

Biogenesis of *Leishmania*-harbouring parasitophorous vacuoles following phagocytosis of the metacyclic promastigote or amastigote stages of the parasites

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Summary

Protozoan parasites *Leishmania* alternate between a flagellated promastigote form and an amastigote form. In their mammalian hosts, *Leishmania* survive and multiply in macrophages. Both forms can be internalized by these host cells at different stages of the infectious process and eventually establish themselves within parasitophorous vacuoles exhibiting phagolysosomal properties. To determine whether the biogenesis of these organelles differs according to the parasitic stage used to initiate infection, we compared their formation kinetics after phagocytosis of either metacyclic promastigotes or amastigotes of *L. amazonensis* or of *L. major* by mouse bone-marrow-derived macrophages pre-exposed or not to IFN- γ . After 10 minutes of contact, an accumulation of F-actin was observed around the promastigotes and amastigotes undergoing phagocytosis or those that had already been internalized. This accumulation was transient and rapidly disappeared at later times. At 30 minutes, most of the promastigotes were located in long, narrow organelles that were exactly the same shape as the parasites. The latter were elongated with their cell bodies near to the macrophage nucleus and their flagella towards the periphery. This suggests that promastigote phagocytosis mainly occurs in a polarized manner, with the cell body entering the macrophages first. Most, if not all, of the phagocytosed promastigotes were located in organelles that rapidly acquired phagolysosomal properties. At 30 minutes, lamp-1, macrosialin, cathepsins B and D were detected in 70-98% of these compartments and about 70% of them were surrounded by rab7p. These late endosome/lysosome 'markers' were recruited through fusion with late endocytic compartments. Indeed, when late

endosomes/lysosomes were loaded with fluorescein dextran, 81-98% of the promastigote-harbouring compartments contained the endocytic tracer 30 minutes after infection. Electron microscopy of infected macrophages previously loaded with peroxidase confirmed that the phagosomes rapidly fused with late endocytic compartments. When the amastigote stage of *L. amazonensis* was used to initiate infection, the kinetics of acquisition of the different late endosome/lysosome 'markers' by the phagosomes were similar to those measured after infection with metacyclics. However, more rab7p⁺-phagosomes were observed at early time points (e.g. 90% were rab7p⁺ at 30 minutes). The early endosome 'markers', EEA1 and the transferrin receptor, were hardly detected in parasite-containing compartments regardless of the parasitic stage used to infect macrophages and the time after infection. In conclusion, both metacyclic- and amastigote-containing phagosomes fuse with late endosomes/lysosomes within 30 minutes. However, with *L. amazonensis*, the time required for the formation of the huge parasitophorous vacuoles, which are characteristic of this species, was much shorter after infection with amastigotes than after infection with metacyclic promastigotes. This indicates that the initial fusions with late endosomes/lysosomes are followed by a stage-specific sequence of events.

Movies available on-line

Key words: *Leishmania*, Promastigote, Amastigote, Macrophage, Phagosome, Phagolysosome, Parasitophorous vacuole

Introduction

Leishmania are protozoan parasites that cause several human diseases called leishmaniasis, which can display very different clinical aspects (Peters and Killick-Kendrick, 1987b). The life cycle of *Leishmania* involves two kinds of hosts: dipteran insects (sandflies) and several mammals, in which they adopt

a motile, flagellated, promastigote form and a non-motile, amastigote form with a very short flagellum, respectively (Peters and Killick-Kendrick, 1987a). The promastigotes colonize the lumen of the digestive tract of sandflies, where they multiply and differentiate into metacyclic promastigotes, which are infectious for mammals (Sacks, 1989). Metacyclic

promastigotes are inoculated into the dermis of mammals during the bloodmeal of infected sandflies. They are then phagocytosed by macrophages where they transform into amastigotes within membrane-bound organelles of the endocytic pathway progressively acquiring late endosomal/lysosomal characteristics. This differentiation process starts in the hours following phagocytosis and takes at least 5 days (Galvao-Quintao et al., 1990; Courret et al., 2001). Amastigotes are the disseminating form in mammals and it is commonly admitted that, after their release from infected macrophages, they can be phagocytosed by adjacent macrophages.

The morphologies of mature parasite-harboring compartments, known as parasitophorous vacuoles (PVs), vary depending upon the *Leishmania* species. Large communal PVs (*L. amazonensis*, *L. mexicana*) and tight individual PVs (*L. major*, *L. donovani*) have been identified. In spite of their very different aspects, they share some properties and features (for a review, see Antoine et al., 1998). PVs are acidic compartments containing certain lysosomal enzymes. They are surrounded by a membrane enriched with late endosomal/lysosomal proteins, such as rab7p, macrosialin, lamp-1, lamp-2 and vacuolar H⁺-ATPase, and with molecules of the antigen-presentation machinery (MHC class II and H-2M molecules) in IFN- γ -treated macrophages.

Compared with our knowledge of the events following internalization of inert particles such as latex beads (for reviews, see Desjardins, 1995; Garin et al., 2001), the biogenesis of *Leishmania*-harboring PVs is still poorly understood. However, it has been recently described that the formation of PVs occurs differently according to the stage of the parasites internalized, at least in terms of kinetics (Desjardins and Descoteaux, 1997; Dermine et al., 2000). Thus, it has been shown that, after the phagocytosis of cultured stationary phase *L. donovani* promastigotes by the macrophage-like cells J774, the parasites are transiently located in phagosomes with poor fusogenic properties towards late endocytic compartments (Desjardins and Descoteaux, 1997). In contrast, after their internalization, amastigotes are found in compartments that rapidly fuse with late endocytic organelles (Lang et al., 1994b; Dermine et al., 2000). These distinctive features of the early phagosomes could be linked to the stage-specific expression of a high molecular weight glycolipid, the lipophosphoglycan (LPG), on the plasma membrane of the promastigotes, which may modify the fusion capacity of the phagosomal membrane, at least temporarily. (Desjardins and Descoteaux, 1997; Scianimanico et al., 1999; Dermine et al., 2000). Furthermore, it has been shown in these studies that most of the phagosomes containing LPG-bearing promastigotes display an impaired recruitment of the small GTPase rab7p, which is involved in the homotypic fusions of late endosomes or lysosomes and in the heterotypic fusions of late endosomes with lysosomes (Scianimanico et al., 1999). A long-lasting (more than 1 hour) accumulation of filamentous (F) actin has also been noted around these phagosomes (Holm et al., 2001). It is suspected that these anomalies reflect the transient lack of phagosome maturation or are involved in the maintenance of this property. This fusion restriction lasts several hours, which may allow the parasites to initiate their differentiation into amastigotes, which are more adapted to the lysosomal compartment. Such a proposal is consistent with the

fact that *L. major* promastigotes that are unable to synthesize LPG survive poorly within mouse peritoneal macrophages (Späth et al., 2000).

The generality of this model was recently questioned by a study showing that stationary phase LPG-deficient *L. mexicana* promastigotes bind to and multiply within mouse peritoneal macrophages as efficiently as, or even more efficiently than, wild-type promastigotes. This indicates that, at least for this *Leishmania* species, LPG is not a determining factor for the differentiation of promastigotes into amastigotes (Ilg, 2000). Consequently, the biological role of the transient restriction of fusion described for phagosomes containing *L. major* or *L. donovani* is not obvious, but it suggests that each *Leishmania* species has developed its own establishment strategy for mammalian macrophages (Turco et al., 2001).

We used different parasite-host cell combinations to study the maturation of *Leishmania* phagosomes. Previous studies on this topic were carried out on unselected and thus heterogeneous (especially in terms of virulence) stationary phase promastigotes. In contrast, we mainly focused on the early events following the phagocytosis of metacyclic promastigotes, which are pre-adapted to the encounter with mammals, in particular to intracellular conditions. We used immunofluorescence confocal microscopy and quantitative analyses to determine whether the kinetics of association of late endosomal/lysosomal molecules to *Leishmania*-harboring phagosomes varies according to the *Leishmania* species or the parasitic stage put into contact with the macrophages. We examined the association with phagosomes of the following molecules: the TfR, which, at steady state, is localized in early sorting and recycling endosomes (for a review, see Gruenberg and Maxfield, 1995); EEA1, a rab5p effector that is mainly detected on the cytosolic side of early endosomes (Mu et al., 1995); rab7p, which appears to control the aggregation and fusion of late endocytic structures/lysosomes (Chavrier et al., 1990; Bucci et al., 2000); macrosialin, a macrophage-specific membrane glycoprotein belonging to the lamp family and mainly expressed in late endosomes (Rabinowitz et al., 1992; Holness et al., 1993); lamp-1, a major protein constituent of late endosomal and lysosomal membrane (for a review, see Hunziker and Geuze, 1996); cathepsins B and D, two acid hydrolases mainly concentrated in macrophage lysosomes (Rodman et al., 1990; Claus et al., 1998); and MHC class II molecules, which are localized within antigen-presenting cells, in compartments called MIIC that have all of the characteristics of the late endosomes or lysosomes (for a review, see Geuze, 1998).

We conclude that young phagosomes containing *L. amazonensis* promastigotes or amastigotes or *L. major* promastigotes rapidly acquire a competence to fuse with late endosomes/lysosomes.

Materials and Methods

Mice and parasites

Two- to four-month-old female BALB/c and Swiss nu/nu mice were obtained from the breeding center of the Institut Pasteur (Paris, France) and Iffa Credo (St Germain-sur-l'Arbresle, France), respectively.

