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Leishmania chagasi:

Homogenous metacyclic promastigotes isolated by buoyant density are highly virulent in a mouse model

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Abstract

Homogenous metacyclic promastigotes of *Leishmania chagasi* were isolated by buoyant density from *in vitro* heterogeneous cultures and used for biochemical characterization of isoforms of the major surface protease (MSP). Compared to stationary phase promastigotes, metacyclic cells had three times more MSP, produced three-fold higher parasite loads in a mouse model *in vivo*, and were more resistant to complement-mediated lysis *in vitro*. These metacyclic *L. chagasi* expressed both the virulence-associated 59-kDa, and the constitutively expressed 63-kDa, isoforms of MSP.

Keywords

Leishmania; metacyclic promastigote; virulence; buoyant density

Metacyclogenesis of *Leishmania* spp. is the development of procyclic into metacyclic promastigotes in the sand fly vector. It can be mimicked *in vitro* by promastigote growth from logarithmic to stationary phase (Sacks, 1989). Metacyclic organisms have been isolated from *in vitro* heterogeneous cultures of stationary phase cells of several *Leishmania* species in sufficient quantity and purity to perform biochemical studies (da Silva and Sacks, 1987; Sacks and da Silva, 1987; Sacks et al., 1995; Lira et al., 1998; Courret et al., 1999; Pinto-da-Silva et al., 2002). Techniques to purify metacyclic organisms can be classified as either lipophosphoglycan (LPG)-dependent or LPG-independent. The LPG-based methods depend on the observation that LPG side groups become modified during development, and utilize a lectin or antibody to select the metacyclic organisms. For example, monoclonal antibody (mAb) 3F12 reacts specifically with metacyclic promastigotes of *L. major* (Sacks and da Silva, 1987). The lectin peanut agglutinin (PNA) binds the terminal galactose on logarithmic LPG,

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but does not bind its metacyclic LPG counterpart which terminates in arabinose (McConville et al., 1992). *Bauhinia purpurea* lectin (BPL), which specifically recognizes β -gal(1 $\rightarrow$ 3)-galNAC, and the lentil lectin, which is specific for D-Mannose-terminal glycoconjugates, negatively and positively select for metacyclic organisms of *L. braziliensis*, respectively (Almeida et al., 1993; Pinto-da-Silva et al., 2002). The mAbs XCIV 1H2-A8 (T11) and 3A1 negatively select for metacyclic forms of *L. tropica* and *L. amazonensis*, respectively (Lira et al., 1998; Courret et al., 1999).

Due to physicochemical differences between LPGs of the various *Leishmania* spp, a lectin or antibody used to bind logarithmic or metacyclic LPG of one species often does not recognize the corresponding LPG of other species. An LPG-independent method was developed to purify metacyclic organisms from LPG biosynthetic gene-knockout promastigote cultures, using the buoyant density characteristics of *L. major* (Spath and Beverley, 2001). One would expect this method to be less species-specific than LPG-based methods. This method has been adapted to *L. donovani* and *L. chagasi* (Svensjo et al., 2006; Akilov et al., 2007). We herein further modified this method to isolate a homogenous population of cells that contained 98% metacyclic promastigotes from heterogeneous cultures of *L. chagasi*. We then determined whether a 59-kDa isoform of the major surface protease (MSP), which is associated with virulence (Roberts et al., 1995), was the exclusive MSP isoform expressed by metacyclic promastigotes or whether it was expressed constitutively by all parasite forms.

Isolation of metacyclic promastigotes

A Brazilian strain (MHOM/BR/00/1669) of *L. chagasi* was continuously passaged in golden hamsters to maintain virulence (Yao et al., 2002). Promastigotes transformed from spleen-derived amastigotes were cultured in hemoflagellate-modified minimal essential medium (HOMEM) (Berens et al., 1976; Yao et al., 2004; Yao et al., 2005). The Ficoll step-gradient originally developed for *L. major* (Spath and Beverley, 2001) was modified to maximize the yield of *L. chagasi* metacyclic promastigotes. Briefly, stationary-phase promastigotes of day 7-9 cultures were collected and washed in DMEM (GIBCO, Carlsbad, CA). Cells (2 ml) at a density of 2×10^8 cells/ml in DMEM were overlaid on a step gradient of 2 ml of 40% Ficoll in phosphate buffered saline (PBS) and 2 ml of 10% Ficoll in M199 medium (Amersham, Piscataway, NJ). The tubes were then centrifuged at room temperature for 10 min at $365 \times g$ with the brake off. Metacyclic promastigotes, identified by morphological criteria, *i.e.*, short and slender with a long flagellum twice the body length (Howard et al., 1987; Bandyopadhyay et al., 1991; Nieves and Pimenta, 2000; Pinto-da-Silva et al., 2002), were collected from the band located at the interface between the 0% and 10% Ficoll layers.

