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POPULATION CHANGES IN *LEISHMANIA CHAGASI* PROMASTIGOTE DEVELOPMENTAL STAGES DUE TO SERIAL PASSAGE

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Abstract

Leishmania chagasi causes visceral leishmaniasis, a potentially fatal disease of humans. Within the sand fly vector, *L. chagasi* replicates as promastigotes which undergo complex changes in morphology as they progress from early stage procyclic promastigotes, to intermediate stage leptomonad and nectomonad promastigotes, and ultimately to terminal stage metacyclic promastigotes that are highly infective to vertebrates. This developmental progression is largely recapitulated in vitro using axenic promastigote cultures that have been passaged only a few times. Within a single passage (which takes about a week), axenic cultures progress from logarithmic to stationary growth phases; parasites within those growth phases progress from stages that do not have metacyclic cell properties to ones that do. Interestingly, repeated serial passage of promastigote cultures will result in cell populations that exhibit perturbations in developmental progression, in expression levels of surface macromolecules (major surface protease, MSP, and promastigote surface antigen, PSA), and in virulence properties, including resistance to serum lysis. Experiments were performed to determine whether there exists a direct relationship between promastigote developmental form and perturbations associated with repeated serial passage. Passage 2 to passage 4 *L. chagasi* cultures at stationary growth phase were predominately (>85%) comprised of metacyclic promastigotes and exhibited high resistance to serum lysis and high levels of MSP and PSA. Serial passaging 8, or more, times resulted in a stationary phase population that was largely (>85%) comprised of nectomonad promastigotes, almost completely devoid (<2%) of metacyclic promastigotes, and that exhibited low resistance to serum lysis and low levels of MSP and PSA. The study suggests that the loss of particular cell properties seen in cells from serially passaged cultures is principally due to a dramatic reduction in the proportion of metacyclic promastigotes. Additionally, the study suggests that serially passaged cultures may be a highly enriched source of nectomonad-stage promastigotes, a stage that has largely been characterized only in mixtures containing other promastigote forms.

The leishmaniasis are a disease group that, in humans, varies in severity from self-healing cutaneous lesions to potentially fatal visceral infections. The causal agents of the leishmaniasis are protozoan parasites, *Leishmania* spp., that cycle between a sessile amastigote form that primarily develops within phagocytic cells of the vertebrate immune system and a motile promastigote form that develops within the alimentary tract of the phlebotomine (sand fly) vector. Sand flies acquire amastigotes while blood-feeding on infected vertebrates. Ingested sessile amastigotes quickly differentiate into the motile promastigote form within the fly midgut. Over a period of 1, to several, weeks within the

fly, promastigotes replicate and undergo developmental changes that give rise to morphologically distinct stages (Walters, Chapman et al., 1989; Walters, Modi et al., 1989; Walters et al., 1993; Bates, 1994; Rogers et al., 2002; Gossage et al., 2003). The procyclic promastigote is the first stage that develops from the amastigote within the fly gut. The endpoint stage, the metacyclic promastigote, is highly infectious to the vertebrate host and is produced later in the infection. Other promastigote stages, including leptomonad and nectomonad promastigotes, also develop during the fly infection. These 4 stages have been identified in fly-parasite infection models (Walters, Chapman et al., 1989; Walters et al., 1993; Rogers et al., 2002; Gossage et al., 2003) as well as in axenic promastigote culture systems (Gossage et al., 2003; Lei et al., 2010).

