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THE ULTRASTRUCTURE OF THE PARASITOPHOROUS VACUOLE FORMED BY *LEISHMANIA MAJOR*

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ABSTRACT: Protozoan parasites of *Leishmania* spp. invade macrophages as promastigotes and differentiate into replicative amastigotes within parasitophorous vacuoles. Infection of inbred strains of mice with *Leishmania major* is a well-studied model of the mammalian immune response to *Leishmania* species, but the ultrastructure and biochemical properties of the parasitophorous vacuole occupied by this parasite have been best characterized for other species of *Leishmania*. We examined the parasitophorous vacuole occupied by *L. major* in lymph nodes of infected mice and in bone marrow–derived macrophages infected in vitro. At all time points after infection, single *L. major* amastigotes were wrapped tightly by host membrane, suggesting that amastigotes segregate into separate vacuoles during replication. This small, individual vacuole contrasts sharply with the large, communal vacuoles occupied by *Leishmania amazonensis*. An extensive survey of the literature revealed that the single vacuoles occupied by *L. major* are characteristic of those formed by Old World species of *Leishmania*, while New World species of *Leishmania* form large vacuoles occupied by many amastigotes.

Leishmania species are dimorphic protozoan parasites transmitted between mammalian hosts by phlebotomine sand flies (Alexander et al., 1999; Handman, 2000). Flagellated *Leishmania* spp. promastigotes develop into an infective stage in the digestive tract of sand flies and are transmitted to a mammalian host during a blood meal. In the vertebrate host, promastigotes are phagocytosed by macrophages and differentiate into unflagellated amastigotes, which replicate within lysosomal compartments. Dissemination within the host most likely occurs through secondary infections of macrophages and other phagocytic cells. Human infections occur in countries surrounding the Mediterranean Sea, in East Africa, the Middle East, south Asia, and in Central and South America (Davies et al., 2003). Diseases caused by *Leishmania* spp. include self-healing cutaneous lesions, disfiguring mucocutaneous infections, and fully disseminating visceral leishmaniases, which may be fatal unless treated. The severity of disease clearly depends both upon the species of *Leishmania* and the immunogenetics of the host (Colmenares et al., 2002).

The parasitophorous vacuole (PV) inhabited by intracellular *Leishmania* spp. amastigotes is a modified, lysosomal compartment (Antoine et al., 1998). Lysosomal hydrolases appear to be fully active within the PV, and *Leishmania* spp. antigens are most likely processed within this compartment for presentation to T cells. When properly activated, macrophages are capable of killing amastigotes residing within this compartment (Liew and O'Donnell, 1993). This adaptation to life within the very compartment responsible for its destruction suggests that *Leishmania* spp. may directly manipulate the host immune response. Thus, experimental infection of macrophages or mice with *Leishmania* spp. offers opportunities for investigating the role of macrophages in initiating and effecting immune responses to intracellular parasites.

While more than 10 species of *Leishmania* pathogenic to humans have been described, just a few have been extensively used as experimental models of infection. The immune response to *Leishmania major* in mice has been especially well studied (Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002), but characterization of the PV occupied by amastigotes has mostly been performed with macrophages infected with other species of *Leishmania*. The earliest descriptions of the PV were the result of electron microscopic examinations of spleens from hamsters heavily infected with *Leishmania donovani* (Chang, 1956; Rudzinska et al., 1964) or *Leishmania mexicana* (Creemers and Jadin, 1967), and skin biopsies of human patients infected with *L. donovani* or *Leishmania tropica* (Sanyal and Sen Gupta, 1967; Pham et al., 1970). Soon thereafter, investigators produced electron micrographs of macrophages infected in vitro with *L. donovani* (Alexander and Vickerman, 1975) or *L. mexicana* (Akiyama and McQuillen, 1972). Subsequent biochemical and immunoelectron microscopic analyses of the PV have largely been limited to macrophages infected with the latter 2 species (Chang, 1983; Alexander and Russell, 1992; Antoine et al., 1998; Alexander et al., 1999). We report here our initial ultrastructural characterization of the PV formed by *L. major*. In infected lymph node cells of mice, as well as in mouse bone marrow–derived macrophages infected in vitro, *L. major* amastigotes reside within small vacuoles in which the host membrane is tightly wrapped around a single parasite. These vacuoles are similar in morphology to those occupied by other Old World species of *Leishmania* and contrast sharply with the large vacuoles formed by New World species of *Leishmania*. Because actively replicating parasites occupy single, small vacuoles at all time points after infection, it can be concluded that *L. major* amastigotes segregate into separate vacuoles during replication.

MATERIALS AND METHODS

Parasites

Leishmania major (WHOM/IR/-/173) or *Leishmania amazonensis* (LV78) promastigotes were maintained at 28 C in M199 media supplemented with 20% fetal bovine serum (Sigma, St. Louis, Missouri), glutamine, and penicillin-streptomycin (Invitrogen, Carlsbad, California). To maintain infectivity, parasites were passaged through BALB/c mice. Amastigotes were harvested from the feet or lymph nodes of infected mice, differentiated into promastigotes in culture, and frozen in 10% DMSO in fetal bovine serum. Frozen stocks of promastigotes were thawed and used within 6 in vitro passages.

Mice

Colonies of BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) were maintained in the animal facility at Chicago State University. Mice were fed and watered ad libitum.

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Macrophages

Bone marrow–derived macrophages (BMMØs) were obtained by flushing the femurs of mice with 1% FCS/PBS and culturing the resulting cells in L cell-conditioned media (LCM; 25% supernatant from L929 cells cultured in Iscove's supplemented with glutamine, pen-strep and 2-ME). Bone marrow cells from a single mouse were routinely plated in a 100-mm tissue culture dish for 1 day, after which nonadherent cells were harvested and replated in tissue culture dishes or 6-well tissue culture plates. After 5–10 days, LCM was removed from the adherent macrophages and media lacking macrophage-colony stimulating factor (M-CSF) was added at least 2 hr prior to in vitro infections.

