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A Lipophosphoglycan-Independent Method for Isolation of Infective *Leishmania* Metacyclic Promastigotes by Density Gradient Centrifugation

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Späth, G. F. and Beverley, S. M. 2001. A lipophosphoglycan-independent method for isolation of infective *Leishmania* metacyclic promastigotes by density gradient centrifugation. *Experimental Parasitology* **99,** 97–103. At the end of their growth in the sand fly, *Leishmania* parasites differentiate into the infective metacyclic promastigote stage, which is transmitted to the mammalian host. Thus, in experimental studies of parasite infectivity toward animals or macrophages, the use of purified metacyclics is generally preferred. While metacyclics of several *Leishmania* species can be efficiently purified with the aid of lectins or monoclonal antibodies, which differentially exploit stage-specific differences in the structure of the abundant surface glycolipid lipophosphoglycan (LPG), such reagents are unavailable for most species and they are unsuitable for studies involving LPG-deficient mutants. Here we describe a simple density gradient centrifugation method, which allows the rapid purification of infective metacyclic parasites from both wild-type and LPG-deficient *Leishmania major*. The purified metacyclic promastigotes are authentic, as judged by criteria such as their morphology, expression of the metacyclic-specific gene *SHERP*, and ability to invade and replicate within macrophages *in vitro*. Preliminary studies suggest that this method is applicable to other *Leishmania* species including *L. donovani*.

Index Descriptors and Abbreviations: trypanosomatid parastic protozoa; virulence; phlebotomine sand fly; disease transmission; LPG, lipophosphoglycan; PNA, peanut agglutinin; Gal, galactose; Ara, arabinose; Man, mannose.

INTRODUCTION

Protozoans of the genus *Leishmania* undergo several developmental transitions during their infectious cycle. Following ingestion of infected macrophages by the sand fly, parasites are released and differentiate from the intracellular amastigote stage to the extracellular procyclic promastigote stage, which resides within the sand fly alimentary tract. After a period of replication terminating approximately upon the complete digestion of the blood meal, a proportion of the parasites undergo a second developmental transition, yielding the metacyclic promastigote parasite which is now infective to the mammalian macrophage (Sacks and Perkins 1984). This differentiation step can be conveniently replicated during *in vitro* culture: log-phase parasites resemble procyclic parasites and, following entry into stationary phase, a fraction of the parasites differentiate into the metacyclic form, with properties resembling those of authentic sand fly metacyclic promastigotes (Sacks and Perkins 1984, 1985). Following the discovery of the metacyclic stage and its role as the infective form transmitted by the sand fly, researchers have generally found it preferable to use purified metacyclic parasites in studies of parasite infectivity to mammals and macrophages.

Metacyclogenesis is accompanied by changes in parasite morphology, gene expression, and composition of the parasite surface glycocalyx, which is composed of a variety of GPI-anchored molecules including the abundant glycolipid lipophosphoglycan (LPG) (McConville *et al.* 1993; Turco

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and Descoteaux 1992). LPG undergoes changes in size and carbohydrate structure during development: procyclic promastigotes express a smaller LPG capable of binding to the sand fly midgut, while metacyclic promastigotes express a larger LPG typically accompanied by modifications which prevent midgut binding (reviewed in Sacks, 2001). In *Leishmania major*, procyclic promastigote LPG is modified by galactosyl side chains, which in the metacyclic-stage LPG are capped with arabinosyl residues (Fig. 1) (McConville *et al.* 1992; Sacks *et al.* 1990), while in other species analogous stage-specific changes in LPG structure have been described

