

Transepithelial Migration of *Toxoplasma gondii* Is Linked to Parasite Motility and Virulence

Antonio Barragan^{1,2} and L. David Sibley¹

¹Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110

²Center for Infectious Medicine, Karolinska Institutet, Department of Medicine, Huddinge University Hospital, SE-141 86 Stockholm, Sweden

Abstract

After oral ingestion, *Toxoplasma gondii* crosses the intestinal epithelium, disseminates into the deep tissues, and traverses biological barriers such as the placenta and the blood-brain barrier to reach sites where it causes severe pathology. To examine the cellular basis of these processes, migration of *T. gondii* was studied in vitro using polarized host cell monolayers and extracellular matrix. Transmigration required active parasite motility and the highly virulent type I strains consistently exhibited a superior migratory capacity than the nonvirulent type II and type III strains. Type I strain parasites also demonstrated a greater capacity for transmigration across mouse intestine ex vivo, and directly penetrated into the lamina propria and vascular endothelium. A subpopulation of virulent type I parasites exhibited a long distance migration (LDM) phenotype in vitro, that was not expressed by nonvirulent type II and type III strains. Cloning of parasites expressing the LDM phenotype resulted in substantial increase of migratory capacity in vitro and in vivo. The potential to up-regulate migratory capacity in *T. gondii* likely plays an important role in establishing new infections and in dissemination upon reactivation of chronic infections.

Key words: apicomplexa • dissemination • invasion • epithelium • barrier

Introduction

Toxoplasma gondii is an important opportunistic pathogen causing disseminated congenital infections in the developing fetus (1), severe neurological complications in immunocompromised individuals (2), and ocular pathology in otherwise healthy individuals (3). Worldwide genotypic analysis of *T. gondii* isolates has identified a population structure consisting of three widespread clonal lineages, termed type I, II, and III (4). Although type II strains have a higher overall prevalence in human infections, type I strains are over represented in studies of congenital toxoplasmosis (4, 5), and a perfect correlation between acute virulence in mice and type I strains exists (6).

T. gondii is an obligate intracellular parasite of the phylum Apicomplexa. It enters a wide range of host cells by active penetration, a rapid process that is dependent on the actin-myosin cytoskeleton of the parasite, and does not rely on the host cell machinery for uptake (7, 8). Once inside the host cell, the parasite resides in a vacuole that avoids fu-

sion with host cell endocytic and exocytic vesicular trafficking pathways (9, 10). Apicomplexan parasites lack cilia or flagella and their mode of locomotion, termed gliding motility, relies on the actin-myosin motor of the parasite (8). Gliding motility also plays determinant roles in infections by other apicomplexan that are important causative agents of human and animal diseases, e.g., Plasmodium (11), Cryptosporidium (12), and Eimeria (13).

In natural oral infections, *Toxoplasma* initially crosses the intestinal epithelium, disseminates into the deep tissues, and traverses biological barriers such as the placenta and the blood-brain barrier to reach immunologically privileged sites where it causes the most severe pathology. Histopathological studies are consistent with invasion of a variety of cell types in the intestine, including intra-epithelial leukocytes (14), and with rapid hematogenous spread of parasites (15–17). Once in the circulation, it remains unknown whether *Toxoplasma* disseminates as free, extracellular parasites or if it resides inside infected leukocytes, which are permissive hosts that can cross many biological barriers.

Previous study of migration by *Toxoplasma* has relied on gliding assays on coated surfaces in vitro (8) and focused on the role of gliding motility for host cell invasion. The rapid

Address correspondence to L. David Sibley, Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Phone: 314-362-8873; Fax: 314-362-3203; E-mail: sibley@borcim.wustl.edu

spread of primary infections beyond the gut (15–17) led us to hypothesize that this mode of locomotion plays a role in another crucial step in infection, i.e., the ability to disseminate in the host by crossing nonpermissive biological barriers. Using *in vitro* and *ex vivo* models for migration, we now demonstrate that *T. gondii* has substantial capacity to cross epithelial barriers. Transmigration is correlated with virulence and likely contributes to dissemination during *in vivo* infections.

Materials and Methods

Parasites and Host Cell Lines. Toxoplasma tachyzoites were maintained by serial 2-d passage in human foreskin fibroblast (HFF)* cell monolayers as described previously (7). Parasite strains used in this study include RH, RH-*lacZ* (8), PTG, and the green fluorescent protein-expressing lines PTG-GFPS65T and RH-GFPS65T, provided by Dr. K. Kim, Albert Einstein College of Medicine, Bronx, NY (18). The various strains and clinical isolates of genotype I, II, and III (4, 6) used in this study have been deposited at the American Type Culture Collection. Viability of parasites was determined by plaque formation on HFF monolayers (19) and was defined as the ratio of the number of intracellular replicating parasites/number of parasites inoculated. Madin Darby Canine Kidney cells (MDCK, CCL-34) were obtained from the American Type Culture Collection and cultured according to guidelines.

Reagents and Antibodies. Cytochalasin D was obtained from Calbiochem. Low gelling temperature agarose was obtained from Sigma-Aldrich. Matrigel® was obtained from Becton Dickinson. For detection of parasites in tissue sections and gliding trails, polyclonal rabbit anti-toxoplasma tachyzoite serum or mAb DG52 directly conjugated to Alexa 488 fluorophore (Molecular Probes Inc.) were used. Goat anti-rabbit antibodies conjugated to Alexa 488 or to Alexa 594 (Molecular Probes Inc.) were used as secondary antibodies.

Migration Assays on HFF Monolayers Under Agarose. Freshly harvested parasites were plated onto HFF monolayers to yield an infection density of ~1 infected HFF cell per 4 fields of vision ($\times 20$). After 24 h, the monolayer was washed twice and the media substituted by 0.75% low-melting temperature agarose in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, and 20 $\mu\text{g}/\text{ml}$ gentamicin. Assessment of migration under agarose was performed ~18–24 h after egress of parasites from the initial host cell to allow migration and invasion into new host cells. The monolayers were examined using a ZEISS Axioscope equipped for phase-contrast and epifluorescence microscopy. Foci were photographed randomly and distances migrated by individual, intracellular replicating parasites were assessed using image analysis software by measurement of the shortest distance between the original focus of lysis (host cell) and the intracellular parasites (Openlab, Improvision Inc.; and Adobe Photoshop version 5.5, Adobe Systems Inc.).

Cloning by Micromanipulation. Parasites migrating a distance >110 μm on HFF monolayers were cloned using micromanipulation technique. Glass micropipettes (Stoelting) were stretched to have a distal diameter of 25 μm and an Eppendorf Micromanipulator 1570 system and ZEISS Axioscope equipped for phase

contrast and epifluorescence microscopy were used. Individual vacuoles containing intracellular replicating parasites located >110 μm from the original focus of egress were picked and plated onto HFF monolayers in 96-well plates.