Infections

Stationary phase cultures (4–5 days after passage) of *L. major* promastigotes were used for infections. *Leishmania major* promastigotes were enriched for infectious metacyclic organisms by negative selection with peanut agglutinin-coated beads (Sigma), as described (Sacks et al., 1985). Promastigotes that did not bind to the beads were washed 3 times in cell media for in vitro infections or in PBS for infections of mice. BMMØs were infected at a ratio of 5:1 or 10:1. BALB/c mice were infected with 1×10^6 to 2×10^6 *L. major* promastigotes or 5×10^6 *L. amazonensis* promastigotes in the right footpad under methoxyflurane or nembutal anesthesia.

Quantification of in vitro infection

Macrophages were cocultured with promastigotes overnight, washed extensively with cell media to remove uninternalized parasites, and then incubated in normal cell media in the presence of 10 ng/ml recombinant IL-4 (R&D Systems, Minneapolis, Minnesota) for 1–4 days with addition of fresh media and cytokine each day (Iniesta et al., 2002). Individual cover slips were removed at indicated time points and stained with a LeukoStat staining kit (Fisher Scientific, Pittsburgh, Pennsylvania). At 400× magnification, individual amastigotes were clearly visible in the cytoplasm of infected macrophages. More than 100 infected macrophages were individually scored for the number of amastigotes contained within the cytoplasm. The number of infected macrophages in a field of at least 100 macrophages was counted to obtain the percentage infected.

Transmission electron microscopy

Macrophages infected in vitro were fixed by adding an equal volume of 2× fixative (4% paraformaldehyde, 0.2% glutaraldehyde in phosphate buffer) directly to the cell media. After 15 min at room temperature, fixative was removed and cells were washed 3 times with 0.1% glycine/PBS. The cells were scraped directly into wash buffer and pelleted by centrifugation in 15-ml Falcon tubes. The pellet was resuspended in 10% gelatin/PBS at 37 C. After 10 min incubation at 37 C, the cells were transferred to a microcentrifuge tube, pelleted by centrifugation, and placed on ice for at least 30 min to allow the gelatin to harden. The tip of the tube was cut with a razor to remove the cell pellet lodged in gelatin. This gelatin block was diced into 1-mm^3 pieces and fixed with 2% osmium in 0.1 M phosphate buffer for 1 hr. The blocks were dehydrated by successive incubations in 50%, 70%, 85%, 95%, and 100% EtOH and embedded in Epon (LADD Research Industries, Williston, Vermont). Thin sections were cut with a Diatome diamond knife on an RMC ultramicrotome. Sections were stained with uranyl acetate and lead citrate and viewed with a JEOL TEM 1200EX at an accelerating voltage of 80 kV.

The draining popliteal lymph nodes of infected mice were removed at various times after infection and fixed immediately (2% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer). Fixed tissue was trimmed and cut into 1-mm^3 pieces, then postfixed, dehydrated, embedded, sectioned, stained, and viewed as described above.

All images were acquired and stored on Kodak Electron microscope film with a Gatan CCD camera. Negatives were scanned at 400–800 dpi and manipulated with Adobe Photoshop 6.0.1.

RESULTS

Because we were interested in correlating the murine immune response to *L. major* infection with the cell biology of the interaction between amastigotes and mouse macrophages, we initiated a systematic examination of the ultrastructure of lymph node cells infected with *L. major*. BALB/c mice were infected in the hind footpad, and the draining lymph nodes were removed at various times after infection and processed for transmission electron microscopy (TEM). Within 2 wk after infection, we could locate infected cells in lymph node sections; at later times, infected cells were more easily located. Infected cells displayed classic characteristics of macrophages, with irregularly shaped nuclei and large, vacuolated cytoplasm. They were invariably surrounded by lymphocytes, smaller cells with large nuclei and scant cytoplasm. We did not note any obvious differences in the morphology of lymph node cells from uninfected and infected mice.

Infected cells harbored 1 to several amastigotes in the cytoplasm. Typical transmission electron micrographs of *L. major*–infected macrophages from draining lymph nodes are shown in Figures 1–4. Amastigotes were readily identified as membrane-bound entities ~2 μm in diameter with a prominent nucleus. The kinetoplast was often visible and the flagellum, with its characteristic organization of microtubules, was sometimes obtained in cross section. A row of subpellicular microtubules was usually evident immediately inside the plasma membrane of the amastigote. The plasma membrane was surrounded tightly by another membrane, the host parasitophorous vacuole membrane (Fig. 1B). In Figure 2, a vacuole is shown in which the host membrane is distended in 2 places. However, such spaces between parasite and host membrane were seldom observed.

We examined transmission electron micrographs of lymph nodes at many different time points after infection. With increasing time after infection, there were usually more amastigotes per macrophage. In nearly all cases, however, each of the amastigotes was contained within a single, tight parasitophorous vacuole. It is evident that even when 2 amastigotes are close together within the cytoplasm, they are contained within separate host vacuoles (Figs. 1, 2, 4). Another less common profile encountered is shown in Figure 3. In these cells, pairs of amastigotes are tightly wrapped together within a single vacuole. These amastigotes appear to be replicating, and the parasitophorous vacuole membrane appears to be dividing synchronously with the parasite.