Amastigotes of *L. amazonensis* strain LV79 (MPRO/BR/1972/M1841) and of *L. major* strain NIH173 (MHOM/IR/-/173) were purified from the feet of infected nude mice as described previously

(Antoine et al., 1989). Metacyclic promastigotes of these two *Leishmania* strains were obtained from amastigotes cultured at 26°C (Courret et al., 1999). They were purified by negative selection using the peanut agglutinin (NIH173) (Vector Laboratories, Burlingame, CA) or the monoclonal antibody (mAb) 3A1 (LV79) (Sacks et al., 1985; Courret et al., 1999). *L. amazonensis* stationary phase promastigotes submitted to the same cycle of washings and centrifugations as metacyclic promastigotes during their purification, but not incubated with the mAb 3A1, were also prepared.

Macrophage infections

Macrophages were obtained from BALB/c mice by in vitro differentiation of bone marrow precursor cells in 24-well plates containing 12 mm diameter round glass coverslips for light microscopy and scanning electron microscopy studies, or in 35 mm culture dishes for transmission electron microscopy studies. For microcinematography, precursors were deposited in 60 mm cultures dishes containing 34×34 mm square coverslips. Cells were cultured in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Dutscher, Brumath, France), 50 U/ml penicillin, 50 µg/ml streptomycin and 20% L-929 fibroblast-conditioned medium. After 5 days at 37°C in a 5% CO₂/95% air atmosphere, non-adherent cells were removed and adherent macrophages were further incubated in culture medium containing only 3% of conditioned medium. At this step, cells were treated or not with rIFN-γ (10–25 U/ml, Genentech, San Francisco, CA) for 18–24 hours before the addition of the parasites. Macrophages were infected at a stationary phase promastigote-, metacyclic promastigote- or amastigote-host cell ratio of 3:1 to 5:1. The parasites and macrophages were quickly brought into contact by centrifugation at 20°C (130 g, 5 minutes). Cultures were then incubated at 34°C (*L. amazonensis* infections) or 37°C (*L. major* infections) for 30 minutes before being washed with Dulbecco's phosphate-buffered saline (PBS, Seromed) to remove free parasites. Macrophages were fixed either immediately (time point 30 minutes) or after various times (between 1 and 48 hours post-infection). In some experiments, cultures were centrifuged as above after the addition of the parasites and only incubated at 34 or 37°C for 5 minutes before fixation (time point 10 minutes). In this case, the cultures were not washed before fixation. Control experiments ensured that centrifugation of the parasites did not modify the characteristics of the early events analysed in this study.

Loading of macrophages with endocytic tracers

Before infection, macrophages were incubated either for 2 hours at 37°C with 2–3 mg/ml anionic, lysine fixable fluorescein dextran (FDex, average *M_r* 10,000, Molecular Probes, Eugene, OR), or for 30 minutes at 37°C with 25 µg/ml horseradish peroxidase (HRP, RZ 3, Sigma Chemical Co., St Louis, MO). Cells were then thoroughly washed with cold PBS and chased for 140–160 minutes or overnight (17–20 hours) in tracer-free medium.

Antibodies and fluorescent reagents

The mAb 3A1, a mouse IgG2b specific to the LPG of *L. amazonensis* log phase promastigotes (Courret et al., 1999), was provided by D. L. Sacks (Laboratory of Parasitic Diseases, NIAID, Bethesda, MD). A rabbit immune serum raised against *L. mexicana* leishmanolysin was obtained from P. Overath [Max Planck Institute for Biology, Tübingen, Germany (Bahr et al., 1993)]. The mAb RI7 217.1.3, a rat IgG2a specific for the mouse transferrin receptor [TfR (Lesley et al., 1984)], was a gift from D. Ojcius (Institut Pasteur, Paris, France). Rabbit immune sera specific for EEA1 and rab7p were provided by M. Zerial (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany). Before use, the anti-rab7p immune

serum was adsorbed on a *L. amazonensis* amastigote lysate. Hybridoma cells secreting the FA/11 mAb, a rat anti-mouse macrosialin IgG2a (Smith and Koch, 1987), were obtained from G. Koch (MRC Laboratory of Molecular Biology, Cambridge, UK). The anti-mouse lamp-1 (CD107a) mAb 1D4B, a rat IgG2a (Chen et al., 1985), and the biotin-conjugated anti-mouse I-A^d/I-E^d mAb 2G9 [rat IgG2a (Becker et al., 1992)] were purchased from Pharmingen (San Diego, CA). Rabbit IgG specific to rat cathepsin B or D and crossreacting with mouse cathepsin B or D were obtained from B. Wiederanders (Friedrich-Schiller University, Jena, Germany) and H. Kirschke (University of Halle, Halle, Germany). A *Leishmania*-specific immune serum was prepared from *L. amazonensis*-infected BALB/c mice. The mAb JES6-1A12, a rat IgG2a specific to mouse IL-2 (Abrams et al., 1992), and a normal rabbit serum, were used as controls for the specific primary Abs or immune sera described above. Primary Abs associated with cell preparations were detected by use of the following conjugates: fluorescein-labeled F(ab')₂ fragments of donkey anti-rat or anti-rabbit Ig; Texas Red-labeled F(ab')₂ fragments of donkey anti-mouse Ig (Jackson ImmunoResearch Laboratories, West Grove, PA); and fluorescein-labeled ExtrAvidin (Sigma). Alexa Fluor 488-phalloidin (Molecular Probes) was used to stain F-actin.

Fluorescence microscopy

Macrophages were fixed with paraformaldehyde and then permeabilized (Lang et al., 1994a). They were labeled with primary Abs and fluorescent conjugates according to standard procedures (Lang et al., 1994a). After simple immunolabelings, nucleic acids were stained with propidium iodide (Lang et al., 1994a). Cell preparations were mounted in Mowiol (Calbiochem, San Diego, CA) before observation under an Axiophot Zeiss epifluorescence microscope or under a LSM 510 Zeiss confocal microscope (Carl Zeiss Microscopy, Jena, Germany). Confocal microscopy images were acquired and analysed by use of the 2.5 version of the LSM 510 software before being exported to Adobe PhotoShop (Mountain View, CA).

Transmission electron microscopy

Infected macrophages exposed to HRP were fixed for 1 hour at room temperature with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, HCl buffer, pH 7.2, containing 0.1 M sucrose. Cells were washed overnight at 4°C with sucrose-containing cacodylate buffer and incubated with 3,3'-diaminobenzidine tetrachlorhydrate and H₂O₂ (Malmgren and Olsson, 1977) before post-fixation with osmium and Epon embedding. Sections were examined with a Jeol 100CXII electron microscope (Jeol Ltd., Akishima, Japan).

Scanning electron microscopy

Macrophages were exposed to *L. amazonensis* metacyclic promastigotes (five parasites/host cell) for 10 minutes at 34°C. Cells were then fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, HCl buffer, pH 7.2, containing 0.1 M sucrose. After three washes with sucrose-containing cacodylate buffer, cells were dehydrated in ethanol and processed for scanning electron microscopy according to standard protocols. Cells were examined at the Centre Inter-Universitaire de Microscopie Electronique (CIME) Jussieu (Paris, France).

Time-lapse microcinematography

Coverslips with macrophages were mounted in observation microchambers known as Rose's chambers (Rose, 1954), which were then placed on the stage of a thermostated (34°C) Zeiss inverted microscope linked to an automated 16 mm Arriflex camera. The camera was programmed to take one or two pictures per second for 30–60 minutes. The grabbing sequences started when the

promastigotes were placed in the Rose's chambers. The images were analyzed on a NAC projector.

Online supplemental material

Movies 1 and 2 (see <http://jcs.biologists.org/supplemental>) correspond to Fig. 1A and B and contain QuickTime sequences depicting the phagocytosis of metacyclic promastigotes by macrophages. Images were captured every 0.5 seconds over the course of 367 seconds (Movie 1) or 125 seconds (Movie 2).

Results

Experimental conditions and time course of infection

We studied the biogenesis of PVs between 10 minutes and 48 hours after the infection of mouse bone-marrow-derived macrophages with *L. amazonensis* or *L. major* metacyclic promastigotes or amastigotes. Unlike the other parasitic stages (stationary phase promastigotes, amastigotes), purified metacyclic promastigotes have never been used in such a study. Thus, most of the data presented below concern the interactions between the macrophages and the metacyclics. Amastigotes were used only in some experiments for comparison. In many experiments, to enable us to follow the appearance of MHC class II molecules in the membrane of the phagocytic compartments, macrophages were pre-exposed to a low dose of IFN- γ . We checked that this cytokine, at the concentration used, had no effect on the course of infection (data not shown).

Furthermore, for most of the analyzed parameters, very similar results were obtained with untreated or IFN- γ -pre-treated macrophages (see below). Compared with the initial values, determined 30 minutes after infection, the percentage of infected macrophages and the parasite load decreased only slightly at 48 hours when metacyclic promastigotes were used to initiate infection (e.g. 70-75 and 75-80% of the initial values, respectively, in experiments using *L. amazonensis* as infectious agents). As shown before, intermediate parasitic stages started to divide after 24-48 hours (Courret et al., 2001). The percentage of infected macrophages was the same 30 minutes and 48 hours after infection with amastigotes of *L. amazonensis*, but the number of parasites increased during the period of observation. These data indicate that our experimental conditions allowed the intracellular establishment of most parasites regardless of the parasitic stage used.

