Preparation of highly infective Leishmania promastigotes by cultivation on SNB-9 biphasic medium

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A B S T R A C T

Protozoan hemoflagellates Leishmania are causative agents of leishmaniases and an important biological model for study of host–pathogen interaction. A wide range of methods of Leishmania cultivation on both biphasic and liquid media is available. Biphasic media are considered to be superior for initial isolation of the parasites and obtaining high promastigote infectivity; however, liquid media are more suitable for large-scale experiments. The aim of the present study was the adaptation and optimization of the cultivation of Leishmania promastigotes on a biphasic SNB-9 (saline–neopeptone–blood 9) medium that was originally developed for Trypanosoma cultivation and combines the advantages of biphasic and liquid media. SNB-9 medium is characterized with a large volume of the liquid phase, which facilitates the manipulation with the culture and provides parasite yields comparable to parasite yields on such liquid medium as Schneider’s Insect Medium. We demonstrate that SNB-9 very considerably surpasses Schneider’s Insect Medium in in vitro infectivity of the parasites. Additionally, we show that the ratio of apoptotic parasites, which are important for the infectivity of the inoculum, in Leishmania culture in SNB-9 is higher than in Leishmania culture in Schneider’s Insect Medium. Thus, we demonstrate that the cultivation of Leishmania on SNB-9 reliably yields highly infective promastigotes suitable for experimental infection.

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1. Introduction

Leishmania spp. have a great clinical significance as the causative agent of leishmaniases. Twelve million people are infected with Leishmania and 350 million people are under risk of infection in 88 countries (Desjeux, 1996). Yet, no vaccine has been developed (Kedzierski, 2010) and the treatment needs significant improvement (Goto and Lindoso, 2010; Moore and Lockwood, 2010). Animal models of leishmaniases play a key role in understanding the mechanisms of the disease and in finding ways to treat and prevent it (De Oliveira et al., 2004; Lipoldová and Demant, 2006). However, one of the most important preconditions for obtaining reliable results is the long lasting availability of cultured infective Leishmania parasites with constant properties.

The media used for parasites’ cultivation can be divided into biphasic and monophasic (liquid). Generally, handling biphasic media is substantially more technically demanding than handling liquid media, which are more suitable for the mass culture of Leishmania (Chang and Fish, 1983). However, biphasic media are strongly recommended for initial isolation of Leishmania parasites (Schuster and Sullivan, 2002). Additionally, there is evidence that biphasic media are more favorable for the infectivity of Leishmania parasites (Dey et al., 2002). Here, we describe the cultivation of Leishmania parasites on biphasic SNB-9 (saline–neopeptone–blood 9) medium that combines the advantages of biphasic and liquid media. This medium was originally used for isolation of trypanosomes from avian bone marrow (Diamond and Herman, 1954) and was adapted for cultivation of Leishmania for the study of Leishmania physiology (Svobodová et al., 1997) and genetic control of susceptibility to L. major parasites (Lipoldová et al., 2000). However, details of this adaptation have not been described before. A part of this adaptation and optimization was the change of the ratio of solid phase to liquid phase from 4:1 (Diamond and Herman, 1954) to 1:1 for flat tubes and 1:2 for flat flasks. Using a higher volume of the liquid phase in SNB-9 rather than in the classic NNN (Nicolle–Novy–MacNeal) and USMARU (Difco blood agar) biphasic media (WHO, 1996) facilitates the manipulation of the culture. Obviously, it might have also an impact on the total yields of the parasites. Thus, we analyzed the growth dynamics of Leishmania on SNB-9 and compared it with the growth dynamics on Complete Schneider’s Medium (Lima et al., 1997). To estimate the infectious potential of the stationary
phase of the culture, we performed in vitro infection of mouse intraperitoneal macrophages. As it was recently shown that the presence of apoptotic Leishmania parasites in the inoculum increases its infectivity (van Zandbergen et al., 2006; Wanderley et al., 2009), we additionally analyzed the proportion of apoptotic parasites in the stationary phase of cultivation.