These tight, individual vacuoles contrast sharply with the large, swollen vacuoles found in macrophages infected in vitro with species of the *L. mexicana* complex (Antoine et al., 1991; Russell et al., 1992; Veras et al., 1992). To confirm that large vacuoles are the property of *L. mexicana* and not of in vitro cultured macrophages, we infected mice with *L. amazonensis* promastigotes and processed the lymph nodes for TEM. Vacuoles formed by this species were almost never tight vacuoles and often contained many parasites within a single vacuole. A typical vacuole containing a single amastigote is shown in Figure 5A. Only a small proportion of the vacuolar membrane is closely associated with the amastigote. Opposite to this attachment site, the membrane is expanded and diffuse, granular material has accumulated between the host membrane and the cell membrane of the parasite. Figure 5B shows a typical large vacuole (~10 μM in diameter) with 7 amastigotes residing within it. Several of the amastigotes are bound to the host membrane, but the majority of the cell membrane of the amastigote is unattached and instead is exposed to the lumen of the vacuole.

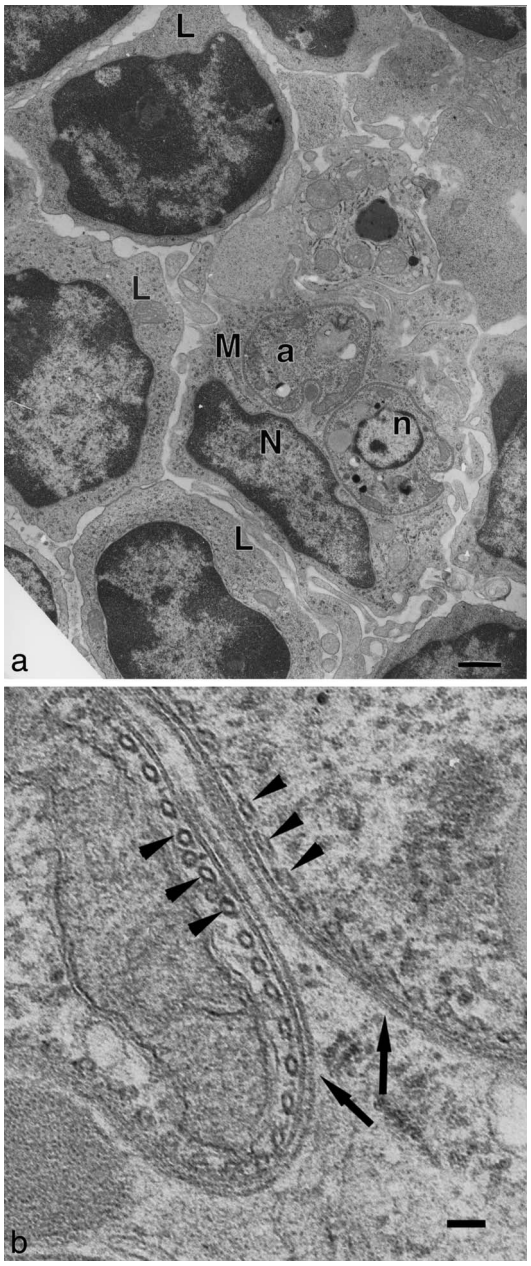


FIGURE 1. Infected macrophage from the draining lymph node of a BALB/c mouse 13 days after infection with *L. major*. The hind footpad of a mouse was injected with *L. major* promastigotes and the draining lymph node was removed 13 days later and processed for TEM. (**A**) An infected macrophage is surrounded by lymphocytes. Two amastigotes are clearly visible in the cytoplasm of the macrophage. (**B**) Magnification of an area where the 2 amastigotes lie close together reveals that each parasite is in a separate compartment and tightly surrounded by host membrane. Note that both the host membrane and the amastigote membrane have the familiar ''railroad track'' appearance of a lipid bilayer. a, amastigote; L, lymphocyte; M, macrophage; N, macrophage nucleus; n, amastigote nucleus; arrows, host PV membrane; arrowheads, subpellicular microtubules. Scale bar in A = 500 nm; scale bar in B = 25 nm.

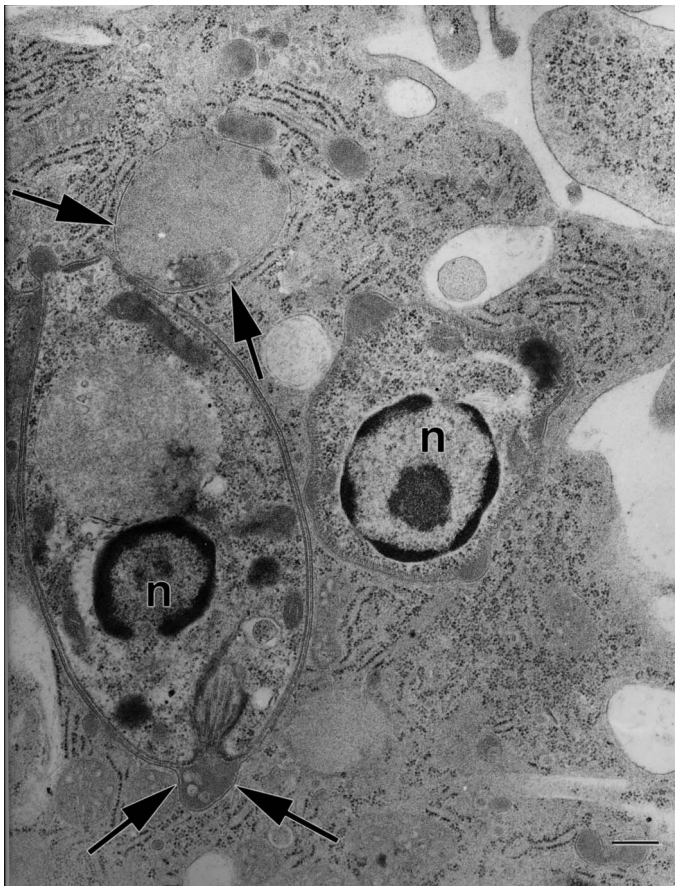
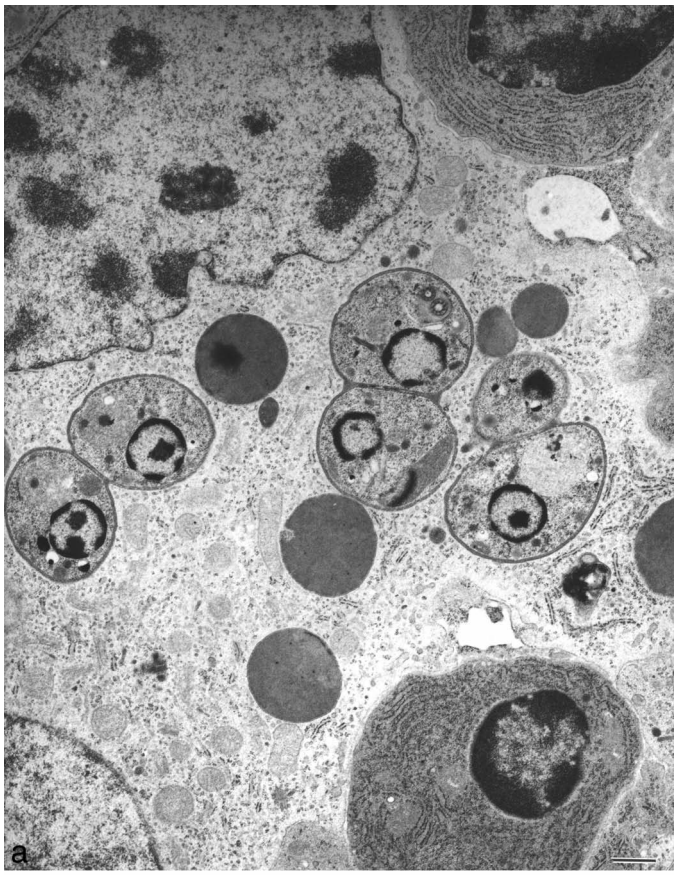


