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Characterization and optimization of animal and culture models of Leishmania chagasi

Soi Meng Lei
Iowa State University

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by

Soi Meng Lei

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Program of Study Committee:
Jeffrey K. Beetham, Major Professor
Lyric Colleen Bartholomay
Douglas E. Jones

Iowa State University

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ABSTRACT

Leishmania spp. are an important pathogen of humans and other vertebrate animals, with significant global prevalence and impact on human and animal health. *Leishmania chagasi* is one species that causes visceral leishmaniasis, a potentially fatal disease of humans: annual incidence ≈ 0.5 mil. These insect-vector borne protozoan pathogens undergo complex development within the sand fly as they progress from morphological forms having low infectivity (to vertebrates) to a form that has high infectivity. Repeated serial passage of promastigote cultures results 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. One area of study presented within this thesis is a determination of the temporal abundance of morphologically distinct parasite stages that appear within axenic *in vitro* cultures of *L. chagasi* parasites. The principal finding is that nectomonad promastigotes predominate, while metacyclic promastigotes diminish, in cultures that have been serially passaged more than seven times and that are at stationary growth phase. A second, related area of study also presented within this thesis was designed to optimize the animal-model culture system used to produce and propagate infectious parasites. The principal finding on this was that parasite development and characteristics were equivalent in cell cultures regardless of whether cultures were initiated using never-stored or cryopreserved cells. The second study also validated the use of saphenous vein inoculation for the inoculation/infection of *L. chagasi* parasites into hamsters, a common animal model of

visceral leishmaniasis; saphenous vein inoculation is considered a more humane procedure than the alternative and commonly used cardiac puncture method. As a result, the second study increases the utility and efficiency of the *in vitro* model of visceralizing *Leishmania spp.* and decreases the number of animals needed to maintain the animal model.

CHAPTER 1. LITERATURE REVIEW

Overview and Impact

Leishmania spp. are an important pathogen of humans and other vertebrate animals, with significant global prevalence and impact on human and animal health. These insect-vector-borne protozoan pathogens undergo complex development within the sand fly as they progress from morphological forms having low infectivity (to vertebrates) to a form that has high infectivity. Of these morphological forms, only one, the infectious metacyclic promastigote, has been extensively characterized in a relatively "pure" state. The other forms (i.e. procyclic, nectomonad, and leptomonad) have largely been characterized as mixtures containing all three non-infectious forms.

My main interest was to determine the temporal abundance of morphologically distinct parasite stages that appear within axenic *in vitro* cultures of *L. chagasi* parasites. By doing so, I hoped to identify specific culture conditions by which to isolate parasite populations that would be highly enriched for specific parasite forms/stages that have heretofore not been characterized in a highly enriched state. Such isolation of specific parasite stages would facilitate future studies of these stages. The principal finding of this study, as presented within Chapter 2, is the determination that nectomonad promastigotes predominate, while metacyclic promastigotes diminish, in cultures that have been serially passaged more than seven times and that are at stationary growth phase.

A related interest was to determine whether the dynamics and characteristics of parasite development were equivalent in cultures that had been established using

hamster-derived parasites that had either never been stored/frozen or had been cryopreserved in liquid nitrogen. Establishing such equivalency would have potential to (i) increase the utility and efficiency of the *in vitro Leishmania spp.* and (ii) decrease the number of animals needed to maintain the animal model. The principal finding of this study, as detailed within Chapter 3 and the corresponding publication, was that parasite development and characteristics were equivalent in the cell cultures, regardless of whether cultures were initiated using never-stored or cryopreserved cells. The study also validated the use of saphenous vein inoculation for the inoculation/infection of *L. chagasi* parasites into hamsters, a common animal model of visceral leishmaniasis; saphenous vein inoculation is considered a more humane procedure than the alternative and commonly used cardiac puncture method.

Background

Epidemiology of leishmaniasis

Leishmania spp. are protozoan obligate intracellular parasites that cause leishmaniasis. Leishmaniasis is a disease group that affects human and other vertebrates such as dogs and rodents. Leishmaniasis is endemic in 88 countries, threatening 350 million people located predominately in tropic and sub-tropic regions (1). Each year there are more than two million new cases of human leishmaniasis. Among the new cases, three quarters are cutaneous leishmaniasis (CL) and one quarter are visceral leishmaniasis (VL) (1, 2). Approximately 90% of CL infections occur in central Asia and Latin America, while 90% of VL infections occur in subcontinent India, southwest and central Asia, South America, Africa, and along the Mediterranean (2).

The genus *Leishmania* contains more than two dozen species (3). In general, certain species cause specific disease forms in human. *L. donovani*, *L. infantum*, and *L. chagasi* are three of the species that cause VL; *L. major*, *L. pifanoi* and *L. mexicana* are three of the species that cause CL; *L. braziliensis, L. panamensis*, and *L. guyanensis* are three of the species that cause mucocutaneous leishmaniasis. In VL, parasites spread to organs of the reticulohistiocytic system (3), infecting bone marrow, spleen, and liver. If untreated, the fatality rate associated with VL can be greater than 90% (1). On the contrary, CL usually generates a localized self-healing lesion (papule, nodule, or nodule-ulcer) at the site of the sand fly bite that initiated the infection (1). Although the lesion may heal, it leaves a permanent scar and parasites remain in the proximal area. Most commonly in mucocutaneous leishmaniasis, symptoms of mucosal dissemination of parasites develops sometime (typically 1-5 years) after the cutaneous lesion has healed. Physical evidence of mucosal leishmaniasis begins with ulcerations at the nares, and eventually produces major disfigurement of the nasal pharyngeal system (2).

Life cycle

Despite the wide range of disease manifestations, different species of *Leishmania* share the same general life cycle (reviewed in (4-6) and described briefly herein). *Leishmania* have a heteroxenous life cycle, existing as sessile amastigotes within phagocytic cells in vertebrate hosts and as flagellated promastigotes in the alimentary tract of sand fly vectors. When a parasitized female sand fly takes a bloodmeal from the vertebrate host, metacyclic promastigotes are regurgitated into the capillary bed and encounter the host innate immune defense system comprised of the complement system

and phagocytes (neutrophils, dendritic cells, and macrophages). Metacyclic promastigotes resist complement-mediated lysis (CML) (7-9) but are opsonized with derivatives of complement protein C3 and become the targets for receptor-mediated phagocytosis *via* receptors including CR1, CR3, mannose fucose receptors, fibronectin receptor and Fc receptor (10, 11).

Although parasite promastigotes can be phagocytosed by neutrophils (12, 13), dendritic cells (14), and macrophages (15, 16), and do differentiate into amastigotes in all three cell types, parasite replication occurs only within macrophages (15, 16). Promastigote phagocytosed by macrophages are enclosed within a phagosome, a vacuole formed from the host cell membrane. The phagosome fuses with late endosomes and lysosomes and ultimately forms a parasitophorous vacuole (PV). Depending on species and animal/cell-infection system studied, *Leishmania* parasites interactions with the host cell at different points during the cell-infection process can perturb the cell's capacity to mount an effective anti-microbial defense, as is described within the introduction section entitled "*Leishmania* interaction with host". Within the PV, promastigotes differentiate into amastigotes, the non-motile parasite stage that is responsible for parasite replication within the infected vertebrate and that is adapted to the acidic and proteolytic environment of the PV. While progression of infection involves amastigote infection of additional macrophages, the mechanism by which this occurs is unclear. One hypothesis is that uninfected macrophages become infected by engulfing infected macrophages (17). Another hypothesis is that an infected macrophage bursts or otherwise releases its PVs

(e.g. *via* exocytosis), releasing amastigotes that are subsequently phagocytosed by uninfected macrophages (18, 19).

The general aspects of parasite development within the fly that are described here draw from several reviews (4-6, 20, 21). When a sand fly takes a bloodmeal from an infected host, amastigotes within monocytes pass with the bloodmeal into the digestive tract of the vector. Amastigotes are released from the monocytes and transform into procyclic promastigotes in the midgut within 24 hours. During the next 1-2 weeks, promastigotes replicate and undergo developmental changes, passing through morphologically distinct intermediate stages (nectomonad and leptomonad promastigotes), before eventually reaching the metacyclic promastigotes stage, which is the virulent stage. Metacyclic promastigotes are found in increased concentrations in the anterior portion of the digestive tract (22). Parasites are inoculated into the vertebrate host when the fly takes a bloodmeal and in so doing regurgitates contents of the gut. Studies have indicated two factors that possibly influence regurgitation. One factor is that the chitin lining of the fly cardiac valve is damaged by promastigote-secreted chitinase, as has been shown in electron micrographs of infected flies (23). The other factor is that a parasite-secreted filamentous proteophosphoglycan forms a gel plug within the fly foregut that promotes reflux of both parasites and gel during blood feeding (24-26). Both factors may result in reduced efficiency of blood intake during feeding, with consequent increases in the duration and/or frequency of blood feeding that may promote parasite transmission (24).

Leishmania interactions with host

Complement cascade and *Leishmania* evasion of complement-mediated lysis

Complement is a primary component of innate immunity and is the first host defense mechanism that *Leishmania* promastigotes face upon inoculation into the vertebrate host. The complement cascade consists of a group of soluble serum proteins that interact with the pathogen surface and function to (i) identify/label the pathogen for uptake by phagocytic immune cells, (ii) activate other components of the immune system, and (iii) form a membrane attack complex (MAC) in the cell membrane that results in cell lyses. The complement system must be activated in order to productively interact with a pathogen. There are three pathways for complement activation: classical, alternative and mannose-binding lectin pathway. The classical pathway is activated when complement C1q binds to antibodies adsorbed on the pathogen; the alternative pathway is activated when spontaneous hydrolyzed complement C3 adsorbs on the pathogen; and the mannose-binding lectin pathway is activated when mannose-binding-protein adsorbs to mannose that is present on the pathogen (27). *Leishmania* parasites activate complement *via* all three pathways, although some studies support the idea that activation occurs mainly *via* the alternative (28) or the classical (30, 31) pathway. Regardless of the mechanism of activation, all three pathways merge subsequent to activation at the point at which C3 convertase cleavage of complement protein C3 into C3a and C3b. C3b is part of a C5 convertase that cleaves C5 into C5a and C5b. The MAC is a heteromeric complex consisting of C5b, C6, C7, C8, and multiple C9 units; MAC inserts into the

surface membrane of the pathogen. C3a and C5a have anaphylactic properties that produce a local inflammatory response (27).