Entry of *L. amazonensis* metacyclic promastigotes into macrophages

After the parasites bound to the macrophages, either long tubular pseudopods tightly encircling their cell body or their flagellum (Fig. 1A,C) or ruffles (Fig. 1B,D) were formed. Very often, parasites were phagocytosed with the cell body entering first (Fig. 1; see also Movies 1 and 2 at <http://jcs.biologists.org/supplemental>), but ingestion starting by the flagellum was also observed (data not shown). In some cases, the parasites first

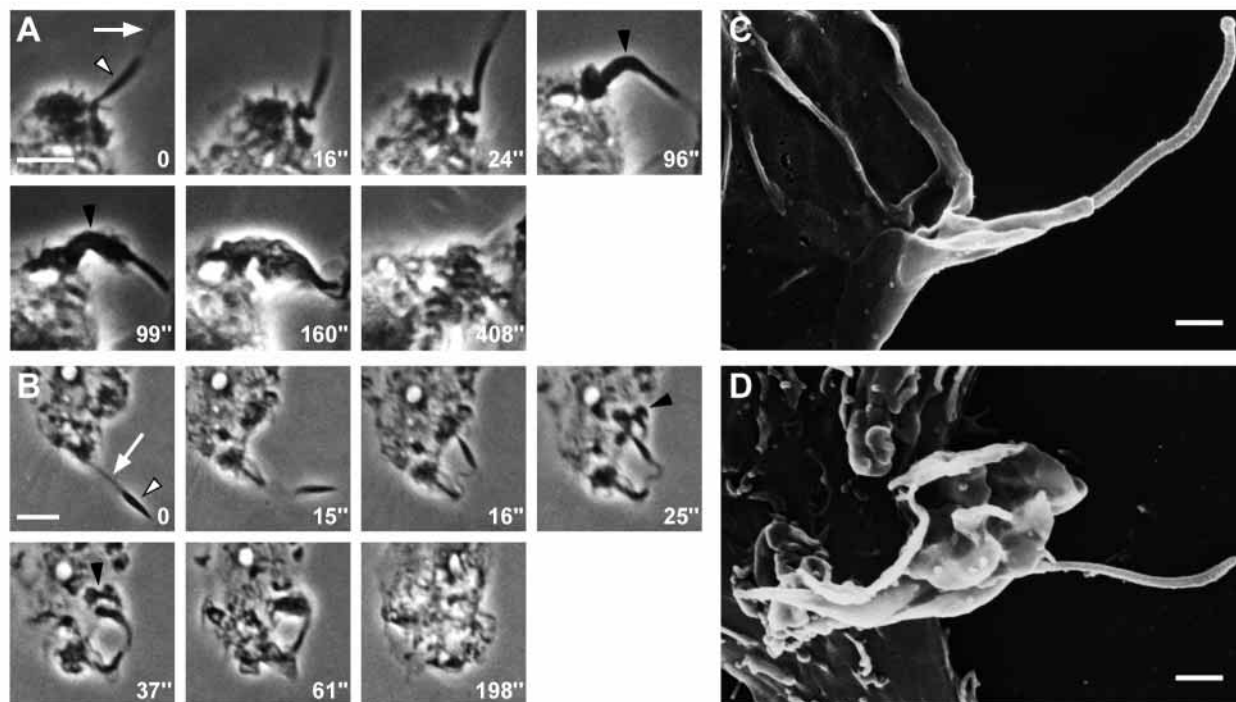


Fig. 1. Mechanisms of entry of *L. amazonensis* metacyclic promastigotes into macrophages. (A,B) Microcinematography of phagocytic events. Promastigote cell bodies and flagella are indicated by white arrowheads and white arrows, respectively. (A) Parasite attachment occurs by the cell body. The promastigote is then progressively internalized through the formation of a long tubular pseudopod (black arrowheads). (B) The parasite binds to the macrophage via the tip of the flagellum. It then turns around, so that its cell body comes into contact with the cell surface of the macrophage. Ruffles are formed at this site (black arrowheads). The promastigote is finally ingested via the cell body. The numbers indicated in the right hand corners correspond to the time (in seconds) elapsed from the first contact of promastigotes with macrophages (time 0). QuickTime movie sequence versions of A and B are available online (<http://jcs.biologists.org/supplemental>). (C,D) Scanning electron micrographs showing the internalization of promastigotes via the formation of a closely apposed pseudopod (C) or of ruffles (D). Macrophages were fixed after 10 minutes of contact with the parasites. Bars, 10 μ m (A,B), 1 μ m (C,D).

interacted with the macrophages via their flagellum (Fig. 1B, time points 0 to 15 seconds) but they rapidly turned around (Fig. 1B, time point 16 seconds) and finally entered macrophages by the cell body (Fig. 1B, time points 37 seconds to 61 seconds). We also observed the lateral attachment of the parasites to the macrophage cell surface. In these cases, the plasma membrane folds wrapped themselves around the parasites, and the different parts of the latter were simultaneously internalized (data not shown). Complete phagocytosis took about 3–9 minutes.

Polymerization of macrophage actin is a transient event during metacyclic promastigote or amastigote phagocytosis

The metacyclic promastigotes and amastigotes of *L. amazonensis* were internalized by an actin-dependent process as shown by the accumulation of F-actin around the parasites during and after phagocytosis. To determine whether the duration of F-actin accumulation varied with the parasitic

stage, macrophages were fixed at different times after infection, and F-actin was stained with fluorescent phalloidin. At 10 minutes, about 40–55% of metacyclics or amastigotes were surrounded by F-actin. At this stage, most of the F-actin⁺-parasites were still in the process of phagocytosis. After completion of the internalization process, the percentage of F-actin⁺-parasites rapidly dropped to reach 5–10% at 1–2 hours post-infection (Fig. 2A). At the early time point, F-actin appeared generally as a thick ring around the promastigote cell bodies and amastigotes or adopted the shape of a sleeve around the promastigote flagella (Fig. 2B,C). With promastigotes, F-actin was often concentrated around either the cell body or the flagellum and less frequently around both parts. This suggests that F-actin sequentially polymerizes and depolymerizes along the parasites during their internalization. Presence of F-actin in the ruffles formed at the entry point of some parasites was also noted (data not shown).

As with *L. amazonensis*, F-actin was detected around *L. major* metacyclic promastigotes in the process of phagocytosis. Thereafter, parasite-associated F-actin disappeared with a kinetics slower than that observed for *L. amazonensis*. Thus, 10 minutes, 30 minutes and 2 hours after adding parasites, 77.2, 48.0 and 24.7% of them were F-actin⁺, respectively. This, apparently, did not slow down the recruitment of endosome/lysosome 'markers' into phagosomes (see below).

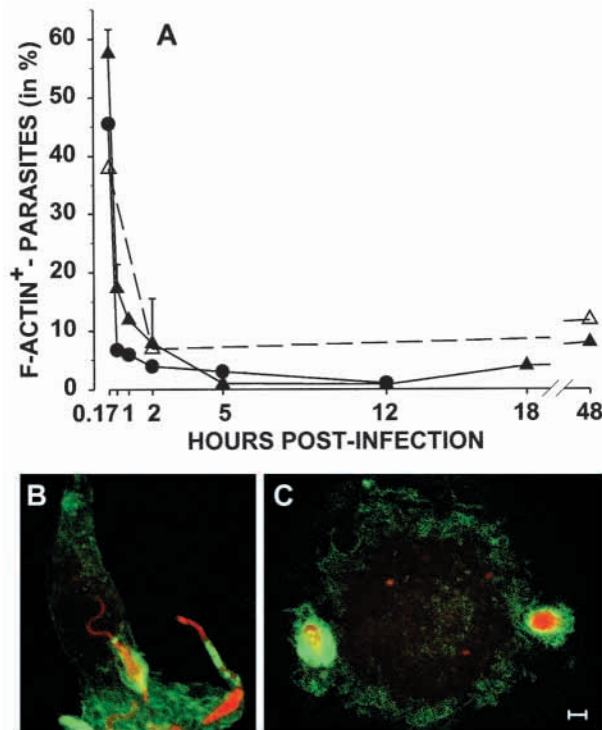


Fig. 2. F-actin accumulation around parasites during or following phagocytosis. (A) Macrophages untreated (Δ) or treated with IFN- γ (\blacktriangle , \bullet) were infected with *L. amazonensis* metacyclic promastigotes (Δ , \blacktriangle) or amastigotes (\bullet) and then fixed and permeabilized at the different time points indicated. F-actin was stained with Alexa Fluor 488 phalloidin (green staining) and parasites with a mouse anti-*Leishmania* immune serum and a Texas Red conjugate (red staining) before examination of the cells by fluorescence microscopy. The percentages of promastigotes or amastigotes surrounded by F-actin were determined after counting about 100 parasites for each time point. Data are the means \pm s.d. of three experiments (\blacktriangle) or are from a single experiment (Δ , \bullet). (B,C) Fluorescence confocal microscopy of macrophages incubated for 10 minutes with *L. amazonensis* metacyclic promastigotes (B) or amastigotes (C). F-actin and parasites were stained as in A. A 3D reconstruction and an optical section (0.5 μ m thickness) are shown in B and C, respectively. Bar, 2 μ m.