FIGURE 2. Amastigotes in a macrophage from a draining lymph node 28 days after infection with *L. major*. As in Figure 1, the 2 amastigotes visible here are clearly segregated in separate compartments. In 2 locations, the vacuolar membrane is distended (arrows). n, amastigote nucleus. Scale bar = 200 nm.

While some amastigotes appear to be suspended in the lumen of the vacuole, it cannot be concluded that they are not attached to the membrane, since the attachment site may not be within the plane of the section.

If *L. major* amastigotes are replicating within macrophages after infection and intracellular amastigotes are always contained within a single vacuole, then it can be concluded that daughter parasites segregate into separate vacuoles after each round of replication. *Leishmania major* amastigotes appeared to be replicating within lymph nodes. No signs of degradation of amastigotes were apparent, and some amastigotes contained duplicated flagella (Fig. 3), one of the first steps in amastigote replication. Statistical analysis of the infection and replication of amastigotes in lymph node sections was technically challenging, so we repeated these experiments with in vitro infected macrophages. Bone marrow–derived macrophages were infected with *L. major* promastigotes at infection ratios and times that produced ~1–2 amastigotes per macrophage shortly after infection. Macrophages were then incubated for 1–4 days to allow replication of the parasites. IL-4 was added to the media to suppress macrophage activation and assure reproduction of the parasites (Iniesta et al., 2002). The cells were fixed and stained for light microscopic determination of the infection rate and the numbers of amastigotes per macrophage. Parallel dishes



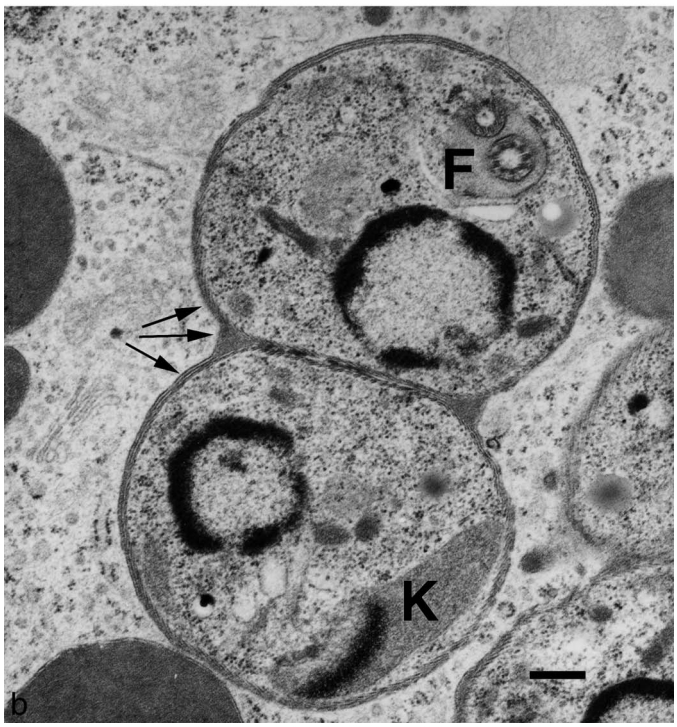


FIGURE 3. Replicating amastigotes in a macrophage from a draining lymph node 4 wk after infection with *L. major*. (**A**) Three pairs of fused amastigotes are visible in the cytoplasm. (**B**) The central pair is magnified to show how the host membrane is tightly associated with both amastigote membranes, except at the point where the amastigotes are most closely associated with each other (arrows). The amastigote at the top of this pair is clearly dividing, as 2 flagella (F) are visible in cross section. K, kinetoplast. Scale bar in A = 500 nm; scale bar in B = 200 nm.