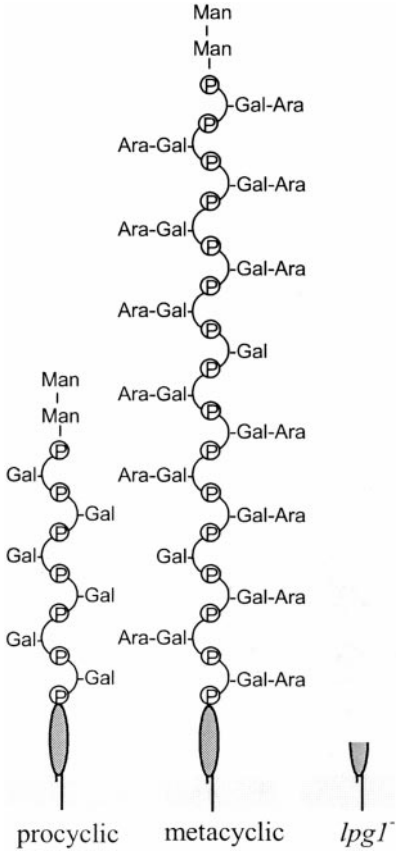

FIG. 1. Schematic representation of LPG structures in procyclic and metacyclic *L. major*, and the *lpg1*$^-$ mutant. LPG is inserted into the membrane by a 1-*O*-alkyl-2-lyso-phosphatidylinositol lipid anchor that is linked to a heptasaccharide glycan core (symbolized by the shaded oval) followed by a phosphodisaccharide repeating unit of Gal-Man-P (symbolized by the half circles) and a terminal mannose cap. In the Friedlin line of *L. major*, LPG shows several modifications during metacyclogenesis including an approximate doubling of the average number of repeat units per molecule and the capping of galactose (Gal) side chains with arabinose (Ara). *lpg1*$^-$ cells express a truncated form of LPG, due to defective addition of the galactofuranosyl residue within the glycan core (Ryan *et al.* 1993).

(Sacks 2001). The developmental differences in LPG structure are the basis of current methods for metacyclic purification. For *L. major*, lectins such as peanut agglutinin (PNA) bind procyclic but not metacyclic LPG and can be used in negative selection protocols to purify metacyclic parasites (Sacks *et al.* 1985). Similar protocols have been developed using *Leishmania tropica* procyclic LPG-specific monoclonal antibodies (Lira *et al.* 1998) or proposed for *Leishmania donovani* (Almeida *et al.* 1993). However, for most *Leishmania* species knowledge of stage-dependent changes in LPG is limited, and the available lectin or anti-LPG monoclonal antibody reagents work poorly, if at all. Moreover, these methods are unsuitable in studies focused on the biological properties of LPG-deficient parasites (Beverley and Turco 1998; Turco *et al.* 2001). Thus, a method for purifying metacyclic promastigotes that does not depend exclusively upon LPG-based reagents or structural knowledge would be valuable.

The infective metacyclic stage of all *Leishmania* species is morphologically distinct from noninfective promastigotes, with a short, narrow body and a long flagellum more than twice the body size (Sacks and Perkins 1984; Zakai *et al.* 1998). This suggested that metacyclics might exhibit different sedimentation properties under appropriate conditions. Here we describe an LPG-independent method for the purification of infective metacyclic parasites by Ficoll density gradient centrifugation and validate its efficacy for wild type and an LPG-deficient line of *L. major* (Späth *et al.* 2000).

MATERIALS AND METHODS

Leishmania culture. *L. major* strains Friedlin V1 (MHOM/JL/80/Friedlin) and LV39clone5 (Rho/SU/59/P; Marchand *et al.* 1987) cells were grown in M199 medium at 26°C as previously described (Kapler *et al.* 1990). The *lpg1*$^-$ mutant was obtained by targeted gene disruption and was maintained in M199 medium supplemented with 16 μg/ml hygromycin B and 20 μM puromycin as described (Späth *et al.* 2000). Dulbecco's modified Eagle's medium (DMEM, with 1 mg glucose/ml) was prepared without serum for use in these studies. In this work, stationary-phase parasites were obtained from cultures that had reached a constant density for 4 days.