2. Materials and methods

2.1. Media formulations

SNB-9 consists of the blood agar base and the overlay. For the blood agar base, 2% BactoTM Agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 2% BactoTM Neopeptone (Becton, Dickinson and Company) and 0.6% NaCl water solution were prepared and autoclaved for 20 min at 120 °C and 1 atm. Being shaken gently at 15 min intervals, the solution was let to cool down in a water bath 2219 MULTITEMP II (KLB, Bromma, Sweden) set for 60 °C. Then, rabbit defibrinated blood (Bioveta, a.s., Ivanovice na Hané, Czech Republic) was added to the final concentration 20% and thoroughly mixed. After that, the bottle with agar was kept in a thermally insulated tank with hot water (56 °C) and the tubes/flasks were filled as quickly as possible to prevent the solidification of agar in the pipette. Flat tubes were filled with 1–1.5 ml agar and 25 cm² flat flasks with 5 ml. Agar was poured into the very end of the tubes/flasks and then spread over the bottom. Around 2 cm of the flat part of the bottom was left clear to facilitate microscopic observation of the parasites. The tubes were put in a slightly inclined position and the flasks in a horizontal position. The remnants of agar were left in the bottle and incubated at 23 °C for two days to control sterility. The tubes/flasks with blood agar base were stored at +4 °C not more than for two months. For the overlay, 2% neopeptone and 0.6% NaCl water solution was prepared and autoclaved for 20 min at 120 °C and 1 atm. The overlay can be stored at +4 °C for at least 6 months. Immediately before the cultivation of Leishmania parasites, 1.5 ml of the overlay was added to flat tubes with blood agar base and 10 ml of the overlay was added to 25 cm² flat flasks with blood agar base.

To prepare 2 formulations of Complete Schneider's Medium (in the text, Schneider's 10% and Schneider's 20%), Schneider's Insect Medium (Sigma-Aldrich) was supplemented with 50 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA), 63.7 μg/ml penicillin G potassium salt (Sigma-Aldrich) and 100 μg/ml streptomycin sulfate salt (Sigma-Aldrich); for basic cultivation, 50 μg/ml gentamicin (Sigma-Aldrich). The overlay can be stored at +4 °C for at least 6 months. Immediately before the cultivation of Leishmania parasites, 1.5 ml of the overlay was added to flat tubes with blood agar base and 10 ml of the overlay was added to 25 cm² flat flasks with blood agar base.

To prepare 2 formulations of Complete Schneider's Medium (in the text, Schneider's 10% and Schneider's 20%), Schneider's Insect Medium (Sigma-Aldrich) was supplemented with 50 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA), 63.7 μg/ml penicillin G potassium salt (Sigma-Aldrich) and 100 μg/ml streptomycin sulfate salt (Sigma-Aldrich), 2% human urine and 10% or 20% heat-inactivated Fetal Bovine Serum (FBS) (Sigma-Aldrich). Human urine was filtered using 0.2 μm pore filters immediately after obtaining. The next day, urine was frozen and stored at −20 °C. FBS was inactivated by heating to 56 °C for 30 min.

The peritoneal exudate cells were harvested and maintained using Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 50 μg/ml gentamicin (Sigma-Aldrich), 63.7 μg/ml penicillin G potassium salt (Sigma-Aldrich), 100 μg/ml streptomycin sulfate salt (Sigma-Aldrich), 25 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid buffer (Sigma-Aldrich), 6.4 mM 2-mercaptoethanol (SERVA Electrophoresis GmbH, Heidelberg, Germany) and 10% heat-inactivated FBS (Sigma-Aldrich).

2.2. Isolation of the parasites from lesion and tissues of Leishmania-infected mice

Wild-type Leishmania tropica SU23 (MHOM/TR/98/HM) were isolated from infected BALB/cHeA (BALB/c) mice. Shortly before isolation, 1.5 ml overlay was added to flat tubes with blood agar base. Mice infected with Leishmania were killed with cervical dislocation. The border of the lesion and the skin around were disinfected with alcohol. 100–200 μl of the overlay was injected into the border of the lesion and aspirated (Fig. 1). Ejection and subsequent aspiration were repeated several times. After that, the syringe barrel cavity was washed by repeated aspiration and ejection of the overlay from a single flat tube. The procedure was repeated with 2–3 more syringes, each with a new flat tube (totally, 3–4 tubes). Then, inguinal and lesion lymph nodes were obtained. Each lymph node was put into a separate flat tube. If lymph nodes were larger than 2 mm in diameter, they were cut and 2 mm pieces of each lymph node were used.

