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Soi Meng Lei
Iowa State University, samlei@iastate.edu

Amanda E. Ramer-Tait
University of Nebraska-Lincoln, aramer-tait2@unl.edu

Rebecca R. Dahlin-Laborde
The Mayo Clinic, Rochester, MN, DahlinLaborde.Rebecca@mayo.edu

Kathleen Mullin
Iowa State University, kmullin@iastate.edu

Jeffrey K. Beetham
Iowa State University, jbeetham@iastate.edu

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REDUCED HAMSTER USAGE AND STRESS IN PROPAGATING *LEISHMANIA CHAGASI* PROMASTIGOTES USING CRYOPRESERVATION AND SAPHENOUS VEIN INOCULATION

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

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, because *L. chagasi* parasites newly isolated from an infected hamster can be grown in culture for only several weeks before loss of function/phenotype occurs. In an effort to decrease animal usage and animal stress, 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), 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 *Leishmania donovani* infection 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 major surface protease and promastigote surface antigen.

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 weeks, 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 several criteria in addition to morphology, i.e., 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 exhibit modified glycosylation states of lipophosphoglycan, 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 the animal model 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, hemorrhage in hamster for cardiac puncture, and very slow onset of symptoms and frequent need for repeated inoculation after 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 passage 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 previously (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 previously (Pearson and Steigbigel, 1980; Zarley et al., 1991;

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*Departments of Veterinary Pathology and Entomology, Iowa State University, Ames, Iowa 50011. *e-mail: jbeetham@iastate.edu*
†Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa 50011.
‡Department of Experimental Medicine and Pathology, The Mayo Clinic, Rochester, Minnesota 55902.
§Lab Animal Resources, Iowa State University, Ames, Iowa 50011.
‖To whom correspondence should be addressed.
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Ramamoorthy et al., 1992; Dahlin-Laborde et al., 2005). In brief, axenic promastigote culture densities increased throughout logarithmic culture phase until reaching a maximum (stationary) phase concentration of 2–5 $\times 10^7$ cells/ml at approximately day 5 of culture; cultures were passaged by dilution to 1.0×10^6 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); if not fully anesthetized within 5 min, they 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 (because anesthetized hamsters do not blink), and the 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 1-ml tuberculin syringe fitted with a 26-gauge, 2.5-cm-length needle, and containing 0.2 ml of inoculum (2–10×10^7 stationary phase promastigotes in sterile phosphate-buffered saline [PBS], pH 7.4), was inserted bevel-up into the vein; digital pressure on the upper thigh was removed, and then the inoculum was delivered over a 15- to 30-sec range. Inoculum was derived from low passage cultures initiated either with parasites freshly isolated from infected hamsters or with cryostored parasites. After 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 were 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 (Thermo Fisher Scientific, Waltham, Massachusetts) or Giemsa, and visualized (×1,000, oil). For animals killed from 2004 through the study's end, the degree of infection was quantified as *Leishmania donovani* infection units (L.D. units), which were calculated as (amastigotes per nucleated host cell) × spleen weight (in milligrams) (Stauber, 1958; Wilson et al., 1989).

Cryopreservation

Promastigotes from cultures at late logarithmic growth phase ($1.0–1.5 \times 10^7$ cells/ml) were washed twice in sterile PBS and resuspended at 1×10^7 cells/ml in HOMEM containing 7.5% dimethyl sulfoxide (Thermo 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, and 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) and then diluted in a 25-cm^2 cell culture flask (Corning Life Sciences, 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 3.5×10^6 promastigotes in PBS (50 µl) to equal volume of 24% human serum in PBS and then incubated at 37 C for 30 min as described previously (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 (Thermo Fisher Scientific), visualized via light microscopy, and measured using NIS-Elements D software (Nikon Instruments, Melville, New York). Cells were categorized into 1 of 4 different promastigote stages (procyclic, leptomonad, nectomonad, or metacyclic) based upon morphology as described previously (Rogers et al., 2002; Gossage et al., 2003; Yao et al., 2008). In brief, procyclic, leptomonad, and metacyclic promastigotes all have similar body lengths (6.5–11.5 µm) 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, whereas 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 *g* for 10 min at 4 C, resuspended in 0.1 M potassium phosphate buffer, pH 7.8, containing 1% Triton X-100, and 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-semi-dry-transferred to polyvinylidene fluoride membranes using standard procedures. Gel loadings were equivalent to 0.5×10^6 and 3.0×10^6 parasites/lane for assessment of MSP and PSA abundance, respectively. Nonfat 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 Chemical, Rockford, Illinois) diluted 1:20,000. Antibody binding was visualized via enzyme-linked chemiluminescence (SuperSignal®, Pierce Chemical).