Kinetics of PV formation following metacyclic promastigote or amastigote phagocytosis

All the parasites still present in the cultures were completely internalized 30–60 minutes after the addition of *L. amazonensis* promastigotes to macrophage monolayers pre-exposed or not to IFN- γ . Most of them (about 70%) were elongated, with the cell body directed towards the macrophage nucleus and the flagellum directed towards the periphery (Fig. 3, group 1; Fig. 4A). About 3% of the parasites were in the opposite direction (Fig. 3, group 3). The remainder (about 30%) displayed no clear orientation and were in vacuoles already located near to the macrophage nucleus (Fig. 3, group 2). At this step of the infectious process, most of the parasites were located in very long (several tens of microns), narrow compartments, the membrane of which adopted their exact shape (see below). The lumen of these organelles could not normally be seen under the light microscope (Fig. 4A). Similar compartments were observed after infection of macrophages with *L. major* metacyclic promastigotes (data not shown). At 5 hours post-infection, most of the parasites had lost their long flagella and were located in smaller compartments gathered around the macrophage nucleus (Fig. 4C).

After amastigote phagocytosis, parasites were initially localized in peripheral, tight, ovoid vacuoles that then rapidly reached the macrophage cell center (Fig. 4E). With *L. amazonensis*, which induces the formation of huge PVs, we noted that the time elapsed before the enlargement process began varied according to the parasitic stage used. The first signs of dilatation were seen about 12–18 hours after infection with metacyclic promastigotes and 2 hours after infection with amastigotes (Fig. 4F). At later times, large vacuoles containing numerous parasites began to appear. They displayed a similar size in macrophages infected for 18/24 hours with metacyclics or for 5 hours with amastigotes (Fig. 4D,G).

Kinetics of endosome/lysosome 'marker' recruitment into phagosomes containing initially metacyclic promastigotes

It has been reported that huge communal PVs housing *L. mexicana* or *L. amazonensis* are formed by the fusion of small individual vacuoles between them and with compartments of the endocytic pathway (for a review, see Antoine et al., 1998). To determine whether the different kinetics of PV formation described above were linked to the capacity of the early phagosomes to fuse with endocytic compartments, we studied the acquisition by these organelles of various soluble and membrane molecules known to be preferentially associated with early endosomes or with late endosomes/lysosomes (TfR, EEA1, rab7p, macrosialin, lamp-1, cathepsins B and D, and MHC class II molecules). We initially focused on the association of these molecules with phagosomes formed after metacyclic entry.

In macrophages that had been pre-treated with IFN- γ , no more than 10–15% of the phagosomes formed after internalization of *L. amazonensis* metacyclics displayed the early endosome 'markers' TfR and EEA1, even at the earliest time points (10–30 minutes) (Fig. 5A,B). In contrast, at 30 minutes, about 95% of these compartments already contained macrosialin and lamp-1, about 75 and 90% of them had acquired cathepsins B and D, respectively and 70% were rab7p-positive (Fig. 5A,B; Fig. 6A–D). In macrophages expressing MHC class II molecules, about 50% of the phagosomes/phagolysosomes contained class II at 30 minutes post-infection. At this infection stage, the soluble and membrane 'markers' detected in phagosomes were both closely associated with the promastigotes and surrounded the entire parasites, including the flagella (Fig. 6). Later on,

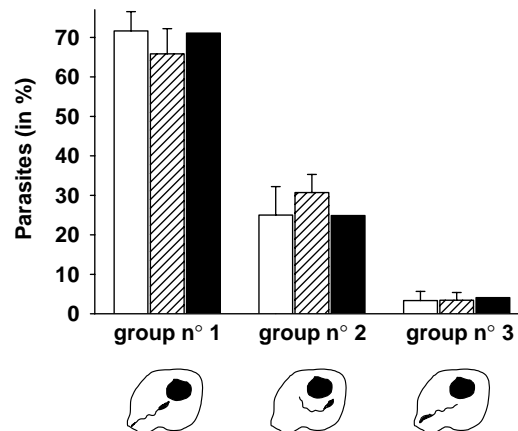


Fig. 3. Promastigote orientation shortly after internalization.

Macrophages untreated (white bars) or treated with IFN- γ (hatched and black bars) were infected with *L. amazonensis* metacyclic promastigotes. They were fixed 30 minutes (white and hatched bars) or 60 minutes (black bars) after the addition of the parasites and permeabilized before staining with an anti-leishmanolysin or anti-*Leishmania* immune serum and adequate fluorochrome conjugates. Cell preparations were then examined by fluorescence microscopy. Three groups of intracellular parasites were distinguished according to their orientation as shown in the schemes below the histograms. Results are expressed as the means+s.d. of two (white bars) or three experiments (hatched bars) or are from a single experiment (black bars). Percentages were determined after counting 703, 2200 and 712 parasites, respectively.

phagolysosomes were virtually all positive for macrosialin and lamp-1 from 1 hour post-infection (Fig. 5A,B; Fig. 6E). The percentage of rab7p⁺ and of class II⁺ parasite-containing

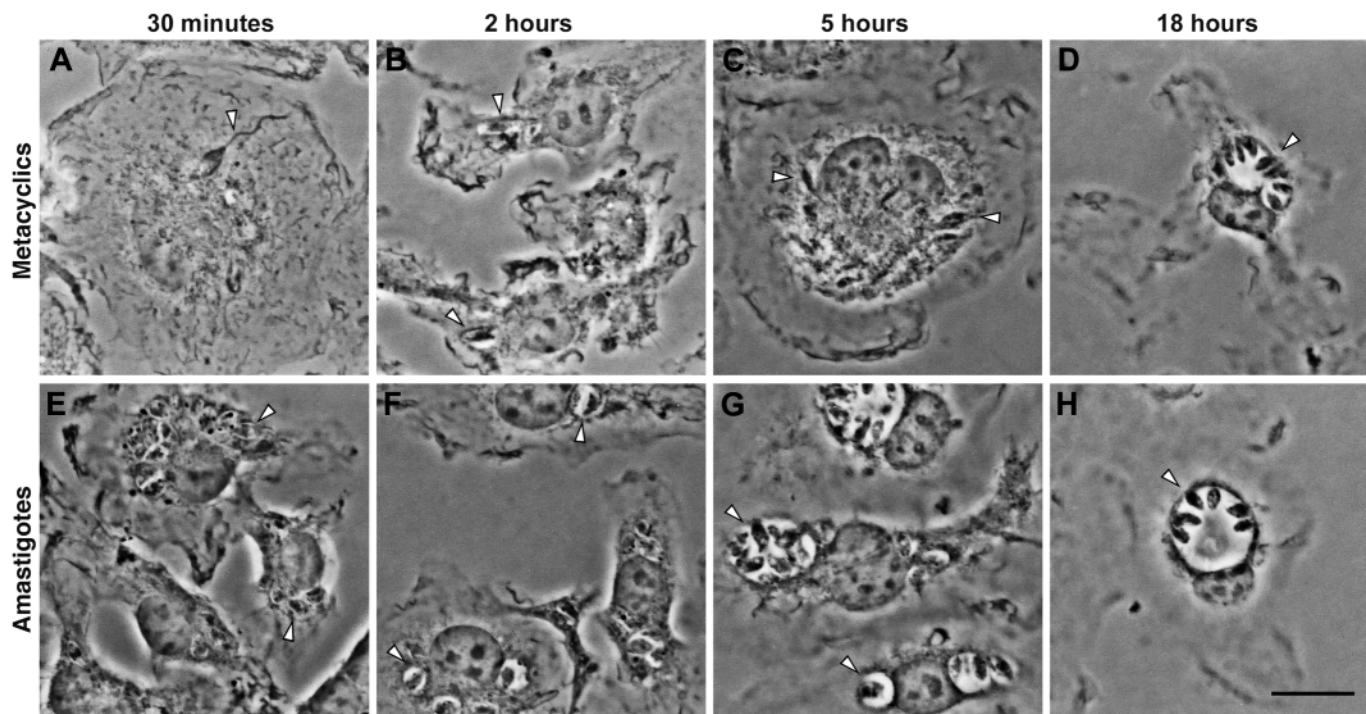


Fig. 4. Light microscopy analysis of PV formation. Macrophages treated with IFN- γ were infected with *L. amazonensis* metacyclic promastigotes (A–D) or amastigotes (E–H). At the time points indicated, macrophages were fixed and then examined by phase-contrast microscopy. In each panel, some parasites are indicated by white arrowheads. Bar, 10 μ m.

