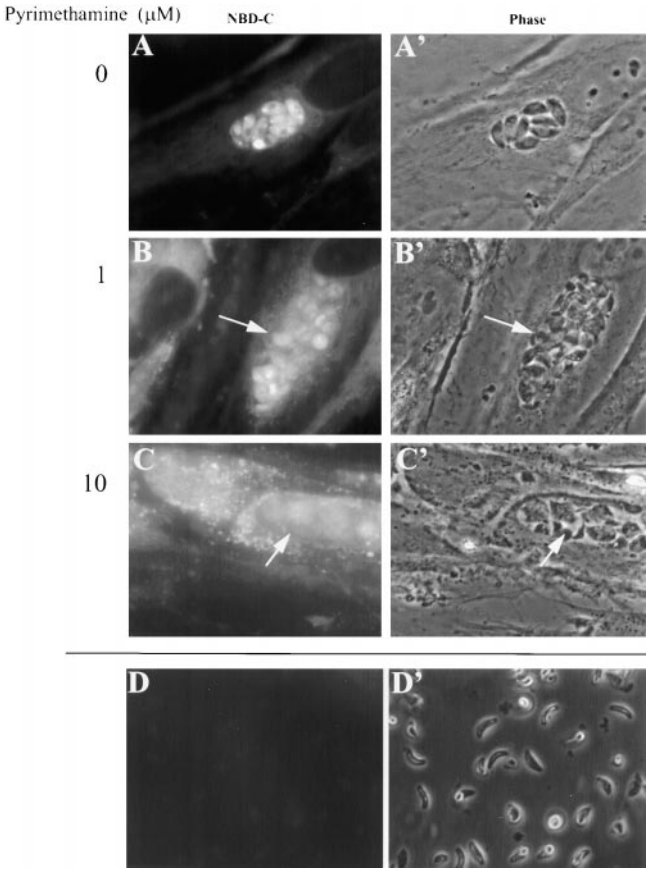

Figure 6. Absence of [NBD-C]-LDL acquisition in pyrimethamine-treated or extracellular parasites. 24 h after infection in medium containing 10% LPDS, infected fibroblasts were preincubated for 20 h without (A) or with pyrimethamine at 1 (B) or 10 (C) μM. As observed in B′ and C′, the pyrimethamine-treated cells contain vacuoles with dying or dead parasites (arrows), having abnormal shapes. Cells were then pulse-labeled at 37°C with 0.1 mg/ml [NBD-C]-LDL for 60 min, washed, and processed for fluorescence observation. Extracellular tachyzoites were pulse-labeled at 37°C with 0.1 mg/ml of [NBD-C]-LDL for 60 min, washed, and directly observed by fluorescence microscopy.

Inhibitors of the LDL Endocytic Pathway Block Cholesterol Transport to the Parasite

We next wanted to elucidate whether delivery of exogenous cholesterol to the parasite involves the endocytic pathway of the host cell. The transport of LDL-derived cholesterol from the external medium was measured in the presence of agents known to block steps in LDL-cholesterol transport in mammalian cells. These treatments target LDL internalization and degradation, as well as cholesterol egress from lysosomes, by both vesicular and nonvesicular transport. Since the viability of parasites is required for cholesterol uptake, we monitored the levels of uracil incorporated into the parasites for each inhibitor tested, and found incorporation of ≥94% of control without inhibitor.

Preincubation of infected cells with anti–LDL receptor antibodies inhibited LDL binding to the host cell and abrogated by 90% of control the delivery of radioactive cholesteryl esters to parasites after [^{3}H-CO]-LDL endocytosis (Table III). This inhibition of accessibility of exogenous cholesterol by anti–LDL receptor antibodies was confirmed by the absence of fluorescent labeling in the host cell and in intravacuolar parasites after short pulse-labeling with [NBD-C]-LDL (Fig. 7, A and B).

The effects of the lysosomotropic agent chloroquine that impairs acid hydrolase activities (de Duve et al., 1974; Brown et al., 1975; de Duve, 1983) or of the indigestible solute sucrose that interferes with the fusion competence of mature lysosomes (Montgomery et al., 1991) on cholesterol acquisition by *T. gondii*, were investigated. In their presence, the inhibition of tritiated cholesteryl oleate (Table III) or fluorescent cholesterol (Fig. 7, C–E) transport was obvious. At 100 μM chloroquine, LDL degradation was partial, as shown by a slight fluorescent staining of cellular compartments and parasites, whereas at 200 μM the drug caused an extensive vacuolization of lysosomes (and acidic endosomes) leading to a complete blockade of LDL proteolysis and an absence of release of cholesterol trapped within the particles. Under these conditions, infected cells showed a bright punctate fluorescent pattern

Table III. Effect of Treatments on the Acquisition of [^{3}H-CO]-LDL by Intracellular T. gondii

	[^{3}H-C] associated with parasites
Control	100
Blockade of LDL receptors	
Anti–LDL receptor antibodies	10 ± 4
Defective lysosomal function	
Chloroquine	10 ± 6
Sucrose	17 ± 7
Lysosomal cholesterol sequestration	
U18666A	28 ± 11
Progesterone	23 ± 8
U18666A + progesterone	15 ± 7
Cytoskeletal disorganization	
Cytochalasin B	101 ± 21
Colchicine	99 ± 8
Disruption of the Golgi	
Brefeldin A	93 ± 7

[^{3}H-C] uptake by intracellular parasites was determined 24 h after infection using 5 mg/ml [^{3}H-CO]-LDL for 1 h. This LDL pulse-labeling followed inhibitor treatment of the infected monolayers, except for cytoskeleton inhibitor conditions, where a 10-min pulse preceded the drug incubation. Infected cells were washed after each treatment (except for lysosomal function inhibition and Golgi disruption) before purification of parasites and the determination of the levels of [^{3}H-C] in the parasite sterol fraction. Data are expressed in percent relative to control (infected cells without treatment), taken as 100% ± SD from three separate experiments done in duplicate. The specific inhibitor treatments used were: 400 μg/ml anti–LDL receptor antibodies for 1 h at 37°C, for 30 min at 4°C; 300 μm chloroquine for 15 min; 20 mg/ml sucrose for 2 h; 1 μg/ml U18666A for 90 min; 10 μg/ml progesterone for 24 h; 50 μm cytochalasin B for 30 min; 50 μm colchicine for 30 min; 1 μg/ml Brefeldin A for 90 min.