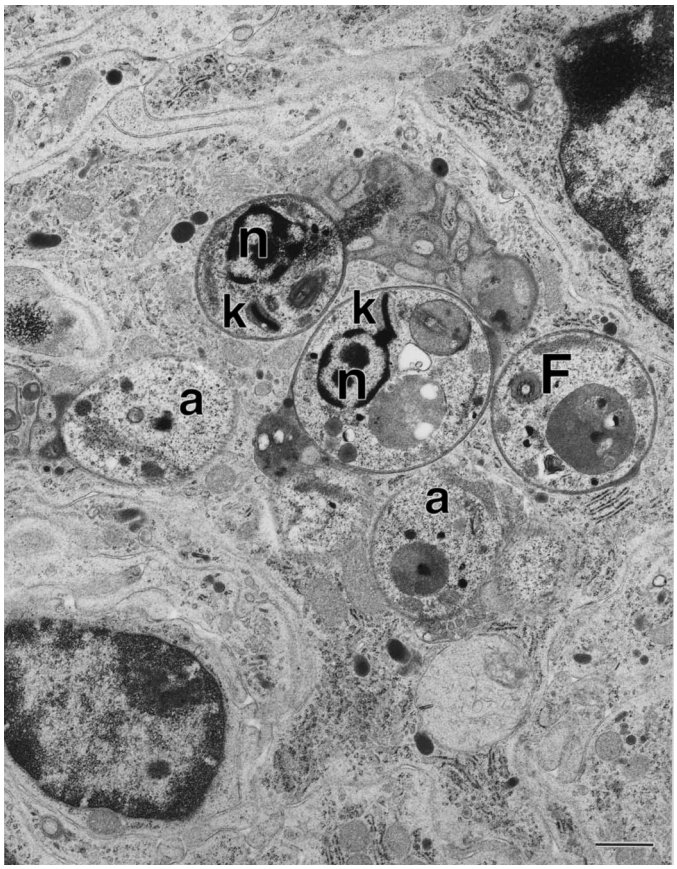


FIGURE 4. Amastigotes in a macrophage from a draining lymph node 6 wk after infection with *L. major*. At least 5 amastigotes are visible in the cytoplasm of this macrophage. Each is clearly in a separate compartment. F, flagellum; k, kinetoplast; a, amastigote; n, amastigote nucleus. Scale bar = 1 μm.

of macrophages were processed for TEM to determine the ultrastructure of the parasitophorous vacuole.

Leishmania major amastigotes were replicating under the in vitro conditions used in the experiment (Fig. 6). Each day after infection, the number of amastigotes per macrophage increased, with a 12-fold increase occurring over 4 days. Moreover, the initial infection rate of ~50% did not change significantly during this time period, indicating that secondary infection of macrophages was not occurring.

Electron micrographs revealed that macrophages infected in vitro harbored *L. major* amastigotes within parasitophorous vacuoles similar to those observed in infected lymph nodes. Each PV housed a single amastigote, with the membrane of the vacuole closely associated with the entire parasite cell membrane. This was true whether 1 amastigote (Fig. 7A) or several amastigotes (Fig. 7B) were present in a single macrophage. Large, swollen vacuoles harboring many parasites were never observed in >10 independent in vitro infections. Nearly all amastigotes observed in these macrophages were intact and thus most likely replicating, as indicated by the analysis of the infection rate in Figure 6. We conclude that *L. major* amastigotes segregate into separate parasitophorous vacuoles during replication.

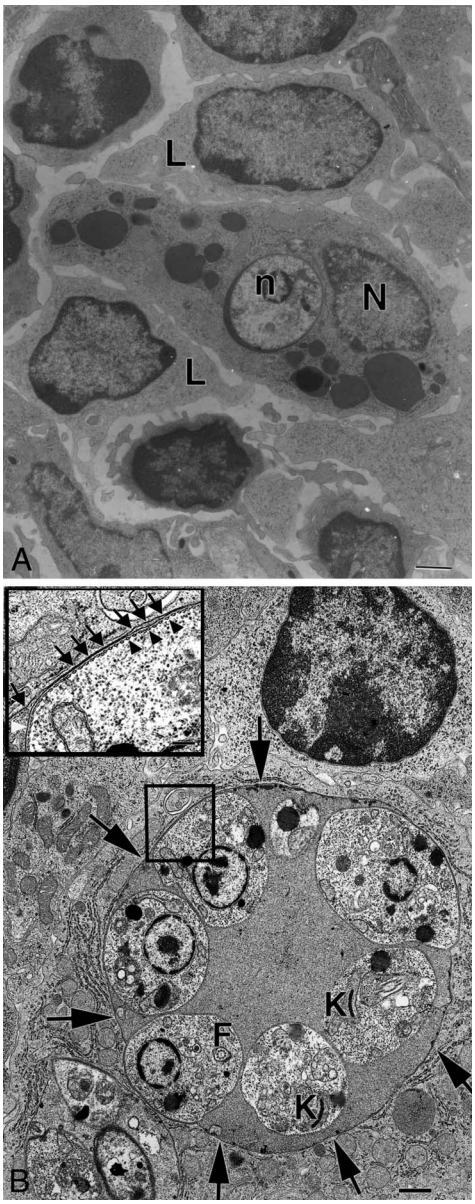


FIGURE 5. Infected macrophages from a draining lymph node 4 wk after infection with *L. amazonensis*. (**A**) A vacuole with a single amastigote is shown. In contrast to parasitophorous vacuoles housing *L. major* amastigotes, the membrane of this vacuole is not wrapped completely around the amastigote cell membrane. (**B**) The parasitophorous vacuole shown here is large and spacious and contains at least 7 amastigotes. The host membrane (arrows) is clearly continuous around the entire circumference of the vacuole. Several of the amastigotes are closely associated with the PV membrane, but the attachment is limited to a small portion of each amastigote membrane. The higher magnification in the inset reveals the close association of the host and amastigote membrane (arrows). L, lymphocyte; F, flagellum; K, kinetoplast; N, macrophage nucleus; n, amastigote nucleus; black arrowheads, subpellicular microtubules; white arrowhead, amastigote membrane. Scale bar in A = 500 nm; scale bar in B = 1 μm; scale bar in inset = 200 nm.

