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Leishmania (L.) amazonensis: Fusion between parasitophorous vacuoles in infected bone-marrow derived mouse macrophages

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Abstract

[*Leishmania (L.)*] *amazonensis* amastigotes reside in macrophages within spacious parasitophorous vacuoles (PVs) which may contain numerous parasites. After sporadic fusion events were detected by time-lapse cinemicrography, PV fusion was examined in two different models. In single infections, it was inferred from the reduction in PV numbers per cell. In a reinfection model, macrophages infected with unlabeled amastigotes were reinfected with GFP-transfected- or carboxyfluorescein diacetate succinimidyl ester-labeled parasites, and fusion was detected by the colocalization of labeled and unlabeled amastigotes in the same PVs. The main findings were: (1) as expected, fusion frequency increased with the multiplicity of infection; (2) most fusion events took place in the first 24 h of infection or reinfection, prior to the multiplication of incoming parasites; (3) resident and incoming parasites multiplied at similar rates in fused PVs. The model should be useful in studies of parasite and host cell factors and mechanisms involved in PV fusogenicity.
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Index Descriptors and Abbreviations: Protozoa; *Leishmania*; *Leishmania (L.) amazonensis*; Amastigote; Macrophages; PV, parasitophorous vacuole; Vacuolar fusion; CFDA-se, carboxyfluorescein diacetate succinimidyl ester; GFP, green fluorescent protein; MOI, multiplicity of infection

1. Introduction

Leishmania are parasitic protozoa transmitted by hematophagous phlebotomine sandflies which cause human and zoonotic diseases with significant worldwide morbidity and mortality (Desjeux, 2004). In the course of insect blood meal, metacyclic promastigotes regurgitated in the dermis of a mammalian host, are taken up by macrophages, dendritic or other cells, in which they transform into amastigotes that multiply within phagolysosome-like parasitophorous vacuoles (PVs) (Antoine et al., 1998; Alexander et al., 1999; Rittig and Bogdan, 2000; Burchmore and Barrett, 2001; Chang et al., 2003; Kima and Dunn, 2005; Kima, 2007; Peacock et al., 2007; McConville et al., 2007). Infection is maintained in the lesions by re-uptake of amastigotes released from disrupted host cells.

Macrophages, or other cells in culture, can be infected with lesion-derived amastigotes, axenically grown amastigotes, or promastigotes. *In vitro* uptake of lesion-derived amastigotes by mouse macrophages appears to mainly rely on Fc- and phosphatidylserine receptors (Wanderley et al., 2005; Kima, 2007).

Most species of *Leishmania* are sheltered in small, single- or double-occupancy "tight" PVs. In contrast, parasites of the *mexicana* complex lodge predominantly in large PVs which may enclose many parasites, and have been particularly useful in studies of PV biogenesis, composition and fusogenicity.

In the relatively few species studied, *Leishmania* PVs are acidified, rich in lysosomal enzymes, and their membranes display the phagolysosomal markers proton-ATPases, LAMP-1, rab7p, macrosialin, cation-independent mannose phosphate receptor, and calnexin.

In the course of their growth and maturation, *Leishmania* PVs fuse with endocytic, phagocytic, autophagic, endoplasmic reticulum, or other elements, which could

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bring membrane and contents to the vacuoles (Shepherd et al., 1983; Barbieri et al., 1985; Antoine et al., 1990; Veras et al., 1992; Russell et al., 1992; Veras et al., 1994; Collins et al., 1997; Schaible et al., 1999; Courret et al., 2002). Although the relative contribution of the different potential membrane sources is unknown, late endosomes and lysosomes appear to be the most likely candidates. However, fusion with endocytic or other vesicles has been often inferred from PV time-dependent acquisition of electron opaque colloids, fluorescent or other markers internalized by the host cells prior to or after infection. To our knowledge, the actual fusion of PVs with well characterized vesicles, with delivery of a specific cargo to the vacuoles, has not yet been documented in living cells.