(Fig. 7 E), corresponding to lysosomal structures retaining intact [NBC-C]-LDL, and no fluorescence was observed elsewhere in infected cells, including the parasite vacuole.

We next tested the influence of U18666A, one of a number of hydrophobic amines that are reported to induce an accumulation of free exogenous cholesterol in late endosomes/lysosomes during the endocytic processing of LDL (Roff et al., 1991; Liscum and Faust, 1983; Kobayashi et al., 1999). Progesterone also sequesters cholesterol in lysosomes by an unknown and reversible mechanism (Butler et al., 1992). Progesterone and U18666A inhibited the transfer of tritiated cholesteryl oleate into the parasite sterol fractions after endocytosis of the reconstituted radioactive LDL (Table III); moreover, the effects of U18666A and progesterone are cumulative. This inhibition is coincident with a weak fluorescent pattern of parasites preincubated with these drugs, alone or in combination, followed by exposure to [NBD-C]-LDL (Fig. 8). After a 24-h chase in a progesterone-free medium, the free cholesterol pool leaves lysosomes to be redistributed intracellularly, as demonstrated by fluorescence throughout the host cell and parasites (Fig. 8, B and C).

Agents that disrupt the cytoskeletal network, cytochalasin B or colchicine, caused noticeable cell rounding but no inhibition of LDL-derived cholesterol delivery to intravacuolar parasites (Table III). This result demonstrates that no vesicular transport is involved in cholesterol traffic from the lysosomes to the ER, and then to the PV, suggesting a route independent of the ER.

Brefeldin A affecting the Golgi apparatus organization (Misumi et al., 1986; Klausner et al., 1992; Torii et al., 1995) had no significant effect on cholesterol transport to

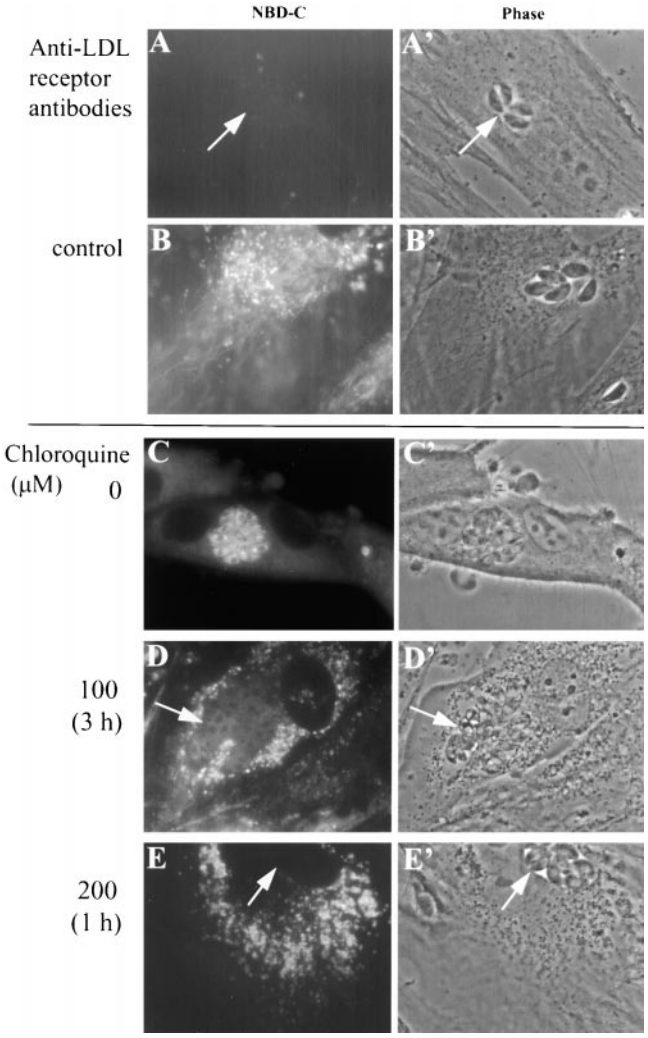

Figure 7. Blockade of [NBD-C]-LDL acquisition by parasites with anti–LDL receptor antibodies or chloroquine. 24 h after infection in medium containing 10% FBS, fibroblasts infected by *T. gondii* were preincubated without (A) or with (B) 400 μg/ml anti–LDL receptor antibodies for 1 h at 37°C, and then for 30 min at 4°C, washed, pulse-labeled at 37°C for 10 min with 0.1 mg/ml [NBD-C]-LDL, washed, and processed for fluorescence observation. In another set of experiments, 24 h after infection in medium containing 10% LPDS, infected fibroblasts were incubated without drug (C) or with chloroquine either at 100 μM for 150 min (D) or at 200 μM for 30 min (E), pulse-labeled at 37°C for 30 min with 0.1 mg/ml of [NBD-C]-LDL, washed, and processed for fluorescence observation. Vacuoles containing parasites are illustrated by arrows.

T. gondii, suggesting the use of a Golgi-independent pathway (Table III).

Parasite Replication Is Reduced in a Host Cell Line with Defective Mobilization of Cholesterol from Lysosomes

The cellular and biochemical consequences of the progesterone-induced block in lysosomal cholesterol transport strongly resemble the phenotypic manifestations of the Niemann-Pick type C (NPC) mutation (Pentchev et al., 1986;