Agglutination assay. Agglutination tests were performed with parasites at a density of $1–2 \times 10^8$/ml and 50 μg/ml peanut agglutinin (da Silva and Sacks 1987). After a 30-min incubation at room temperature in DMEM, cells were separated by centrifugation for 10 min at 200*g* to yield the agglutinated PNA$^+$ cells, and 1990*g* to yield the free PNA$^-$ metacyclic parasites. Parasite fractions were washed once in 10 ml DMEM supplemented with 20 mM galactose. For agglutination tests on cells isolated by density gradient centrifugation, recovered parasite fractions were washed once in DMEM, and PNA$^-$ and PNA$^+$ cells were isolated as described above.

Microscopy. Parasites from various growth phases or purified by different methods were washed once in cold phosphate-buffered saline (PBS), fixed with PBS containing 3.5% paraformaldehyde at room temperature for 5 min, immobilized on poly-L-lysine-coated glass cover slips, and viewed under phase contrast in a Zeiss Axiophot system.

Macrophage isolation and infection. Starch-elicited peritoneal exudate cells were obtained from female BALB/c mice (The Jackson Laboratory) (Titus *et al.* 1984) and seeded onto 18-mm glass cover slips (approximately 1.5×10^5 cells/well). Prior to infection, parasites were opsonized for 30 min in DMEM with 4% C5-deficient mouse serum (Racoosin and Beverley 1997). Infection was performed for 2 h at 33°C under serum-free conditions in DMEM containing 0.7% bovine serum albumin, at a ratio of 10 parasites per macrophage. Free parasites were removed by multiple washing steps with DMEM, and the washing was repeated daily to remove residual extracellular parasites. The number of intracellular parasites/100 macrophages was monitored at 2, 24, 48, and 120 h postinfection by fluorescence microscopy as described (Späth *et al.* 2000).

Northern blot analysis. Total cellular RNA was prepared by the guanidinium thiocyanate method (Chomczynski and Sacchi 1987), subjected to electrophoresis in denaturing agarose gel, blotted onto a nylon membrane (Hybond-N; Amersham), and hybridized with a ^{32}P-labeled probe made from DNA corresponding to the protein-coding region of the metacyclic promastigote-specific gene *SHERP* (Knuepfer *et al.* 2001). The extent of hybridization was quantified by a Bio-Rad PhosphorImager and normalized to the expression level in cells of logarithmic growth phase and to total RNA levels, as determined by ethidium bromide staining of ribosomal RNAs.

Ficoll density gradients and metacyclic purification. A 40% stock solution of Ficoll Type 400 (Sigma, St. Louis, MO) in sterile, endotoxin-free water was prepared. Dilutions were established using five-fold concentrated M199 medium (without serum) to obtain the final desired Ficoll concentration in 1× M199 medium, and solutions were filtered through a 0.22-μm cellulose acetate filter. Stock solutions were kept at 4°C in darkness for no longer than 1 month, and working dilutions were prepared on the day that they were used.

For preliminary tests, we used 15-ml Falcon conical centrifuge tubes containing 2 ml of 40% Ficoll at the bottom, overlaid by 2 ml of a solution containing from 2 to 30% Ficoll (a single concentration was used in a given tube) in M199 medium lacking serum, which was finally overlaid by 2 ml of stationary-phase parasites (2×10^8/ml) suspended in DMEM (Fig. 2A). These step gradients were centrifuged for 10 min at 1300*g* at room temperature, and the parasites were recovered from the upper interface with a sterile Pasteur pipette.

For large-scale preparations of "top 10 metacyclics," 10^9 stationary-phase parasites were collected by centrifugation for 10 min at 1900*g* and resuspended in 6 ml DMEM. Two milliliters of this suspension was layered on a step gradient in a 15-ml conical Falcon centrifuge tube containing 2 ml 40% Ficoll stock solution, 2 ml 10% Ficoll (in M199 medium without serum), and, in some cases, 2 ml of 5% Ficoll (in M199 medium without serum). The gradients were centrifuged at 1300*g* for 10 min at room temperature; parasites located at the upper 10% Ficoll boundary were recovered with a sterile Pasteur pipette, diluted with one volume DMEM, and collected by centrifugation at 1900*g* for 10 min at room temperature.