The metacyclic promastigote stage has been the focus of a number of studies because of its presumptive role as the etiologic disease agent. Investigations in a number of *Leishmania* species have determined that metacyclic promastigotes, relative to non-metacyclic promastigotes, exhibit increased resistance to the lytic effects of serum complement, upregulated expression of the surface proteins promastigote surface antigen (PSA, also known as GP46 [Beetham et al., 1997]) and major surface protease (MSP; Wilson et al., 1989; Roberts et al., 1995), and differential glycosylation of surface lipophosphoglycan (LPG; Saraiva et al., 1995). Most of these studies of metacyclic promastigotes were undertaken using axenic promastigote culture systems comprised of low-passage cultures established from either animal-derived amastigotes (Wilson et al., 1989; Roberts et al., 1995) or from frozen low-passage promastigotes (Saraiva et al., 1995; Lei et al., 2010). Promastigote characteristics and development within axenic culture recapitulates in vivo development in a number of ways, including morphology, sequence of progression through developmental stages, and temporal progression from promastigote forms having low infection to vertebrates, to promastigote forms that are highly infectious (Sacks et al., 1984; Gossage et al., 2003; Lei et al., 2010).

For many *Leishmania* species, axenic cultures at stationary growth phase contain only a small proportion of metacyclic promastigotes. Consequently, for these species, the identification and characterization of metacyclic stage promastigotes have required the establishment of techniques with which to enrich for metacyclic promastigotes. Many of these are lectin-based enrichment techniques that capitalize on characteristics of surface glycosylation that vary between metacyclic and non-metacyclic promastigote stages (Saraiva et al., 1986; Pinto-da-Silva et al., 2002), while others use alternative procedures, including density gradient centrifugation (Spath and Beverley, 2001). The need for such enrichment techniques in *L. chagasi* are greatly reduced, given that studies of axenic cultured *L. chagasi* have determined that metacyclic promastigotes comprise a relatively large proportion of the promastigote forms found within cultures at stationary growth phase (Dahlin-Laborde et al., 2005; Yao et al., 2008; Lei et al., 2010).

In earlier studies of *L. chagasi*, it was shown that PSA is upregulated in metacyclic stage promastigotes derived from cultures at stationary phase, and that the regulation involved varied stability of PSA RNA effected by elements present within the 3'-untranslated region of the mRNA (Beetham et al., 1997; Myung et al., 2002). Others have characterized a similar regulation of 1 class of MSP (Wilson et al., 1989). Interestingly, it was found that serial passage greatly reduced the upregulation of GP46 and MSP, and that this reduction was determined to involve RNA stability (Brittingham et al., 2001; Beetham et al., 2003). In the present study, we have extended these earlier observations by determining that serial passage results in parasite cultures at stationary growth phase that exhibit a dramatic loss of metacyclic stage promastigotes and a corresponding increase in the proportion of nectomonad-stage promastigotes.

MATERIALS AND METHODS

Parasites

Infectious *L. chagasi* amastigotes (strain MHOM/BR/00/1669, originally isolated in Brazil from a patient with visceral leishmaniasis), isolated from spleens of infected golden Syrian hamsters, differentiated into promastigotes by incubation at 26 C in a modified minimum essential media (HOMEM) supplemented with hemin and heat-inactivated calf serum (Ramamoorthy et al., 1992). Promastigote cultures utilized the same medium seeded at 1.0×10^6 cells ml^{-1} . Cells within such cultures typically multiply logarithmically for 4–5 days until reaching a maximum cell density of $\sim 4\text{--}7 \times 10^7$ ml^{-1} . Cultures were considered to be in stationary phase 48 hr after reaching maximum cell density (day 7). Serial passaged cultures were split every 7 days from a cell density of $\sim 4\text{--}7 \times 10^7$ ml^{-1} down to 1.0×10^6 ml^{-1} . Parasite evaluations reported here were for cultures at their 2nd, and higher, passages after isolation from hamsters.

Human serum and complement assay

Human serum from multiple naïve donors was pooled and stored at -80 C. Complement assays were performed by incubating 3.5×10^6 promastigotes for 30 min at 37 C in 100 μl phosphate-buffered saline (PBS, pH 7.4) or in PBS supplemented with human serum, as described (Dahlin-Laborde et al., 2005). Afterwards, cells were diluted 1:10 in PBS and motile cells were counted by hemocytometer. Cell survival was reported as the percent of motile cells in the serum-treatment group relative to the number of cells in the no-serum control.