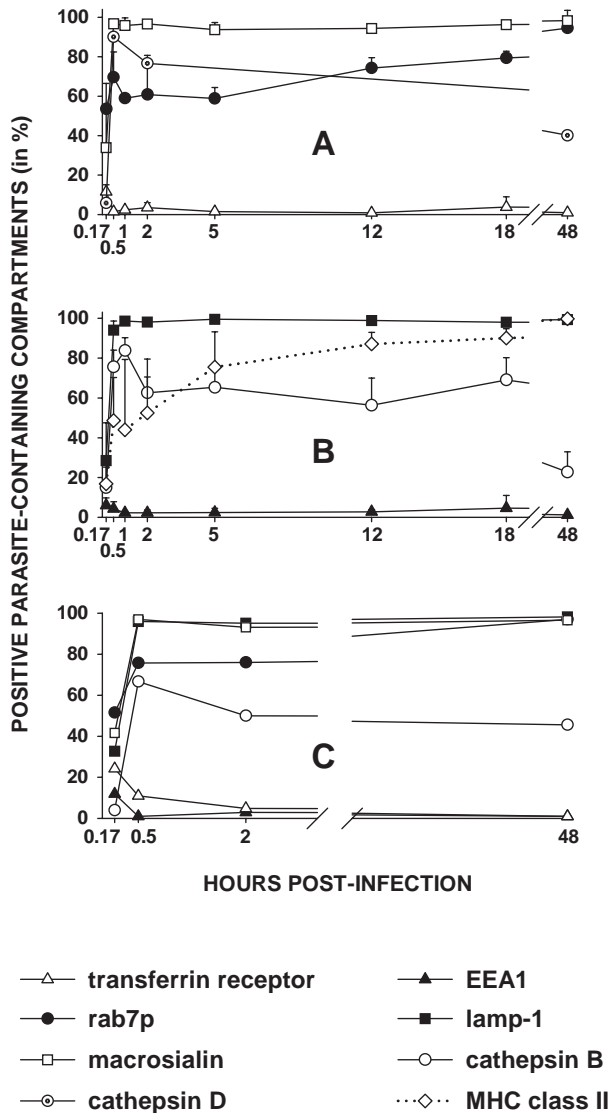


Fig. 5. Kinetics of endocytic 'marker' association with phagosomes containing initially *L. amazonensis* metacyclic promastigotes. Macrophages pre-exposed (A,B) or not exposed (C) to IFN- γ were infected at a multiplicity of four parasites/host cell. Association of the following molecules with parasite-containing phagosomes was examined as a function of time: transferrin receptor, EEA1, rab7p, macrosialin, lamp-1, cathepsin B, cathepsin D and MHC class II molecules. At the time points indicated, cell preparations were fixed, permeabilized and incubated with immunological reagents before analysis by fluorescence microscopy. For each experiment and at each time point, the percentages of parasite-containing compartments displaying the molecules listed above were determined after counting about 100 to 200 organelles. Each value represents the mean \pm s.d. of two to eight experiments (A,B) or is from a single experiment representative of two separate experiments (C).

organelles gradually increased, reaching 100% at 48 hours post-infection. About 60–80% of the phagosomal compartments were cathepsin-positive between 2 and 18 hours, a time period during which enzymes were clearly detected in the lumen of the still small PVs (Fig. 5A,B; Fig. 6F). The percentage of cathepsin⁺-compartments dropped considerably at 48 hours (Fig. 5A,B) but this can easily be explained by the

fact that soluble molecules like these enzymes are not retained in the large PVs during fixation.

As the results described above were obtained using IFN- γ -pre-treated macrophages as host cells, we next examined whether the IFN- γ treatment could modify the kinetics of PV maturation. Comparison of Fig. 5A, B and C clearly indicates that, at the dose used, IFN- γ had no effect on the analyzed parameters.

The metacyclic promastigotes used in the preceding experiments were prepared from stationary phase promastigotes by negative selection using the mAb 3A1 (Courret et al., 1999). To see whether the treatment applied to the parasites could influence the biogenesis of the phagosomal compartments, a side-by-side comparison of phagosomes containing initially either stationary phase or metacyclic promastigotes of *L. amazonensis* was undertaken. Fig. 7 shows that phagosome formation, measured by the appearance in the phagosomal membrane or lumen of the transferrin receptor, rab7p, lamp-1 or cathepsin B, was strictly similar in both conditions of infection. Study of the association of EEA1, macrosialin and MHC class II molecules ended at the same conclusion (data not shown).

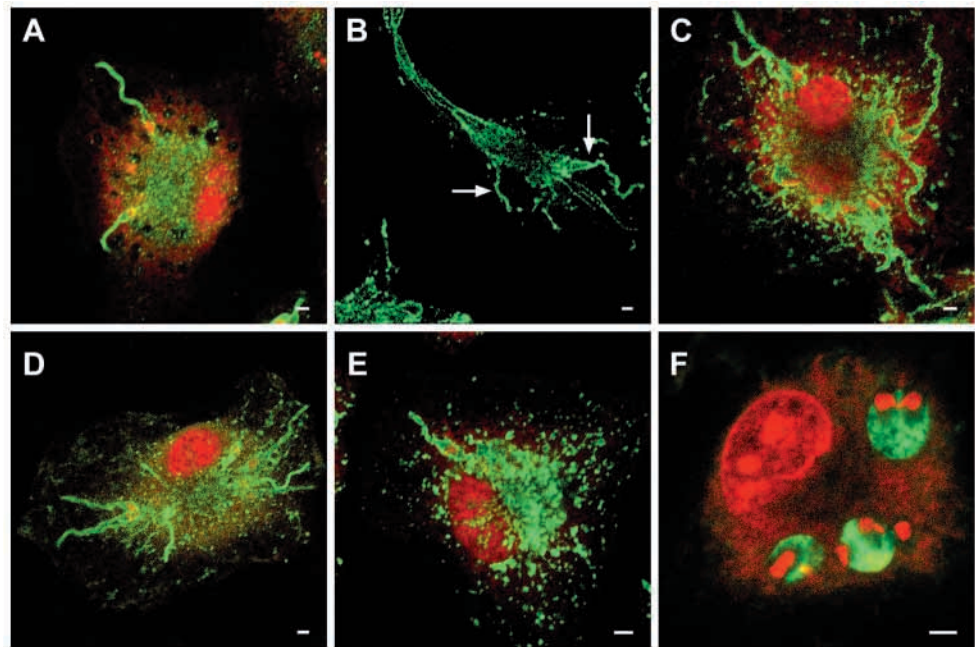
Likewise, similar data were obtained when metacyclic promastigotes of *L. major*, a species that does not induce the formation of large PVs, were used to initiate infection (Fig. 8). This suggests that the rapid acquisition of late endosome/lysosome 'markers' by promastigote-containing organelles is a *Leishmania* species-independent process.

As negative controls, promastigote-infected macrophages were incubated with an irrelevant mAb or normal rabbit serum instead of specific reagents. We also incubated purified metacyclic promastigotes with macrophage organelle-specific Abs. No staining or a very weak background staining could be detected on these preparations (data not shown).

Fusion of FDex- or HRP-loaded late endocytic compartments with phagosomes harbouring initially metacyclic promastigotes

Previous data clearly showed that, at 30 minutes post-infection with metacyclic promastigotes, most parasite-containing organelles displayed molecules of the late endocytic compartments. We demonstrated that the recruitment of these molecules occurred through the fusion of late endocytic compartments with phagosomes as follows. Before infection with metacyclics, macrophages were pre-incubated with FDex for 2 hours, washed and then incubated for 2–3 hours or overnight to allow the preferential accumulation of the fluorescent molecules in late endosomes or lysosomes, respectively. Regardless of the time of chase, FDex rapidly appeared in compartments containing initially metacyclic promastigotes. For example, at 30 minutes, 90–100% and 80–85% of these organelles were fluorescent after infection with *L. amazonensis* and *L. major* metacyclics, respectively (Fig. 9A). At this stage, phagosome-associated FDex was localized around the cell body and the flagellum of the parasites and adopted their exact shape (Fig. 9B,C). The percentage of fluorescent parasite-containing organelles decreased slightly at later times of infection, which could be due to a redistribution of fluorescent molecules or, in the case of *L. amazonensis*-containing compartments, to the loss of soluble FDex from the

Fig. 6. Immunofluorescence labeling of late endosome/lysosome ‘markers’ associated with parasite-containing organelles at different time points after infection of IFN- γ -treated macrophages with *L. amazonensis* metacyclic promastigotes. Macrophages were processed for fluorescence microscopy 10 minutes (A), 30 minutes (B–D), 2 hours (E) or 18 hours (F) after the addition of the parasites. Cell preparations were incubated with immune sera or Abs directed against rab7p (A), macrosialin (B), lamp-1 (C,E), cathepsin B (D,F) and then with adequate fluorescein conjugates (green staining). Except in B, macrophage nuclei and parasite nuclei and kinetoplasts were stained with propidium iodide (red staining). In B, the parasites are indicated by arrows. Sections (0.3–0.5 μ m thickness) obtained by confocal microscopy are shown. The micrographs are representative of three to eight experiments. Bars, 2 μ m.



enlarging organelles during fixation. At this stage, FDex associated with the small individual PVs was clearly localized in the lumen of the organelles (Fig. 9D,E). Very similar results were obtained with macrophages that had been pre-incubated for 30 minutes with HRP, chased for 160 minutes or overnight and then infected with *L. amazonensis* metacyclic promastigotes. Electron microscopy analysis of these cells showed that HRP was present in the lumen of the parasite-containing compartments 30 minutes after infection (Fig. 10A). Typical images of fusion of HRP-

loaded late endosomes/lysosomes with phagosomes were observed (Fig. 10B,C). As controls, macrophages that had not been incubated with HRP were infected and then processed as above. No staining could be detected under these conditions.