Transmigration Assays In Vitro. MDCK cells were plated onto 24-well transwell filters (3 μm pore; Becton Dickinson) and grown for at least 7 d to form a polarized monolayer (resistance >2,500 Ω/cm^2 using an Ohmmeter; Millipore). Freshly egressed parasites ($0.5–1 \times 10^6$) were added to the upper well of the Transwell system and incubated at 37°C at indicated times. Alternatively, a layer of 18–22 μm of Matrigel® mixed with an equal volume of DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, and 20 $\mu\text{g}/\text{ml}$ gentamicin was added on top of transwell filter and allowed to solidify. Transmigration of parasites was quantified by colorimetric detection of β -galactosidase activity immediately after incubation as described previously (8) or by visual counting of parasite vacuoles, expressed as CFU in the underlying HFF monolayer using epifluorescence microscopy (green fluorescent protein [GFP]-transfected parasites) or phase-contrast microscopy. Visual counting of vacuoles with intracellular replicating parasites was performed before lysis of the host cells, i.e., 24 to 48 h after invasion. For inhibition of motility, parasite suspensions were pretreated for 10 min at room temperature with 1 μM cytochalasin D and incubated in the presence of 1 μM cytochalasin D during the assay. For heat inactivation, parasites were incubated 30 min at 50°C before use.

Transmigration Assays Ex Vivo. Adult female CD1 mice (Charles River Laboratories) were used to obtain ileum that was extensively washed and placed, luminal side up, into a modified Ussing 2-chamber system. Both the mucosal and serosal reservoirs of the chamber contained DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, and 20 $\mu\text{g}/\text{ml}$ gentamicin (pH 7.4). The buffer was gassed (21% O₂–5% CO₂) continuously during the experiment and kept at 37°C. Parasites were introduced in the upper chamber and incubated at 37°C for 8 h. Samples were fixed in 3.7% formaldehyde in PBS, paraffin-embedded, and sections processed for immunohistochemistry as described below. Penetration of parasites to the different histological layers was assessed using phase-contrast or epifluorescence microscopy by scoring parasites in the epithelial cell layer and subepithelial cells layers including the lamina propria, rich in connective tissue, and the submucosa, where the post-capillary circulation is found. All protocols involving animals were approved by the Animal Studies Committee of Washington University.

Immunohistochemistry. Serial sections from mouse ileum were deparaffinized, rehydrated, and exposed to primary antibody (polyclonal rabbit anti-toxoplasma tachyzoite serum). Detection was provided using a Vectastain Elite ABC HRP kit, visualized by diaminobenzidine tetrahydrochloride (Vector Laboratories), and counterstained with hematoxylin. In immunofluorescence (IF) experiments, sections were exposed to a polyclonal rabbit anti-toxoplasma serum or mouse mAb DG52 followed by goat anti-rabbit conjugated to Alexa 594 or goat anti-mouse conjugated to FITC, respectively (Molecular Probes). Fluorescein-conjugated Wheat Germ Agglutinin (Vector Laboratories) was used as a surface cell marker when indicated, and nuclear staining was with 4',6 diamidino-2-phenylindole (DAPI; Vector Laboratories).

Parasite Dissemination In Vivo. Adult female CD1 mice (Charles River Laboratories) were challenged intra-peritoneally with 10⁴ parasites. Parasite numbers in the spleen were determined as described by Mordue et al. (20). Briefly, mice were killed and the spleen was extracted and homogenized under conditions

*Abbreviations used in this paper: DAPI, 4',6 diamidino-2-phenylindole; HFF, human foreskin fibroblast; IF, immunofluorescence; LDM, long distance migration; MDCK, Madin Darby Canine Kidney.

that did not affect parasite viability. The number of viable parasites in the tissue was determined by plaque formation on HFF monolayers.

Gliding Assays. Gliding assays were performed as described (8). Briefly, glass chamber slides (Lab-Tek; Nunc) were coated by incubation in 50% fetal bovine serum in PBS (pH 7.4) for 1 h at 37°C. Recently egressed parasites were added to the chamber slides and incubated at 37°C for 30 min before fixation in 3.7% formalin-PBS. Detection of trails was performed using mAb DG52 directly conjugated to Alexa 488 fluorochrome. For time-lapse video microscopy, parasites were allowed to glide on pre-coated glass-bottom microwells and motion was recorded using a ZEISS Axioscope, an ORCA-ER digital camera (Hamamatsu Photonics), and image capture software Openlab version 3.02 (Improvision Inc.).

Statistical Analyses. Statistical analyses were performed using the Statistical Analysis Systems (SAS, version 8.1; SAS Institute).

Results

The Migratory Capacity of *Toxoplasma* on Host Cell Monolayers Is Strain Specific. To determine the capacity of *Toxoplasma* to migrate away from a focus of infection, the

spread of parasites under soft agarose was measured in vitro. Quantitative differences were observed between the ability of the virulent type I strain RH and the nonvirulent type II strain PTG to migrate in this assay. While PTG parasites always migrated <70 μm, a small but reproducible subpopulation of the RH strain distinguished itself by migrating >50% further than the maximum distance observed for PTG (i.e., >110 μm; Fig. 1, A and B, and Table I). Parasites exhibiting the long distance migration phenotype, termed LDM, were cloned by micromanipulation (Fig. 1, B and B', see arrows). When tested in the HFF spreading assay, these clones generated exhibited a higher frequency of LDM parasites, reaching levels ≥20% of the parasite population (Fig. 1, C and C', and Table I). In contrast, type II PTG parasites cloned from the periphery of an infection focus (~70 μm migration) did not exhibit increased migratory capacity, i.e., no parasites migrated distances >70 μm and no shift in the distribution of migrated distances was observed (data not shown). Collectively, these results show (a) important differences in migration exist between the type I strain RH and the type II