Promastigote morphology

Promastigotes in culture medium were applied to glass slides, air dried, stained with HEMA 3 stain set (Fisher Scientific, Chicago, Illinois), then visualized by light microscopy and measured using Nikon NIS-Elements D software (Nikon Instruments, Melville, New York). Promastigotes were categorized as procyclic, leptomonad, nectomonad, and metacyclic, based upon morphological characteristics (detailed in Fig. 2) as described in results and previous studies (Rogers et al., 2002; Gossage et al., 2003; Lei et al., 2010).

Protein detection

Cells pelleted at 2,000 g for 10 min at 4 C were resuspended in 0.1 M potassium phosphate buffer, pH 7.8, containing 1% triton $\times$ 100, then lysed via 3 cycles of freeze–thaw using liquid nitrogen and immersion in a 37 C water bath. Total lysates (0.5 μg for MSP, 5.0 μg for PSA, per lane) were separated by SDS-PAGE and electro-semi-dry-transferred to polyvinylidene fluoride membranes using standard procedures. After membrane blocking, using 5% non-fat powdered milk in tris-buffered saline (0.05 M Tris pH 7.4, 0.15 M NaCl, 0.1% Tween-20), membranes were exposed to primary and secondary anti-sera diluted in blocking solution: sheep anti-MSP (Wilson et al., 1989) diluted 1:10,000; rabbit anti-PSA (Beetham et al., 2003) diluted 1:1,500; mouse anti- α Tubulin (Calbiochem, EMD Chemicals, Gibbstown, New Jersey) diluted 1:1,000; horseradish-peroxidase conjugated anti-sheep antibody (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) diluted 1:30,000; horseradish-peroxidase conjugated anti-rabbit antibody (Pierce, Rockford, Illinois) diluted 1:1,000; and horseradish-peroxidase conjugated goat anti-mouse antibody (Pierce) diluted 1:1,000. Antibody binding was visualized via enzyme-linked chemiluminescence (SuperSignal[®], Pierce).

RESULTS

Serial passage results in progressive loss of serum resistance in parasites from cultures at stationary growth phase

Parasites within 7 independently initiated low-passage (passage 2 to 3) cultures exhibited low serum survival (12.1%, ± 3.4 SE) when in cultures at logarithmic growth phase, and high survival (94.7%, ± 3.3 SE) in cultures at stationary growth phase (Fig. 1A); independently initiated cultures are those initiated using amastigotes derived from separate parasitized hamsters. To evaluate the effect of serial passage on serum sensitivity, 3 cultures were followed for multiple passages; each passage spanned 1 wk (Fig. 1B). For all cultures, resistance to serum progressively decreased with successive culture passages, although the passage number at which the first major reduction was seen varied from passage 4 to passage 6. Although more highly resolved in the data presented here that deals with observations within and among independent cultures, these observations are in general agreement with previous studies reporting loss of complement resistance in *L. chagasi* due to serial passage (Lincoln et al., 2004; Dahlin-Laborde et al., 2005).

Serial passage results in a diminished proportion of metacyclic promastigotes and a greatly increased proportion of nectomonad promastigotes in cultures at stationary growth phase

Parasites within the cultures that were assessed for serum sensitivity were also assessed for assignment to presumptive promastigote developmental stages, i.e., procyclic, leptomonad, nectomonad, and metacyclic. Figure 2 provides information on the morphological parameters (body and flagellum size and ratios) used for stage assignment. The parameters used here for *L. chagasi* were based (with minor modification) upon those previously used by others in assessing developmental stages in promastigotes of *Leishmania mexicana* (Rogers et al., 2002). While the body length of <11.5 μm reported here for 3 procyclic, leptomonad, and metacyclic forms are similar, the simultaneous consideration of body width and of flagellum length relative to body length allows discrimination of the separate forms; procyclic and leptomonad forms have widths >1.5 μm and flagella that are shorter, or longer, respectively, relative to body length. The width of metacyclic promastigotes is 1.5 μm and the flagella are 1.5 to 2 times the body length. Nectomonad promastigotes have the longest body lengths (>12 μm) of any promastigote form.