Kinetics of endosome/lysosome ‘marker’ acquisition by phagosomes formed after amastigote ingestion
PV maturation assessed by the acquisition of early endosome, late endosome or lysosome ‘markers’ was studied after the

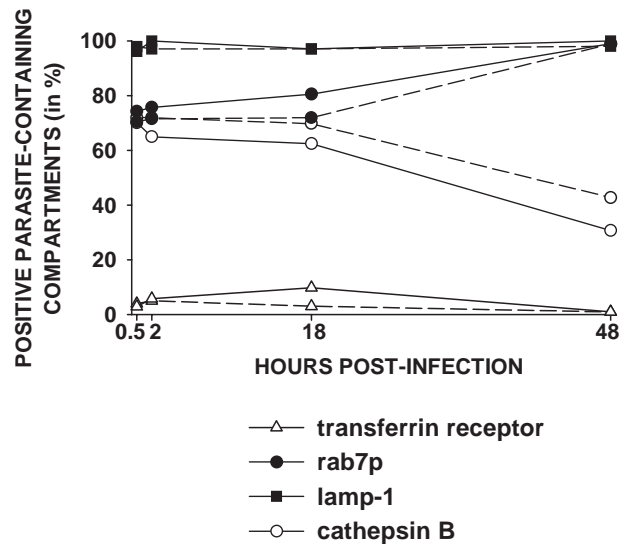


Fig. 7. PV biogenesis in IFN- γ -treated macrophages infected with either *L. amazonensis* stationary phase or metacyclic promastigotes. Macrophages were processed as described in the legend to Fig. 5 to determine the association of the transferrin receptor, rab7p, lamp-1 and cathepsin B with phagosomes as a function of time. Results obtained after phagocytosis of stationary phase and metacyclic promastigotes are represented by broken and solid lines, respectively.

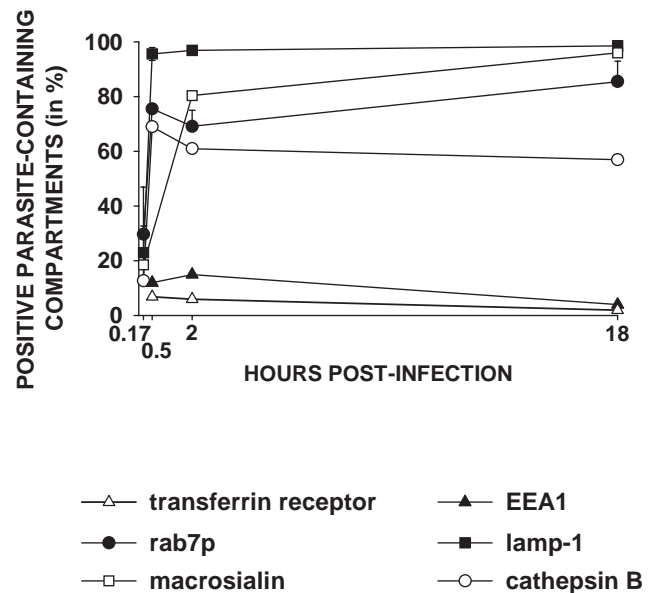


Fig. 8. PV biogenesis in IFN- γ -treated macrophages infected with *L. major* metacyclic promastigotes. Macrophages were processed as described in Fig. 5 to determine the association of the different endosome/lysosome ‘markers’ with phagosomes as a function of time. Results are the means \pm range of two experiments.

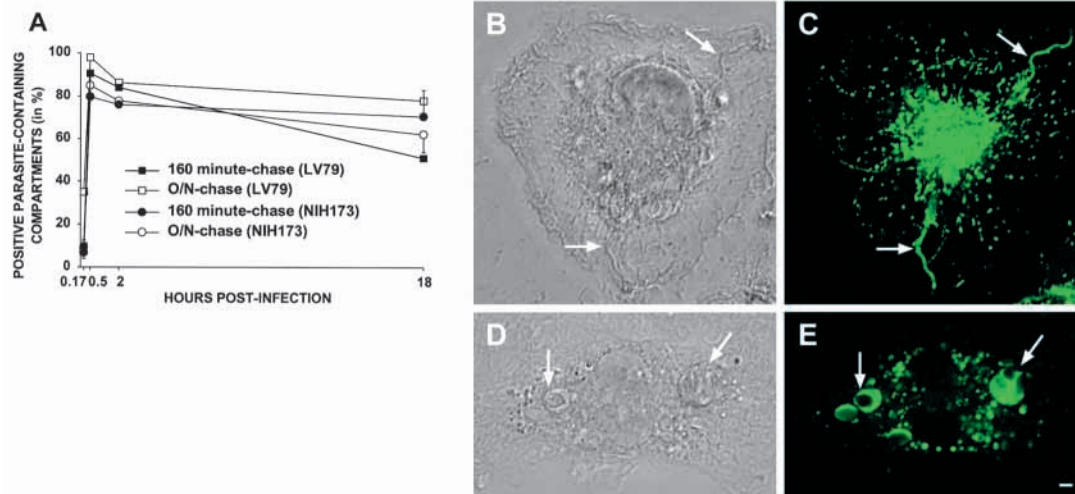


Fig. 9. (A) Kinetics of delivery of late endosome/lysosome-associated FDex into parasite-containing organelles formed after phagocytosis of metacyclic promastigotes. IFN- γ -treated macrophages were incubated with FDex for 2 hours. After washings, they were chased either for 160 minutes or overnight before infection with *L. amazonensis* (LV79) or *L. major* (NIH173) metacyclic promastigotes. Macrophages were fixed and permeabilized at various times post-infection and the parasites they contained were counted after staining with either a mouse anti-*Leishmania* immune serum and a Texas Red conjugate or propidium iodide. For each experiment and at each time point, the percentage of FDex-positive, parasite-containing compartments was determined after counting about 100 organelles. Results are from a single experiment (LV79) or are the means \pm range of two experiments (NIH173). (B-E) Confocal microscopy of macrophages loaded with FDex, chased overnight in FDex-free medium and then infected with *L. amazonensis* metacyclics. Analysis was done 30 minutes (B,C) or 18 hours (D,E) after adding parasites. (B,D) and (C,E) are the differential interference contrast (DIC) and the fluorescence images of the same cells, respectively. Optical sections (0.3–0.5 μ m thickness) are shown. The position of the parasites is indicated by arrows. Bar, 2 μ m.

internalization of *L. amazonensis* amastigotes. The kinetics of 'marker' recruitment were rather similar to those we measured after the ingestion of metacyclic promastigotes (Fig. 11). However, small differences were noted (compare Figs 5 and 7 with Fig. 11). The percentage of rab7p⁺ phagocytic compartments was slightly higher after amastigote internalization. For example, at 30 minutes and 12 hours post-infection, 90% and 100% of the amastigote-harboring compartments displayed rab7p in their membrane (Fig. 11; Fig. 12A), respectively, whereas, at the same times after infection with metacyclics, rab7p was detected on only 70 and 75% of the parasite-containing compartments, respectively. Similar results were obtained for lamp-1 and macrosialin with both parasitic stages (Fig. 11; Fig. 12B), except that the percentages of positive compartments were higher at 10 minutes post-infection with amastigotes, indicating a slightly faster acquisition of the molecules. Likewise, class II molecules were acquired more quickly after infection with amastigotes. In contrast, although most amastigote-harboring phagosomes had cathepsin B in their lumen at 30 minutes post-infection (Fig. 11, Fig. 12C), this enzyme, at later times, was more difficult to detect in these organelles than in compartments formed after phagocytosis of metacyclics. This is probably because of an earlier enlargement of the PVs which, during fixation, lose a part of their soluble content. Negative controls performed as described previously for promastigote-infected macrophages gave only weak background staining (data not shown).

Discussion

We examined early interactions between macrophages and the two *Leishmania* parasitic stages encountered in mammals,

including the binding and phagocytosis of parasites and the formation of PVs, to determine whether these stages behaved similarly during these processes. We focused on metacyclic-macrophage interactions, which, so far, have been poorly investigated.

Most of the studies on parasite entry into macrophages have concerned the multiple receptor-ligand systems involved in the binding and internalization of promastigotes and amastigotes (Alexander and Russell, 1992; Guy and Belosevic, 1993; Love et al., 1993; Peters et al., 1995) and only a few reports have looked at the phagocytic events (for a review, see Chang, 1983; Rittig et al., 1998). In particular, it is not yet clear whether *Leishmania*, which are strongly polarized cells, are bound preferentially by a pole or not. Likewise, it is not known whether the primary binding sites are the first to be internalized. Our microcinematographic data indicate that the first interactions between *L. amazonensis* metacyclic promastigotes and mouse bone-marrow-derived macrophages occur through the parasite flagellum, the cell body or the entire parasite. We also found that the binding of the flagellum can be followed by phagocytosis starting by the cell body. Scanning electron microscopy of similar cell preparations further indicated that 10 minutes after adding parasites to macrophage monolayers, flagella are free or have established contacts with macrophage plasma membranes, but the only partial internalization processes observed involved parasite bodies. The fact that shortly after promastigote internalization (30–60 minutes), most of the parasites (~70%) are elongated with their cell bodies directed towards the macrophage nucleus and their flagella directed towards the plasma membrane also suggests that promastigote phagocytosis mainly occurs in a polarized manner with the cell body entering macrophages first. Although unlikely, a re-orientation of the parasites after