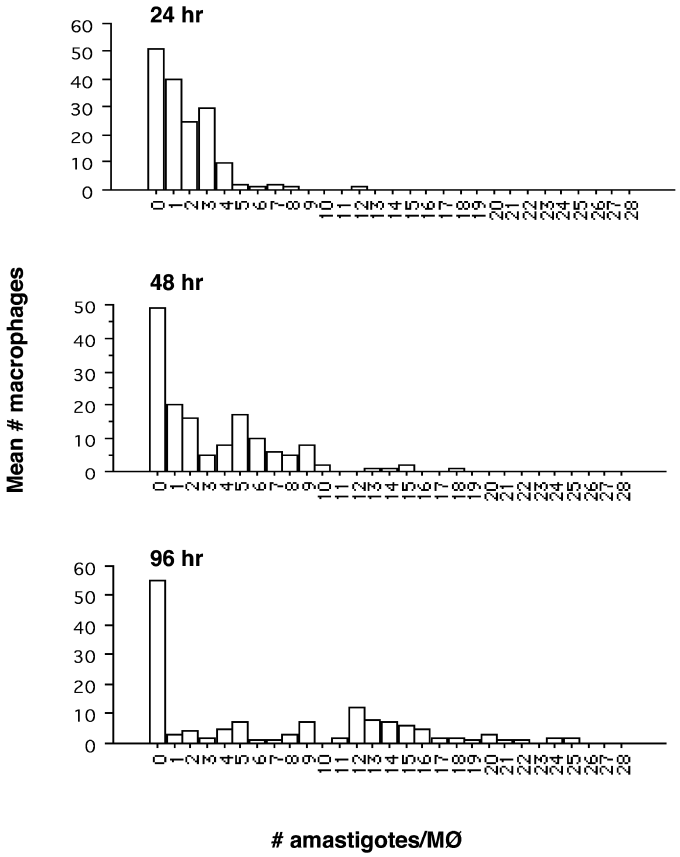


FIGURE 6. Replication of *L. major* amastigotes after infection in vitro. BMMØs growing on glass cover slips were infected with *L. major* promastigotes at 5:1 or 10:1 ratio. Individual coverslips were removed at 24, 48, and 96 hr after infection, and cells were fixed and stained for quantification of the infection rate. ANOVA showed no significant difference in the percentage of uninfected macrophages at the different time points ($F = 0.056$; $P = 0.82$), indicating that cross-infections of macrophages by released amastigotes did not occur over time. Chi square tests of the frequency distributions of the number of amastigotes per infected macrophage showed increases in the number of amastigotes in the samples of infected cells, both from 24 to 48 hr and from 48 to 96 hr ($P < 0.001$ for both intervals).

DISCUSSION

Since the first electron micrograph of the intracellular amastigote of *Leishmania* sp. was published (Chang, 1956), the morphology of parasitophorous vacuoles formed by many species of *Leishmania* has been extensively characterized. It is often stated that at least 2 types of vacuoles exist, depending upon the species of *Leishmania* harbored in the vacuole (Chang, 1983; Antoine et al., 1998; Schaible et al., 1999; Handman, 2000; Rittig and Bogdan, 2000; Sacks and Sher, 2002). Old World species of *Leishmania* occupy small, individual vacuoles, with extensive association between the parasite membrane and the vacuolar membrane. In contrast, New World species occupy large, spacious vacuoles, which contain 1, or many, amastigotes that are usually attached to the vacuolar membrane through a limited interaction with the parasite membrane. An extensive review of published electron micrographs of *Leishmania* PVs formed in vivo (Table I) and in vitro (Table II) generally supports this conclusion. Adopting terminology first used by Chang and Dwyer (1978), we refer to small, individual vacuoles as type I vacuoles and large, communal vacuoles as type II vacuoles. The major distinction between these 2 types of vacuoles is the degree to which the parasite cell membrane is associated with the host PV membrane. In our analysis of the literature,

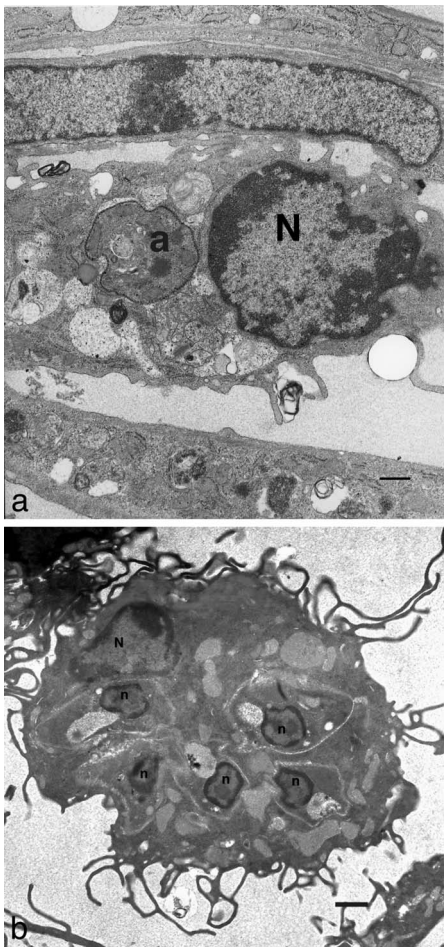


FIGURE 7. The parasitophorous vacuole of *L. major* after infection of macrophages in vitro. BMMØs growing in Petri dishes in parallel with those described in Figure 6 were harvested at (**A**) 24 and (B) 72 hr after infection, then fixed, stained, and processed for TEM. N, macrophage nucleus; n, amastigote nucleus; a, amastigote. Scale bar in A = 1 μM; scale bar in B = 500 nm.