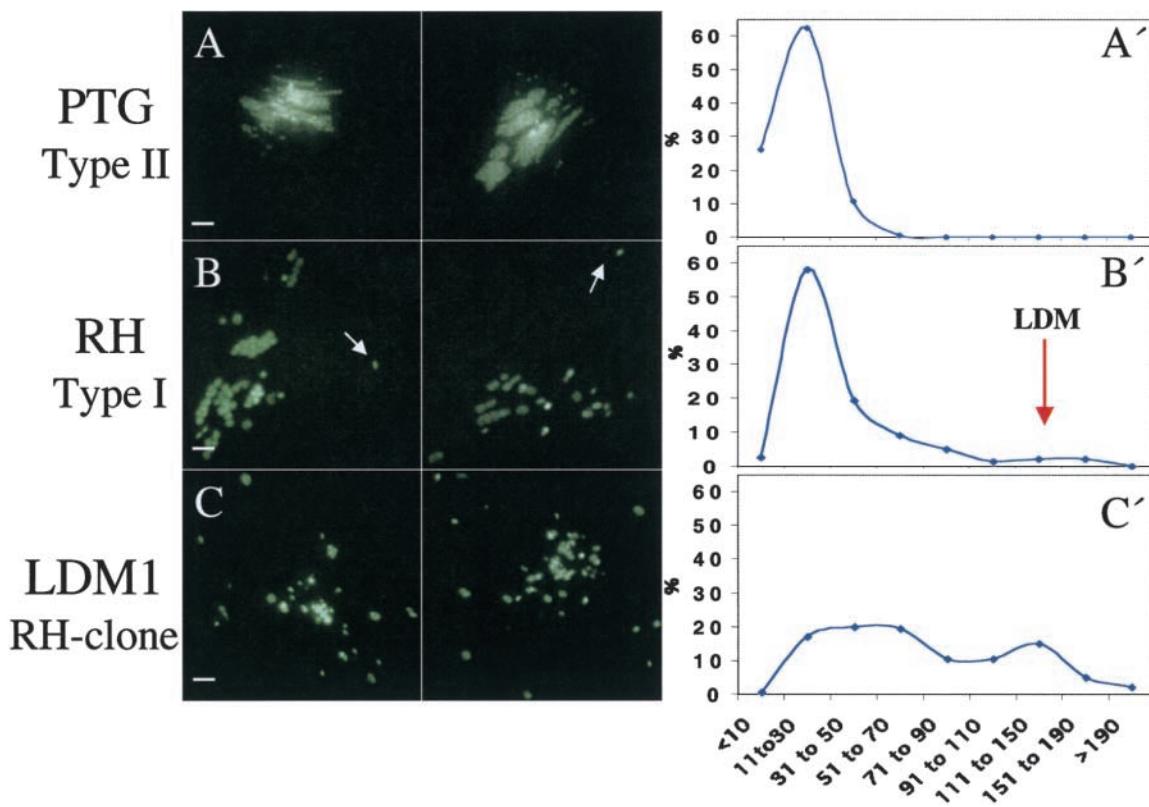


Figure 1. Characteristic migration patterns of the type I (RH) and the type II (PTG) strains and the LDM1 clonal line. Parasites were cultured on HFF monolayers with an overlay of 0.75% agarose in culture medium as indicated in Materials and Methods. Migration was assessed 24 h after egress from the original infected cell. Characteristic dispersion patterns of PTG strain (A) and RH strain (B, arrows indicate parasites migrating >110 μm) and the clone LDM1 (C), respectively. Scale bar = 20 μm. Type I (RH) parasites migrating >110 μm were termed LDM and surpassed by >50% the longest migrated distances by type II (PTG) parasites. Graphics show the relative distribution of migrated distances (μm) by different parasite populations calculated using image analysis software as described in Materials and Methods (PTG [A'], n = 306; RH [B'], n = 296; LDM1 [C'], n = 303). The y-axis indicates the relative portion (%) of each category related to the total of the population examined. The distribution of distances migrated by individual parasites was analyzed with the Shapiro-Wilk test of normality, showing that the PTG population approached a normal distribution ($W = 0.94$; $P \leq 0.0001$), whereas RH ($W = 0.81$; $P \leq 0.0001$), and LDM1 ($W = 0.76$; $P \leq 0.0001$) were not normally distributed.

Table I. Migratory Characteristics of the Type I (RH) and Type II (PTG) *T. gondii* Strains and the LDM1 Clone

Strain	Migration away from foci % > 110 μm ^a	Transmigration across polarized MDCK cells		Migration through ECM	Gliding on substrate		Ex vivo penetration
		Parasites traversing ^b	Parasites traversing ^c		Mean distance ^d	Max distance ^e	
Type I (RH)	2.7 (1.2)	1.333 (0.211)	0.892 (0.245)	4.79 (0.30)	32	2.2 (1.18)	0.8 (0.38)
Type II (PTG)	0 (0)	0.025 (0.006)	0.062 (0.023)	1.56 (0.44)	9	0.38 (0.37)	0 (0)
LDM1 (RH clone)	22.1 (5.7)	3.14 (0.023)	ND	5.36 (0.32)	33	ND	ND

Values represent mean (\pm SD) from three or four independent experiments. ND, not done.

^aMean percentage of parasites migrating on HFF monolayers.

^bMean number of parasites $\times 10^{-3}$ penetrating MDCK monolayers.

^cMean number of parasites $\times 10^{-3}$ migrating across extracellular matrix (ECM).

^dTrail lengths measured in number of parasite body lengths (5–7 μm).

^eTrail lengths measured in number of parasite body lengths (5–7 μm).

^fParasite penetration in mouse ileum ex vivo scored as percentage of the total population. The type I (RH) and the type II (PTG) strains exhibited significant differences in penetration to the lamina propria ($P \leq 0.05$, Student's *t* test) and to the submucosa ($P \leq 0.005$).

strain PTG, (b) the presence of at least two distinct populations with different migratory behavior in the type I strain RH, and (c) migration can be up-regulated in the virulent RH strain.

Transmigration of Toxoplasma Across Polarized Cell Monolayers and Extracellular Matrix Is Strain-specific and Requires Active Motility. During in vivo infections, Toxoplasma crosses epithelial barriers by an unknown process. To address the question whether Toxoplasma tachyzoites are capable of actively crossing cellular barriers, migration across

polarized MDCK cell monolayers or extracellular matrix in a Transwell system was tested in vitro. Parasites readily crossed the polarized monolayer in a time-dependent fashion with type I strain RH being 10–100-fold more effective than type II strain PTG (Fig. 2, A and C, and Table I). The increased migratory capacity of RH versus PTG was confirmed using extracellular matrix (Matrigel®), where transit also required active motility on the part of the parasite (Fig. 2, C and D, and Table I). Transmigration across MDCK monolayers was totally inhibited by heat inactiva-