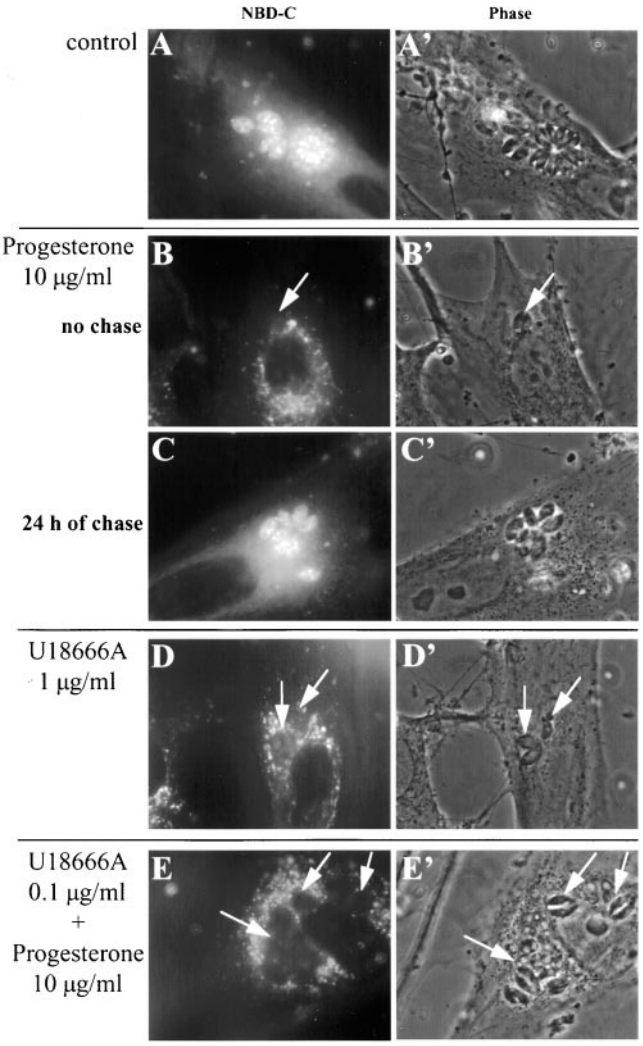

Figure 8. Blockade and reversibility of [NBD-C]-LDL acquisition by parasites with progesterone and U18666A. 24 h after infection in medium containing 10% LPDS, fibroblasts infected by *T. gondii* (arrows) were preincubated without drug (A), with progesterone at 10 μg/ml for 24 h (B and C), with U18666A at 1 μg/ml for 90 min (D), or with progesterone at 10 μg/ml for 20 h, and then with U18666A at 0.1 μg/ml for 90 min. After washing, cells were pulse-labeled at 37°C for 10 min with 0.1 mg/ml of [NBD-C]-LDL, washed, and processed for fluorescence observation. A chase of 24 h in medium containing 10% LPDS was performed in the experiment in C before analysis of the fluorescence pattern.

Sokol et al., 1988; Liscum et al., 1989). NPC fibroblasts exhibit lysosomal storage of unesterified cholesterol due to a single gene defect that affects the egress of LDL-cholesterol from late endosomes/lysosomes and LDL-mediated regulation of cellular cholesterol homeostasis (reviewed in Liscum and Klansek, 1998). The somatic 2-2 mutant derived from CHO cells has the same biochemical phenotype of the NPC mutation (Dahl et al., 1992, 1993).

A direct comparison of lysosomal cholesterol processing and transport to the parasite was carried out between the 2-2 mutant and progesterone-treated cells. Parasite growth in the 2-2 mutant is reduced by ~15 and 25% in comparison with control after 24 and 48 h, respectively, as assessed by uracil incorporation (Fig. 9 A). Concomitant with the impaired parasite replication in these mutant cells, the parasite cholesterol content revealed by filipin is weak, indicating a lower cholesterol content due to the retention of cholesterol inside host lysosomes (compare Fig. 9 B with Figs. 1 B and 3 C for CHO cells). In addition, incubation of infected 2-2 mutant cells with [^{3}H-CO]-LDL led to a lower incorporation of radioactive cholesteryl oleate into parasites (64 ± 6% of control). Incubation of fibroblasts with progesterone induced a more drastic decrease of parasite replication of ~50 and 70% after 24 and 48 h, respectively (Fig. 9 C), as compared with untreated cells, probably consistent with a more profound block in cholesterol delivery to intracellular parasites (see Table III).

A Large Excess of Host Cholesterol Does Not Modify Parasite Growth

The SRD-1 mutant has an increase of cholesterol input from exogenous LDL and endogenous synthesis due to the loss of suppression of the sterol-regulated genes in response to sterol overload. This mutant also has a stimulation of cholesterol esterification upon addition of 25-hydroxycholesterol (Metherall et al., 1989). In medium containing 5% LPDS, the intravacuolar development of parasites within the SRD-1 mutant is comparable with that of parasites infecting CHO cells without LP (uracil incorporation: 101 ± 3% of control). The higher number of LDL receptors exposed at the surface of the SRD-1 mutant did not increase the amount of tritiated cholesteryl oleate associated with the parasite sterol fraction after endocytosis of [^{3}H-CO]-LDL, representing 96 ± 4% of the parasite control in CHO cells. This suggests that the ability to acquire exogenous cholesterol by *T. gondii* might be a saturable process.

Discussion

The obligate intracellular *T. gondii* replicates inside a nonfusogenic vacuole within nucleated cells. The membrane of the vacuole forms a barrier between the nutrient rich cytosol and the parasite surface, and prevents access of host proteins to the vacuole space. The intravacuolar space is likely full of low molecular weight compounds originating from the host cell since the PVM contains pores facilitating passive transfer of molecules (Schwab et al., 1994). The current data dramatically illustrate a new feature regarding nutrient acquisition by *T. gondii*, demonstrating that the parasite can efficiently access cholesterol from host lysosomal compartments by an active mechanism, which is independent of vesicular fusion, and requires parasite viability.

The putative pathway underlying cholesterol-derived LDL acquisition by *T. gondii* is the pathway IIIa depicted in the schematic model in Fig. 10. First, a large contribution of the receptor-mediated endocytosis of LDL appears to be the primary mechanism as antibodies against LDL receptors can block the accessibility of LDL to intracellular compartments, including *Toxoplasma* vacuoles (Fig. 10, 1).