Metacyclic promastigotes, but not non-metacyclic promastigotes, are highly resistant to CML (7-9). Several studies suggest multiple mechanisms for this resistance. In one, resistance is due to surface lipophosphoglycan (LPG) that is significantly longer than on complement-sensitive cells (32) and causes MAC formation distal to the membrane (33). In another mechanism, major surface protease (MSP), up-regulated in metacyclic promastigotes (34, 35), cleaves complement factor C3b to the iC3b form that is unable to promote assembly of a MAC (10, 36). An additional benefit (to the parasite) of the MSP-dependent formation of iC3b is that iC3b serves as an effective opsonin that can promote macrophage phagocytosis (*via* CR3) without triggering an antimicrobial oxidative burst response (10).

Leishmania macrophage interactions

The general process of *Leishmania* parasite uptake by macrophages and subsequent residence within the PV that potentially matures into a phagolysosome was described within the "Life cycle" section of this chapter. The following paragraphs detail specific *Leishmania* parasite-macrophage interactions that influence the capacity of macrophages to mount effective anti-microbial responses.

Parasite LPG has been shown to delay or otherwise perturb the capacity of the phagosome to fuse with other microbodies/vacuoles (e.g. late endosomes and lysosomes), thereby preventing formation of a fully acidified phagolysosome (37-39); LPG was shown in one study to inhibit phagosome acquisition of rab7p, a GTPase found on

phagosomes only after fusion with late endosomes or lysosomes (38). The delayed maturation of the phagosome presumably allows time for promastigotes to transform into amastigotes, which are more resistant to acidic and proteolytic environments (40).

Parasite LPG has also been shown to act by two different mechanisms to reduce the ability of the infected macrophage to produce an anti-microbial oxidative burst (41). The "normal" anti-microbial oxidative burst requires activation of a signal cascade that includes protein kinase C (PKC) and protein tyrosine kinases, and that results in increased NADPH-oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) activity that generates reactive oxygen intermediates (42, 43). In the first mechanism, LPG alters the physical properties of the lipid bilayer to inhibit PKC membrane translocation (44). In the second mechanism, LPG competes antagonistically with the diacylglycerol-activation of PKC (45). Moreover, *Leishmania* produce superoxide dismutase which can deplete reactive oxygen intermediates (46).

The parasite can also interfere with macrophage production of cytokines. For example, parasite infection of a macrophage can inhibit macrophage production of IL-12, an important cytokine involved in T-lymphocyte activation (43). Activated T-lymphocytes can secrete INF-γ (interferon-gamma), which in turn can activate infected macrophages to mount an antimicrobial response (47). Parasite interference with macrophage cytokine production is not limited to inhibitory effects. Parasite-infected macrophages can also exhibit higher expression of anti-inflammatory cytokine IL-10 and TGF-β (transforming growth factor- beta) that serve to suppress an effective anti-leishmania immune response (48). *Leishmania* can also prolong the life of an infected

macrophage by delaying apoptosis *via* induced secretion of TNF-α (tumor necrosis factor-alpha) and GM-CSF (granulocyte-macrophage colony-stimulating factor) (49).

Leishmania neutrophil interactions

Polymorphonuclear neutrophils (PMN) are the first group of cells recruited in the first few hours to the site of *Leishmania* promastigotes entry (50). Their primary function is to phagocytose and kill microorganisms and to recruit other immune cells. PMN are recruited by complement factor C3a and cytokines IL-8, IL-17, and trans-membrane tumor necrosis factor (51) and by promastigote-secreted chemotactic factor (52). Live parasites can be found within PMN *in vitro* and *in vivo* (53). As in macrophages, PMN that contain parasites have perturbed antimicrobial capacity. Parasitized PMN been shown to exist in a reduced state of activation (i.e. have reduced anti-microbial activity). The PV within PMN have reduced lytic activity (13). Parasitized PMN undergo delayed apoptosis (54). As a result of these activities, even though data indicate a lack of parasite replication within the PMN, the infected PMN may serve as a temporary shelter for the parasite early during the infection process.

Leishmania dendritic cell interactions

Dendritic cells (DC) are professional antigen presenting cells that serve as an important bridge between the innate and adaptive immune response (55) DC exist in two functionally distinct phenotypes, immature or mature. Immature DC are highly endocytic and in peripheral tissues sense indicators of infection or tissue damage. Immature DC can phagocytose opsonized or non-opsonized promastigotes and amastigotes (58, 61).

Upon antigen encounter, DC can become activated and undergo a process of maturation whereby they lose their endocytic capacity and become antigen-presenting cells. Mature DC are characterized by upregulation of chemokine receptors that allow cell migration to lymph nodes, and by surface expression of major histocompatability complex (MHC) and co-stimulatory molecules that directly interact with naïve T cells. In addition, mature DC produce and secrete cytokines necessary for T cell polarization (55).

Due to the critical role that DC play in the initiation of primary adaptive immune responses, alterations to normal DC maturation can interfere with a productive adaptive immune response towards a pathogen. DC-*Leishmania* interaction involves many host-cell surface receptors including Toll-Like Receptors (TLR) 2,4,7 and 9 (57), and DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) (58), although not all interactions are involved in phagocytosis of the parasite. DC exhibit differing responses to *Leishmania* infection depending on species/strains and on parasite developmental form/stage; some interactions lead to maturation/activation, while others lead to reduced DC activation with consequent reduced expression of MHC and co-stimulatory molecules that results in reduced adaptive immune responses required for control of infection (36, 59, 60). In example, the DC interactions with amastigotes and metacyclic promastigotes of *L. infantum* and *L. pifanoi* involved receptor DC-SIGN and did not result in DC maturation (58). Interestingly, non-metacyclic forms of *L. infantum*, *L. pifanoi*, or *L. major* promastigotes did not bind DC-SIGN on DC (58).

Leishmania interactions with sand fly vector

Only seven percent of all the 1000 or more known sand fly species are competent vectors to transmit *Leishmania* parasite (2). The parasite is faced with a number of challenges in successfully infecting and being transmitted by the sand fly. Upon ingestion of the parasite-containing blood meal, parasites must survive the proteolytic environment of the gut. Parasites then need to get around or through the peritrophic matrix (PM) that surrounds the bloodmeal in order to attach to the gut surface and thereby be retained within the gut when the gut contents are excreted. Still later during the course of infection parasites must be present in a non-bound state in order to be available for transmission when the sand fly takes a blood meal. Additionally, parasites modify the vector environment towards increasing the vectorial capacity of the vector.

Proteolytic environment within gut

Leishmania parasites need to survive the biochemically hostile environment that exists during bloodmeal digestion. Sand flies secrete a variety of digestive enzymes, including trypsin, chymotrypsin, aminopeptidase, carboxypeptidases, and alpha-glucosidase, with peak enzymatic activities occurring 18-48 hours post bloodmeal ingestion (62). Parasites are especially vulnerable to these conditions during transformation from amastigote to procyclic promastigote, which occurs within the first 24 hours (63, 64). Several studies have shown that inhibition of gut trypsin activity greatly increased parasite survival in the sand fly (63, 65). Studies of *L. major* and *L. donovani* within their natural vectors showed that trypsin and chymotrypsin activities were suppressed in parasite-containing sand flies during the first 30 hours of bloodmeal

intake (66, 67). This suggests the parasites modulate the digestive activity of the vectors in favor of parasite survival.

Peritrophic matrix

The peritrophic matrix (PM) is composed of a meshwork of protein and chitin that encases the ingested food and is produced by midgut epithelium. The PM is semi-permeable; digestive enzymes and nutrient can diffuse in and out of the PM (68, 69). The PM does limit the rate at which digestive enzymes can pass into the gut lumen where the blood meal is located, and by doing so can limit initial parasite exposure to proteolytic enzymes (62). Parasites within the gut lumen are surrounded by the PM and need to get around the PM to allow binding to the gut wall and thereby avoid excretion. Chitinase produced by *Leishmania* promastigotes, MSP, and endogenous trypsin within the digestive bloodmeal are thought to contribute to the degradation of the PM (63, 64, 70).

Parasite persistence in the sand fly gut and "strategy" for successful transmission

Electron micrographs depict promastigote-gut interactions that are characterized by insertion of the parasite flagella between gut membrane microvilli (71, 72). The interaction is mediated by LPG (32) and other gut-associated lectins or lectin-like molecules on the midgut lumen (73, 74) Having survived the first period of about 3-4 days post-infection during which the blood meal is digested then defecated, promastigotes move to the anterior region of the midgut and multiply, in preparation for transmission to a vertebrate host. A gel-like plug composed of parasite-secreted filamentous proteophosphoglycans (fPPG) then forms in the thoracic midgut and cardiac

valve region of the sand fly (75, 76). A large portion of the parasites detected at this time of infection are embedded in the gel plug and are not bound to the midgut epithelium (22). The predominant form of parasites within the population of promastigotes in the anterior region is the most infectious form, the metacyclic promastigote, which is pre-adapted for host infection (75, 76).

Leishmania promastigotes have been shown to modulate the sand fly gut in several ways that are thought to enhance transmission to the vertebrate. Promastigote secreted chitinase damages the fly cardiac valve, perturbing the one-way flow of the blood-meal into the fly, and consequently promoting regurgitation of the parasite into the vector during blood-feeding (63). In a similar way, the presence of fPPG at the cardiac valve region prevents valve closure and promotes regurgitation (75). Also, increase feeding persistence and other perturbed feeding behaviors are attributed to the presence of the gel-plug and its impairment of fly mechanoreceptors that detect blood flow in the foregut (77, 78).

Promastigote morphological forms

Leishmania undergo a complex series of transformations within the sand fly gut that are characterized by morphologically distinct forms of promastigotes. The following is a detailed description of promastigote forms: procyclic, nectomonad, leptomonad, metacyclic, haptomonad, and paramastigote.

Procyclic promastigote

Procyclic promastigotes are the first promastigote form to appear after amastigotes are ingested by a sand fly (6). Procyclics develop in the midgut within 24 hours post blood meal ingestion (22, 62). They are ellipsoid, short, weakly motile, and are often found in rosettes within the blood meal (5). As characterized in two reports, their flagellar length are shorter than body length (6-11.5 μm) and their body width ranges from 2 to 2.5 μm (6, 76). Procyclics are responsible for the first round of population increase that occurs in the sand fly (22).