2.3. Cultivation of the parasites

The cultures were maintained with an air access at 23 °C for 7 days. On the 7th day of cultivation, the cultures were checked for contamination using light microscopy. 10⁶ parasites per 1 ml of the fresh overlay were passed into the new tubes/flasks with blood agar base. Serial passaging leads to the loss of the infectivity of the promastigotes (da Silva and Sacks, 1987), so culture #2 was used for all the types of analysis.

2.4. Counting parasites

Parasites were counted in duplicates with 22 Coulter Counter (Beckman Coulter, Inc., Brea, CA, USA) every 24 h. In the instrument settings, the lower size of the objects was set to 5 femtoliters (fl) and the upper size to 50 fl.

2.5. In vitro infectivity assay

Macrophages were obtained from 19 week old BALB/c female mice 4 days after intraperitoneal injection of 1 ml of 3% thioglycolate (Sigma-Aldrich). The peritoneal exudate cells were harvested using supplemented DMEM and subsequently maintained in this medium at 37 °C and 5% CO₂. Macrophages were infected with GFP-expressing L. major in the stationary phase of growth at the ratio of 1:10. The cells were coincubated at the density of 60,000 macrophages/100 μl/well in 96 well plates (Nunc, Waltham, MA) for 24 h. Subsequently, the medium containing non-adherent macrophages and extracellular parasites was removed and the plates were washed 3 times with Hanks’ Balanced Salt solution (HBSS) (Sigma-Aldrich). Finally, HBSS was replaced with

![Fig. 1. Isolation of Leishmania from the lesion of infected mice. Examples of correct places (red cross) and directions (black arrows) of inserting a syringe needle. These parts contain the largest numbers of viable parasites.](image)
supplemented DMEM and macrophages were incubated for additional 12 h. After that, the medium was aspirated and the macrophages were detached from the plates by washing with cold phosphate buffered saline (PBS) containing 10 mM glucose (Sigma-Aldrich) and 3 mM ethylenediaminetetraacetic acid (Sigma-Aldrich). Then, the cells were stained with 5 μg/ml of propidium iodide (PI) (Sigma-Aldrich) in the dark at room temperature for 30 min. Forward Scatter (FSC), Side Scatter (SSC), fluorescence in FL1 (530 ± 15 nm bandpass filter) channel for GFP (green, excitation/emission = 489/510 nm) and in FL2 channel (585 ± 21 nm bandpass filter) for PI (orange, excitation/emission = 351/617 nm) were analyzed using FACS Calibur (Becton, Dickinson and Company) and software CellQuest Pro (Becton, Dickinson and Company). Acquisition stopped when 10,000 events in the gate corresponding to macrophages (based on FSC and SSC) were acquired. Macrophages stained with PI (necrotic and late apoptotic cells with the penetrable plasma membrane) were excluded from the analysis.

2.6. Apoptosis assay

The percentage of apoptotic forms of *Leishmania* was assessed on the 7th day of cultivation. Promastigotes were washed and incubated with 25 μg/ml PI in PBS for 10 min in the dark at room temperature. After that 1 μg/ml laser dye steryl (LDS)-751 (Invitrogen Corporation, Carlsbad, CA, USA) was added and the samples were incubated for additional 20 min under the same conditions. Then the samples were washed, resuspended in PBS and analyzed for fluorescence using flow cytometer FACS Calibur (Becton, Dickinson and Company). PI is a dye staining necrotic and late apoptotic cells with the penetrable plasma membrane. PI positive cells generate an average angle of 45° as PI (orange, excitation/emission = 351/617 nm) is fluorescent in both FL2 channel (585 ± 21 nm bandpass filter) and FL3 channel (>670 nm longpass filter), which is primarily used to detect staining with LDS-751 (red, excitation/emission = 543/712 nm), a cell permeable dye accumulated in polarized mitochondria. Positive LDS-751 staining indicated non-apoptotic cells and negative LDS-751 staining indicated early to late apoptotic as well as necrotic cells (Fig. 2). The samples were also analyzed for light scatter. 20,000 events were acquired.