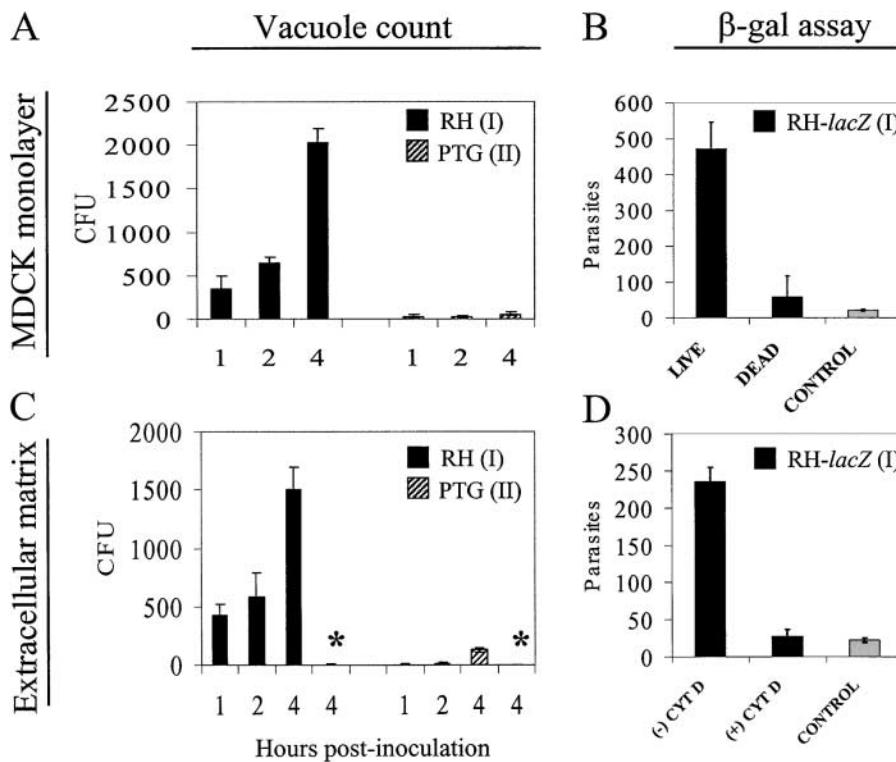


Figure 2. Transmigration across polarized cell monolayers and extracellular matrix by the type I strain (RH) and the type II strain (PTG). Transmigration assays across polarized MDCK monolayers (A and B) or Matrigel® (C and D) were performed as indicated in Materials and Methods. Quantification was done by counting CFU (intra-cellular vacuoles containing parasites) in an underlying HFF monolayer (A and C) or by colorimetric detection of β -galactosidase (β -gal) activity immediately after transmigration using the parasite strain RH-lacZ (B and D). Results are means from one representative experiment done in triplicate. (A) Kinetics of transmigration across polarized MDCK cell monolayers revealed differences between type I (RH) and type II (PTG) strains. (B) Heat inactivation (Dead) of type I (RH-lacZ) strain resulted in inhibition of transmigration across polarized MDCK cell monolayers. Parasite culture supernatant was used as control. (C) Kinetics of transmigration across matrigel® showed differences between type I (RH) and type II (PTG) strains. Transmigration was totally inhibited in the presence of 1 μM cytochalasin D (*). (D) Inhibition of migration across Matrigel® by 1 μM cytochalasin D (CYT D). Parasite culture supernatant was used as control.

tion of parasites (Fig. 2 B) and migration across extracellular matrix was blocked by cytochalasin D treatment (Fig. 2, C and D), indicating it depends on active motility by the parasite.

Upregulation of Migration Also Enhances Traversal of Biological Barriers. To examine whether increased migratory capacity of LDM-parasites on HFF monolayers would also confer increased transmigratory capacity, LDM-clones were assessed for their ability to cross-polarized monolayers. For comparison, we analyzed a clone called LDM1 that had an enhanced capacity for long distance migration on HFF monolayers where up to 20% of parasites migrated >110 μm from foci of infection (Fig. 3 A). The LDM1 clone also exhibited an enhanced capacity to traverse MDCK cell monolayers *in vitro* relative to the type I RH strain from which it was derived (Fig. 3 B, and Table I). As described above, the nonvirulent strain PTG did not display a subpopulation of LDM parasites (Fig. 3 A), and showed significantly lower levels of transmigration (Fig. 3 B). The differences in transmigration were not due to differences in viability or infectivity between the strains, which varied by <20% as shown by plaque formation on HFF monolayers (Fig. 3 C). We conclude from these findings that (a) *Toxoplasma* is able to actively cross polarized cell monolayers and extracellular matrix, (b) these abilities are greatly enhanced in the virulent type I (RH) strain, and (c) transmigratory capacity of type I strains can be up-regulated.

Gliding Motility Features Are Conserved But Quantitative Differences Exist Between Strains. To investigate whether the difference in migration observed between strains was

due to differences in motility, the strains were compared using well established gliding assays (8). Significant quantitative differences in gliding distance were observed between the RH strain and the PTG strain (Fig. 3 D, and Table I). Furthermore, the frequency of gliding parasites was significantly higher for RH (1.3 trails/adhered parasite, SD = 0.20) than PTG (0.047 trails/adhered parasite, SD = 0.012). Time-lapse video microscopy of the PTG strain confirmed that the three types of movement previously described for gliding motility in RH, i.e. upright twirling, circular and helical gliding, were present (data not shown). Thus, the lower overall motility of PTG is not due to qualitative differences in motility. Importantly, the LDM1 clone was only slightly more motile in this *in vitro* assay when compared with the RH strain (Fig. 3 D). Thus, while the enhanced migration ability of type I strains may be due to greater motility, this cannot account for the increase in transmigration seen in LDM clones.

Ex Vivo Transmigration Is Strain Specific. During *in vivo* infection, *Toxoplasma* crosses the intestinal epithelium to lead to disseminated infection. The capacity of virulent and nonvirulent strains to penetrate tissue was assessed using mouse intestine in a modified Ussing two-chamber system. Parasites readily invaded the intestinal mucosa, where a majority of them (>96%) took up residence in epithelial cells (Fig. 4). However, significantly more type I strain RH parasites penetrated into deeper tissue layers than type II PTG parasites (Table I). RH parasites were more successful in entering the lamina propria, penetrating the submucosa, and in some cases were able to reach the vascular endothelium (Fig. 4 D). The notable capacity for transmigration of