RESULTS

Enrichment of metacyclic Leishmania *promastigotes by density gradient centrifugation.* We prepared a series of Ficoll density-step gradients to test whether conditions that selectively enriched for metacyclic parasites could be found. Initially, these gradients contained on the bottom a 40% Ficoll cushion, in the middle a Ficoll "step" solution containing varying amounts of Ficoll (2–30%) in medium, and on top parasites suspended in serum-free medium (Fig. 2A). Following centrifugation, parasites were recovered from

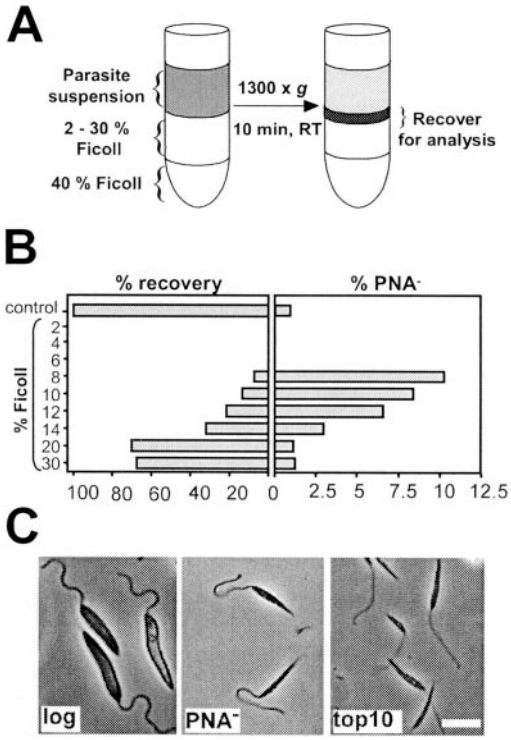

FIG. 2. Method and analysis of Ficoll density gradient-purified *Leishmania* metacyclics. (A) *Schematic representation of the density gradient.* Parasites were separated using a step gradient of 40% Ficoll, overlayed with solutions of 2–30% Ficoll. After centrifugation, cells were isolated from the interface (indicated by the bracket on the right side of the figure). (B) *Analysis of Ficoll density fractions.* Cells isolated from the interface shown in (A) were counted (left panel) and the recovery was normalized to the number of applied cells ("control;" 4×10^8 stationary-phase *L. major*). The right panel shows the fraction of PNA$^-$ parasites. The data are representative for at least three independent experiments. (C) *Microscopy.* Phase-contrast micrographs of procyclic promastigotes (left panel), PNA$^-$ metacyclic promastigotes (center panel), and top 10 metacyclics prepared by density gradient centrifugation (right panel). The bar corresponds to 10 μM.

the upper medium/Ficoll interface and washed by further centrifugation.

At Ficoll step concentrations from 2 to 6%, only cellular debris without intact cells was recovered at the interface. Above 8% Ficoll step concentrations, progressively higher numbers of cells were recovered (Fig. 2B). Notably, at densities from 8 to 12% Ficoll, the recovered parasites appeared morphologically similar to metacyclic parasites prepared by the standard PNA agglutination method (Fig. 2C, middle panel). At Ficoll step concentrations of 14% a heterogeneous cell fraction was obtained, containing procyclic and metacyclic parasites. At 20% Ficoll, most of the parasites (>75%) were retained at the interface, with a small fraction of aggregated cells sedimenting further to the bottom (Fig. 2B).