2.7. Statistical analysis

To analyze the results, GraphPad Prism 5 for Windows (GraphPad Software, USA) was used. The growth of *Leishmania* promastigotes on different media was analyzed using two-way ANOVA with Bonferroni post-tests. In the assay of infectivity, first, sample variances were compared using F test. For SNB-9 and Schneider’s 10%, but not SNB-9 and Schneider’s 20%, the variances were significantly different \( P<0.01 \). So, to estimate the differences in the infectivity of *L. major* cultivated on SNB-9 and Schneider’s 10%, we used unpaired \( t \) test with Welch’s correction. To estimate the differences in the infectivity of *L. major* cultivated in SNB-9 and Schneider’s 20%, we used unpaired \( t \) test. To analyze the results of apoptosis assay, unpaired \( t \) test was used.

3. Results

3.1. Parasite growth dynamics

We compared growth of *L. major* and *L. tropica* in SNB-9, Schneider’s 10% and Schneider’s 20%. The differences in *L. major* concentration between SNB-9 and Schneider’s 10% were not significant and the differences in parasite concentrations between SNB-9 and Schneider’s 20% were significant on the 4th \( P<0.01 \), 5th, 6th and 7th \( P<0.001 \) days of cultivation (Fig. 3A). On the 7th day of cultivation, *L. major* mean promastigote concentration in SNB-9, Schneider’s 10% and Schneider’s 20% was 27.9 ± 6.5 million/ml, 31.7 ± 4.3 million/ml and 93.1 ± 21.8 million/ml, respectively.

The differences in *L. tropica* concentration between SNB-9 and Schneider’s 10% were not significant and the differences in parasite concentrations between SNB-9 and Schneider’s 20% were significant on the 5th, 6th \( P<0.001 \) and 7th \( P<0.001 \) days of cultivation (Fig. 3B). On the 7th day of cultivation, *L. tropica* promastigote concentration in SNB-9, Schneider’s 10% and Schneider’s 20% was 20.5 ± 8.2 million/ml, 32.8 ± 15.7 million/ml and 58.1 ± 28.7 million/ml, respectively.

![Fig. 2. Two-parameter, two-color analysis of apoptosis in *L. major* promastigotes cultured in SNB-9. PI (orange, excitation/emission = 351/617 nm) stains positively apoptotic cells with penetrable plasma membrane (fluorescent both in FL2 channel (<670 nm longpass filter) and FL3 channel (>670 nm longpass filter), in the present figure 10.8%). LDS-751 (red, excitation/emission = 543/712 nm) stains positively non-apoptotic cells with polarized mitochondria (fluorescent in FL3 channel only (>670 nm longpass filter), in the present figure 76.9%). Double-negative cells (in the present figure 10.4%) correspond to apoptotic cells with depolarized mitochondria but an integral plasma membrane.](image)

![Fig. 3. Comparison of *L. major* (A) and *L. tropica* (B) growth in biphasic and liquid media. *Leishmania* growth in SNB-9 is shown with circles, in Schneider’s 10% — with squares and in Schneider’s 20% — with triangles. The graphs summarize data of 5 independent experiments with *L. major* and 4 independent experiments with *L. tropica*.](image)
3.2. In vitro infectivity of the promastigotes

Flow cytometry data demonstrated that *L. major* promastigotes cultured on SNB-9 were two times more infective for murine macrophages in comparison with promastigotes cultured on Schneider’s 10% and Schneider’s 20% (Fig. 4).