Nectomonad promastigote

Nectomonad promastigotes appear later in the infection, *e.g*. in *L. chagasi* infected *Lutzomyia longipalpis*, nectomonads were detected beginning at 48 hours post-ingestion (6). In that study, nectomonads were longer that other promastigote forms (13-23μm) and were slender (average width 1.6 μm); in all studies, nectomads have been the longest of any promastigote form (6, 22, 72). Based upon their temporal appearance that follows procyclics, nectomonads are considered to be responsible for much of the sand fly infection that follows initial blood-meal ingestion and amastigote-promastigote differentiation; nectomonads are thought to responsible for escaping the PM and promoting the anterior migration of parasites (62, 76). Nectomonads are strongly motile and first found to have escaped the PM and to be accumulated at the anterior end of the PM beginning at day 3. At subsequent times post ingestion they are still found in the anterior midgut, with some attaching to the microvilli of the midgut epithelium, and are found at the cardiac valve (78). Electron micrographs provide evidence that

nectomonads attach to the midgut by insertion of their flagella into the gaps between the microvilli of the gut epithelium (71, 72). The attachment has been shown to involve LPG (5, 62). At least one study suggests that nectomonads are a non-replicating promastigote form; Gossage et al inferred that nectomonads are essentially a non-divisional form based on analysis of *L. mexicana* and *L. infantum* in which they observed 1-5% and 0.1-0.2% (respectively) dividing nectomonads among all dividing cells (22).

Leptomonad promastigote

Leptomonad promastigotes are small, as the name indicates ("lepto"= Greek prefix for slender, small). They are shorter than nectomonads, 6-11.5 μm in length, with flagellum longer than the cell body (76). Leptomonad forms are found in sand flies 3 days after blood feeding (22). Based on their location within the region of the gel-plug, one study speculates that leptomonads derive from the non-attached sub-population of nectomonad promastigotes that reach the barrier of the cardiac valve (5). Leptomonads are divisional and are thought to be the precursor of metacyclic promastigotes (22). One major activity of leptomonads is the production of promastigote secretory gel (5, 78), which as noted earlier, plays an important role in transmission of the infection.

Metacyclic promastigote

The metacyclic promastigote has been the most well characterized form among all promastigote forms largely because it is the form that is highly infectious to vertebrates, and because techniques have been developed that allow for its enrichment/purification from among the mixed populations of promastigote forms that are present in axenic

promastigote cultures (79-82). However, *in vivo* studies have also produced important descriptive information about metacyclics.

Metacyclic promastigotes have been detected free swimming in the anterior portion of the midgut and foregut (5), the cardia lumen, and the pharynx (6), and have been detected imbedded in the anterior pole of the gel plug (78). They are short and slender, with flagella that are long, about twice the body length. One study showed, based on 25 metacyclics measured, that *L. major* metacyclic had an average length of 8.22±0.9 μm and width of 1.15±0.2 μm (6). They are a "strictly non-dividing stage" (22) and are pre-adapted for host-cell entry (83).

Metacyclogenesis is the poorly understood process by which cells develop from non-metacyclics to metacyclics. A number of factors have been characterized by *in vitro* studies to have an influence over metacyclogenesis, including decreased pteridine availability (84) and low pH (e.g. below pH 5.5) (85).

Critical towards studies requiring a relatively large number of cells, developmental properties of metacyclics have been shown to be equivalent regardless of whether the cell source was *in vivo* or *in vitro* (22). In general, the concentration of metacyclic promastigotes is the highest in stationary phase of low passage cultures. Several genes have been identified (by analysis of low passage cultured cells) that exhibit increased RNA and protein expression in metacyclics, relative to non-metacyclics, including glycoproteins GP46 and MSP (34, 35, 86). Their up-regulation has been shown to be due to increased mRNA stability rather than to increased rates of transcription (35,

86). Also upregulated in metacyclics is the small hydrophilic endoplasmic reticulum protein (SHERP) that was characterized in *L. major* (83).

Haptomonad promastigote and paramastigote

Haptomonad promastigotes and paramastigotes are two morphologic forms of promastigotes that are thought to either be of very low abundance, or to be found only within a subset of *Leishmania spp*. (76). Haptomonad promastigotes are characterized by a disc-like expansion of the flagellar tip, a very short flagellum, and (usually) a tear- or pear-shaped cell body (76). Where described, the haptomonad flagellum was found to be attached to the cuticular surfaces of the midgut *via* hemidesmosome-like attachment plaques (87). Paramastigotes are also not commonly observed, and are characterized by their kinetoplast being located immediately proximal to the nucleus (76).

Effect of serial passage during axenic culture

Prolonged *in vitro* cultivation causes attenuation in many viral, bacterial, and eukaryote pathogen laboratory models such as West Nile virus (88), *Mycobacterium bovis* (89), and *Neospora caninum* (90). Long term cultivation of *L. chagasi* can also lead to attenuation (91), and in addition, to decreased expression of MSP (35) and GP46 (92). This loss of GP46 and MSP expression has been attributed to reduced mRNA stability (35, 92).

Animal models for visceral leishmaniasis

Production of infectious *Leishmania spp*. parasites requires their regular passage through an animal such as mice, hamsters, Guinea pigs, or gerbil (93) because their long

term *in vitro* culture results in attenuation. While mice and hamsters are both commonly used as animal models for *L. chagasi* (94), hamsters are more commonly used as a source for infectious parasites because of similarities between the infection in hamster and that in dog or human. Hamsters are similar in susceptibility to infection with *L. chagasi* and also display similar symptoms during late stage chronic infection (e.g. hepatosplenomegaly) (95).

In the hamster model, inoculation is routinely by cardiac puncture (93, 96) or less frequently by intraperitoneal injection (97). The cardiac puncture procedure is accompanied by a modest risk of complication from cardiac arrest, cardiac tamponade, or hemorrhage, while intraperitoneal injection is accompanied by an extremely very slow onset of symptoms and a frequent need for repeated inoculations to achieve assurance of infection (97-99).

The significance of the study

Work presented in Chapter 2 examines the temporal abundance of morphologically distinct parasite stages that appear within axenic *in vitro* cultures of *L. chagasi* parasites. Our principal finding is that repeated serial passage results in a profound loss of metacyclic promastigotes from cultures at stationary growth phase, and a concomitant increase in nectomonad promastigotes. Thus, this study identifies a potential source for parasite populations that are highly enriched in the nectomonad form. In addition, the study clarifies studies by others that determined that serial passage results in mRNA-abundance perturbations that affect metacyclic-stage cell properties including expression of specific surface proteins and surface glycosylation state. The study

presented in Chapter 2 provides the broader explanation that serial-passage induced loss of metacyclic cell properties is due to changes in population structure characterized by reduced numbers of metacyclic stage cells within culture at stationary growth phase. There is growing interest in using live attenuated parasites (100) or attenuated parasites with target gene deletion (101, 102) as vaccine candidate for leishmaniasis. The data from Chapter 2 can provide valuable background information on attenuated parasites.

Work presented in Chapter 3 establish the equivalency between cultures established using parasites freshly isolated from infected hamsters versus established using cryopreserved parasites, and validates the utility of inoculating hamsters *via* the saphenous vein. Results of this study may benefit future studies; variability within experiments may be decreased in experiments conducted at different times by using cells from cultures initiated with equivalent cryo-preserved cells, *i.e.* cryo-preserved parasites isolated from the same hamster. Study results have potential to improve aspects of animal care, based upon the positive aspects of saphenous vein inoculation relative to the traditional inoculation method of cardiac puncture. Study results also have potential to increase the efficiency of animal usage, given that the use of cryo-preserved parasites can decrease the number of hamsters that are needed to serve as sources of parasites with which to initiate cultures.

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CHAPTER 2. POPULATION CHANGES IN *LEISHMANIA CHAGASI* PROMASTIGOTE DEVELOPMENTAL STAGES DUE TO SERIAL PASSAGE

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Soi Meng Lei, Nathan M. Romine, Jeffrey K. Beetham

Departments of Veterinary Pathology and Entomology, Iowa State University, Ames, IA USA

Abstract

Leishmania chagasi are one species of *Leishmania* that cause visceral leishmaniasis, a potentially fatal disease of humans: annual incidence ≈ 0.5 mil. Within the sand fly vector *Leishmania* replicate as promastigote-form parasites that 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 more 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), such axenic cultures progress from logarithmic to stationary growth phases, and parasites within those growth phases progress from parasites that do not have metacyclic cell properties to ones that do. Interestingly, repeated serial passage of promastigote cultures results 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 these perturbations associated with repeated serial passage. Passage 2-4 *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 exhibited low resistance to serum-lysis and low levels of MSP and PSA. This 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, this study suggests that serially passaged cultures may be a highly enriched source nectomonad promastigotes, a parasite form that has largely been characterized in mixtures containing other promastigote forms.

Introduction

The leishmaniases 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 leishmaniases 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 take in 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 (1-6). The procyclic promastigote is the first stage that the amastigote gives rise to 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 the leptomonad promastigote and the nectomonad promastigote, occur during the fly infection. These four stages have been identified in fly-parasite infection models (2-5) as well as in axenic promastigote culture systems (4,7).

The metacyclic promastigote stage has been the focus of a number of studies because of its presumptive role as the etiologic disease agent. Studies 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 surface proteins Promastigote Surface Antigen (PSA, also known as GP46 (8)) and Major Surface Protease (MSP) (9,10), and differential glycosylation of surface lipophosphoglycan (LPG (11)). Most of these studies of metacyclic promastigotes were enabled by use of axenic promastigote culture systems comprised of low passage cultures established from either animal-derived amastigotes (9,10) or from frozen low passage promastigotes (7,11). 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 promastigotes forms having low infection to vertebrates to promastigote forms that are highly infectious (4,7,12).

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 has 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 (13,14), while others use alternative procedures including density gradient centrifugation (15). The need for such enrichment techniques in *Leishmania 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 (7,16,17).

In earlier studies of *L. chagasi*, we and collaborators showed 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 RNA elements present within the 3'-untranslated region of the mRNA (8,18). Studies by others have characterized a similar regulation of one class of MSP (9). Interestingly, serial passage was found to greatly reduce the upregulation of GP46 and MSP, and this loss of upregulation was determined to involve RNA stability (19, 20). The study presented here extends 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 proportion of nectomonad-stage promastigotes.