we have not distinguished between tight and loose vacuoles, since parasites that appear to be suspended within the lumen of the vacuole may in fact be attached. The PVs of New World species, *L. mexicana* and *L. amazonensis*, always appear to be type II vacuoles, which are characteristic enough to be easily seen even with a light microscope (Chang, 1980; Veras et al., 1992; Chang et al., 2003). *Leishmania tropica* and *L. donovani* usually occupy type I vacuoles, but more than 1 investigator has found type II vacuoles harboring *L. donovani*, especially when infections were done in vitro. This may be a property of *L. donovani*, since vacuolar space between parasite and host membrane was more likely to be seen with this species, even in vacuoles formed in vivo. Alternatively, the apparent intermediate phenotype of *L. donovani* vacuoles may have to do with the activation state of the macrophages (Chang and Dwyer, 1978).

The micrographs presented here, the first published of *Leishmania* sp. amastigotes in the lymph nodes of infected mice, are consistent with the general conclusion regarding the morphology of Old World and New World PVs described above. The appearance of lymph nodes infected with *L. major* is very similar to that of hamster spleens heavily infected with *L. donovani* (Rudzinska et al., 1964) and representative of the general structure of Type I vacuoles. Most intracellular amastigotes visualized were wrapped tightly by host membranes, though at times membrane extensions were present (Fig. 2), possibly because of fusion of the vacuole with late endosomal compartments or secondary lysosomes. Heavily infected cells sometimes had clusters of PVs, which were crowded together in what has been termed a ''phagosomal gel'' (Ridley and Wells, 1986). In contrast, the PVs in the lymph nodes of mice infected with *L. amazonensis* resembled type II vacuoles, enlarged vacuoles with 1 (Fig. 5A), or several (Fig. 5B), amastigotes per vacuole. As noted by many earlier investigators, amastigotes in large vacuoles were often attached to the membrane at 1 end of the parasite.

The fact that *L. major*, *L. tropica*, and *L. donovani* amastigotes are almost always found in type I vacuoles suggests that the vacuole replicates as amastigotes replicate. We examined *L. major*–infected lymph nodes at many different time points after infection. Lymph nodes at later time points tended to be more heavily infected, i.e., more amastigotes per macrophage, but the amastigotes were always enclosed in type I vacuoles. Similarly, when BMMØs infected in vitro were analyzed at various time points after infection, amastigotes were definitely proliferating over time, but the morphology of the vacuoles did not change. Similar results have been obtained with *L. donovani* infections in vivo and in vitro (Rudzinska et al., 1964; Berman et al., 1979; Pearson et al., 1981). The simplest explanation for this observation is that there is a close interaction between molecules of the parasite and host membranes, and cytokinesis of the parasite leads to division of the PV membrane as well. In support of this idea, we observed dividing amastigotes that appeared to be at different stages of this division process (Fig. 3). Interestingly, *L. amazonensis* amastigotes are usually attached to the PV membrane, but the attachment is most often limited to a localized region of the parasite. It is possible that the same molecules mediate the interaction between the PV membrane and the membranes of Old World and New World parasites, but that these molecules are restricted to a particular region of the membrane of New World species, while being evenly distributed throughout the cell membrane of Old World species. Antoine et al. (1999) noted that the distribution of MHC class II molecules in the host membrane of *Leishmania* spp. PVs correlates with the extent of attachment of the parasite to the membrane. MHC molecules are localized to the site of attachment between *L. amazonensis* amastigotes and the host membrane, while MHC molecules are evenly distributed around the periphery of the host vacuole surrounding *L. major* amastigotes (Antoine et al., 1999). *Leishmania donovani* PVs had an intermediate distribution of MHC molecules (Antoine et al., 1999). We are currently trying to identify the molecules mediating the interaction between *Leishmania* spp. amastigotes and PV membranes.

Our literature review has also revealed that very few micrographs of *L. major* parasitophorous vacuoles have been published. This is curious, since the immune response to *Leishmania* spp. has been especially well characterized by experimental infection of inbred strains of mice with this particular species of *Leishmania* (Sacks and Noben-Trauth, 2002). Adaptive immune responses are initiated by MHC class II presen-

TABLE I. Electron micrographs of parasitophorous vacuoles formed after *Leishmania* infection in vivo. References to Old World species are listed chronologically, followed by references to New World species.