3.3. The ratio of apoptotic promastigotes in the stationary phase culture

In *L. major* cultures, the level of apoptosis in cultures in SNB-9 and Schneider’s 10% was comparable (22.68% and 23.04%), whereas in Schneider’s 20% it was much lower (11.76%) (Fig. 5A). For SNB-9 and Schneider’s 10%, we observed similar ratios of early apoptotic (10.96% and 10.956%, respectively) and late apoptotic (9.682% and 9.484%, respectively) parasites. However, in Schneider’s 20%, late apoptotic parasites slightly prevailed over early apoptotic parasites (5.32% in comparison with 4.208%). For *L. tropica*, the percentage of apoptotic forms was much higher in SNB-9 (23.7%) than in Schneider’s 10% (12.6%) and Schneider’s 20% (9.825%) (Fig. 5B). Noticeably, very few early apoptotic parasites could be observed in Schneider’s 10% and Schneider’s 20% (3.268% and 3.465%, respectively). The ratio of late apoptotic parasites was high in SNB-9 (13.058%), low in Schneider’s 20% (6.068%) and intermediate in Schneider’s 10% (8.988%).

4. Discussion

Pursuing the goal of developing a biphasic medium suitable for large-scale experiments with *Leishmania* spp., we successfully adapted and optimized SNB-9 biphasic medium originally developed for the cultivation of *Trypanosoma*. The medium proved to be suitable for the study of *Leishmania* and both insect (Volf et al., 1998) and mammalian host interactions (Demant et al. 1996; Lipoldová et al., 2002; Vladimirov et al., 2003; Havelková et al., 2006; Kurey et al., 2009), for the analysis of promastigotes (Svobodová et al., 1997), for obtaining *Leishmania*-related biological material (Sádlová et al., 2006; Myskova et al. 2008; Kobets et al. 2010), and for primary isolation of *Leishmania* strains (Svobodová et al., 2009).

The yield of the parasites is one of the key parameters which determine the applicability of a medium for mass cultivation. SNB-9 allowed reaching the same parasite concentration as liquid Schneider’s 10%. Taken together with the large volume of liquid phase of optimized SNB-9, it makes the medium suitable for large-scale experiments. Though, Schneider’s 20% provided even higher parasite concentrations, particularly for *L. major*. Similar yields as those obtained in Schneider’s 20% were described by Merlen et al. (1999) for CDM/LP (completely defined medium for the cultivation of *Leishmania* promastigotes). *L. major* and *L. tropica* reached the stationary phase concentration of 50 millions/ml and 68 millions/ml, respectively (Merlen et al. 1999). However infectivity of parasites was not tested.

In vitro experimental infection of murine macrophages revealed that the infectivity of *L. major* cultured on SNB-9 was two times higher in comparison with *L. major* cultured on Schneider’s 10% and Schneider’s 20%. We supplemented the results by determination of the ratio of apoptotic promastigotes as an indirect infectivity assay. The ratio of apoptotic parasites in *Leishmania* cultures in SNB-9 was much higher than in *L. tropica* cultures in Schneider’s 10% and Schneider’s 20% and *L. major* cultures in Schneider’s 20%. To sum up, our findings support the data about superior infectivity of *Leishmania* parasites cultured on biphasic media.

In conclusion, we have shown that *Leishmania* cultivation in SNB-9 provides a) parasite yields comparable to the yields obtained in liquid media, b) higher *in vitro* infectivity than liquid media and c) increased ratio of apoptotic parasites, important for the overall infectivity, in comparison with liquid media. Thus, in comparison with liquid media, SNB-9 gives similar yields, but is highly superior in infectivity of the parasites.

**Competing interests statement**

The authors declare that they have no competing financial interests in this investigation.

**Author contributions**

E.N. and M.S. conceived and designed the method, I.G. and M.S. extended and refined the method, M.S., I.G. and M.L. performed parasitological experiments, and I.G. and M.L. wrote this paper.

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**Figure 4.** Comparison of infectivity of *L. major* promastigotes cultured on biphasic and liquid media with 6 replicates for each medium. Data are presented as mean ± SD.

**Figure 5.** The percentage of alive (LDS-751−PI−), early apoptotic (LDS-751−PI+), and late apoptotic (LDS-751+PI−) parasites in the stationary phase culture of *L. major* (A) (5 independent experiments) and *L. tropica* (B) (4 independent experiments). Data are presented as mean ± SD.
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