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 before reaching a non-divisional state (stationary phase). Associated with this progression to stationary culture phase, promastigotes undergo several 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 Fig. 1A, B). Similarly, as cultures progressed from logarithmic to stationary phase, cells became increasingly and equivalently resistant to complement/serum lysis, reaching more than 100% survival relative to control cells not incubated in serum. (Stationary culture survival in excess

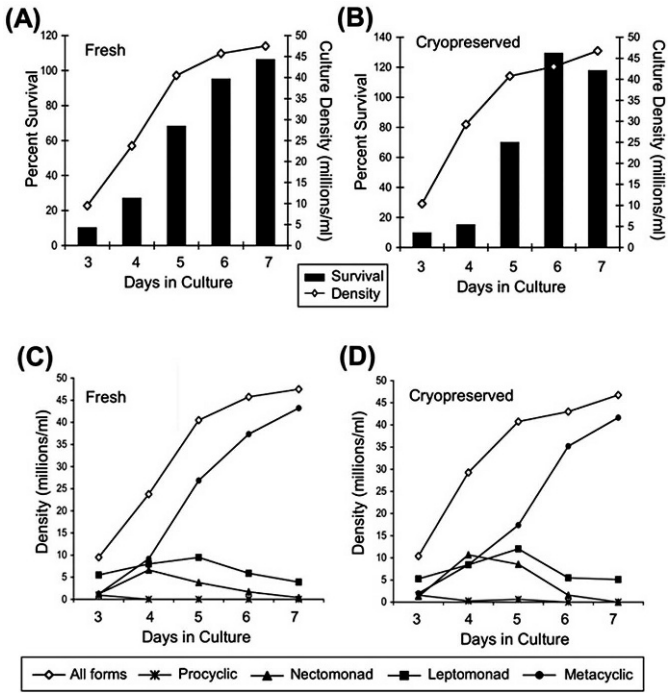

FIGURE 1. Complement survival rates and developmental profiles are equivalent between fresh and cryopreserved *Leishmania chagasi* promastigotes in vitro. (**A**, **B**) Culture density and percent survival in normal human serum were determined daily via enumeration on a hemocytometer. (**C**, **D**) A minimum of 100 parasites was 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 8 independent experiments.

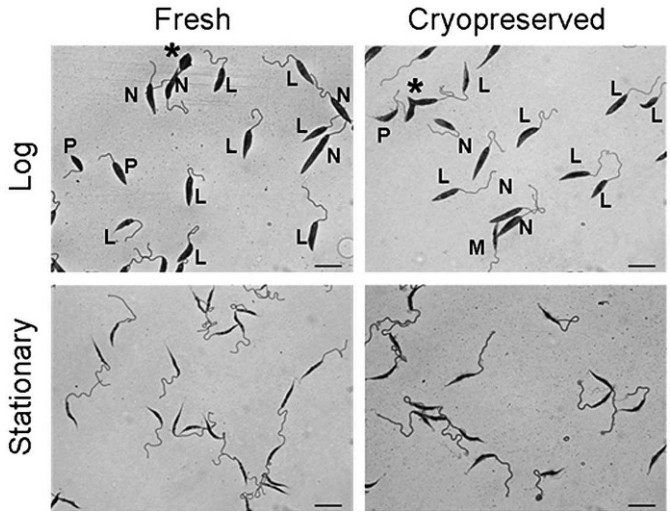

FIGURE 2. Equivalent distributions of different morphological forms between fresh and cryopreserved cultures in logarithmic or stationary culture growth phase. Logarithmic (top) and stationary (bottom) cultures were taken from day 3 and 7 cultures, respectively. Promastigote developmental stages were determined in the fresh cells (left) and in cells from cultures initiated with cryopreserved cells (right). 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 an asterisk (*). Logarithmic cultures contained a mixed population of all 4 populations. All promastigotes in the bottom panels are metacyclic cells (×1,000 magnification; bars = 10 μm).