The percentage of parasites that were PNA$^-$ was determined for the parasites located at the step boundaries (Fig. 2B). Parasites recovered from the 8 or 10% Ficoll steps showed the highest percentage of PNA$^-$ cells, 10.3 and 8.4%, corresponding to nearly a 10-fold enrichment of PNA$^-$ metacyclic parasites over the starting culture (1.1% metacyclics). Remarkably, all of the cells at the 8 or 10% Ficoll interfaces showed the typical metacyclic morphology (Fig. 2C, right panel), given the fact that only ~10% were PNA$^-$. In the following studies we characterized further the metacyclic-like promastigotes retained at the 10% Ficoll interface, which will be referred to as "top 10" metacyclics.

Expression of a metacyclic-specific marker gene in gradient-purified metacyclic L. major *promastigotes.* We performed Northern blot analysis with RNAs prepared from log- and stationary-phase *L. major* promastigotes, and conventional PNA$^-$ or top 10 metacyclic promastigotes, using a hybridization probe for the metacyclic marker gene *SHERP* (Knuepfer *et al.* 2001). As expected, *SHERP* was expressed at low levels in log-phase procyclic promastigotes and at higher levels in stationary-phase (3-fold) or PNA$^-$ metacyclic promastigotes (20-fold; Fig. 3A). Expression of *SHERP* in the top 10 metacyclics was elevated 15-fold, comparable to that seen in the PNA$^-$ metacyclics (Fig. 3A).

Analysis of the top 10 metacyclics prepared from LPG-deficient L. major. We examined a line of LPG-deficient *L. major*, arising from genetic inactivation of the *LPG1* gene (Späth *et al.* 2000). These parasites specifically lack LPG but not related glycoconjugates, due to the loss of the putative galactofuranosyltransferase encoded by *LPG1* (Ryan *et al.* 1993). Morphologically, the *lpg1*$^-$ top 10 metacyclics appeared indistinguishable from conventional wild-type PNA$^-$ or top 10 metacyclics (data not shown; Fig. 2C), and were recovered in comparable numbers. Northern blot analysis of *SHERP* expression in the *lpg1*$^-$ parasites showed that, as with wild-type parasites, *SHERP* expression rose 5-fold in stationary phase, while the top 10 metacyclics showed 25-fold log-phase levels of increased SHERP expression (Fig. 3B). Thus, by morphology and metacyclic-specific gene expression criteria, top 10 metacyclics from the LPG-deficient *lpg1*$^-$ line appear to be bona fide metacyclics.

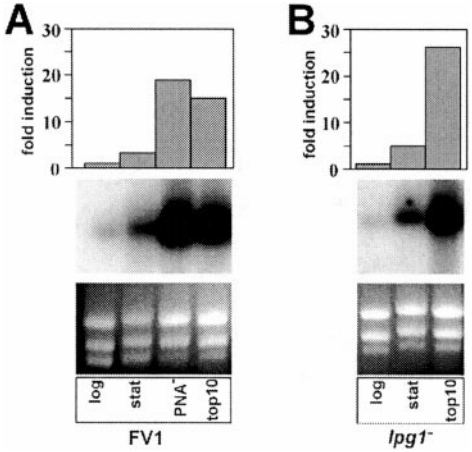

FIG. 3. Expression of a metacyclic-stage-specific marker in wild-type and LPG-deficient top 10 metacyclic *L. major.* Expression of the metacyclic marker *SHERP* was analyzed in wild-type Friedlin V1 *L. major* (A) and the LPG-deficient *lpg1*$^-$ line (B) by Northern blot analysis with a *SHERP* hybridization probe. RNAs were prepared from logarithmic phase (log), 4-day stationary phase (stat), and PNA$^-$ or top 10 metacyclics (PNA$^-$ or top 10, respectively) of each cell line (where appropriate). The top panel shows the results of quantitation by phosphorimaging (normalized to log-phase levels), the middle panel shows the autoradiogram, and the lower panel shows staining of the rRNA region of the ethidium bromide-stained gel (loading control).