Materials and Methods

Parasites

L. chagasi amastigotes 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 (21). 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 ~$4\text{-}7 \times 10^7$ ml^{-1}. Cultures were considered to be in stationary phase 48 hrs after reaching maximum cell density (day 7). Serial passaged cultures were split every seven days from a cell density of 4×10^7 ml^{-1} down to 1.0×10^6 cells ml^{-1}. Parasites 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 -80C. Complement assays were performed by incubating 3.5×10^6 promastigotes for 30 min at 37 C in 100 ul PBS or PBS supplemented with human serum as described (16). Afterwards, cells were diluted 1:10 in ice-cold 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 morphology as described previously by ourselves and other (4,5,7). While the body length of < 11.5 μm for three of these forms (procyclic, leptomonad, and metacyclic) are similar, 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 promastigote have the longest body lengths (> 12 μm) of any promastigote form.

Protein detection

Cells pelleted at 2,000 x g for 10 min at 4 C were resuspended in 0.1M potassium phosphate buffer, pH 7.8 containing 1% triton x 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, 0.15M NaCl, 0.1% Tween-20), membranes were exposed to primary and secondary antisera; sheep anti-MSP (9) diluted 1:10,000 in blocking solution, rabbit anti-

PSA (20) diluted 1:1,500, mouse anti-αTubulin (Calbiochem, California) diluted 1:1000, 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, Rockford, Illinois) diluted 1:1000. 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 seven independently initiated low passage (P2-P3) 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 cultures initiated using amastigotes derived from separate parasitized hamsters. To evaluate the effect of serial passage on serum sensitivity, three cultures were followed for multiple passages; each passage spanned one week (Fig. 1B). For all cultures, resistance to 12 or 50% 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 within and among independent cultures, these data are in general agreement with previous studies by ourselves and others reporting loss of complement resistance in *L chagasi* due to serial passage (16, 22).

Serial passage results in stationary growth phase cultures comprised of a diminished proportion of metacyclic stage promastigotes, and a greatly increased proportion of nectomonad stage promastigotes, relative to low passage cultures.

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. Table 1 provides information on the morphological parameters (body and flagellum size and ratios) used for stage assignment. The morphological parameters used here for *L. chagasi* were based (with minor modification) upon those used previously by others in assessing developmental stages in promastigotes of *L. mexicana* (5).

Paralleling the observation that compliment 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 form progressively increased in stationary phase cultures. Figure 2A demonstrates these changes in metacyclic and nectomonad proportions in passage 2, 3 and 9 cultures, and is based upon analysis of 7 independent low passage cultures, and of three high passage cultures derived (serially passaged) from three of those low passage cultures.

An analysis of the promastigote forms present within a single culture during passage 3 and passage 9 is presented in Figure 2B and C and is representative of the 3 independent cultures that were closely analyzed. As shown in 2B, 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, e.g. 2 daughter cells still joined together (data not shown). These relatively early time points are associated with a high proportion, albeit low absolute number, of metacyclic cells result from the fact that the cultures are initiated with cells from the prior passage at stationary growth. Leptomonads predominated during the logarithmic growth phase that consistently occurred during days 2-4 of culture, and nectomonads also increase in proportion through 96 hr. The onset of increased proportions of metacyclic promastigotes was in mid logarithmic culture phase. In comparison, a culture at passage 9 (Fig. 2C) seeded with cells from a previous high passage culture contained almost no metacyclic cells initially or at any other culture growth phase, exhibited increased proportion of procyclic and (to a lesser degree) leptomonad promastigotes during the first 48-72 hrs, then an increased nectomonad proportion beginning sometime around 72 hrs and on.

Photomicrographs of cells in a single culture at passage 3, 9, and 15 (all at stationary culture phase) are shown in Figure 2D, E, F, 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. 2D) emphasizes the size difference between these two 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 (Fig. 2E, 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 (19,20) levels of these proteins were also assessed. Similar to those previous studies, levels of MSP and GP46 were found to increase in promastigotes as low passage cultures progress from logarithmic into stationary growth phase, and serial passage resulted in a dramatic loss of MSP and PSA expression (Fig. 3).

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 phenomena (i) is associated with reduced MSP and PSA mRNA levels, (ii) is not associated with changes in transcription rate of the corresponding mRNAs, and (iii) 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 phase of growth 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 is incomplete concerning much of the developmental biology of promastigotes including what comprises a promastigote developmental stage, how cells progress from one stage to the next, and what the progenitor cell is for a given stages. 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; those studies do provide some support, albeit non-definitive, towards predictions of cell hierarchy.

Studies of developmental biology of *Leishmania spp*. will benefit from increased characterizations of all developmental promastigote forms. Ease of isolation and enrichment are attributes of high passage nectomonad promastigotes as a model for fly-derived and low passage (culture-derived) nectomonads. 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, for its unusually high proportion of metacyclic cells (approaching 100%, Fig. 2a) that are found in low passage promastigote cultures. By comparison, reports in other species have been of stationary culture metacyclic promastigote of much lower proportions of (23, 24). The high proportion of metacyclic promastigotes greatly accentuated the difference seen in metacyclic proportions during serial passage, and greatly enabled the observation reported here that serial passage is associated with a loss of 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 vertebrate 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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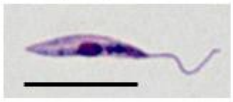
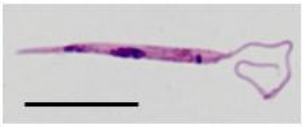
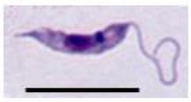
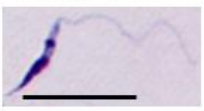
Procyclic	Nectomonad	Leptomonad	Metacyclic
			

	Procyclic	Nectomonad	Leptomonad	Metacyclic
Body length (μm)	6.5-11.5	≥12	6.5-11.5	6.5-11.5
Body width (μm)	>1.5	>1.5	>1.5	≤ 1.5
Flagellum (F) versus Body (B) length	F<B	Variable	F>B	F>B

Table 1. **Morphological criteria used to discriminate between promastigote stages.** Scale bars = 10 μm

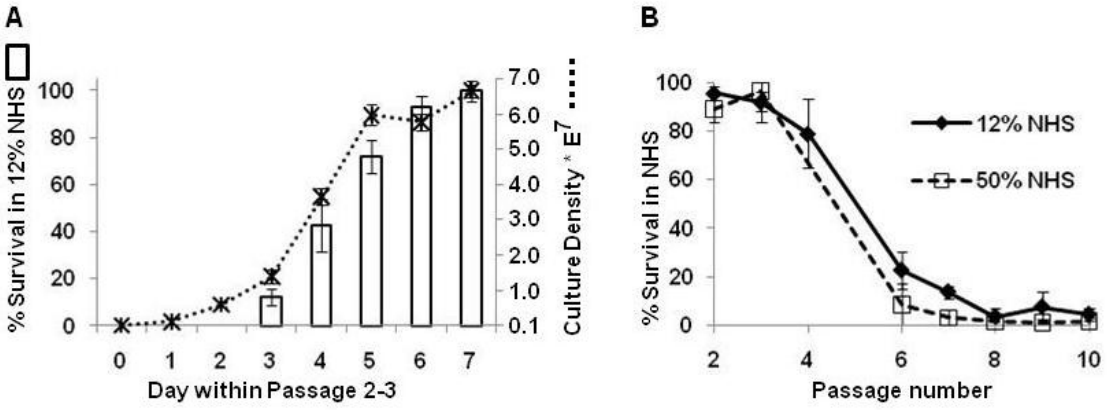


Figure 1. Promastigote sensitivity to serum lysis during serial passage. Promastigotes from ≥ 7 independently initiated cultures at passage 2 or 3 were assessed for survival in 12% NHS (A). Promastigotes from 3 independently initiated cultures were passaged serially, and within each passage the parasites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

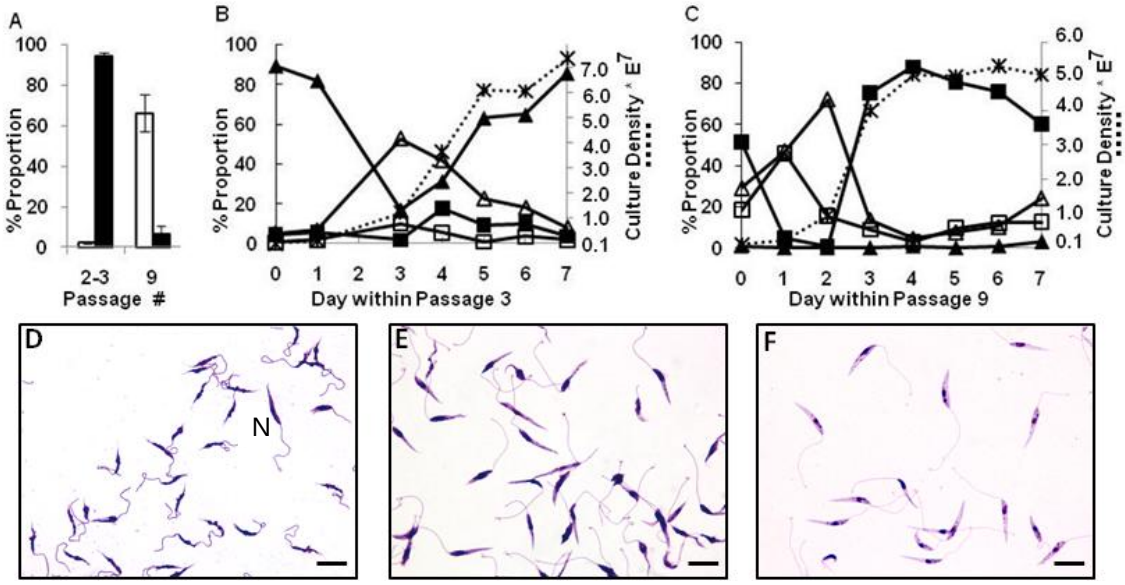


Figure 2. Changes in predominant promastigote morphologic forms during serial passage.

A: Promastigotes within seven stationary phase independent cultures at passage 2-3 or within a subset of three 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 abundance 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. Scale bars represent 10 μm.