Fusion of *Leishmania (L.) amazonensis* and *L. (L.) mexicana* PVs with incoming phagosomes depends on the nature of the enclosed particles (Veras et al., 1992, 1996; Collins et al., 1997). Newly formed, but not aged, PVs of *L. (L.) amazonensis* in fibroblasts and macrophages, also fuse with vacuoles which shelter phase II *Coxiella burnetii*, a bacterium which normally occupies similar vacuoles, albeit with autophagic characteristics (Veras et al., 1995; Rabinovitch et al., 1999; Schaible et al., 1999; Romano et al., 2007).

The present study examines the fusion between PVs, which could provide an additional mechanism for PV membrane acquisition and vacuolar growth.

We show here that *L. (L.) amazonensis* PVs fuse with each other in infected mouse macrophage cultures, and examine the kinetics of the fusion. Fusion between the PVs was investigated in two kinds of experiments. In a single infection model, fusion, initially detected in time-lapse cinematographic recordings, was inferred from the reduction in the number of PVs in cultures fixed at different times after infection. In reinfection experiments, macrophages, infected with unlabeled parasites, were reinfected 30 min or 48 h later, with parasites transfected with GFP or labeled with CFDA-se, a cell-permeant, amine-reactive probe. In these experiments, fusion was detected by the colocalization of labeled and unlabeled parasites in the same vacuoles.

2. Materials and methods

2.1. Mice and parasites

Female Balb/c mice, 8 week of age (Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia, CEDEME, UNIFESP-EPM), were used as hosts for the parasites and as bone-marrow donors. *L. (L.) amazonensis* (LV79 strain, MPRO/BR/72/M1841) were isolated from footpad lesions as described previously (Veras et al., 1992), and used immediately for *in vitro* infection experiments. GFP-transfected *L. (L.) amazonensis* (MHOM/BR/1973/M2269), generously donated by Dr. Silvia R. Uliana, ICB-USP, Brazil), were cultivated *in vitro* in M199 medium with 10% FBS and 30 μg/ml Hygromicin B at 26 °C; stationary phase promastigotes were used for infection with GFP parasites.

2.2. Labeling of parasites

Amastigotes were incubated for 10 min at room temperature with 5 μM carboxyfluorescein diacetate, succinimidyl ester (CFDA-se, Molecular Probes). After two washes with HBSS, the parasites were immediately used for reinfection experiments. Forty-eight hours post-reinfection, fluorescence was reduced, possibly by dilution due to parasite division. However, CFDA-se fluorescence of intracellular parasites could be detected for at least 80 h.

For studies with killed parasites, freshly labeled amastigotes, suspended in HBSS were kept for 15 min at 60 °C, or fixed with formaldehyde 3.5% for 30 min at room temperature and washed 5× in HBSS.

2.3. Macrophage culture

For time-lapse cinemicrography recordings, resident or thioglycollate-induced peritoneal macrophages were obtained from Balb/c mice; cell suspensions, plated on 25 mm diameter round coverslips, were kept for 2–3 days in culture; prior to infection coverslips were mounted in Sykes-Moore chambers (Veras et al., 1996).

In all other experiments, macrophages were derived *in vitro* from mouse bone-marrow precursors (Zamboni and Rabinovitch, 2003). After 7 days in culture macrophage suspensions were plated on round 12 mm coverslips (5×10^4 macrophages per coverslip) in 24 well plates containing RPMI 1640 medium with 10% fetal bovine serum, 5% L929 cell conditioned medium, 2g/l sodium bicarbonate and 20 mM HEPES (“complete medium”), and allowed to adhere overnight at 37 °C, 5% CO_2.

2.4. Infection of macrophage cultures

For cinemicrography, macrophages were infected in Sykes-Moore chambers at multiplicities of about 10 parasites per macrophage, and the medium changed after 30 min. In the other experiments, parasites were added at parasite–host cell ratios of 3:1, 10:1 or 20:1, and incubated at 37 °C, 5% CO_2 for 2 h or 30 min in complete medium. Cultures were washed twice with HBSS to remove free parasites, and replaced in complete medium with 40 μg/ml of gentamicin at 34 °C, 5% CO_2 in air atmosphere.

2.5. Microscopy

Cinemicrography was performed as previously reported (Rabinovitch et al., 1986); the sequence shown was obtained at 2 exposures per min. For the remaining single infection experiments, at each time, cultures on coverslips were washed twice with HBSS, fixed with methanol for 30 min and stained with Giemsa. Counts were performed

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