Virulence of density gradient-purified metacyclic promastigotes. Metacyclic promastigotes have been shown to be superior to procyclic parasites in their ability to infect and replicate in cultured macrophages (Sacks *et al.* 1985). We examined the ability of procyclic promastigotes from day 4 of stationary growth or PNA$^-$ metacyclic and top 10 metacyclic promastigotes to infect murine peritoneal macrophages (Fig. 4). As expected, procyclic promastigotes entered and showed a modest degree of replication as amastigotes, increasing about two-fold over a 5-day period (Fig. 4A). Both PNA$^-$ and top 10 metacyclics entered macrophages efficiently and replicated extensively as amastigotes, increasing about eight-fold over the 5-day incubation (Figs. 4B and 4C). Thus, top 10 metacyclics appear as infective to macrophages as conventional PNA$^-$ metacyclics.

As noted earlier, only 10% of the top 10 metacyclics are PNA$^-$ (Fig. 2B). We recovered the PNA$^+$ parasites from the top 10 metacyclic preparation and tested them in the

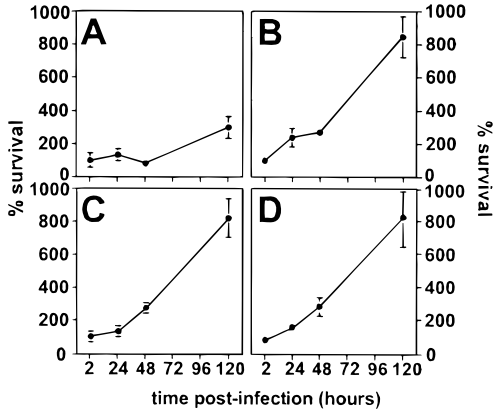

FIG. 4. Infectivity of *L. major* developmental stages and preparations to macrophages *in vitro*. Infections were established in peritoneal macrophages using stationary parasites (A), PNA$^-$ metacyclics (B), total top 10 metacyclics (C), and top 10 PNA$^+$ metacyclics (D). All infections were performed with C3b-opsonized parasites at multiplicity of 10 parasites/macrophage. Survival was calculated as the number of parasites/100 macrophages and normalized to the initial value at 2 h postinfection. Three independent experiments were performed, each with triplicate points; one representative experiment is shown. The mean and standard deviations are shown.

macrophage infection assay (Fig. 4D). These parasites replicated as amastigotes and increased about eight-fold in number over the 5-day incubation, comparable to the PNA$^-$ metacyclic and top 10 metacyclic preparations. We conclude that the top 10 metacyclic population is as highly infective to macrophages as conventional PNA$^-$ metacyclics and that this is independent of their PNA agglutination phenotype.

DISCUSSION

To study the ability of *Leishmania* parasites to establish infections in animals or cultured macrophages, it is considered advisable to use the stage normally transmitted by the sand fly vector, namely the metacyclic promastigote. Previously metacyclics were defined by LPG-based agglutination and morphological criteria, and for *L. major*, methods based upon a negative selection with PNA have been used widely to provide metacyclic parasites for experimental analysis. In this report we describe an alternative method for purifying a fraction of promastigotes from stationary-phase cultures of *L. major* that closely resembles the classic PNA$^-$ metacyclics in key criteria such as morphology, metacyclic-specific gene expression, and their ability to invade macrophages (Figs. 2–4).