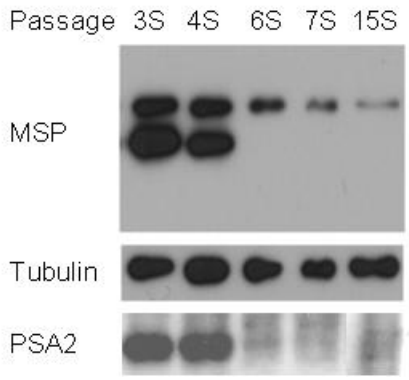


Figure 3. Changes in MSP and PSA expression due to serial passage.

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) and Promastigote Surface Antigen (PSA). Gels were loaded by constant mass of protein, and as an additional loading control were assessed for tubulin abundance following assessment of MSP or PSA; control panel shown represents tubulin control for MSP loading.

CHAPTER 3. REDUCED HAMSTER USAGE AND STRESS IN POPAGATING *LEISHMANIA CHAGASI* PROMASTIGOTES USING CRYOPRESERVATION AND SAPHENOUS VEIN INOCULATION

Modified from a paper published in *The Journal of Parasitology*[1]

Soi Meng Lei, Amanda E. Ramer-Tait[*], Rebecca R. Dahlin-Laborde[†], Kathleen Mullin[‡], Jeffrey K. Beetham[§]

Departments of Veterinary Pathology and Entomology, Iowa State University, Ames, Iowa USA

Abstract

Leishmania chagasi, a causal agent of visceral leishmaniasis, requires passage through lab animals such as hamsters to maintain its virulence. Hamster infection is typically accomplished *via* cardiac puncture or intraperitoneal injection, procedures accompanied by risks of increased animal stress and death. The use of the hamster model also necessitates a regular supply of infected animals, since *L. chagasi* parasites newly isolated from an infected hamster can be grown in culture for only several wk before loss of function/phenotype occurs. Towards decreasing animal usage and animal stress,

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[*] Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa IA 50011.

[†] Department of Experimental Medicine and Pathology, The Mayo Clinic, Rochester, Minnesota 55902.

[‡] Lab Animal Resources, Iowa State University, Ames, Iowa 50011.

[§]Corresponding author

experiments were performed to assess a more gentle inoculation procedure (saphenous vein inoculation) and the use of cryopreserved parasite cells for research experiments. Of 81 hamsters inoculated by the saphenous vein, 80 became infected as determined ante mortem by display of clinical symptoms of leishmaniasis (onset of symptoms at 105 ± 22 days post-inoculation [PI]) and postmortem by the presence of parasites within the spleen. Splenic parasite load calculated for a subset (n=34) of infected hamsters was 124 to 26,177 LD units. Cryopreserved and never-stored cells were equivalent in all properties evaluated, including developmental changes in morphology during culture, culture growth rates, parasite resistance to serum-mediated lysis, and expression of developmentally regulated surface proteins MSP and PSA.

Introduction

Leishmania spp. (Trypanosomatidae) are protozoan parasites that cause leishmaniasis, a disease group that in humans varies in severity from self-healing cutaneous lesions to potentially fatal visceral infections. The parasites have a heteroxenous life cycle, existing as flagellated promastigotes within the alimentary system of the sand fly vectors, or as ovoid, sessile amastigotes primarily found within macrophages of the vertebrate hosts. Amastigotes within a blood meal ingested by a sand fly enter the midgut and within 24 hr transform into procyclic promastigotes. Over a period of 1, to several, wk, promastigotes initially replicate and generate a mixed population consisting of several morphologically defined developmental stages, and eventually generate a population predominated by the metacyclic promastigote parasite stage (Gossage et al., 2003). Studies of axenic-culture or fly-derived promastigotes

representing a number of *Leishmania* species have shown that metacyclic promastigotes are distinguishable from other promastigote forms by a number of criteria in addition to morphology; metacyclic promastigotes are highly infectious to vertebrates (Sacks and Perkins, 1984), resist complement mediated lysis (Pinto-da-Silva et al., 2002; Dahlin-Laborde et al., 2005), display increased levels of surface glycoproteins including major surface protease (MSP) (Yao et al., 2008) and promastigote surface antigen (PSA) (Beetham et al., 2003), and display modified glycosylation states of lipophosphoglycan (LPG), the most abundant surface macromolecule on promastigotes.

The process by which promastigotes mature into the metacyclic promastigote stage within the sand fly is recapitulated in axenic cultures initiated with parasites derived from infected animals (Pearson and Steigbigel, 1980; Gossage et al., 2003); such cultures progress from a logarithmic growth phase to a stationary phase in which the parasites have properties of metacyclic promastigotes. One limitation in the utility of such axenic cultures is that serial passage results in stationary phase cells that lose some of the properties of cells found in low passage stationary phase cultures. Studies with *Leishmania chagasi*, one of the species causing visceral leishmaniasis, have shown that promastigotes from serially passaged cultures at stationary growth phase do not resist complement lysis, and do not exhibit upregulated abundance of MSP and PSA (Wilson et al., 1989; Beetham et al., 2003). For this reason, most experiments utilizing axenic promastigotes use cultures passaged 5, or fewer, times. This necessitates maintaining a constant supply of animal-derived parasites with which to initiate axenic cultures.

Mice and hamsters are the 2 common animal models for visceral leishmaniasis (Handman, 2001). Hamsters are frequently the preferred model because they are more susceptible and display symptoms such as hepatosplenomegaly during late stage chronic infection that are also seen in dog and human infections (Requena et al., 2000). Regardless of which animal model is used, inoculation is routinely achieved using either cardiac puncture or intraperitoneal injection (Stauber, 1958; Pearson and Steigbigel, 1980; Wyllie and Fairlamb, 2006), procedures accompanied by modest risk of complications, including cardiac arrest, cardiac tamponade, or hemorrhage in hamster for cardiac puncture, and very slow onset of symptoms and frequent need for repeated inoculation following the initial inoculation for intraperitoneal injection (Wyllie and Fairlamb, 2006; Moreno et al., 2007).

As with any animal model, ethical and economic concerns encourage efforts aimed at minimizing animal usage and animal stress. Consequently, experiments were undertaken that sought to minimize these factors. One experimental aim was to establish the equivalency between low passages cultures initiated using parasites freshly isolated from hamsters and parasites recovered from cryopreserved low passage promastigotes. The other aim was to determine the utility of saphenous vein inoculation to inoculate hamsters.

Materials and methods

Parasites

Infectious *L. chagasi* amastigotes (strain MHOM/BR/00/1669, originally isolated in Brazil from a patient with visceral leishmaniasis) were maintained in golden Syrian hamsters as described (Pearson and Steigbigel, 1980). Axenic promastigote cultures in supplemented modified minimum essential media (HOMEM) were initiated with amastigotes isolated from hamster spleen, and subsequently passaged as described (Pearson and Steigbigel, 1980; Zarley et al., 1991; Ramamoorthy et al., 1992; Dahlin-Laborde et al., 2005). Briefly, axenic promastigote culture densities increased throughout logarithmic culture phase until reaching a maximum (stationary) phase concentration of 2-5 x 10^7 cells/ml at about day 5 of culture; cultures were passaged by dilution to 1.0 x 10^6 cells/ml 48 hr after reaching stationary phase. Parasite cultures used were serially passaged for < 5 wk.

Hamster inoculation

All animal work was approved by the Iowa State University Institutional Animal and Care and Use Committee and was conducted between 1999 and 2007. Outbred 10- to 16-wk-old male golden Syrian hamsters weighing 88-145 g were anesthetized by intraperitoneal administration of ketamine (120 mg/kg) with acepromazine (1.2 mg/kg), and if not fully anesthetized within 5 min, were given up to an additional dose of anesthesia. Immediately upon exhibiting full sedation, triple antibiotic ointment (containing polymyxin B sulfate, bacitracin zinc, and neomycin) was topically applied to corneas to maintain eye moistness and prevent eye ulcers (since anesthetized hamsters do not blink), and hind legs were shaved to visualize the lateral saphenous veins. Blotting 70% ethanol onto the shaved area increased vein visibility. Moderate digital pressure

applied on the upper thigh along with slight tension stretching the skin caused blood retention and the vein to stand out and be stabilized. A tuberculin 1-ml syringe fitted with a 26-gauge, 2.5 cm length needle, and containing 0.2-ml inoculum (2-10 x 10^7 stationary phase promastigotes in sterile phosphate-buffed saline [PBS], pH 7.4) was inserted bevel-up into the vein, digital pressure on the upper thigh was removed, then the inoculum was delivered over a 15- to 30-sec range of time. Inoculum was derived from low passage cultures initiated either with parasites freshly isolated from infected hamsters or with cryostored parasites. Following removal of the needle and gentle compression at the site of injection to stop any bleeding, animals were observed to verify full and non-complicated recovery from the anesthesia and procedure.

Spleen impression smears and L.D. units

Hamsters were killed within 7 days of exhibiting symptoms of advanced leishmaniasis, i.e., ascites fluid buildup in abdominal cavity, dull coat, rough (ruffled) coat, general slow activity, and loss of ear turgor. Animals were then weighed, and the spleens aseptically removed, weighed, and processed for isolation of amastigotes as described previously (Pearson and Steigbigel, 1980). Spleen impression smears were made by lightly touching a small cut piece of spleen to a glass slide. Tissues on slides were stained with HEMA 3 stain set (Fisher Scientific, Pittsburg, Pennsylvania) or Giemsa, and visualized (1,000X, oil). For animals killed from 2004 through the study's end, the degree of infection was quantified as *Leishmania donovani* infection units (L.D. unit), which were calculated as (amastigotes per nucleated host cell) x spleen weight (in mg) (Stauber, 1958; Wilson et al., 1989).

Cryopreservation

Promastigotes from cultures at late logarithmic growth phase (1.0 - 1.5 x 10^7 cells/ml) were washed twice in sterile PBS and resuspended at 1 x 10^7 cells/ml in HOMEM containing 7.5% dimethyl sulfoxide (Fisher Scientific). Aliquots (1 ml) within 2.0-ml cryogenic vials were placed into room temperature Cryo 1C Freezing containers (Nalgene, Rochester, New York), stored 12-24 hr at –80 C, then stored in the vapor phase of liquid nitrogen. Stored cells within vials were recovered by thawing (immersion in 26 C water until fully thawed, approximately 1 min) then diluted in a 25-cm^2 cell culture flask (Corning, Lowell, Massachusetts) containing 2 ml of HOMEM. Cultures reached stationary phase 2-5 days after initiation and were ready for serial passage and/or expansion.