Uptake of LDL by host cells infected by *T. gondii* occurs

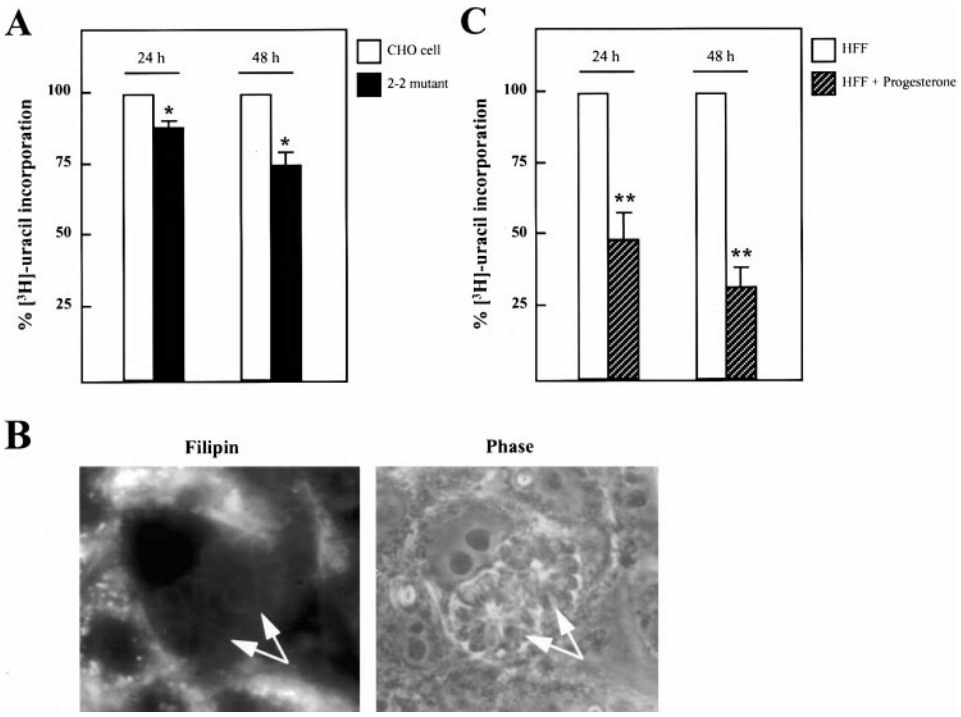

Figure 9. Reduction of parasite replication and cholesterol content in the 2-2 mutant or in fibroblasts treated with progesterone. (A) Uracil incorporation was assayed using the 2-2 mutant or CHO cells as control infected for 24 or 48 h in medium containing 10% FBS. Data in percent are expressed relative to control taken as 100% ± SEM from three separate experiments done in duplicate. Differences between values of control and experimental groups were statistically significant (*$P < 0.005$). (B) The 2-2 mutant cells infected with parasites (arrows) were cultivated for 24 h in medium containing 10% FCS, fixed, cytochemically stained with filipin for cholesterol detection, and observed by fluorescence microscopy. (C) Uracil incorporation was assayed using fibroblasts infected for 24 or 48 h in medium containing 10% FBS plus 10 μg/ml of progesterone. Data in percent are expressed relative to control (infected fibroblasts without progesterone) taken as 100% ± SEM from three separate experiments done in duplicate. Differences between values of control and experimental groups were statistically significant (**$P < 0.0005$).

in an enhanced fashion over uninfected cells. Results do not distinguish, however, whether this phenomenon is directly induced by intravacuolar parasites or is a more general response of the host cell as a consequence of parasitization. Since endocytosis of other ligands is not modified after infection, the putative mechanism may involve a higher density of cell-surface molecules or receptors capable of internalizing lipoproteins. If upregulation of the classical apoprotein B/E LDL receptor is occurring in the presence of unchanged HMG-CoA reductase activity upon infection with *T. gondii* (Table II), this would suggest an unusual pathway of cholesterol regulation (Goldstein and Brown, 1990). For another intravacuolar pathogen, exposure of macrophages to *Chlamydia pneumoniae* specifically increases the LDL uptake and foam cell formation by accumulation of cholesteryl ester droplets by a mechanism not involving the apoprotein B/E LDL receptor (Kalayoglu et al., 1999).

Endocytosed lipoproteins are delivered to endosomes and their protein/phospholipid coat degraded in late endosomes/lysosomes, yielding free cholesteryl esters that will be hydrolyzed to cholesterol. In the presence of chloroquine or sucrose impairing lysosomal function and provoking an accumulation of intact LDL, minimal molecules of cholesterol reach the PV, implicating host LDL proteolysis in the parasite acquisition of this lipid (Fig. 10, 2). Incubation of *T. gondii* in host cells where cholesterol translocation from late endosomes/lysosomes (2-2 mutant or treated with progesterone and U18666A) is impaired leads to a dramatic reduction of cholesterol associated with the parasites, concomitant with a slowing of parasite replication (Fig. 10, 3). The higher inhibition of parasite growth by progesterone compared with the 2-2 mutant might be related to a direct toxic effect of the steroid on *T. gondii*. Alternatively, progesterone is known to sequester in lysosomes cholesterol derived not only directly from endocytosed LDL, but also from other existing sterol pools, such as lipid droplets or organelle membranes (McGookey and Anderson, 1983; Butler et al., 1992), and could therefore also remove cholesterol from PV-containing parasites.

In mammalian cells, the mechanisms and precise routes of transport of cholesterol from late endosomes/lysosomes to the other cellular compartments are areas of intense investigation. From the cell biological point of view, the *Toxoplasma* vacuole may be considered as such a compartment in the infected cell and it could be used as a tool for analysis of intracellular cholesterol movement in mammalian cells. A portion of cholesterol is mobilized to the plasma membrane through the Golgi apparatus (reviewed in Liscum and Munn, 1999). The pathway of cholesterol acquisition by *T. gondii* is clearly not altered by incubation of infected cells with Brefeldin A (no use of the pathway IIIb in Fig. 10), indicating a Golgi-independent route despite the location of mature PVs adjacent to the host cell Golgi. This absence of delivery of host lipids from the Golgi to the *Toxoplasma* vacuole, a matter of some controversy (Melo and de Souza, 1996), has been recently confirmed by the observation of PV free of fluorescent lipids in experiments using NDB-ceramide loaded in fibroblasts before infection with parasites (Mordue et al., 1999). By comparison, the pathogen *Chlamydia trachomatis*, which similarly resides in a nonfusogenic vacuole, inter-