While the density gradient-purified top 10 metacyclics appear identical to conventional PNA$^-$ metacyclics by all criteria applied, only about 10% of these cells were PNA$^-$ (Fig. 2). Nonetheless, the top 10 PNA$^+$ metacyclics appeared morphologically identical to conventional PNA$^-$ metacyclics and were fully infective to macrophages (Fig. 4D). We advance two explanations. In the "transitional" model, we postulate that the PNA$^+$ cells in the top 10 promastigote fraction are metacyclics that have not yet completed the process of replacing the PNA-reactive procyclic surface LPG (containing exposed Gal residues) with metacyclic LPG (where the Gal residues are capped by Ara; see Fig. 1). Since LPG is continually synthesized and then shed from the *Leishmania* surface with a half-life of about 6 h (King *et al.* 1987; McConville *et al.* 1993), and agglutination requires only low levels of surface LPG expression (Ryan *et al.* 1993), residual levels of procyclic LPG with its terminal Gal residues could lead to a PNA$^+$ phenotype, despite the cell having otherwise completed the metacyclogenesis program. Alternatively, the "distinct" model suggests that the top 10 PNA$^+$ parasites represent another stage, occurring between the procyclic and the bona fide metacyclic stages. This possibility arises from that fact that a number of other parasites morphotypes (nectomonads, haptomonads) have been described in sand fly hosts (Walters 1993). However, we did not see any clear morphological differences between conventional PNA$^-$ and top 10 metacyclics (Fig. 2), and the top 10 metacyclic parasites were as infective as standard PNA$^-$ metacyclics (Fig. 4). Thus, at present we favor the "transitional" model. If correct, this implies that the percentage of metacyclic parasites (as defined by morphology and infectivity to macrophages) in stationary-phase *Leishmania* populations may be underestimated by the standard PNA negative selection protocol. Thus, the top 10 metacyclic protocol would have the experimental advantage of yielding increased numbers of infective parasites from a given volume of stationary-phase parasites.

A key advantage of the density-gradient centrifugation protocol is that it does not utilize LPG-based reagents to separate procyclic from metacyclic parasites. This was established by generating metacyclic promastigotes from LPG-deficient *Leishmania*, an observation which establishes that metacyclogenesis does not depend upon expression of intact LPG. As described elsewhere, the new metacyclic purification procedure has allowed us for the first time to characterize the (in)ability of LPG-deficient metacyclics to execute key steps required for successful establishment of infections in macrophages and mice (G. F. Späth *et al*, unpublished). Preliminary studies suggest that the Ficoll density gradient method can also be used to purify metacyclic-like fractions

from other species of *Leishmania* including *L. donovani*. Since the size, shape, and density of *Leishmania* parasites vary somewhat, it is necessary to determine the Ficoll concentrations that are most effective for each parasite species.

In summary, we have developed a new, LPG-independent protocol for the purification of highly infective metacyclic promastigotes from *L. major*. This method can be scaled up to yield large quantities, which should facilitate molecular and biochemical studies of metacyclogenesis. This method also shows great promise for use in the study of other species of *Leishmania* and LPG-deficient lines.

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REFERENCES

Almeida, M. C., Cuba, C. A., de Sa, C. M., Pharoah, M. M., Howard, K. M., and Miles, M. A. 1993. Metacyclogenesis of *Leishmania (Viannia) braziliensis in vitro*: Evidence that lentil lectin is a marker of complement resistance and enhanced infectivity. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **87,** 325–329.

Beverley, S. M., and Turco, S. J. 1998. Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite *Leishmania*. *Trends in Microbiology* **6,** 35–40.

Chomczynski, P., and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162,** 156–159.

da Silva, R., and Sacks, D. L. 1987. Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. *Infection and Immunity* **55,** 2802–2806.

Kapler, G. M., Coburn, C. M., and Beverley, S. M. 1990. Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. *Molecular and Cellular Biology* **10,** 1084–1094.

King, D. L., Chang, Y. D., and Turco, S. J. 1987. Cell surface lipophosphoglycan of *Leishmania donovani*. *Molecular and Biochemical Parasitology* **24,** 47–53.

Knuepfer, E., Stierhof, Y. D., McKean, P. G., and Smith, D. F. 2001. Characterization of a differentially expressed protein that shows an unusual localization to intracellular membranes in *Leishmania major*. *Biochemistry Journal* **356,** 335–344.