Human serum and complement assay

Human serum from multiple naïve donors was pooled and stored at –80 C in < 1.0-ml aliquots. Complement assays were performed by exposing 3.5 x 10^6 promastigotes in PBS (50 µl) to equal volume of 24% human serum in PBS, then incubated at 37 C for 30 min as described (Dahlin-Laborde et al., 2005).

Promastigote morphology study

Promastigotes within 10 µl culture samples were applied to glass slides, and then air dried, stained with HEMA 3 stain set (Fisher Scientific), visualized *via* light microscopy and measured using Nikon NIS-Elements D software (Nikon Instruments, Melville, New York). Cells were categorized into 1 of 4 different promastigote stages

(procyclic, leptomonad, nectomonad, metacyclic) based upon morphology as described previously (Rogers et al., 2002; Gossage et al., 2003; Yao et al., 2008). Briefly, procyclic, leptomonad, and metacyclic promastigotes all have similar body lengths (6.5-11.5 μm in length) but are individually distinguishable by simultaneous consideration of body width and of flagellum length relative to body length. Procyclic and leptomonad forms both have width > 1.5 μm, but the procyclic flagella are shorter than body length while the leptomonad flagella are longer than body length. The widths of metacyclic promastigotes are ≤1.5 μm and the flagella are 1.5 to 2 times the body length. The fourth group, nectomonad promastigotes, have longer body lengths (> 12 μm).

Protein detection

Promastigote culture (10 ml) was pelletted at 2,000 x g for 10 min at 4 C, resuspended in 0.1M potassium phosphate buffer, pH 7.8 containing 1% triton x100, then lysed *via* 3 cycles of freeze/thaw using liquid nitrogen and immersion in a 37 C water bath. Total cell lysates were separated by SDS-PAGE and electro-semi-dry-transferred to polyvinylidene fluoride membranes using standard procedures. Gel loadings were equivalent to 0.5 x 10^6 and 3.0 x 10^6 parasites/lane for assessment of MSP and PSA abundance, respectively. Non-fat powdered milk (5%) was used as the blocking agent. Reagents used included sheep antisera to MSP (Wilson et al., 1989) diluted 1:10,000, rabbit antisera to PSA (Beetham et al., 2003) diluted 1:1,500, horseradish-peroxidase conjugated anti-sheep antibody (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) diluted 1:30,000, and horseradish-peroxidase conjugated anti-rabbit

antibody (Pierce, Rockford, Illinois) diluted 1:20,000. Antibody binding was visualized *via* enzyme-linked chemiluminescence (SuperSignal®, Pierce).

Results

Comparison of freshly isolated and cryostored *L. chagasi* promastigotes

Under appropriate conditions, in cultures seeded with *L. chagasi* amastigote parasites newly isolated from infected hamsters, the parasites differentiate into promastigotes that divide (logarithmic phase) for several days prior to reaching a non-divisional state (stationary phase). Associated with this progression to stationary culture phase, promastigotes undergo a number of changes in morphology and surface protein expression and also become more resistant to lysis by the complement component of human serum. Therefore, as indicators of the equivalency of cryopreserved versus fresh, i.e., never stored (see Materials and Methods) promastigotes, growth rates, complement resistance, morphology, and surface MSP and PSA protein expression were evaluated during parasite culture. For each analyses presented, fresh parasites and cryopreserved parasites were derived from the same initial culture; that is, they were derived from parasites isolated at one time from a single infected hamster. The duration of cryostorage ranged from 2 to 4 wk.

Parasites within cultures derived from fresh versus cryopreserved cells had very similar growth rates and peak densities (representative data shown as line graph in Figs. 1A, B). Similarly, as cultures progressed from logarithmic to stationary phase, cells became increasingly and equivalently resistant to complement/serum lysis, reaching over

100% survival relative to control cells not incubated in serum. (Stationary culture survival in excess of 100% seen here was commonly obtained; this results from an increased number of cells that died in the no-serum controls and may be due to cells in serum being more heat tolerant than cells not in serum.)

Enumeration of morphological forms within cultures, i.e., procyclic, leptomonad, nectomonad, and metacyclic forms, also yielded very similar data for cultures derived from fresh versus cryopreserved cells (Figs. 1 C, D). The density of metacyclic stage forms increases dramatically at days 4 to 5 before reaching a plateau at about 40 million cells/ml in culture. Based on the total culture density being about 45 million cells/ml in stationary phase, metacyclic promastigotes in both cultures comprised about 90% of the whole population. Analysis of the other parasite forms (procyclic, leptomonad, and nectomonad) is subject to greater between-experiment variability since they are intermediate parasite forms that increase, then decrease, temporally. Still, there appears to be great similarity to their expression patterns in both cultures; e.g., leptomonad and nectomonad promastigote densities increased modestly from day 3 to days 4 and 5 before decreasing. Examples of parasite forms found in day 3 and day 7 cultures are also provided in Figure 2. All 4 promastigote forms were seen in day 3 cultures.

The surface proteins PSA and MSP also exhibited similar changes in expression during culture growth, with MSP isoforms (63 and 58 kDa) and PSA increasing dramatically in cells from stationary phase cultures (Day 7 lanes in Fig. 3). This differential expression of PSA (Beetham et al., 2003) and MSP (Roberts et al., 1995; Yao et al., 2005) during logarithmic and stationary growth phases have been previously

characterized, as has been their decreased expression in cells from cultures subjected to serial passage (Brittingham et al., 2001; Beetham et al., 2003).

Assessment of parasite inoculation *via* saphenous vein

Inoculating *L. chagasi* parasites into hamsters *via* cardiac puncture involves potential complications that may result in increased animal use or discomfort. Consequently, the utility of an alternative method involving intravenous (i.v.) inoculation *via* the saphenous vein was assessed. Inoculum was successfully administered to 78 of 81 hamsters on the first attempt at inoculation, and the remaining 3 were successfully inoculated on a second attempt performed 1 to 2 wk later. Almost 100% of the animals inoculated with *L. chagasi via* the saphenous vein became infected as assessed by clinical symptoms; 80 of 81 injected animals exhibited ruffled fur, abdominal swelling due to hepatosplenomegaly and/or ascites build up within about 15 wk (105 ± 22.2 days) post-inoculation (PI). One of the 81 inoculated hamsters failed to develop parasitemia or clinical symptoms. A small amount of bleeding sometimes occurred at the injection site, but this stopped quickly upon application of mild pressure to the site.

Animals were killed within 7 days of onset of clinical symptoms. Spleens removed from the 80 infected hamsters were almost 1% of total weight (1.02 ± 0.43 g) relative to total body weight (117 ± 30.0 g). By comparison, the spleen and total weight of a single, naive, age-matched hamster were 0.16 g and 106 g, respectively.

The infected hamsters (n = 34) killed in the later time period of the study (see Materials and Methods) were subjected to more extensive examination post mortem to

visualize by microscopy and to quantify by L.D. units the parasite burden within the spleens. Parasites were easily discernable by light microscopy in Giemsa stained impression smears made from the spleens (Fig. 4). A large number of intracellular amastigotes were present within mononuclear phagocytes, and additional extracellular amastigotes were also present in all smears (Fig. 4). In smears representing 20 of the 34 spleens, some extracellular amastigotes appeared to be enclosed by a membrane not associated with any host nuclei (indicated by a circle in Fig. 4A); these possibly derive from phagocytes whose structural integrity is disrupted during sample work-up.

The splenic parasite load calculated for these 34 animals ranged from 124 to 26,177 L.D. units, with a mean of 6,440 (Table I). These values are well above the lower threshold of determination of the assay, which is 1 LD (1 parasite per 1,000 nucleated host cells, which is equivalent to 200 parasites/mg of organ tissue) (Stauber, 1958). Interestingly, in a plot of L.D. units versus hamster terminal weight, there is an apparent clustering of the data points, with spleens of smaller animals tending to have larger parasite loads than spleens of larger animals (Fig. 5). Based upon that observation, the 34 hamsters were separated into 2 sub-groups composed of animals with terminal weights < 115 g or ≥ 115 g (Table I). Neither days of survival, spleen weight, nor weight at inoculation varied significantly within these subgroups. However, as was suggested by the data of Figure 5, L.D. units did vary significantly (P <0.001). The average L.D. unit was five-fold higher in the < 115 g group than the > 115 g group.

Inocula for 18 of the 34 hamsters were from cell cultures initiated with parasites that had been isolated from hamsters and had never been cryostored, while inocula for the

remaining 16 were from cultures initiated with cryostored parasites. No differences were observed between these two groups either in the infection characteristics in hamster, e.g., L.D., or in the properties of parasites isolated from those infected hamsters, e.g., complement survival rates, developmental profiles, and surface protein expression.

Discussion

The use of cryopreserved promastigotes to establish parasite cultures has the potential to reduce animal usage. In addition, use of cryopreserved parasites can enable experiments that require analyzing at different times same-passage parasites derived from a single infected hamster. Previous studies showed that cryopreserved amastigote forms of *L. donovani* produced the same level of infection in juvenile hamsters as did freshly isolated amastigotes (Wyllie and Fairlamb, 2006). Studies presented here demonstrate that *L. chagasi* promastigotes within cultures initiated either with cryopreserved or fresh low passage promastigotes are equivalent in their culture growth dynamics, including ordered appearance of morphologically distinct promastigote stages, their resistance to serum-mediated-lysis, and in their promastigote-stage specific expression of surface proteins MSP and PSA.

Experiments herein utilized cells cryopreserved up to 4 wk. We have also used cells cryostored for 4 yr to successfully initiate promastigotes cultures. While these cultures initiated with the longer-stored cells have not been compared as rigorously to non-stored cells as were the cells/cultures reported here, they were equivalent in complement resistant characteristics (data not shown).