Leishmania species	Host tissue	Type of vacuole	Evidence	Reference
L. donovani	Hamster spleen	Type I	Two micrographs of tight vacuole; 1 micrograph of an extended vacuole with significant vacuolar space between amastigote and membrane	Chang (1956)
L. donovani	Hamster spleen	Type I	Most vacuoles contain single amastigote tightly wrapped in host membrane; some vacuoles with ''capsule'' of granular material accumulated between amastigote membrane and host membrane; some of these vacuoles contain 2 or 4 amastigotes	Rudzinska et al. (1964)
L. donovani	Human cutaneous lesion, India	Type I	All micrographs show single amastigote wrapped by host membrane	Sanyal and Sen Gupta (1967)
L. donovani	Hamster spleen	Type I	Most vacuoles contain single amastigote tightly wrapped in host membrane, but often part of the host membrane is extended and granular and/or membranous material has accumulated between parasite and host membranes	Gardener (1974)
L. donovani	Human cutaneous lesion, India	Type I	Macrophage with 2 amastigotes in cytoplasm, each tightly wrapped in host membrane	Mukherjee et al. (1993)
L. tropica	Human cutaneous lesion, Lebanon	Type I	All vacuoles contain single amastigotes tightly wrapped by host membrane	Pham et al. (1970)
L. tropica	Human cutaneous lesions	Type I	Vacuoles contain single amastigotes, either tightly wrapped by host membrane or attached to 1 side of a large empty vacuole; some amastigotes appeared to be free in cytoplasm unwrapped by host membrane	Sandbank (1976)
L. tropica	Human cutaneous lesions	Type I	Most vacuoles contain single amastigotes tightly wrapped by host membrane; 1 vacuole contains 2 closely apposed amastigotes in center of vacuolar space; some vacuoles accumulate membranous material	Fukuhara and Klingmuller (1976)
L. tropica	Mouse cutaneous lesion	Type I	Heavily parasitized macrophage with cluster of amastigotes, each contained within a single vacuole; colloidal gold injected into skin accumulated between parasite and host membranes	Berman et al. (1981)
Unidentified, probably *L. major*	Human lesions and hamster spleen	Type I	Vacuoles in human lesions contain single amastigotes wrapped by host membrane; in hamster spleen, membrane of some vacuoles is distended, with accumulation of material between parasite and host membranes	Veress et al. (1981)
L. aethiopica	Human cutaneous lesion, Ethiopia	Type I and Type II	Small vacuoles contain 1 amastigote tightly wrapped by host membrane; large, swollen vacuoles contain many amastigotes attached to periphery	Schurr et al. (1987)
L. mexicana	Hamster spleen	Type II	Large spacious vacuoles with several amastigotes per vacuole, usually attached to host membrane	Creemers and Jadin (1967)
L. amazonensis	Hamster cutaneous lesion	Type II	Large spacious vacuoles with 1 or several amastigotes, usually attached to host membrane at 1 limited site	Gardener (1974)
L. amazonensis *L. braciliensis* *L. garnhami*	Hamster, mouse and human cutaneous lesions	Type II	Large vacuoles; parasites attached to vacuole membrane at a site in their membrane opposite the flagellar pocket	Bretana et al. (1983)

tation of parasite antigens to T cells. MHC class II molecules, as well as other molecules necessary for antigen processing and presentation, have been localized, mostly by confocal immunofluorescence microscopy, to the PVs of cells infected with both Old World and New World species of *Leishmania* (Antoine et al., 1991; Lang et al., 1994; Antoine et al., 1998; Antoine et al., 1999). However, this type of characterization of the PV has been limited almost entirely to the PVs of cells infected with *L. amazonensis* and *L. donovani*. The immune responses to *L. amazonensis* and *L. donovani* differ markedly from that to *L. major* (McMahon-Pratt and Alexander, 2004). Further characterization of the *L. major* PV is necessary to determine whether

TABLE II. Electron micrographs of parasitophorous vacuoles formed after *Leishmania* infection in vitro. References are organized as in Table 1.

Leishmania species, stage	Host cell	Type of vacuole	Evidence	Reference
L. donovani promastigotes	Hamster peritoneal MØs	Type I	Intact amastigotes in small, tight vacuoles, but degrading amastigotes found in swollen vacuoles; 1 micrograph of dividing amastigotes in which PV appears to be dividing too	Akiyama and McQuillen (1972)
L. donovani promastigotes	Dog sarcoma cell line	Type I and Type II	One micrograph shows single amastigote wrapped tightly by host membrane; another shows 2 parasites attached to the periphery of a single large vacuole	Akiyama and McQuillen (1972)
L. donovani amastigotes	Hamster peritoneal MØs	Type I and Type II	Mostly single parasites per vacuole, but several examples of vacuoles containing 1 or several parasites either partly attached or unattached to host membrane	Chang and Dwyer (1976, 1978)
L. donovani amastigotes	Mouse peritoneal MØs	Type I	Three to four amastigotes per macrophage all in separate tight compartments, some amastigotes in swollen vacuoles in process of degrading	Chang and Chiao (1981)
L. donovani promastigotes	Human monocyte-derived MØs	Type I	All amastigotes enclosed within single vacuoles, some with loose fitting membranes and granular material accumulated between parasite and host membranes	Pearson et al. (1981)
L. donovani amastigotes	Mouse BMMØs	Type I and Type II	Single tight vacuoles or large vacuole with amastigotes attached at posterior end	Lang et al. (1994)
L. tropica amastigotes	Human monocyte-derived MØs	Type I	Amastigotes contained within single, loose vacuoles that accumulated Thorotrast	Berman et al. (1979)
L. tropica amastigotes	P388 MØ line	Type I	Amastigote dividing within a single loose vacuole that has accumulated colloidal gold	Berman et al. (1981)
L. major amastigotes	Cultured mouse Langerhans cells	Type I	PV with single amastigotes in fairly tight membrane; 1 PV contains 2 amastigotes	Blank et al. (1993)
L. mexicana amastigotes	Mouse peritoneal MØs	Type II	Mostly single amastigotes within loose vacuoles that swell over time	Alexander and Vickerman (1975)
L. mexicana promastigotes and amastigotes	Mouse peritoneal MØs	Type II	Vacuoles were initially loose and swelled over time; those formed by amastigotes swelled more than those formed by promastigotes	Lewis and Peters (1977)
L. amazonensis amastigotes	Mouse BMMØs	Type II	Large swollen PVs with amastigotes attached to membrane at posterior end	Antoine et al. (1991)
L. amazonensis amastigotes	Mouse peritoneal MØs	Type II	Large swollen PVs with amastigotes attached to membrane at posterior end; easily visualized at light microscopic level as well	Veras et al. (1992)
L. mexicana promastigotes	Mouse peritoneal or BMMØs	Type II	Loose, often swollen PVs with single amastigotes, usually attached to membrane but in many different orientations	Russell et al. (1992)

events occurring within the PV are in part responsible for these differences in immune responses.

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