Prior to selection of the above optimal protocol, we tested various Ficoll concentrations from 4% to 20% in the middle layer and different centrifugation forces between 274 - $1,300 \times g$ to maximize recovery of metacyclic organisms and minimize non-metacyclic contamination. Differences between the optimal protocols for purification of *L. chagasi* versus *L. major* metacyclic promastigotes were a lower centrifugal force, *i.e.*, $365 \times g$ as opposed to $1,300 \times g$. This discrepancy is likely due to different physical properties of *L. chagasi* versus *L. major*. Similar to *L. major*, a 10% Ficoll middle layer overlaid on a 40% Ficoll cushion yielded the purest population of metacyclic *L. chagasi* promastigotes and the highest recovery rate. Similarly, another group instead changed the two-step gradient of 40% and 10% for *L. major* to 10% and 5% for *L. chagasi* while keeping $1,300 \times g$ centrifugation. The cells recovered from the 10% step contained 70-90% metacyclic promastigotes (Svensjo et al., 2006).

As showed in Figure 1A, cells recovered from the band at the interface between the 0 and 10% Ficoll layers displayed a homogenous morphology of metacyclic promastigotes. An average of $13.4 \pm 4.0\%$ recovery rates was achieved from ten preparations of *L. chagasi* promastigotes

recently isolated from hamsters. The recovered cells contained $97.6 \pm 0.7\%$ metacyclic promastigotes, a 2.4-fold enrichment compared with the stationary cells, which contained only $41.4 \pm 2.8\%$ ($n=4$) metacyclic organisms. As an additional control, we isolated metacyclic promastigotes from the attenuated L5 strain of *L. chagasi*, a strain with a reduced virulence in a BALB/c mouse model, diminished levels of MSP, and a shorter, simpler LPG compared to its virulent counterpart (Wilson et al., 1989; Miller et al., 2000; Brittingham et al., 2001; Yao et al., 2004). Not surprisingly, the recovery rate of metacyclic organisms from stationary phase promastigotes of the L5 strain was less than 0.1%. We conclude that buoyant density differences using step Ficoll gradients selectively enrich for metacyclic *L. chagasi* promastigotes.

Metacyclic promastigotes are highly virulent

We investigated virulence of the metacyclic promastigotes by measuring their resistance to complement-mediated lysis, and their virulence in a mouse model. To study complement-mediated lysis, fresh sera were collected from the peripheral blood of three healthy individuals who had never been exposed to *Leishmania* spp. infection. 2×10^8 cells logarithmic, stationary or metacyclic promastigotes/ml were incubated in different concentrations of pooled human sera in PBS for 30 min at 37°C. Cells were then subjected to FACS analysis of 100,000 events for cell viability in the presence of 1 µg/ml propidium iodide (Sigma, St. Louis, MO) using a Becton Dickinson FACScan. As showed in Figure 2A, metacyclic, corresponding unpurified stationary and logarithmic promastigotes had the highest, medium and least resistance with 81.6 ± 7.2 , 51.5 ± 14.3 , and $9.5 \pm 4.7\%$ viable cells in 50% human sera, respectively.

Six-week-old female BALB/c mice were injected intravenously with 1×10^7 unpurified stationary phase or purified metacyclic promastigotes through a tail vein. Three weeks later, four mice from each condition were euthanized, and the parasite loads were determined microscopically from impression smears of spleen and liver and their organ weights in milligrams as described (Stauber, 1958; Bradley and Kirkley, 1977). As showed in Figure 2B and C, the parasite loads in mice infected with the metacyclic promastigotes were significantly higher than those infected with unfractionated stationary phase promastigotes when quantified either in the spleen or the liver. These values represent a 3.2- and 2.6-fold increase in parasite load in the spleen and liver, respectively. Similar to prior reports, parasites achieved a much higher burden in the liver than the spleen early in infection (Wilson et al., 1996). As an additional control, stationary promastigotes of the same cultures were subjected to step-Percoll (Sigma) gradients to separate live organisms from cellular debris as described (Ahmed et al., 2003). These cells were more virulent than the unprocessed stationary promastigotes, but less virulent than the metacyclic promastigotes (data not shown).