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 (Fig. 1C, D). The density of metacyclic stage forms increases dramatically at days 4 to 5 before reaching a plateau at approximately 40×10^6 cells/ml in culture. Based on the total culture density being approximately 45×10^6 cells/ml in stationary phase, metacyclic promastigotes in both cultures made up approximately 90% of the whole population. Analysis of the other parasite forms (procyclic, leptomonad, and nectomonad) is subject to greater between-experiment variability because they are intermediate parasite forms that increase, then decrease, temporally. Still, there seems 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 characterized previously, 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

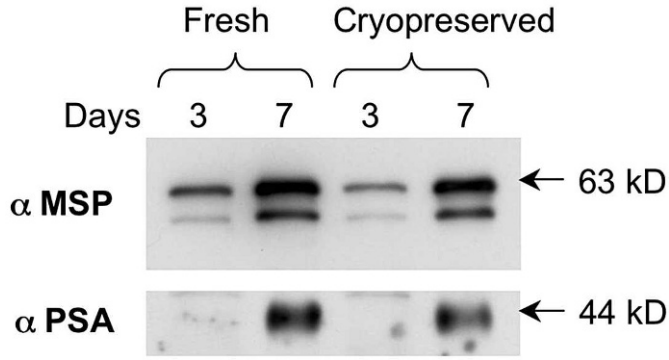

FIGURE 3. MSP and PSA protein levels are equivalent between fresh and cryopreserved *L. chagasi* promastigotes. Whole parasite lysates were generated from parasite cultures on days 3 and 7 and analyzed via Western blotting. Equivalent parasite numbers were loaded in each lane. Separate membranes were probed with antiserum against either MSP or PSA. Blots are representative of 8 independent experiments.

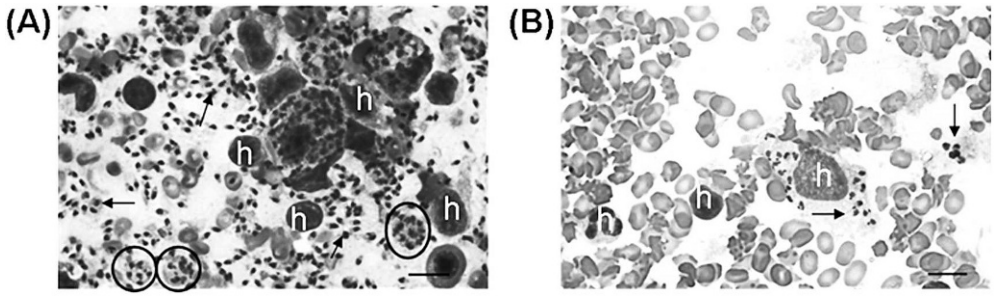

FIGURE 4. Amastigotes are visible in impression smears of spleens from infected hamsters. Splenic tissues from 2 different hamsters (**A**, **B**) were touched to slides, and then Giemsa stained to allow visualization of amastigotes by microscopy (×1,000 magnification). Examples of host cell nuclei and 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. Bar = 10 μm.

increased animal use or discomfort. Consequently, the utility of an alternative method involving intravenous 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 buildup within approximately 15 wk (105 ± 22.2 days) post-inoculation. 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 point of bleeding.

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 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 seemed to be enclosed by a membrane not associated with any host nuclei (indicated by a circle in Fig. 4A); these amastigotes possibly derive from phagocytes whose structural integrity is disrupted during sample workup.