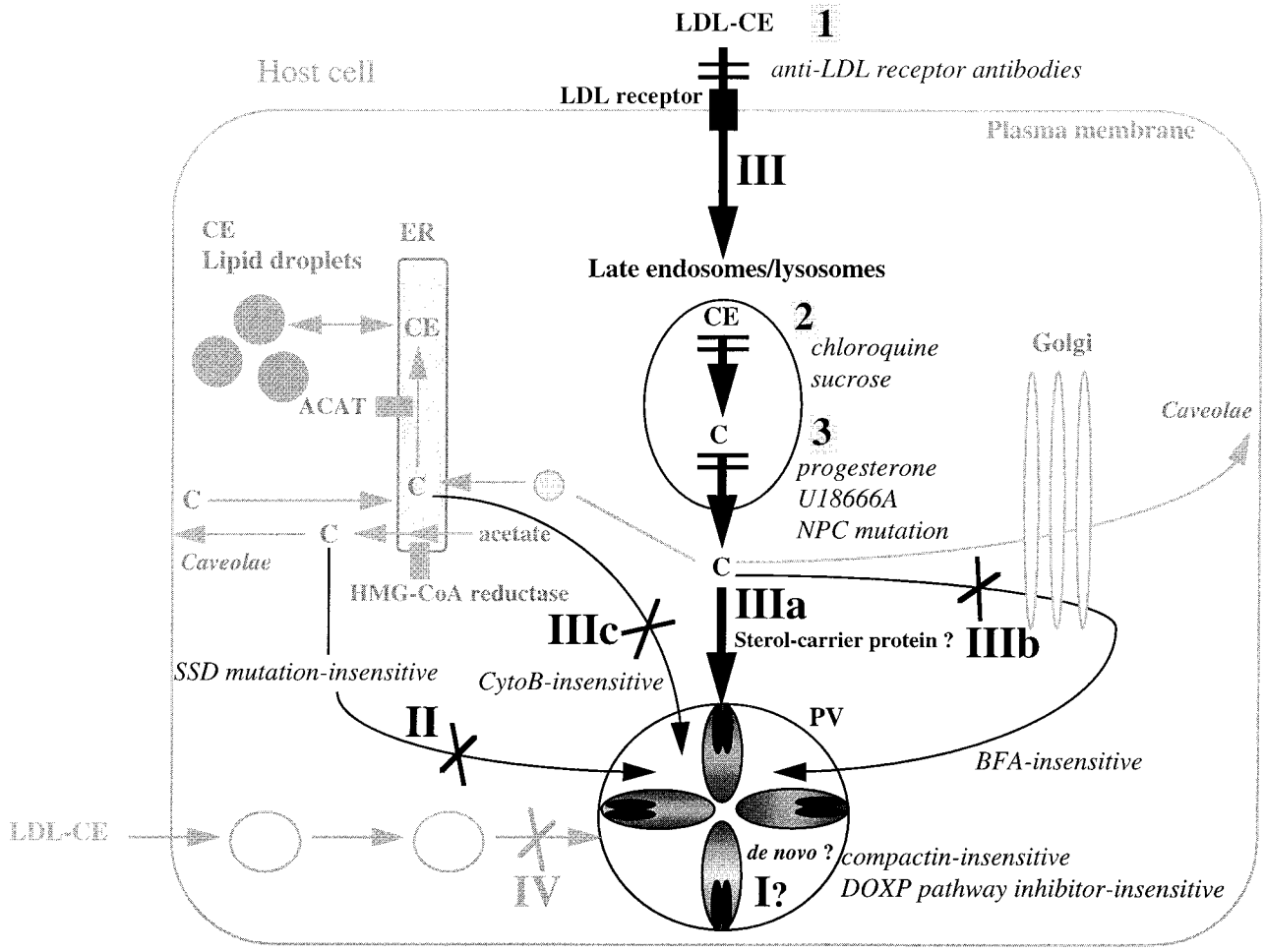

Figure 10. Hypothetical pathway for cholesterol acquisition from endocytosed LDL by intracellular *T. gondii*. This figure depicts the potential source of cholesterol for intracellular *T. gondii* after the binding and endocytosis of LDL via specific receptors as well as the trafficking of cholesterol effluxed from lysosomes to the PV containing four parasites. This pathway III can be blocked at several steps: (1) anti–LDL receptor antibodies block binding to LDL receptors, (2) chloroquine and sucrose affect the lysosomal function, abolishing LDL proteolysis, and (3) progesterone, U18666A, and NPC mutation impair cholesterol translocation from late endosomes/lysosomes, sequestering cholesterol inside these organelles. When blocked, the pathways II in the presence of LP, IIIb, and IIIc have no effect on *T. gondii* replication or cholesterol transport to intravacuolar parasites. The absence of host endo- and lysosomal markers detectable within the PV at any stage during infection excludes a direct access of host intravesicular LDL to the vacuole (pathway IV). No direct experimental evidence is yet available to support parasite cholesterol biosynthesis (pathway I). C, cholesterol; CE, cholesteryl ester; Cyto B, cytochalasin B; DOXP, 1-deoxy-D-xylulose 5-phosphate.

sects with vesicles from the exocytic pathway since this bacteria can acquire endogenously synthesized sphingomyelin in transit from the host Golgi to the plasma membrane (Hackstadt et al., 1995, 1996).

In mammalian cells, a portion of cholesterol effluxed from lysosomes is delivered to the ER by a pathway that is independent of the plasma membrane. For this vesicular transport of cholesterol, actin filaments, but not microtubules, appear to play a role. Cytochalasin B does not alter the cholesterol movement to the PV, suggesting that cholesterol originating from endocytosed LDL does not transit through the host ER to reach the *Toxoplasma* vacuole (no use of the pathway IIIc in Fig. 10). This observation suggests that the PVM-host ER association seen in infected cells (Jones et al., 1972; Melo et al., 1992; Sinai et al., 1997), does not participate in exogenous cholesterol trafficking, at least when LP are present.