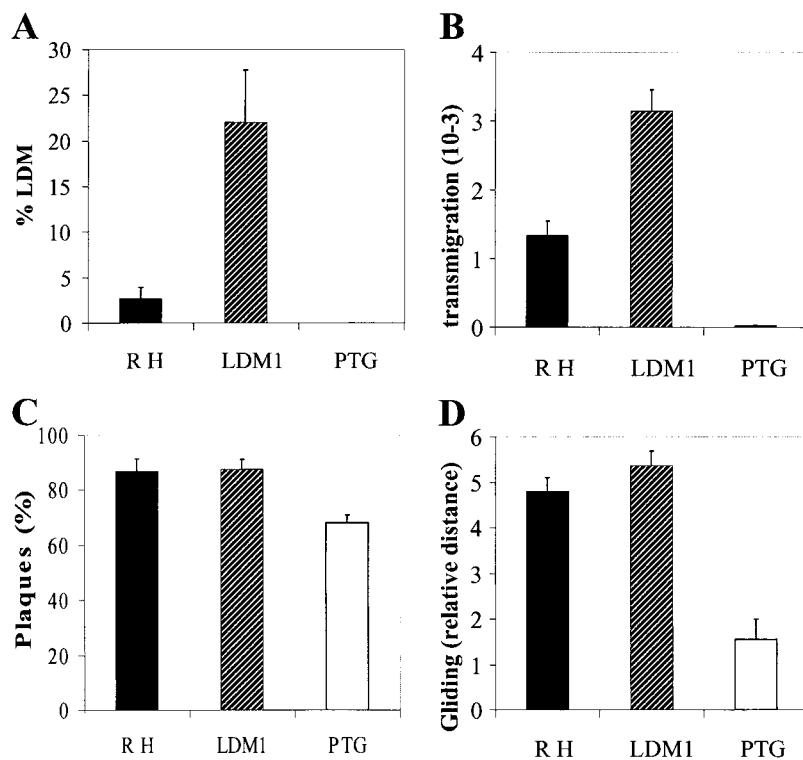


Figure 3. Invasion, motility, and migratory characteristics of the RH (type I) and PTG (type II) strains and the RH-derived clone LDM1. (A) Enhanced long distance migration was observed in the LDM1 clone vs. RH, but was absent in the PTG strain. Frequency of parasites migrating >110 μm (LDM) on an HFF monolayer under agarose as indicated in Materials and Methods. Results are mean (\pm SD) from three independent experiments. (B) Significantly enhanced transmigration capacity was observed in the clone LDM1 vs. RH ($P \leq 0.005$, Student's *t* test). Frequency of parasites (\pm SD) transmigrating across polarized MDCK cell monolayers was assessed as indicated in Materials and Methods. (C) Plaques assays on HFF monolayers showed very similar viability for RH and LDM1, and a slightly reduced viability for PTG. Plaques assays were evaluated at 5–7 d after inoculation and the number of plaques generated was expressed as a percentage of parasites initially added. Average results are shown from three independent experiments (\pm SD). (D) Gliding of the RH strain was similar to that of the LDM1, but significantly shorter trail lengths were observed for PTG vs. RH ($P \leq 0.0005$). Gliding trail lengths were measured as relative parasite body lengths (5–7 μm) and the average trail length from three independent experiments (\pm SD) is shown.

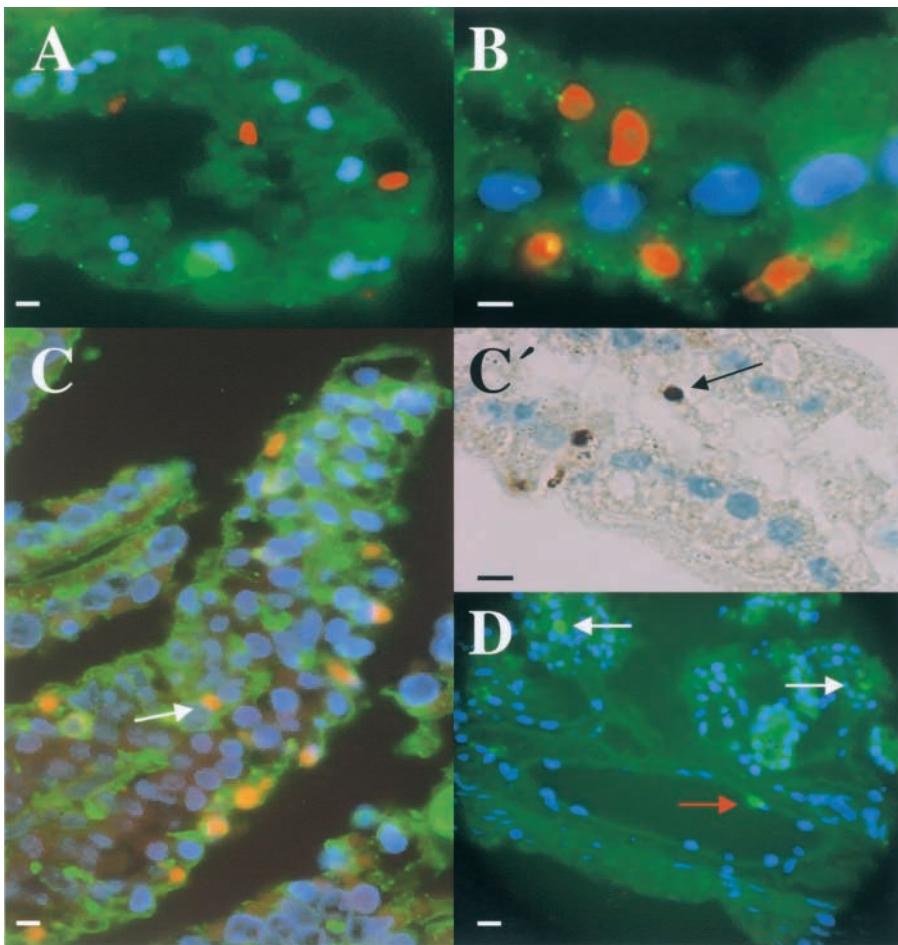


Figure 4. Ex vivo transmigration of *T. gondii* (RH) across intestinal mucosa. Transmigration assays were performed on mouse ileum in a modified Ussing 2-chamber system as described in Materials and Methods. (A) Section of distal villus from mouse ileum showing tachyzoites (red) invading epithelial cells. Immunohistochemistry was performed with polyclonal rabbit anti-RH serum and goat anti-rabbit antibody conjugated to Alexa 594. Epithelial cells were counterstained with fluorescein-conjugated wheat germ agglutinin and nuclear staining was with DAPI. Scale bar = 5 μ m. (B) Close up of epithelial cell invasion by RH tachyzoites (red). In the top part of the micrograph is the apical side of the epithelial cell layer and the lower part shows the limit with the basal lamina (lamina propria). IF was performed as in A. Scale bar = 5 μ m. (C and C') Penetration of tachyzoites to subepithelial cell layers in mouse ileum. (C) Arrows indicate parasite vacuoles in the lamina propria. IF was performed as in A. Scale bar = 5 μ m. (C') Intracellular parasite vacuoles were stained with diaminobenzidine tetrahydrochloride as indicated in Materials and Methods. Epithelial cells were counterstained with hematoxylin. Scale bar = 10 μ m. (D) Penetration of RH tachyzoites (green) to the submucosa (white arrows) and invasion of endothelial cells in the vasculature (red arrow). IF was performed with polyclonal rabbit anti-RH serum and goat anti-rabbit antibody conjugated to Alexa 488. Nuclear staining was with DAPI. Scale bar = 20 μ m.

the RH strain thus enables the parasite to gain rapid transfer across the intestinal epithelium.

Upregulation of Migratory Capacity Results in Faster Dissemination In Vivo. To investigate whether the differences in migration observed between LDM clones and the parental line RH, resulted in differences in dissemination during acute infection, parasites were injected intraperitoneally and the number of viable parasites in the spleen was monitored daily. Both LDM1 and RH parasites were found in the circulation and the spleen as early as 12 h after infection (data not shown). Importantly, LDM1 parasites reached the spleen in higher numbers shortly after the challenge, showing more efficient dissemination at days 2 and 3 (Fig. 5). Despite reaching the spleen more slowly the parental line RH reached similar levels of parasite burden as LDM1 from day 4–5 after infection, suggesting the maximum level of parasite growth is limited by host factors. The extreme lethality of the parental line RH ($LD_{100} = 1$) precluded analysis of differences in mortality. Nevertheless, it is clear that upregulation of the LDM-phenotype results in more rapid dissemination in vivo.