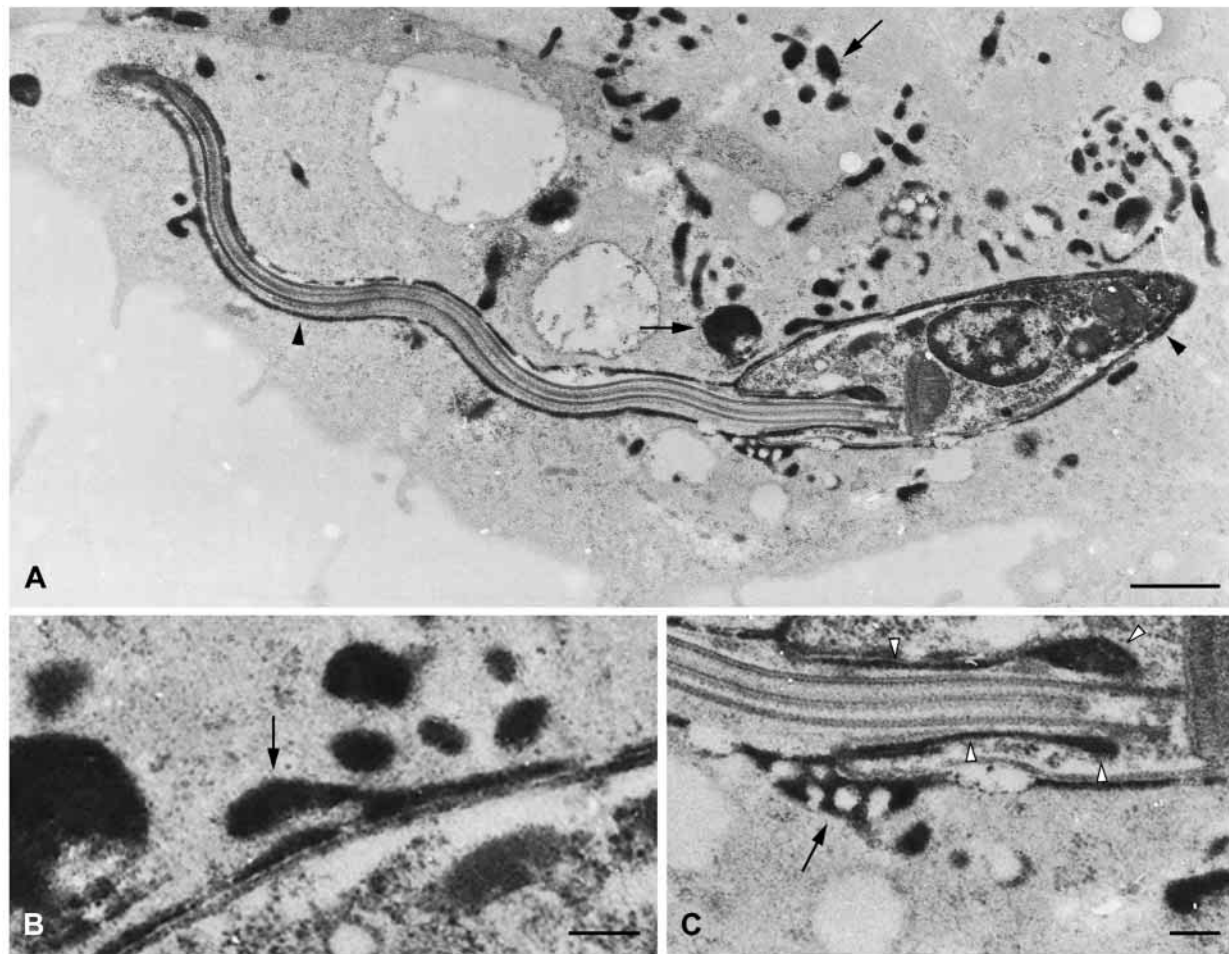


Fig. 10. Fusion of HRP-loaded late endosomes/lysosomes with parasite-containing compartments as observed by electron microscopy. IFN- γ -treated macrophages were incubated with HRP for 30 minutes. After extensive washings, they were chased for 160 minutes before infection with *L. amazonensis* metacyclic promastigotes. Thirty minutes after infection, macrophages were fixed and processed for peroxidase cytochemistry. (A) HRP is located in numerous late endosomes/lysosomes (arrows) as well as in the lumen of the tight promastigote-harboring compartment (arrowheads). (B,C) Details of A showing the fusion of HRP-containing late endosomes/lysosomes with the phagolysosomal compartment (arrows). The presence of HRP in the flagellar pocket of the parasite is also observed (arrowheads). Bars, 1 μ m (A), 0.2 μ m (B,C).

phagocytosis or of the parasite-containing phagosomes just after their formation could also explain this preferential polarity of the intracellular parasites. Similar findings were obtained with *L. major* metacyclics as well as with unselected *L. amazonensis* stationary phase promastigotes. This indicates that this phenomenon is not linked to a particular *Leishmania* species and is not the consequence of the treatments used to obtain homogeneous metacyclics. Furthermore, identical patterns were noted in macrophages that had been pre-treated with IFN- γ and those that had not. This supports the view that binding, phagocytosis and the first steps of phagosome biogenesis are not greatly influenced by this cytokine, at least at low concentrations. Our data are consistent with earlier publications that showed that *L. donovani* promastigotes predominantly enter mouse or hamster macrophages by their posterior end (Pulvertaft and Hoyle, 1960; Akiyama and Haight, 1971).

This method could not be used to determine whether amastigotes also bind and are phagocytosed in a polarized manner because the polarity of this parasitic stage is not as easy to assess by light microscopy as that of promastigotes. A

quantitative electron microscopic study will be needed to examine this point. It is noteworthy that, within PVs, parasites are bound to PV membrane through their posterior pole (Benchimol and De Souza, 1981; Antoine et al., 1998; Courret et al., 2001). It would thus be very interesting to determine whether this particular area of the parasite plasma membrane is also used as a primary binding site for promastigotes and amastigotes with the macrophage plasmalemma or whether this interaction is engaged in the first steps of phagocytosis.

Morphologically distinct phagocytic events were observed after promastigote binding, including the formation of tubular pseudopodia in close contact with the parasites, as already noted by others (Chang, 1979; Rittig et al., 1998), and ruffles. The fact that, shortly after internalization, most of the parasites are in very long, close-fitting phagosomes that exactly follow their outline suggests that ingestion mainly occurs by a zipper mechanism sequentially engulfing the different parts of the parasites. Otherwise, phagocytosis following ruffle extension could be at the origin of the rare phagosomes displaying a distinct lumen, early after their formation (such a phagosome is visible in Movie 1). It is not yet known whether different

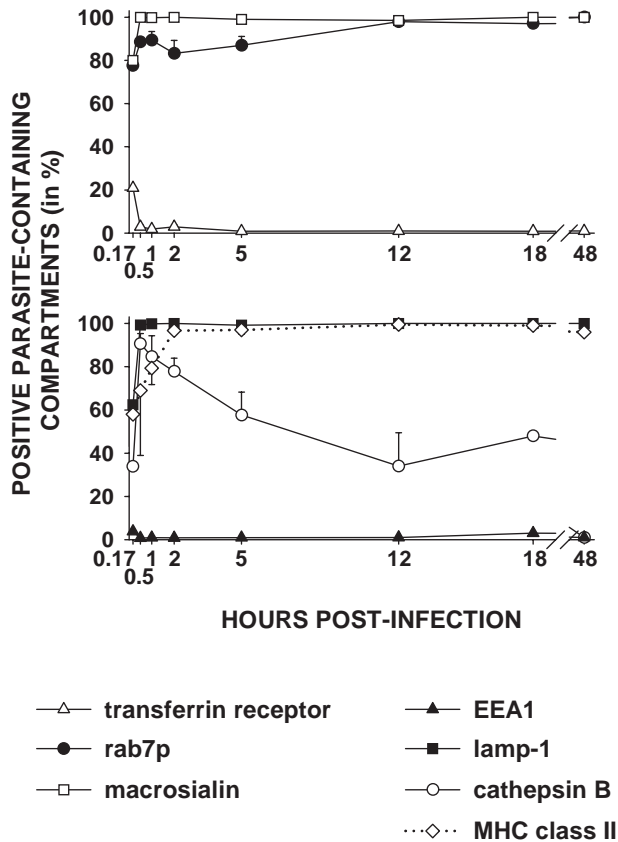


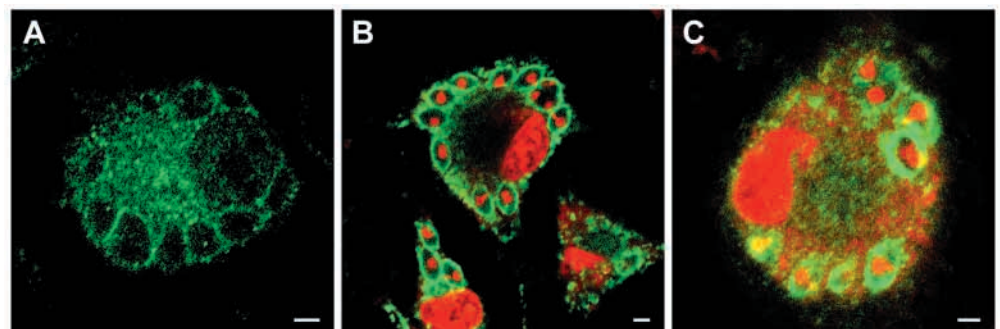
Fig. 11. Kinetics of endocytic 'marker' association with phagosomes formed after internalization of *L. amazonensis* amastigotes. IFN- γ -treated macrophages were infected at a multiplicity of four parasites/host cell. At various times post-infection, they were processed as described in Fig. 5 and the association of the following molecules with parasite-containing compartments was examined: transferrin receptor, EEA1, rab7p, macrosialin, lamp-1, cathepsin B and MHC class II molecules. For each experiment and at each time point, the percentages of parasite-containing organelles displaying the molecules listed above were determined after counting about 100 organelles. Data represent the means \pm or $-$ range of two separate experiments.