Parallel to the observation that complement lysis resistance decreased during serial passage (Fig. 1), the proportion of metacyclic promastigotes that were present in stationary phase cultures progressively decreased during serial passage. At the same time, the proportion of the nectomonad promastigote forms progressively increased in stationary phase cultures. Figure 3A demonstrates these changes in metacyclic and nectomonad proportions in passage 2, 3, and 9 cultures; passage 2 and 3 data were based upon analysis of 7 independent low-passage cultures, and passage 9 data were based upon analysis of 3 of 7 cultures that were subjected to ongoing serial passage.

An analysis of the promastigote forms present within a single culture during passage 3 and 9 is presented in Figures 3B and C and is representative of all 3 independent cultures that were analyzed through passage 9. As shown in Figure 3B, the low-passage culture, seeded with cells from the previous passage, contained mostly metacyclic cells for the first 24 hr, during which time very few cells were observed to be in a state of doubling, i.e., 2 daughter cells still joined together. These relatively early time points are associated with a high proportion, albeit a low absolute number, of metacyclic cells, a consequence of the culture being initiated with cells from the prior passage culture at stationary growth phase in which metacyclic cells predominated. Leptomonads predominated during the logarithmic growth

phase that consistently occurred during 48–96 hr of culture, and nectomonads also increased in proportion through 96 hr. The onset of increased proportions of metacyclic promastigotes was in the mid-logarithmic culture phase. In comparison, a culture at passage 9 (Fig. 3C), seeded with cells from the previous passage 8 culture, contained almost no metacyclic cells initially, or at any other time, exhibited an increased proportion of procyclic and (to a lesser degree) leptomonad promastigotes during the first 48–72 hr, and then an increased nectomonad proportion beginning sometime around 72 hr.

Photomicrographs of cells in a single culture at passage 3, 9, and 15 (all at stationary culture phase) are shown in Figures 3D, E, and F, respectively, and are representative of 3 independent cultures that were serially passaged. The single nectomonad amongst mostly metacyclic promastigotes seen in the low-passage culture (Fig. 3D) emphasizes the size difference between these 2 parasite forms. A repeatable morphological observation was that the flagella in fixed low-passage cells were commonly found to contain many sharp bends and turns, whereas, in high-passage cells, the flagella contained mostly soft bends and curves (Figs. 3E, F). Examination of live cells by microscopy did not reveal an obvious and major motility difference in low- versus high-passage live promastigotes.

Given that serial passage has also been associated with reduced expression of parasite surface glycoproteins, including MSP and PSA (Brittingham et al., 2001; Beetham et al., 2003), levels of these proteins were also assessed. Similar to those previous studies, serial passage resulted in a dramatic loss of MSP and PSA expression (Fig. 4).

DISCUSSION

A number of studies have established that serial passage of *Leishmania* spp. results in perturbed expression of surface glycoproteins PSA and MSP. Related studies attempting to ascertain a mechanism for the reduced protein expression determined that the phenomenon: (1) is associated with reduced MSP and PSA mRNA levels; (2) is not associated with changes in transcription rate of the corresponding mRNAs; and (3) is, therefore, due to perturbed RNA stability. Data presented here suggest that the loss of normal MSP and PSA expression levels in cells from serially passaged cultures at stationary growth phase is due to a major reduction in the abundance of a particular developmental promastigote form, the metacyclic promastigote. Thus, serial passage somehow perturbs the overall developmental progression by which metacyclic cells develop from progenitor cells.

Our understanding concerning much of the developmental biology of promastigotes is quite incomplete. What roles in parasite development within the host do the different stages of parasite play? How do cells progress from one stage to the next? What is the progenitor cell for a given stage? Prior studies of parasites within the fly established that particular forms are enriched at particular sites within the infected fly, and that increased abundance of particular forms are found at specific times post-infection of the fly; these studies provide some support, albeit non-definitive, towards predictions of cell hierarchy.