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 L.D. (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 subgroups composed of animals with terminal weights <115 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 5-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, whereas inocula for the remaining 16 were from cultures initiated with cryostored parasites. No differences were observed between these 2 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.

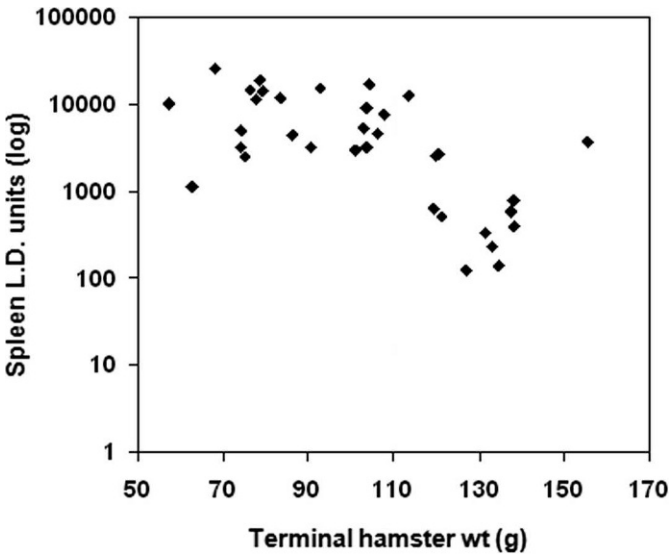

FIGURE 5. Smaller hamsters correspond to higher parasite loads. The degree of infection was quantified as L.D. units in hamsters sacrificed within 7 days of exhibiting enlarged abdomen and ruffled fur.

TABLE I. Mean values for hamsters.

	Terminal wt <115 g		Terminal wt ≥115 g		All hamsters	
No. 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	87	0.94	(0.36)	0.90	(0.37)
Terminal wt (g)	(0.38)	(16)	131	(11)	103	(26)
Mean L.D. units	9,376†	(6,531)	1,058†	(1,206)	6,440	(6,626)
Min. L.D. units	1,142		124		124	
Max. L.D. units	2,6177		3,688		26,177	

* Values in parentheses are standard deviations.
† Indicates significantly different at $P < 0.0001$ using a 2-sample independent t-test.

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). Results 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.

Our experiments used cells cryopreserved up to 4 wk. We also have used cells cryostored for 4 yr to successfully initiate promastigotes cultures. Although 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 ($\times 10^{10}$/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 immunological 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 (<130 g), relative to larger animals. The saphenous veins in these smaller animals (approximately the same size as a mouse lateral tail vein) were easier to locate and access (Fig. 6). Fatty tissue obscures the vein in larger animals.

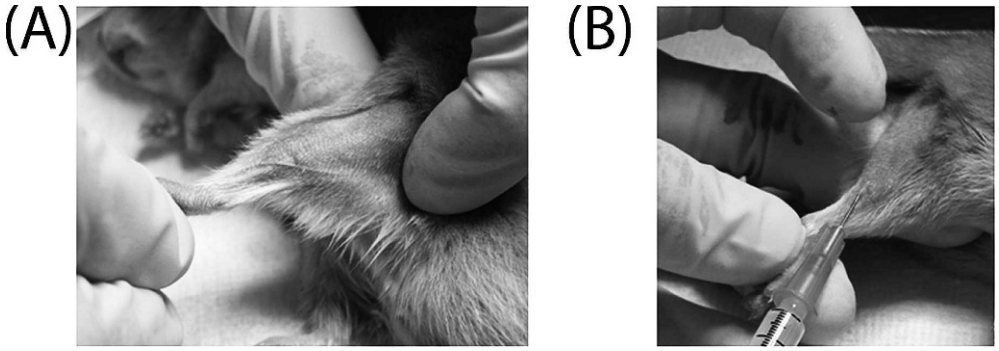

FIGURE 6. Saphenous vein inoculation. The hind leg of the anesthetized golden hamster was shaved, and then mild pressure was applied by compression to the upper lateral thigh to expose the saphenous vein (black arrow in **A**) before insertion of a 26-gauge needle (**B**).

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