Exogenous cholesterol, associated with LDL or freely administrated to cells infected by *T. gondii*, stimulates parasite growth and, in the case of large excess, induces the formation of parasite lipid droplets. Parasites incubated in the SRD-1 mutant, characterized by a high level of cholesterol due to a defect in sterol regulation, have a replication rate comparable with parasites grown in the parental CHO cell. Parasites do not take up cholesteryl esters packaged into LDL more avidly in spite of a high number of LDL receptors exposed at the cell surface of the SRD-1 mutant, suggestive of parasite regulatory mechanisms for sterol homeostasis. These mutant cells also show a high acyl-CoA:cholesterol acyltransferase activity that does not influence parasite growth. This is of interest since host esterification of cholesterol may be involved in the replication of *T. gondii* (Ernst et al., 1999).

Intracellular *T. gondii* do not modify the rate of host de

novo cholesterol biosynthesis. Parasites are capable of replicating in cells altered in their sterol production such as the SSD mutant. These data indicate that parasites are not dependent upon host endogenous cholesterol as long as exogenous sterols are available. They do not intercept the transport of this lipid from the ER in transit to the plasma membrane. These results corroborate the absence of labeled cholesterol associated with the sterol fraction of parasites after incubation of infected cells with radioactive precursors of the mevalonate pathway (no use of the pathway II in Fig. 10). The survival of 30% of the parasite population in the SSD mutant in the absence of LP opens the question on the origin of other potential sources of sterols for *T. gondii*, such as cholesterol from cell debris, stored cholesteryl esters, probably present in their lipid droplets, or cholesterol biosynthesized by the parasite. However, the compactin insensitivity, as well as the absence of sterol synthesis from precursors of the mevalonate pathway in the parasites, suggests the absence of the classical mevalonate pathway functional for isoprenoid biosynthesis in *T. gondii*, as observed for *Plasmodium falciparum*, an apicomplexan parasite related to *T. gondii.* An alternative nonmevalonate pathway for the early steps in the biosynthesis of isoprenoids, the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway, has been proposed in the plasmodial apicoplast, a plastid acquired by members of the phylum *Apicomplexa* by secondary endosymbiosis of an alga (Jomaa et al., 1999; McFadden and Roos, 1999). However, two lines of evidence suggest that sterol synthesis in the *T. gondii* apicoplast is not necessary for parasite replication in the presence of lipoproteins. First, parasites that cannot partition the apicoplast to daughter cells replicate normally in the primary vacuole (D. Roos, personal communication). Second, inhibitors of the DOXP pathway, which block plasmodial replication, do not inhibit *T. gondii* growth in presence of serum (D. Soldati, personal communication).

The accumulation of cholesterol from host lysosomes inside intravacuolar parasites suggests a largely unidirectional influx of this lipid and may be correlated to the rapid division rates of *T. gondii.* Alternatively, the isolation of the PV from the external medium results in the absence of transfer of cholesterol to extracellular cholesterol acceptors. The cholesterol transport pathway in cells infected with *T. gondii* clearly differs from those for the other cell organelles and is characterized by an absence of interaction with vesicles involved in export from the Golgi and ER, as well as those implicated in exchanges between the plasma membrane and the cell interior. The route of delivery of exogenous cholesterol from lysosomes to the PV might involve a sterol-binding protein, mediating a molecular transport of cholesterol towards the PVM. *T. gondii* may actively either divert a host sterol-carrier protein or synthesize such a protein since parasite viability is required for cholesterol acquisition. After crossing the PVM, cholesterol molecules must be translocated to the parasite. The elucidation of the mechanisms of these two sequential steps by generation of resistant mutants forms the basis for our future experiments.

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References

Bradfute, D.L., C.J. Silva, and R.D. Simoni. 1992. Squalene synthase-deficient mutant of Chinese hamster ovary cells. *J. Biol. Chem*. 267:18308–18314.

Brown, M.S., S.E. Dana, and J.L. Goldstein. 1975. Receptor-dependent hydrolysis of cholesteryl esters contained in plasma low density lipoprotein. *Proc. Natl. Acad. Sci. USA*. 72:2925–2929.

Brown, M.S., and J.L. Goldstein. 1999. A proteolytic pathway that controls the cholesterol content of membranes, cell, and blood. *Proc. Natl. Acad. Sci. USA*. 96:11041–11048.

Butler, J.D., J. Blanchette-Mackie, E. Goldin, R.R. O'Neill, G. Carstea, C.F. Roff, M.C. Patterson, S. Patel, M.E. Comly, A. Cooney, M.T. Vanier, R.O. Brady, and P.G. Pentchev. 1992. Progesterone blocks cholesterol translocation from lysosomes. *J. Biol. Chem*. 267:23797–23805.

Coppens, I., M. Andries, J.L. Liu, and M.F. Cesbron-Delauw. 1999. Intracellular trafficking of dense granule proteins in *Toxoplasma gondii* and experimental evidences for a regulated exocytosis. *Eur. J. Cell Biol.* 78:463–472.

Coppens, I., T. Levade, and P.J. Courtoy. 1995. Host plasma low density lipoprotein particles as an essential source of lipids for the bloodstream forms of *Trypanosoma brucei*. *J. Biol. Chem*. 270:5736–5741.

Craig, I.F., D.P. Via, W.W. Mantulin, H.J. Pownall, A.M. Gotto, Jr., and L.C. Smith. 1981. Low density lipoprotein reconstituted with steroids containing the nitrobenzoxadiazole fluorophore. *J. Lipid Res.* 22:687–696.

Dahl, N.K., W.G. Gutheil, and L. Liscum. 1993. Abnormal regulation of low density lipoprotein–sensitive events in a cholesterol transport mutant. *J. Biol. Chem*. 268:16979–16986.

Dahl, N.K., K.L. Reed, M.A. Daunais, J.R. Faust, and L. Liscum. 1992. Isolation and characterization of Chinese hamster ovary cells defective in the intracellular metabolism of LDL-derived cholesterol. *J. Biol. Chem*. 267:4889–4896.

de Duve, C. 1983. Lysosomes revisited. *Eur. J. Biochem*. 137:391–397.

de Duve, C., T. de Barsy, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoof. 1974. Lysosomotropic agents. *Biochem. Pharmacol.* 23:2495–2531.

El-Jack, A.K., K.V. Kandror, and P.F. Pilch. 1999. The formation of an insulin-responsive vesicular cargo compartment is an early event in 3T3-L1 adipocyte differentiation. *Mol. Biol. Cell.* 10:1581–1594.