Lira, R., Mendez, S., Carrera, L., Jaffe, C., Neva, F., and Sacks, D. 1998. *Leishmania tropica*: The identification and purification of metacyclic promastigotes and use in establishing mouse and hamster models of cutaneous and visceral disease. *Experimental Parasitology* **89,** 331–342.

Marchand, M., Daoud, S., Titus, R. G., Louis, J., and Boon, T. 1987. Variants with reduced virulence derived from *Leishmania major* after mutagen treatment. *Parasite Immunology* **9,** 81–92.

McConville, M. J., Turco, S. J., Ferguson, M. A., and Sacks, D. L. 1992. Developmental modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. *EMBO Journal* **11,** 3593–3600.

McConville, M. J., Collidge, T. A., Ferguson, M. A., and Schneider, P. 1993. The glycoinositol phospholipids of *Leishmania mexicana* promastigotes: Evidence for the presence of three distinct pathways of glycolipid biosynthesis. *Journal of Biological Chemistry* **268,** 15595–15604.

Racoosin, E. L., and Beverley, S. M. 1997. *Leishmania major*: Promastigotes induce expression of a subset of chemokine genes in murine macrophages. *Experimental Parasitology* **85,** 283–295.

Ryan, K. A., Garraway, L. A., Descoteaux, A., Turco, S. J., and Beverley, S. M. 1993. Isolation of virulence genes directing surface glycosyl-phosphatidylinositol synthesis by functional complementation of *Leishmania*. *Proceedings of the National Academy of Science USA* **90,** 8609–8613.

Sacks, D. L. 2001. *Leishmania*–sand fly interactions controlling species-specific vector competence. *Cell Microbiology* **3,** 189–196.

Sacks, D. L., and Perkins, P. V. 1984. Identification of an infective stage of *Leishmania* promastigotes. *Science* **223,** 1417–1419.

Sacks, D. L., and Perkins, P. V. 1985. Development of infective stage *Leishmania* promastigotes within phlebotomine sand flies. *American Journal of Tropical Medicine and Hygiene* **34,** 456–459.

Sacks, D. L., Hieny, S., and Sher, A. 1985. Identification of cell surface carbohydrate and antigenic changes between noninfective and infective developmental stages of *Leishmania major* promastigotes. *Journal of Immunology* **135,** 564–569.

Sacks, D. L., Brodin, T. N., and Turco, S. J. 1990. Developmental modification of the lipophosphoglycan from *Leishmania major* promastigotes during metacyclogenesis. *Molecular and Biochemical Parasitology* **42,** 225–233.

Späth, G. F., Epstein, L., Leader, B., Singer, S. M., Avila, H. A., Turco, S. J., and Beverley, S. M. 2000. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. *Proceedings of the National Academy of Science USA* **97,** 9258–9263.

Titus, R. G., Kelso, A., and Louis, J. A. 1984. Intracellular destruction of *Leishmania tropica* by macrophages activated with macrophage

activating factor/interferon. *Clinical and Experimental Immunology* **55,** 157–165.

Turco, S. J., and Descoteaux, A. 1992. The lipophosphoglycan of *Leishmania* parasites. *Annual Review of Microbiology* **46,** 65–94.

Turco, S. J., Späth, G. F., and Beverley, S. M. 2001. Is lipophosphoglycan a virulence factor? A surprising diversity between *Leishmania* species. Trends in Parasitology **17,** 223–226.

Walters, L. L. 1993. *Leishmania* differentiation in natural and unnatural sand fly hosts. *Journal of Eukaryote Microbiology* **40,** 196–206.

Zakai, H. A., Chance, M. L., and Bates, P. A. 1998. *In vitro* stimulation of metacyclogenesis in *Leishmania braziliensis, L. donovani, L. major* and *L. mexicana*. *Parasitology* **116,** 305–309.

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