Saphenous vein inoculation of parasites minimizes stress on the hamster because the animal is sedated during the procedure. The hamster data shown herein reflects an analysis of all *L. chagasi* inoculated hamsters used by our group over more than 7 yr. Therefore, the duration of our usage of the procedure and its efficacy in yielding infected hamsters indicates its utility in maintaining this hamster model of infectious parasites. As reported here, the time from inoculation to onset of severe clinical symptoms requiring termination of the infection is 105 ± 22.2 days; this is similar to values seen by others in studies of hamsters inoculated with amastigotes of the closely related *Leishmania donovani* species *via* intracardiac and intraperitoneal routes, which were 117 ± 32 (n=9) and 139 ± 30 (n=11) days, respectively (Wyllie and Fairlamb, 2006). The same study demonstrated, similar to data presented here, a relatively large variance in infection intensity among animals, with mean values of 1.50 (± 0.76) and 1.90 (± 0.86) amastigotes (x 10^{10}/g spleen) in animals inoculated *via* intracardiac and intraperitoneal routes, respectively. We speculate the large variance in infection intensity seen in this and other studies is partially attributable to variance in physical and immunologic properties among the hamsters that, in turn, are influenced by the outbred nature of golden Syrian hamster colonies maintained by animal providers.

Technically, the procedure does require training; a veterinarian having extensive small animal experience conducts all inoculations for our group. Another point is that saphenous vein inoculation was facilitated by using smaller animals (less than 130 g), relative to larger animals. The saphenous veins in these smaller animals (about the same

size as a mouse lateral tail vein) were easier to locate and access (see Fig. 6). Fatty tissue obscures the vein in larger animals.

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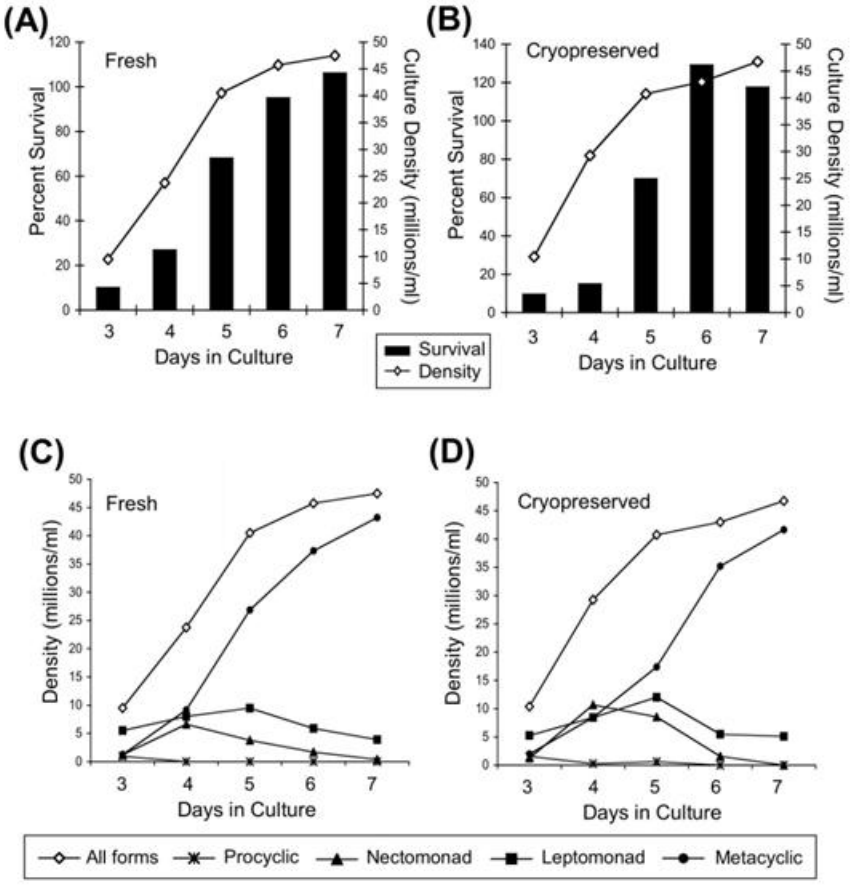

Fig. 1. Complement survival rates and developmental profiles are equivalent between never frozen and frozen and recovered *L. chagasi* promastigotes in vitro.

(A and B) Culture density and percent survival in normal human serum were determined daily *via* enumeration on a hemocytometer. (C and D) A minimum of 100 parasites were examined per day to determine morphology. The density of each parasite form was determined by multiplying the number of each parasite form counted per 100 total parasites by the total culture density. Data are representative of eight independent experiments.

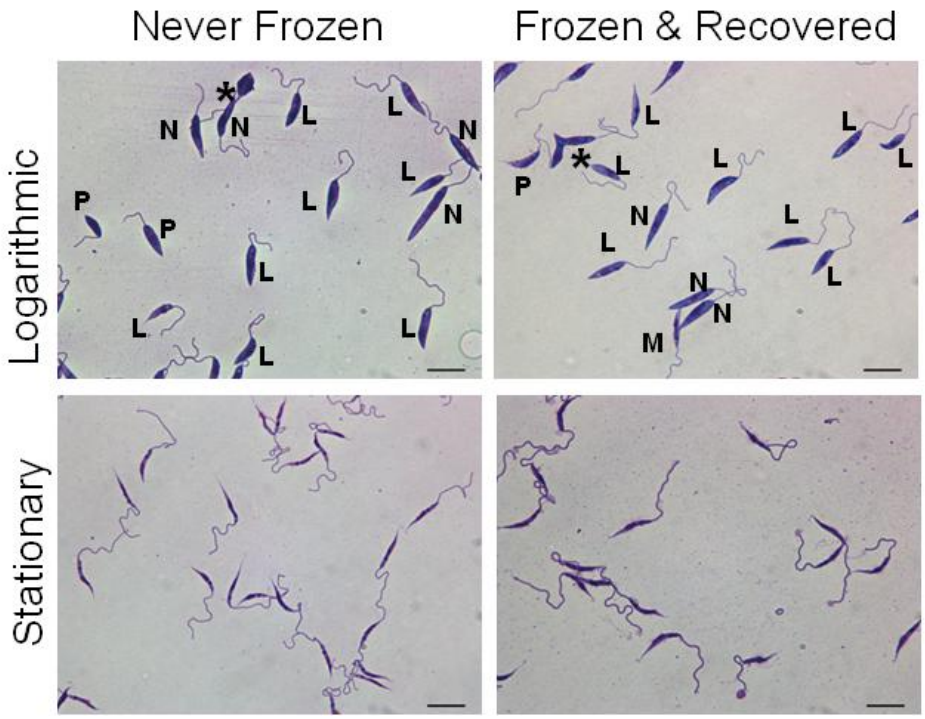

Fig. 2. Promastigotes in logarithmic or stationary culture growth phase.

Logarithmic (top panels) and stationary (bottom panels) cultures were taken from day three and seven cultures respectively. Promastigote developmental stages were determined in the never frozen cells (left panels) and in cells from cultures initiated with cryopreserved cells (right panels). Procyclic (P), Nectomonad (N), Leptomonad (L), and Metacyclic (M) promastigotes were determined by cell size and shape and flagellum length. The promastigotes that were dividing are labeled with *. Logarithmic cultures contained a mix population of all 4 populations. All promastigotes in the bottom panels are metacyclic cells. (Scale bars represent 10 μm).

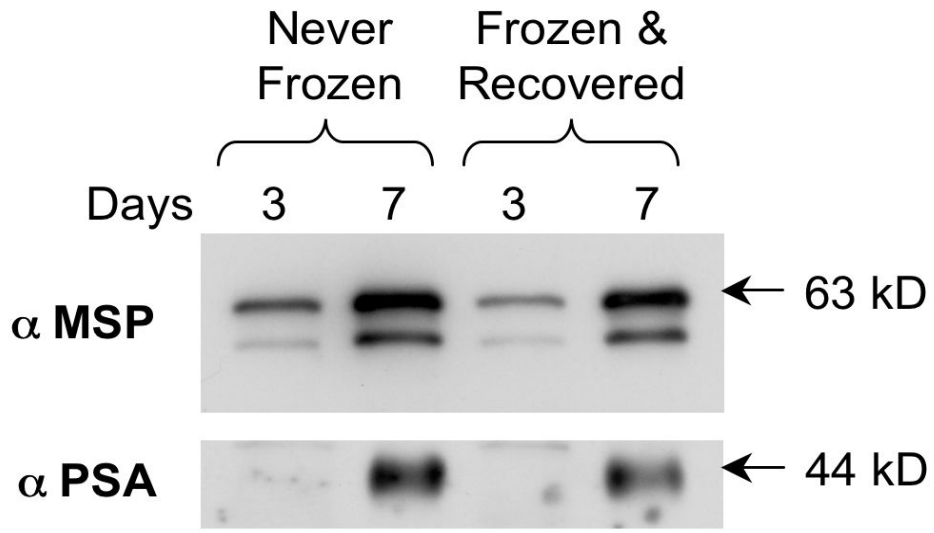

Fig. 3. MSP and PSA protein levels are equivalent between never frozen and frozen and recovered *L. chagasi* promastigotes.

Whole parasite lysates were generated from parasite cultures on days 3 and 7 and analyzed *via* Western blotting. Equivalent parasite numbers were loaded in each lane. Separate membranes were probed with antiserum against either MSP or PSA. Blots are representative of eight independent experiments.

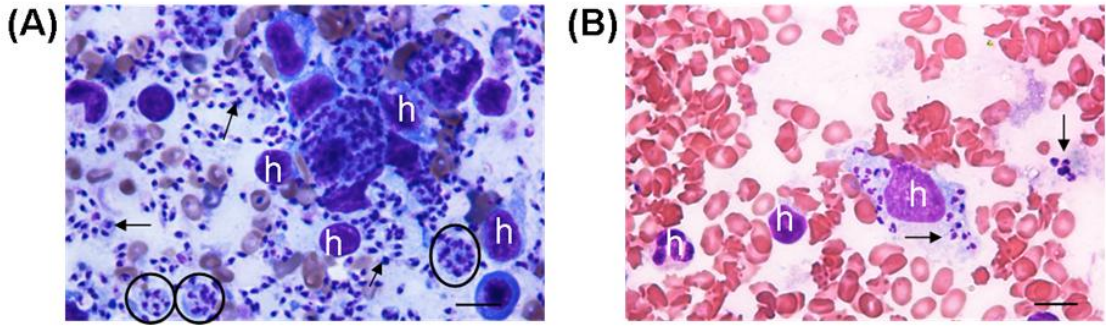

Figure 4. Amastigotes are visible in impression smears of spleens from infected hamsters.