Ernst, J.D., L.-M. Ting, S. Novak, and R.V. Farese. 1999. Fifth Toxoplasmosis Conference. Marshall, CA.

Foussard, F., M.A. Leriche, and J.F. Dubremetz. 1991a. Characterization of the lipid content of *Toxoplasma gondii* rhoptries. *Parasitology*. 102:367–370.

Foussard, F., Y. Gallois, A. Girault, and J.F. Menez. 1991b. Lipids and fatty acids of tachyzoites and purified pellicles of *Toxoplasma gondii*. *Parasitol. Res.* 77:475– 477.

Gallois, Y., F. Foussard, A. Girault, J. Hodberg, A. Tricaud, G. Mauras, and C. Motta. 1988. Membrane fluidity of *Toxoplasma gondii*: a fluorescence polarization study. *Biol. Cell*. 62:11–15.

Geelen, M.J.H., J.S. Papiez, K. Girgis, and D.M. Gibson. 1991. *In situ* measurement of HMG-CoA reductase activity in digitonin-permeabilized hepatocytes. *Biochim. Biophys. Res. Commun.* 180:525–530.

Goldstein, J.L., and M.S. Brown. 1990. Regulation of the mevalonate pathway. *Nature.* 343:425–430.

Greenspan, P., E.P. Mayer, and S.D. Fower. 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol.* 100:965–973.

Hackstadt, T., D.D. Rockey, R.A. Heinzen, and M.A. Scidmore. 1996. *Chlamydia trachomatis* interrupts an exocytic pathway to acquire endogenously synthesized sphingomyelin in transit from the Golgi apparatus to the plasma membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:964–977.

Hackstadt, T., M.A. Scidmore, and D.D. Rockey. 1995. Lipid metabolism in *Chlamydia trachomatis*–infected cell: directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion. *Proc. Natl. Acad. Sci. USA*. 92: 4877–4881.

Havel, R.J., H.A. Eder, and J.H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34:1345–1351.

Joiner, K.A., S.A. Fuhrman, H. Mietinnen, L.L. Kasper, and I. Mellman. 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor transfected fibroblasts. *Science.* 249:641–646.

Jomaa, H., J. Wiesner, S. Sanderbrand, B. Altincicek, C. Weidemeyer, M. Hintz, I. Turbachova, M. Eberl, J. Zeidler, H.K. Lichtenthaler, et al. 1999.

Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science*. 285:1573–1576.
Jones, T.C., S. Yeh, and J.G. Hirsch. 1972. The interaction between *Toxoplasma gondii* and mammalian cells. I. Mechanism of entry and intracellular fate of the parasite. *J. Exp. Med.* 136:1157–1172.
Kalayoglu, M.V., G.S. Miranpuri, D.T. Golenbock, and G.I. Byrne. 1999. Characterization of low-density lipoprotein uptake by murine macrophages exposed to *Chlamydia pneumoniae*. *Microb. Infect.* 1:409–418.
Klausner, R.D., J.G. Donaldson, and J. Lippincott-Schwartz. 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116:1071–1080.
Kobayashi, T., M.H. Beuchat, M. Lindsay, S. Frias, R.D. Palmiter, H. Sakuraba, R.G. Parton, and J. Gruenberg. 1999. Late endosomes membranes rich in lysobisphosphatidic acid regulate cholesterol transport. *Nat. Cell Biol.* 1:113–118.
Lange, Y., and T.L. Steck. 1996. The role of intracellular cholesterol transport in cholesterol homeostasis. *Trends Cell Biol.* 6:205–208.
Leonard, D.A., and H.W. Chen. 1987. ATP-dependent degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in permeabilized cells. *J. Biol. Chem*. 262:7914–7919.
Lindsay, D.S., R.R. Mitscher, M.A. Toivio-Kinnucan, S.J. Upton, J.P. Dubey, and B.L. Blagburn. 1993. Association of host cell mitochondria with developing *Toxoplasma gondii* tissue cysts. *Am. J. Vet. Res.* 54:1663–1667.
Lingelbach, K., and K.A. Joiner. 1998. The parasitophorous vacuole membrane surrounding *Plasmodium* and *Toxoplasma*: an unusual compartment in infected cells. *J. Cell Sci.* 111:1467–1475.
Liscum, L., and J.J. Klansek. 1998. Niemann-Pick disease type C. *Curr. Opin. Lipidol.* 9:131–135.
Liscum, L., and J.R. Faust. 1989. The intracellular transport of low density lipoprotein–derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one. *J. Biol. Chem*. 264:11796–11806.
Liscum, L., and N.J. Munn. 1999. Intracellular cholesterol transport. *Biochim. Biophys. Acta*. 1438:19–37.
Liscum, L., R.M. Ruggiero, and J.R. Faust. 1989. The intracellular transport of low density lipoprotein–derived cholesterol is defective in Niemann-Pick type C fibroblasts. *J. Cell Biol.* 108:1625–1636.
Luskey, K.L., J.R. Faust, D.J. Chin, M.S. Brown, and J.L. Goldstein. 1983. Amplification of the gene for 3-hydroxy-3-methylglutaryl coenzyme A reductase, but not for the 53-kDa protein, in UT-1 cells. *J. Biol. Chem*. 258:8462–8469.
McFadden, G.I., and D.S. Roos. 1999. Apicomplexan plastids as drug targets. *Trends Microbiol.* 7:328–333.
McFarlane, A.S. 1958. Efficient trace-labelling of proteins with iodine. *Nature.* 182:53.
McGookey, D.J., and G.W. Anderson. 1983. Morphological characterization of the cholesteryl ester cycle in cultured mouse macrophage foam cells. *J. Cell Biol.* 97:1156–1165.
Melo, E.J.T., T.U. Carvalho, and W. de Souza. 1992. Penetration of *Toxoplasma gondii* into host cells induces changes in the distribution of mitochondria and the endoplasmic reticulum. *Cell Struct. Funct.* 17:311–317.
Melo, E.J.T, and W. de Souza. 1996. Pathway of C6-NBD-ceramide on the host cell infected with *Toxoplasma gondii. Cell Struct. Funct.* 21:47–52.
Metherall, J.E., J.L. Goldstein, K.L. Luskey, and M.S. Brown. 1989. Loss of transcriptional repression of three-sterol-regulated genes in mutant hamster cells. *J. Biol. Chem*. 264:15634–15641.
Misumi, Y., Y. Misumi, K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara. 1986. Novel blockade by Brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem*. 261:11398–11403.
Monteiro Cintra, W., and W. de Souza. 1985. Distribution of intramembranous particles and filipin-sterol complexes in the cell membranes of *Toxoplasma gondii*. *Eur. J. Cell Biol.* 37:63–69.
Montgomery, R.R., P. Webster, and I. Mellman. 1991. Accumulation of indigestible substances reduces fusion competence of macrophage lysosomes. *J. Immunol.* 147:3087–3095.
Mordue, D.G., S. Hakansson, I. Niesman, and L.D. Sibley. 1999. *Toxoplasma gondii* resides in a vacuole that avoids fusion with host cell endocytic and exocytic vesicular trafficking pathways. *Exp. Parasitol.* 92:87–99.
Mukherjee, S., X. Zha, I. Tabas, and F.R. Maxfield. 1998. Cholesterol distribution in living cells: fluorescence imaging using dehydroergosterol as a fluorescent analog. *Biophys. J.* 75:1915–1925.
Nakaar, V., B.U. Samuel, E.O., and K.A. Joiner. 1999. Targeted reduction of nucleoside triphosphate hydrolase by antisense RNA inhibits *Toxoplasma gondii* proliferation. *J. Biol. Chem*. 274:5083–5087.
Neufeld, E.B., A.M. Cooney, J. Pitha, E.A. Dawidowicz, N.K. Dwyer, P.G. Pentchev, and E.J. Blanchette-Mackie. 1996. Intracellular trafficking of cholesterol monitored with a cyclodextrin. *J. Biol. Chem*. 271:21604–21613.
Ohashi, M., M. Murata, and S. Ohnishi. 1992. A novel fluorescence method to monitor the lysosomal disintegration of low density lipoprotein. *Eur. J. Cell Biol.* 59:116–126.
Pentchev, P.G., H.S. Kruth, M.E. Comly, J.D. Butler, M.T. Vanier, D.A. Wenger, and S. Patel. 1986. Type C Niemann-Pick disease. A parallel loss of regulatory responses in both the uptake and esterification of low density lipoprotein–derived cholesterol in cultured fibroblasts. *J. Biol. Chem*. 261: 16775–16780.
Porchet-Hennere, E., and G. Torpier. 1983. Relations entre *Toxoplasma* et sa cellule-hote. *Protistologica.* 19:357–370.
Poumay, Y., and M.F. Ronveaux-Dupal. 1984. Rapid preparative isolation of concentrated low-density lipoproteins and of lipoprotein-deficient serum using vertical rotor gradient ultracentrifugation. *J. Lipid Res.* 26:1476–1480.
Roff, C.F., E. Goldin, M.E. Comly, A. Cooney, A. Brown, M.T. Vanier, S.P.F. Miller, R.O. Brady, and P.G. Pentchev. 1991. Type C Niemann-Pick disease: use of hydrophobic amines to study defective cholesterol transport. *Dev. Neurosci.* 13:315–320.
Roos, D.S., R.G.K. Donald, N.S. Morissette, and A.L.C. Moulton. 1994. Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Methods Cell. Biol.* 45:27–63.
Schwab, J.C., C.J.M. Beckers, and K.A. Joiner. 1994. The parasitophorous vacuole membrane surrounding intracellular *Toxoplasma gondii* functions as a molecular sieve. *Proc. Natl. Acad. Sci. USA*. 91:509–513.
Sibley, L.D., I.R. Niesman, S.F. Parmley, and M.F. Cesbron-Delauw. 1995. Regulated secretion of multilamellar vesicles leads to formation of a tubulovesicular network in host-cell vacuoles occupied by *Toxoplasma gondii. J. Cell Sci.* 108:1669–1677.
Sibley, L.D., E. Weidner, and J.L. Krahenbulh. 1985. Phagosome acidification blocked by intracellular *Toxoplasma gondii. Nature.* 315:416–419.
Silverman, J.A., M.L. Haynes, B.J. Luft, and K.A. Joiner. 1997. Characterization of anti-*Toxoplasma* activity of SDZ 215-918, a cyclosporin derivative lacking immunosuppressive and peptidyl-prolyl-isomerase-inhibiting activity: possible role of a P glycoprotein in *Toxoplasma* physiology. *Antimicrob. Agents Chemother.* 41:1859–1866.
Sinai, A.P., and K.A. Joiner. 1997. Safe haven: the cell biology of nonfusogenic pathogen vacuoles. *Annu. Rev. Microbiol.* 51:415–462.
Sinai, A.P., P. Webster, and K.A. Joiner. 1997. Association of host cell endoplasmic reticulum and mitochondria with the *Toxoplasma gondii* parasitophorous vacuole membrane: a high affinity interaction. *J. Cell Sci.* 110:2117–2128.
Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, and D.C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem*. 150:76–85.
Sokol, J., J. Blanchette-Mackie, H.S. Kruth, N.K. Dwyer, L.M. Amende, J.D. Butler, E. Robinson, S. Patel, R.O. Brady, M.E. Comly, et al. 1988. Type C Niemann-Pick disease. Lysosomal accumulation and defective intracellular mobilization of low density lipoprotein. *J. Biol. Chem*. 263:3411–3417.
Speer, C.A., S. Clark, and J.P. Dubey. 1998. Ultrastructure of the oocysts, sporocysts, and sporozoites of *Toxoplasma gondii. J. Parasitol.* 84:505–512.
Steinman, R.M., S.E. Brodie, and Z.A. Cohn. 1976. Membrane flow during pinocytosis. *J. Cell Biol.* 68:665–687.
Torii, S., B. Tomohiro, T. Watanabes, Y. Ikehara, K. Murakami, and K. Nakayama. 1995. Cytotoxicity of Brefeldin A correlates with its inhibitory effect on membrane binding of COP coat proteins. *J. Biol. Chem*. 270:11574–11580.