MSP isoforms in metacyclic versus unpurified promastigotes

To investigate MSP isoforms in metacyclic promastigotes, 10 µg of total promastigote protein (10 µg) were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose filters for Western blotting. Filters were probed with sheep polyclonal antiserum (1:10,000 dilution) directed against purified *L. chagasi* MSP (Wilson et al., 1989), mAb against α -tubulin (AB-1, 0.1 µg/ml, Oncogene, San Diego, CA) and goat polyclonal antiserum (1:5,000 dilution) that we generated in our laboratory against recombinant P36, a cytosolic protein of *Leishmania* spp. (Liu and Chang, 1994). Compared to their stationary counterparts, metacyclic promastigotes had a 3.1-fold higher abundance of total MSP, whereas the pelleted metacyclic-depleted cells at the bottom of the tubes had a reduced MSP level that was only 60% of that of the unpurified population (Figure 1B and C). The MSP isoform that serves as a marker for virulent *L. chagasi* stationary phase promastigotes recently derived from mammalian host cells is a 59-kDa isoform (Roberts et al., 1995). We hypothesized that the 59-

kDa MSP would be the only isoform expressed by virulent organisms. To this end, both 59-kDa and 63-kDa isoforms were quantified by densitometry analysis of Western blots. The ratios of 59-kDa to 63-kDa isoforms in metacyclic promastigotes and pelleted metacyclic-depleted cells were compared to that in unpurified stationary promastigotes, which was arbitrarily set as 1. Surprisingly, the ratio in metacyclic promastigotes (1.4 ± 1.0 , $n=7$) was comparable to that in unpurified stationary (1.0 arbitrary unit) and pelleted cells (1.0 ± 0.7). As such, expression of this isoform is not an exclusive property of the most virulent cell type.

This modified Ficoll step-gradient method yielded a 13% recovery rate of metacyclic *L. chagasi* promastigotes. With this recovery rate, $1-2 \times 10^9$ metacyclics were readily recovered from a 200-ml cell culture of stationary phase promastigotes of *L. chagasi* (data not shown). More importantly, the recovered cells are free of non-metacyclic contamination with 98% metacyclic promastigotes.

Another possible non-LPG dependent method for isolation of metacyclic cells involves flow cytometry. Saraiva and colleagues reported that metacyclic promastigotes of four species, *i.e.*, *L. major*, *L. donovani*, *L. amazonensis* and *L. braziliensis*, all displayed low forward-angle light scattering in flow cytometric analysis. The consistency between this method and the negative selection of LPG-dependent agglutination was 52% for *L. donovani* (mAb MG1-), 81% for *L. amazonensis* (mAb 3A1-), 84% for *L. major* (PNA-) and 93% for *L. braziliensis* (BPL-) (Saraiva et al., 2005). Whether this technique would be as efficient as the above density-based method warrants further investigation. Nonetheless the Ficoll-buoyant density method is a simple and efficient means of recovering metacyclic *Leishmania* spp. promastigotes for biochemical and mouse infection studies. It will be important to extend these data and determine whether the contaminating non-metacyclic cells of stationary phase cultures, which constitute 60% of the usually stationary inoculum, are responsible for decreased infectivity of the inoculum due to lower numbers of metacyclics introduced, or due to some immunosuppressive effect due to the increased lysis and decreased survival of non-metacyclic cells.

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abbreviations

LPG, lipophosphoglycan; mAb, monoclonal antibody; PNA, peanut agglutinin; BPL, *Bauhinia purpurea* lectin; MSP, major surface protease.

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Figure 1.

Isolation and characterization of *L. chagasi* metacyclic promastigotes. A. Phase-contrast micrographs of logarithmic (L), stationary (S) and metacyclic (M) promastigotes at 400-fold magnification. Metacyclic promastigotes are identified by arrows, and their percentage is listed on top of each panel (n=4). ND: not determined. B. MSP expression. Total protein from metacyclic (M), stationary (S), or metacyclic-depleted cells (P) were analyzed by western immunoblots. The same filter was probed successively with antiserum against MSP, P36 and α -tubulin. One of seven experiments is presented. (C). MSP quantification. The signal intensities of MSP shown in B were determined by densitometry and are presented with the MSP level in stationary phase promastigotes set as an arbitrary unit of 1.0.

Figure 2.

Metacyclic promastigotes are more virulent than stationary phase promastigotes. A. Human complement-mediated lysis. Data from four experiments using cells of four new *L. chagasi* isolates from hamsters are presented. Parasite loads in the spleens (B) or livers (C) of mice three weeks post-infection with 10^7 metacyclic or stationary promastigotes. *:p<0.01.