Spleenic tissues from 2 different hamsters (**A, B**) were touched to slides, and then Giemsa stained to allow visualization of amastigotes by microscopy. Examples of host cell nuclei and amastigotes are indicated by (h) and arrows (→), respectively. Examples of membrane-bound amastigotes that are not associated with any host cell nuclei are indicated by circles in **A**. Scale bars represent10 micron.

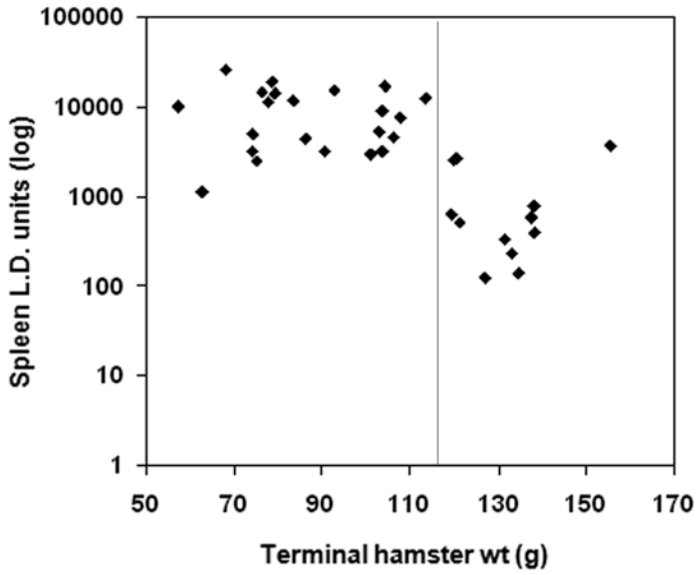

Figure 5. Smaller hamsters correspond to higher parasite loads.

The degree of infection was quantified as L.D. units in hamsters sacrificed within 7 days of exhibiting enlarged abdomen and ruffled fur.

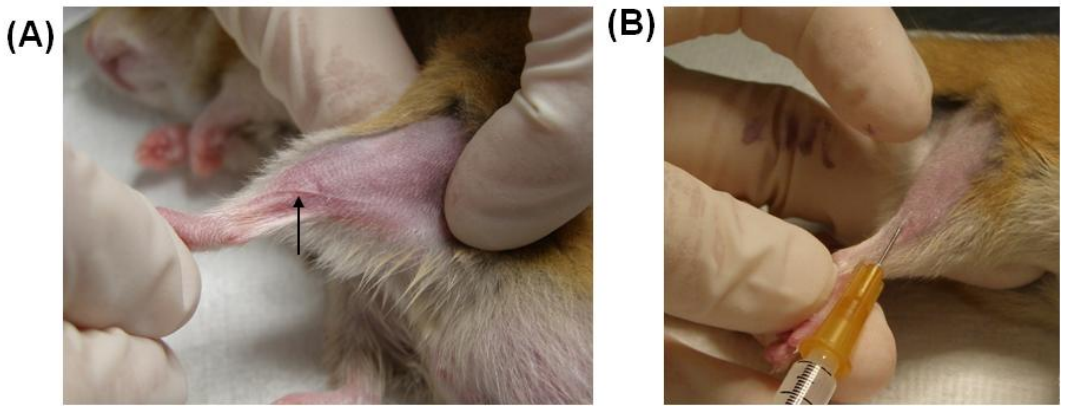

Figure 6. Saphenous vein inoculation.

The hind leg of the anesthetized golden hamster was shaved, and then mild pressure was applied by compression to the upper lateral thigh to expose the saphenous vein (black arrow in **A**) prior to insertion of a 26-gauge needle (**B**).

Table 1. Mean values for hamsters

	Terminal wt < 115 g		Terminal wt ≥ 115 g		All Hamsters	
Number of hamsters	22		12		34	
Days survived	101	(13)	112	(37)	105	(24)
Inoculation wt (g)	116	(13)	119	(12)	117	(13)
Spleen wt (g)	0.88	(0.38)	0.94	(0.36)	0.90	(0.37)
Terminal wt (g)	87	(16)	131	(11)	103	(26)
Mean L.D. Units	9376*	(6531)	1058*	(1206)	6440	(6626)
Min. L.D. Unit	1142		124		124	
Max. L.D. Unit	26177		3688		26177	

Values in ( ) are standard deviations.

* Indicates significantly different at $P < 0.0001$ using a 2 sample independent t-test.

CHAPTER 4. GENERAL CONCLUSIONS AND FUTURE DIRECTION

General conclusions and future direction

The work presented in Chapter 2 examines the temporal abundance of morphologically distinct parasite stages in axenic cultures of *L. chagasi* during serial passage. An important aspect to this work was the finding that the most infectious form, the metacyclic promastigote, which predominated in low passage cultures at stationary growth phase, was greatly diminished within 10 *in vitro* passages. On the other hand, nectomonad promastigotes predominated only within stationary growth phase cultures at higher passage. The nectomonads in high passage are morphologically indistinguishable from nectomonads in low passage; nectomonads in high passage may serve as a potential source of parasites that is highly enriched in this morphological form. Ongoing work is using nectomonads from high passage cultures as well as from low passage cultures for proteomics-based identification of surface proteins. The goal of that work is to identify nectomonad-specific markers for use in cell-labeling and -sorting experiments that would seek a better understanding of the role(s) plays by this cell stage in the overall lifecycle of the parasite.

The second study establishes the equivalency with respect to morphological stages, resistance to complement-mediated lysis, and surface protein expressions (of MSP and GP46), of parasites within low passage culture initiated with parasites that were either freshly isolated from infected hamster or were isolated from hamsters and then cryopreserved. Use of cryostored cells to initiate cultures can enable experiments that require the analysis at different times using same-passage parasites derived from a single

infected hamster, thus reducing variability within and among experiments. Use of cryostored cells also decreases the number of infected hamsters needed to maintain a source of virulent parasites for use in establishing low passage cell cultures of promastigotes. Also presented within the second study are data that support the overall conclusion that inoculation of promastigote *via* the hamster's saphenous vein is efficient, less invasive and more humane than is the cardiac puncture method that is commonly used in the visceral leishmaniasis animal model.

One aspect of potential future study that derives from the nectomonad study is to generate data on promastigote cell lineage, i.e. data that establishes what the progenitor promastigote-stage is for each promastigote morphological form. Other researchers have speculated, based on observations regarding timing and location of promastigotes in the sand fly gut, that procyclics transform into nectomonads, and nectomonads give rise to leptomonads, and leptomonads give rise to metacyclics (1, 2). However, less equivocal evidence demonstrating these linkages between promastigote forms is lacking. It would also be worthwhile to characterize the biochemical properties of individual morphological forms as has previously been done for the metacyclics. The first step toward such characterization would be to obtain a large number of promastigotes that are either pure in form or are highly enriched in a particular promastigote form, as can now be obtained for nectomonad promastigotes as described herein. The next steps would include what also was described earlier, that being to identify specific protein markers for each morphological form.

Another future study is to determine the mechanism or regulation for the process of metacyclogenesis that occurs within the sand fly gut. Perturbing this process in the sand fly could serve as an enabling technique with which to interrupt the parasite life cycle. A possible lead might result from experiments that would determine the mechanism by which parasites within high passage cultures switch to predominant production of nectomonads instead of metacyclics. One possible explanation could be that some subset of procyclic promastigotes must directly derive from amastigotes in order for metacyclogenesis to occur within a cell population.

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APPENDIX

Weight records of infected hamsters

Weekly weight recording of leishmania-infected hamsters suggests that onset of weight loss is an objective early indicator of infection, and that rate of weight loss may have utility as an indicator of advanced disease. After inoculation, hamsters gain weight during the first two months (60 days ±18), reaching average maximum weight of 129 ±7 g (Table 1). After reaching maximum weight hamsters first lose weight gradually for 2-3 weeks, and then lose weight at a much greater rate; the maximum rate loss observed was one gram per day during the 1-2 weeks before euthanasia was performed. Figure 1 shows the weight of two hamsters during their time of infection.

Historically and currently, infected hamsters are killed within one week of exhibiting ruffled fur and enlarged abdomens, indicators that may be measurable but that also are prone to subjective assessment. Monitoring rate of weight loss (as shown in Figure 1 for two hamsters) may enable assessment of the infection earlier post-inoculation than is allowed by present the presently used practice of monitoring for changes in fur and abdominal size. As such, weight monitoring may decrease the duration of infection by allowing the hamster to be euthanized earlier than would be indicated using the historical criteria of fur ruffling and abdomen enlargement.

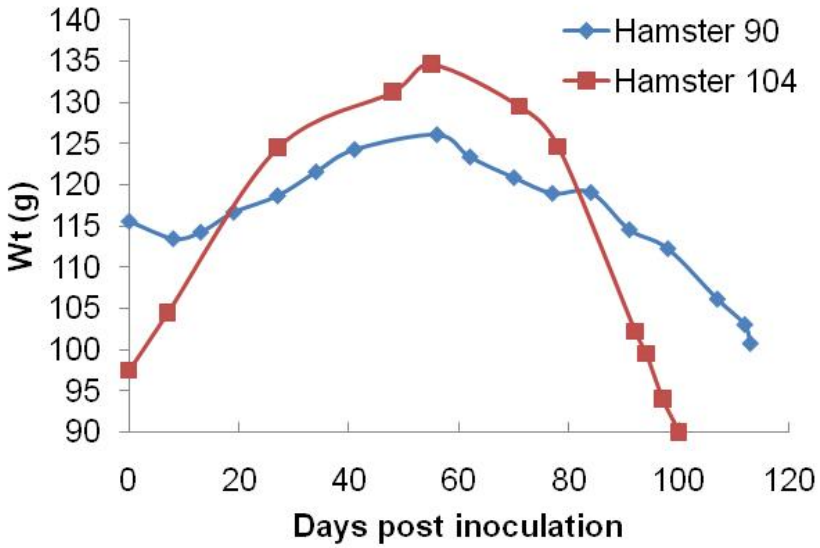

Figure 1. Post inoculation weight records of two representative hamsters.

Number of hamsters	19
Inoculation wt (g)	107±10
Days survived	100±13
Max wt (g)	129±7
Days to reach max wt	60±18
Terminal wt (g)	97±14
Wt loss (g)	32±13
L.D. unit	5473±4179

Table 1. Average value of the hamsters recorded.

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