Type I Strains Exhibit a Superior Migratory Capacity Than Type II and Type III Strains. *T. gondii* strains are highly clonal and distinct biological traits, such as virulence, are characteristic of each clonal type. To investigate if our ob-

servations on differences in migration could be extended to other type I, II, and III strains, 17 different strains and clinical isolates were tested in migration assays. In every case, type I strains showed significantly greater transmigration than types II and III, crossing the barrier on average with up to three orders of magnitude greater efficiency (Fig. 6). Importantly, parasites migrating $>110 \mu$ m (LDM-phenotype) were only found in type I strains, extending our findings on the RH strain to all type I strains tested. In contrast, no type II or type III strain exhibited subpopulations of parasites that migrated $>110 \mu$ m (LDM). Collectively, these results demonstrate a strong association of the virulence trait (type I strains) with increased migratory capacity.

Discussion

During in vivo infections, *Toxoplasma* crosses epithelial barriers to gain access to deeper tissues including the brain, the placenta and the retina. We now demonstrate that *Toxoplasma* tachyzoites use active motility to cross polarized cell layers and extracellular matrix. Using in vitro and ex vivo systems that mimic biological barriers in vivo, we show that the virulent type I strains consistently exhibit a superior migratory capacity compared with the nonvirulent type II and type III strains. The potential for this capacity

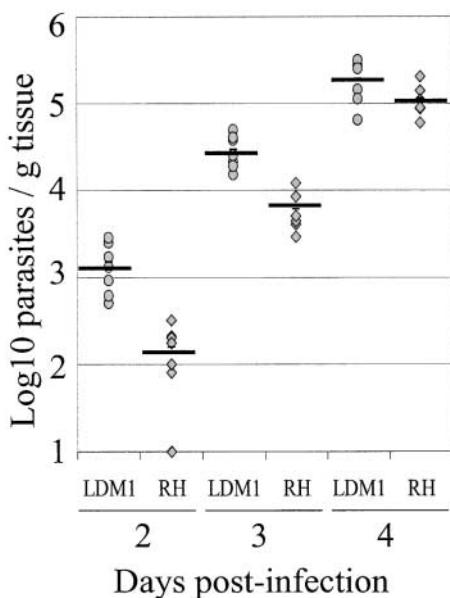


Figure 5. Dissemination of RH (type I) and LDM1(RH clone) parasites *in vivo*. The number of viable parasites retrieved in the spleens of infected mice is plotted separately and the means are indicated by the horizontal bars. Parasite tissue burden was quantified daily after a challenge of 10^6 parasites intraperitoneally as indicated in Materials and Methods. Significantly enhanced dissemination was observed in the clone LDM1 vs. RH at day 2 after infection ($P \leq 0.005$, Student's *t* test) and at day 3 ($P \leq 0.001$), whereas, by day 4, differences in parasite load were nonsignificant ($P \geq 0.05$). The results shown are the mean of two independent experiments consisting of three to four mice per time point.

to be up-regulated, and its association with virulence, suggests that active parasite migration is an important component of dissemination during toxoplasmosis.

Our studies establish that (a) *Toxoplasma* is able to actively migrate across polarized cell monolayers and extracellular matrix *in vitro* and across intestinal epithelium *ex vivo*. Furthermore, we demonstrate that (b) type I strains consistently exhibit a higher migratory capacity than type II and III strains and that (c) the migratory capacity of the highly virulent type I strain RH can be up-regulated. The enhanced transmigration capacity of type I strains, which was up to three orders of magnitude greater, could not be explained by differences in viability or efficiency of invasion. Furthermore, parasites with LDM-phenotype were present in all type I strains tested but not in type II and III strains. The migratory capacity of parasite populations was easily up-regulated in the type I strain (RH) by selecting parasites that migrated further.

Our findings demonstrate that active motility is not only used for cell invasion but also provides the parasite with an effective mechanism of dissemination in its microenvironment within tissues. Moreover, *ex vivo* data indicates important differences in the ability to penetrate tissues, resulting in dramatic differences in the efficiency of reaching the vascular endothelium. The ability to rapidly cross epithelial barriers and reach the circulation may be an important component of dissemination *in vivo*, particularly to sites of immune privilege, e.g., the central nervous system and the developing fetus.

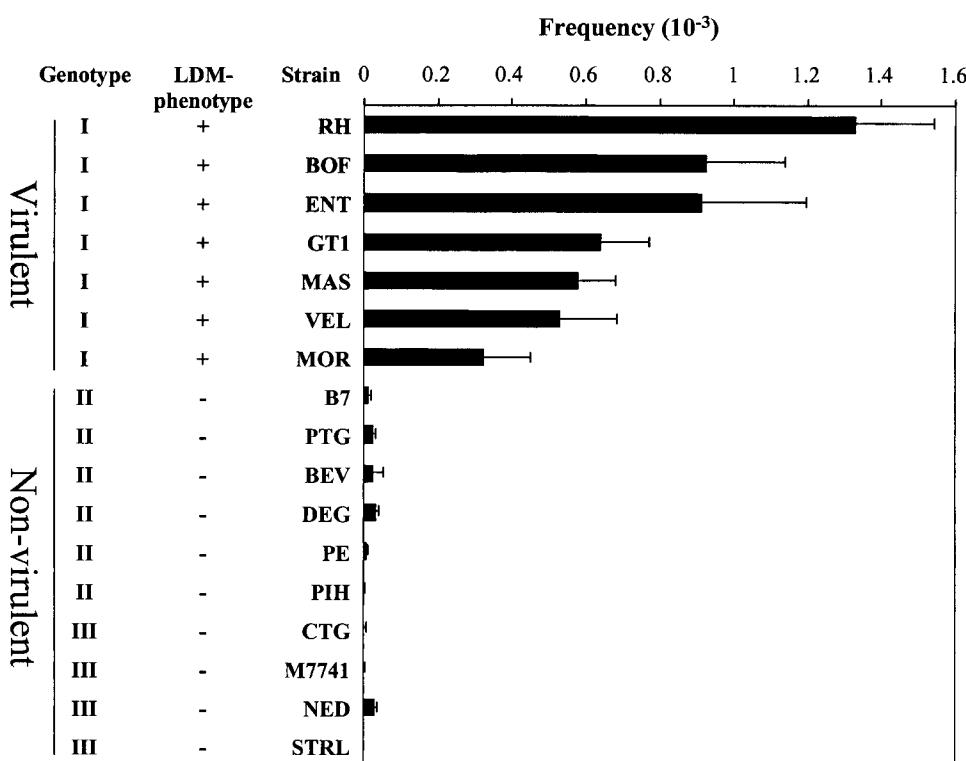


Figure 6. Differences in transmigration capacity between type I, type II, and type III strains and expression of the LDM-phenotype. Virulent and nonvirulent parasite strains and clinical isolates were classified by genotype as described (references 4 and 6). Assessment for the LDM-phenotype (parasite migration $>110 \mu\text{m}$) was performed on HFF cell monolayers under agarose as indicated in Materials and Methods. Graphics show expression (+) of the LDM-phenotype in all type I parasite lines tested and absence (-) of the LDM-phenotype in type II and type III lines. Transmigration of parasites across polarized MDCK cell monolayers was assessed by plaque formation (CFU) as described in Materials and Methods. The transmigration frequency for each strain was calculated as the number of transmigrated parasites (CFU)/1,000 parasites added and represents the mean from three independent experiments (\pm SD). Significant differences in frequency of transmigration were observed between strains ($P \leq 0.001$ for type I vs. type II; $P \leq 0.005$ for type I vs. type III, Mann-Whitney U-test) with transmigration frequency differences superior to three orders of magnitude (10^3).