Studies of the developmental biology of *Leishmania* spp. will benefit from increased characterizations of all developmental promastigote forms. The ease of isolation and enrichment of nectomonads from higher-passage cultures may enable investigations that require higher nectomonad biomass input than is readily obtained from *Leishmania* spp.-infected sand flies or from low-passage promastigote cultures. The degree to which high-passage nectomonads mirror low-passage nectomonads needs to be evaluated. Experiments are planned in which to characterize the nectomonads from higher-passage cultures, in the hope of identifying specific proteins or sugars that are unique to this form, and to validate the presence of such traits in low-passage or fly-derived nectomonads.

Leishmania chagasi is somewhat unique among characterized *Leishmania* species due to its unusually high proportion of metacyclic cells (approaching 100%; Fig. 3A), which are found in low-passage promastigote cultures. By comparison, reports in other species have been of stationary culture metacyclic promastigotes that were present in much lower proportions (Sacks et al., 1985; Zakai et al., 1998). The high proportion of metacyclic promastigotes seen in low-passage *L. chagasi* cultures greatly accentuated the difference seen in metacyclic proportions during serial passage, and enabled the observation reported here that serial passage is associated with a loss of the metacyclic promastigote form.

The process by which serial passage interferes with, or fails to support, metacyclogenesis may involve extrinsic or intrinsic molecules that control normal parasite developmental biology. We speculate that the conditions for culturing promastigotes affect metacyclogenesis via abnormal nutritional inputs. Alternatively, the effect may be due to more general developmental considerations, e.g., a requirement that promastigotes pass through the invertebrate host within some number of cell doublings that is exceeded during moderate serial passage. A loss of metacyclic cells seems unlikely to be something that might occur in naturally infected vectors, given the limited lifespan of the fly. At the same time, an infected fly may well blood-feed multiple times to support multiple rounds of oviposition. In those terms, serial passage in culture, and the consequent cycling from serum-rich to serum-deficient culture conditions, may mirror the in vivo state.

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Figure 1. Promastigote sensitivity to serum-lysis during serial passage. **(A)** Promastigotes from 7 independently initiated cultures at passage 2 or 3 were assessed for survival rate in 12% NHS. **(B)** Promastigotes from 3 independently initiated cultures were passaged serially and, within each passage, the parasites within the stationary culture phase, i.e., day 6 or 7, were assessed for survival in 12% or 50% NHS.

Figure 2.
Morphological criteria used to discriminate between promastigote forms.

Figure 3.

Changes in predominant promastigote morphologic forms during serial passage: (A) Promastigotes within 7 stationary phase independent cultures at passage 2–3, or within a subset of 3 of those cultures at passage 9, were assessed for the proportion of metacyclic (closed bars) and nectomonad (open bars) forms. (B–C) Promastigotes within a single culture were assessed for the presence and proportion of morphologically distinct promastigote forms during the 3rd (B) or 9th (C) passage. Symbols correspond to procyclic (□), nectomonad (■), leptomonad (△), and metacyclic (▲) promastigotes. (D, E, F) Promastigotes within a single culture at stationary growth phase were visualized by light microscopy during the 3rd (D), 9th (E), or 15th (F) passage. In D, all except a single cell (indicated by “N” for nectomonad) are metacyclic form cells. In E and F, almost all cells shown are of the nectomonad form. Bars = 10 μ m.

Figure 4.

Changes in MSP and PSA expression due to serial passage: Total lysates from promastigotes within a single culture at stationary growth phase in passage 3, 4, 6, 7, and 15 were separated by reducing SDS-PAGE and then assessed by western blot analysis for the abundance of major surface protease (MSP, upper panel), promastigote surface antigen (PSA2, lower panel), and tubulin (middle panel) as a loading control. Gel lanes were loaded with equivalent amounts (mass) of protein.