receptor-ligand interactions are at the origin of the various phagocytic mechanisms observed. In any case, these mechanisms as well as amastigote phagocytosis are dependent

upon actin polymerization, as shown by the rapid appearance of F-actin around the parasites. Actin is then rapidly shed from the newly formed phagosomes harbouring either *L. amazonensis* metacyclic promastigotes or amastigotes and at 30 minutes post-infection no more than 10–20% of the phagosomes are still surrounded by F-actin. The data concerning amastigote infections are consistent with those of Love et al., who showed that periphagosomal actin is rapidly lost when parasites are internalized (Love et al., 1998). In contrast, we found no evidence of a lasting accumulation of actin around promastigote-containing compartments (more than 60 minutes) as is the case, in J774 macrophages, for *L. donovani*-harbouring phagosomes (Holm et al., 2001). These authors found that the persistence of actin is LPG-dependent, which is difficult to reconcile with our data showing that the kinetics of periphagosomal actin dissociation are similar after ingestion of *L. amazonensis* promastigotes or amastigotes, which express LPG or are devoid of LPG, respectively (Courret et al., 2001). Whether this discrepancy is due to the different *Leishmania* species/host cells used in these experiments is unclear but it is interesting to note in this respect that we found a higher percentage of *L. major* metacyclic promastigotes surrounded by actin at the three time points examined after adding parasites to macrophages, namely 10 minutes, 30 minutes and 2 hours.

After internalization, all of the promastigotes are located in membrane-bound compartments, including those that are in a phagosome not detected by phase-contrast microscopy. Indeed, at 30 minutes to 1 hour post-infection, all parasites are delineated by the integral membrane proteins lamp-1 and macrosialin, indicating the presence of an endosomal/lysosomal membrane. These data do not agree with previous descriptions of cytosolic promastigotes within phagocytic cells (Akiyama and Haight, 1971; Rittig et al., 1998). Even if such a localization could possibly occur, for example after the rupture of phagosomes, it must be extremely rare and must be formally proven. The formation of very long phagosomes, the membrane of which tightly follows the outline of promastigotes, has never been described before. Nevertheless, these results are reminiscent of the early stages of infection of fibroblasts or epithelial cells by *Trypanosoma cruzi* trypomastigotes, which use a very different mode of entry (Hall et al., 1992). It is currently not known how the integrity of these slender compartments containing highly motile parasites is maintained, but their binding to cytoskeletal elements could be

Fig. 12. Confocal microscopy analysis of the association of late endosome/lysosome 'markers' with early phagosomes formed after internalization of *L. amazonensis* amastigotes. IFN- γ -pre-treated macrophages were infected (four parasites/host cell) and 30 minutes later processed for immunofluorescence microscopy. Cell preparations were incubated with immune sera or Abs directed against rab7p (A), lamp-1 (B) or cathepsin B (C) and then with adequate fluorescein conjugates (green staining). In B and C, cells were also stained with propidium iodide to visualize macrophage nuclei and parasite nuclei and kinetoplasts (red staining). Optical sections (0.3–0.5 μ m thickness) are shown. The micrographs are representative of two separate experiments. Bars, 2 μ m.



involved. At later time points (2-5 hours), the phagosomal membrane remains tightly associated with the parasites but the compartments become shorter. This reduction in size correlates with the progressive loss of the flagellum, suggesting that the parasite remodeling is accompanied by the removal of phagosomal membrane.

Our most important finding is that promastigote-containing phagosomes can fuse with late endocytic compartments very quickly after their formation, as shown by (1) the rapid acquisition by these organelles of both soluble and membrane molecules mainly associated with late endosomes/lysosomes, namely cathepsin B, cathepsin D, macrosialin, lamp-1 and rab7p; and (2) the transfer of the content of late endosomes/lysosomes previously loaded with FDex or HRP in the lumen of these organelles. This fusion capacity is consistent with the fact that F-actin is rapidly shed from the newly formed phagosomes. It is noteworthy that, compared with the other molecules that are acquired almost synchronously, except for MHC class II molecules, rab7p is present on a higher percentage of phagosomes at the earliest time point examined (10 minutes). This implies that rab7p is recruited sooner, and possibly by another way, for instance from the cytosol or from organelles other than late endocytic compartments. The presence of rab7p on a high percentage of early phagosomes (70 to 80%) is consistent with their ability to fuse with late endocytic compartments as recent data indicate that this protein is important for late endosome/lysosome fusion events (Méresse et al., 1995; Papini et al., 1997; Bucci et al., 2000). Whereas rab7p associates only with latex bead phagosomes transiently (between 10 and 200 minutes post-internalization) (Scianimanico et al., 1999), the percentage of parasite-containing compartments with rab7p on their surface progressively increases with time and reaches about 100% at 48 hours. It is not yet known whether this occurs by a deregulation of rab7p function but these results suggest that PVs can retain their ability to fuse with late endocytic compartments for a long time, which could provide the parasites with a means of getting nutrients. The persistent expression of membrane-associated rab proteins has been described for phagosomes harboring other intracellular microorganisms. For example, *Mycobacterium bovis* BCG phagosomal compartments retain rab5p on their surface and, perhaps as a consequence of that, the capacity to fuse with early endosomes. However, these phagosomes do not acquire rab7p and are unable to fuse with late endocytic compartments (Via et al., 1997).

The slower kinetics of class II molecule association with parasite-containing compartments could be due to the fact that these molecules are located in subsets of late endosomes/lysosomes (MIIC) with different fusion capacities towards phagocytic compartments. On the other hand, at all time points, only very low numbers of phagocytic compartments were found to display the early endosome 'markers' TfR and EEA1. This indicates that either low or, more likely, very transient interactions occur with early endocytic compartments just after completion of phagocytosis and that they are followed by rapid recycling of early endosome-associated molecules.

The validity of our conclusions concerning PV biogenesis in promastigote-infected macrophages can be extended to phagocytic compartments containing different *Leishmania* species (*L. amazonensis*, *L. major*) as well as to PVs present

in macrophages under different states of activation (macrophages that were or were not pre-treated with IFN- γ). Finally, the characteristics of PV formation are not biased by the treatment used to purify metacyclic forms. Indeed, we checked that phagosomes harbouring initially either *L. amazonensis* metacyclic or unselected stationary phase promastigotes behaved similarly in terms of late endosome/lysosome 'marker' acquisition.

We also observed an early association of late endosome/lysosome 'markers' with parasite-containing compartments after the internalization of amastigotes. The recruitment of the different molecules examined was slightly more efficient than after ingestion of promastigotes because, at 10 minutes post-infection, a higher percentage of phagosomes displayed rab7p, macrosialin, lamp-1 and MHC class II molecules in their membrane. At 30 minutes, the differences become blurred in terms of percentage of positive phagosomes but the immunolabeling intensity of the different molecules was generally slightly weaker for phagosomes/phagolysosomes harbouring initially promastigotes. Together, these data show that, after internalization of promastigotes and amastigotes, early phagosomes rapidly fuse with late endocytic compartments but that the rate of fusion is higher after amastigote internalization. This difference could be due to a lower association of rab7p with phagocytic compartments harbouring initially metacyclic promastigotes. This was suggested by Scianimanico et al. to explain the fusion properties of phagosomes containing *L. donovani* promastigotes (Scianimanico et al., 1999), despite the fact that their results were clearly different from ours. These authors showed that these organelles, in contrast to phagocytic compartments containing amastigotes or LPG-deficient promastigotes, have limited interaction with late endocytic organelles for several hours (Desjardins and Descoteaux, 1997; Scianimanico et al., 1999; Dermine et al., 2000). The origin of this discrepancy is not clear, but the very different characteristics of the host cells used (J774 macrophages vs bone-marrow-derived macrophages) are an important point to consider.

In conclusion, we have demonstrated that both amastigote- and metacyclic-containing phagosomes interact with late endosomes/lysosomes in the minutes following parasite phagocytosis. Our phase-contrast microscopy study of *L. amazonensis*-infected macrophages also showed that the enlargement of the PVs harbouring this *Leishmania* species is delayed when promastigotes are used to initiate infection. Thus, as suggested by Desjardins, Descoteaux and colleagues, PV formation may be parasite stage-dependent, at least with certain *Leishmania* species. However, in our experimental conditions, the events at the origin of these differences seem to occur after the fusion of early phagosomes with late endosomes/lysosomes. As the formation of the huge PVs is due to the fusion of several individual vacuoles and to the fusion of these vacuoles with compartments of the endocytic pathway, our data suggest that the kinetics of PV formation can be modulated either by the release (1) shortly after phagocytosis, of promastigote-derived molecules that inhibit these processes [e.g. LPG as proposed previously (Dermine et al., 2000)]; or (2) at later times of infection, of amastigote-specific molecules that alter the balance between fusion and fission events in favor of fusions. In this respect, it has been suggested that the proteophosphoglycan secreted by *L. mexicana* amastigotes in

the PV lumen could be involved in the expansion of PVs (Peters et al., 1997). The co-infection of macrophages with promastigotes and amastigotes of *L. amazonensis* should allow us to determine whether the molecules expressed/synthesized by the promastigote/intermediate stages transiently block the enlargement of PVs in our experimental conditions.

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