Traversal of biological barriers, including the intestine, the blood-brain barrier and the placenta, leading to dissemination within the host is a requisite for the establishment of *T. gondii* infections. Ingested parasites (oocysts and bradyzoites in tissue cysts) invade the intestine and differentiate into tachyzoites followed by spread of the organism hematogenously and via lymphatics (15). Tachyzoites play an important role in pathogenesis during acute toxoplasmosis (1, 3) and upon reactivation of chronic infections in humans (21). Thus, our studies on the migratory characteristics of Toxoplasma tachyzoites are directly relevant to the role this stage plays in dissemination-related acute pathology and in reactivation of chronic infections.

T. gondii has a highly unusual population structure comprised of three clonal lineages (I, II, and III) and the parasite genotype influences the pathogenicity of the strain in mice (4, 6). Type I strains have been associated to acute virulence in the murine model and a relative over-representation of type I strains in congenital toxoplasmosis has been described (4, 5). One key feature of infections by type I strains in mice, is that they rapidly disseminate and reach high tissue burdens even from a very low initial inoculum (20, 22). The enhanced ability of type I strains to cross biological barriers likely represents an important advantage in the establishment of disseminated infection.

The strong association of the LDM-phenotype and transmigration efficiency with the parasite genotype I indicates an underlying genetic basis controls this trait. Nonetheless, the degree to which the LDM-phenotype is expressed is clearly influenced by environmental factors as it was up regulated in cloned populations (20%), but waned again over a period of 20–21 infection cycles (~160 mitotic cycles) in the absence of selection (data not shown). LDM clones were also capable of faster migration *in vivo*. After oral infection, it is possible that the ability for deep tissue migration is up-regulated, for example by selection for those parasites that successfully cross the intestinal epithelial barrier. Establishment of this early wave of rapidly migrating parasites may be critically important to dissemination of infection before the onset of an effective immune response.

Natural immunity against *T. gondii* is dependent on the induction of strong parasite-specific immunity in the host (23). Because neutrophils rapidly migrate to a site of infection, this initial response may be important for parasite control before adaptive immunity is established (24, 25). Toxoplasma actively infects leukocytes *in vitro* (26) and tachyzoites have been identified within leukocytes in the murine intestine (14). Thus, trafficking of leukocytes could contribute to dissemination of intracellular parasites via a Trojan horse type of mechanism (27, 28). However, the present finding that Toxoplasma directly penetrates epithelial monolayers and extracellular matrix indicates that migration across barriers may be primarily an active process. Moreover, only active penetration by the parasite can adequately explain transplacental infection, a common feature of acute toxoplasmosis, as maternal cells do not routinely traffic to the fetus. Enhanced migration by LDM parasites

was associated with a specific increase in transmigration and not simply an overall increase in motility or invasion of cells, suggesting that the parasite actively crosses biological barriers using a specific pathway. Future investigations of migration across endothelial and epithelial barriers will be important to establish the mechanism of transit.

A number of invasive bacterial, viral and fungal microorganisms have been described to cross biological barriers in the host and to cause disseminated infections. The precise mechanisms by which microbial pathogens gain entry into the target tissue are only partly understood and include the induction of endocytosis/phagocytosis (29, 30) or host actin-based translocation (31). In contrast, Toxoplasma, and other apicomplexans, actively penetrate their host cells *in vitro* (8) and this process is also instrumental *in vivo*. After subcutaneous injection by an infected mosquito, sporozoites of *Plasmodium* actively cross vascular endothelium and Kupffer cells to reach hepatocytes where they reside (32–36).

Toxoplasma establishes infection following oral ingestion, and consequently, to cause a systemic infection it must cross the intestinal epithelium, basement membrane, and lamina propria. Active traversal of cellular barriers represents a novel means of tissue dissemination used to gain access to biologically restricted organs, e.g., the brain and the placenta. We propose that the inherent property of virulent *T. gondii* type I strains to effectively migrate across barriers and up regulate the migratory capacity is an important component of pathogenesis in toxoplasmosis. Elucidation of mechanisms underlying transmigration may lead to the identification of important virulence factors in infections by *T. gondii* and other apicomplexan parasites.

We are grateful to Jeff Diffenderfer for expert tissue culture, to Dr. Thomas Steinberg, Dr. Jan Andersson, and Dr. Daniel Goldberg for critical evaluation of the manuscript, and to Dr. Eva Varisella for expert advice on micromanipulation.

Supported in part by a grant from the National Institutes of Health (grant no. A134036) to L.D. Sibley. A. Barragan is the recipient of a fellowship from the Swedish Foundation for International Cooperation in Research and Higher Education (STINT). L.D. Sibley is the recipient of a Scholar Award in Molecular Parasitology from the Burroughs Wellcome Fund.

Submitted: 15 February 2002

Revised: 22 April 2002

Accepted: 8 May 2002

References

- Desmonts, G., and J. Couvreur. 1974. Congenital toxoplasmosis: a prospective study of 378 pregnancies. *N. Engl. J. Med.* 290:1110–1116.
- Luft, B.J., R. Hafner, A.H. Korzon, C. Leport, D. Antoniskis, E.M. Bosler, D.D. Bourland, R. Uttamchandani, J. Führer, J. Jacobson, et al. 1993. Toxoplasmic encephalitis in patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 329:995–1000.
- Roberts, F., and R. McLeod. 1999. Pathogenesis of toxoplasmic retinochoroiditis. *Parasitol. Today.* 15:51–57.
- Howe, D.K., and L.D. Sibley. 1995. *Toxoplasma gondii* com-

- prises three clonal lineages: correlation of parasite genotype with human disease. *J. Infect. Dis.* 172:1561–1566.
5. Fuentes, I., J.M. Rubio, C. Ramírez, and J. Alvar. 2001. Genotypic characterization of *Toxoplasma gondii* strains associated with human toxoplasmosis in Spain: direct analysis from clinical samples. *J. Clin. Microbiol.* 39:1566–1570.
 6. Sibley, L.D., and J.C. Boothroyd. 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature*. 359:82–85.
 7. Morisaki, J.H., J.E. Heuser, and L.D. Sibley. 1995. Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell. *J. Cell Sci.* 108:2457–2464.
 8. Dobrowolski, J.M., and L.D. Sibley. 1996. Toxoplasma invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. *Cell*. 84:933–939.
 9. Joiner, K.A., S.A. Furhman, H.M. Miettinen, L.H. Kasper, and I. Mellman. 1990. *Toxoplasma gondii*: Fusion competence of parasitophorous vacuoles in Fc-receptor transfected fibroblasts. *Science*. 249:641–646.
 10. Mordue, D., S. Häkansson, 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.
 11. Vanderberg, J.P. 1974. Studies on the motility of *Plasmodium* sporozoites. *J. Protozool.* 21:527–537.
 12. Arrowood, M.J., C.R. Sterling, and M.C. Healey. 1991. Immunofluorescent microscopical visualization of trails left by gliding *Cryptosporidium parvum* sporozoites. *J. Parasitol.* 77: 315–317.
 13. Russell, D.G., and R.E. Sinden. 1981. The role of the cytoskeleton in the motility of coccidian sporozoites. *J. Cell Sci.* 50:345–359.
 14. Dubey, J.P., C.A. Speer, S.K. Shen, O.C.H. Kwok, and J.A. Blixt. 1997. Oocyst-induced murine toxoplasmosis: life cycle, pathogenicity, and stage conversion in mice fed *Toxoplasma gondii* oocysts. *J. Parasitol.* 83:870–882.
 15. Dubey, J.P. 1997. Bradyzoite-induced murine toxoplasmosis: stage conversion pathogenesis, and tissue cyst formation in mice fed bradyzoites of different strains of *Toxoplasma gondii*. *J. Eukaryot. Microbiol.* 44:592–602.
 16. Derouin, F., and Y.J.F. Garin. 1991. *Toxoplasma gondii*: blood and tissue kinetics during acute and chronic infections in mice. *Exp. Parasitol.* 73:460–468.
 17. Zenner, L., F. Darcy, A. Capron, and M.F. Cesbron-Delaunay. 1998. *Toxoplasma gondii*: Kinetics of the dissemination in the host tissues during the acute phase of infection in rats and mice. *Exp. Parasitol.* 90:86–94.
 18. Kim, K., M.S. Eaton, W. Schubert, S. Wu, and J. Tang. 2001. Optimized expression of green fluorescent protein in *Toxoplasma gondii* using thermostable green fluorescent protein mutants. *Mol. Biochem. Parasitol.* 113:309–313.
 19. Roos, D.S., R.G.K. Donald, N.S. Morrisette, and A.L. Moulton. 1994. Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Meth. Cell Biol.* 45:27–63.
 20. Mordue, D.G., F. Monroy, M. La Regina, C.A. Dinarello, and L.D. Sibley. 2001. Acute toxoplasmosis leads to lethal overproduction of Th1 cytokines. *J. Immunol.* 167:4574–4584.
 21. Reiter-Owona, I., H. Seitz, U. Gross, M. Sahm, J.K. Rockstroh, and H.M. Seitz. 2000. Is stage conversion the initiating event for reactivation of *Toxoplasma gondii* in brain tissue of AIDS patients? *J. Parasitol.* 86:531–536.
 22. Gavrilescu, L.C., and E.Y. Denkers. 2001. IFN- γ overproduction and high level apoptosis are associated with high but not low virulence *Toxoplasma gondii* infection. *J. Immunol.* 167:902–909.
 23. Sher, A., E.Y. Denkers, and R.T. Gazzinelli. 1995. Induction and regulation of host cell-mediated immunity by *Toxoplasma gondii*. *Ciba Found. Symp.* 195:95–104; discussion 104–109.
 24. Khan, I.A., P.M. Murphy, L. Casciotti, J.D. Schwartzman, J. Collins, J.L. Gao, and G.R. Yeaman. 2001. Mice lacking the chemokine receptor CCR1 show increased susceptibility to *Toxoplasma gondii* infection. *J. Immunol.* 166:1930–1937.
 25. Del Rio, L., S. Bennouna, J. Salinas, and E.Y. Denkers. 2001. CXCR2 deficiency confers impaired neutrophil recruitment and increased susceptibility during *Toxoplasma gondii* infection. *J. Immunol.* 167:6503–6509.
 26. Channon, J.Y., R.M. Seguin, and L.H. Kasper. 2000. Differential infectivity and division of *Toxoplasma gondii* in human peripheral blood leukocytes. *Infect. Immun.* 68:4822–4826.
 27. Huang, S.H., and A.Y. Jong. 2001. Cellular mechanisms of microbial proteins contributing to invasion of the blood-brain barrier. *Cell. Microbiol.* 3:277–287.
 28. Shaw, S.K., A. Hermanowski-Vosatka, T. Shibahara, B.A. McCormick, C.A. Parkos, S.L. Carlson, E.C. Ebert, M.B. Brenner, and J.L. Madara. 1998. Migration of intestinal intraepithelial lymphocytes into a polarized epithelial monolayer. *Am. J. Physiol.* 275:G584–G591.
 29. Meresse, S., O. Steele-Mortimer, E. Moreno, M. Desjardins, B. Finlay, and J.P. Gorvel. 1999. Controlling the maturation of pathogen-containing vacuoles: a matter of life and death. *Nat. Cell Biol.* 1: E183–E188.
 30. Koi, H., J. Zhang, and S. Parry. 2001. The mechanisms of placental viral infection. *Ann. NY Acad. Sci.* 943:148–156.
 31. Cossart, P., and H. Bierne. 2001. The use of host cell machinery in the pathogenesis of *Listeria monocytogenes*. *Curr. Opin. Immunol.* 13:96–103.
 32. Sidjanski, S., and J.P. Vanderberg. 1997. Delayed migration of *Plasmodium* sporozoites from the mosquito bite site to the blood. *Am. J. Trop. Med. Hyg.* 57:426–429.
 33. Meiss, J.F., J.P. Verhave, P.H. Jap, and J.H. Meuwissen. 1983. An ultrastructural study on the role of Kupffer cells in the process of infection by *Plasmodium berghei* sporozoites in rats. *Parasitology*. 86:231–242.
 34. Pradel, G., and U. Frevert. 2001. Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion. *Hepatology*. 33:1154–1165.
 35. Vanderberg, J.P., S. Chew, and M.J. Stewart. 1990. Plasmodium sporozoite interactions with macrophages in vitro: a videomicroscopic analysis. *J. Protozool.* 37:528–536.
 36. Mota, M.M., G. Pradel, J.P. Vanderberg, J.C. Hafalla, U. Frevert, R.S. Nussenzweig, V. Nussenzweig, and A. Rodriguez. 2001. Migration of *Plasmodium* sporozoites through cells before infection. *Science*. 291:141–144.