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ORIGINAL PAPER

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A simple and reproducible method to obtain large numbers of axenic amastigotes of different *Leishmania* species

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Abstract This work describes a simple method to yield large amounts of *Leishmania* amastigote-like forms in axenic cultures using promastigotes as the starting population. The method described induced extracellular amastigote transformation of *Leishmania amazonensis* (97%), *Leishmania braziliensis* (98%) and *Leishmania chagasi* (90%). The rounded parasites obtained in axenic cultures were morphologically similar, even at the ultrastructural level, to intracellular amastigotes. Moreover, the axenic amastigotes remained viable as measured by their ability to revert back to promastigotes and to infect BALB/c mice. *L. amazonensis* and *L. braziliensis* promastigotes and axenic amastigotes differed in terms of their Western blot profiles. A 46 kDa protein was recognized by specific antibodies only in axenic and lesion-derived *L. amazonensis* amastigotes and not in promastigotes.

Introduction

Protozoa of the genus *Leishmania* display two distinct morphological and three distinct physiological forms. The promastigotes, the flagellated stages, infect the sand fly vector both as a multiplying and also as a mammal-infective form (Sacks 1992) whereas the non-flagellated amastigote resides in mammalian macrophage phagolysosomes. Both morphological stages are responsible for the pathology in their respective hosts (Chang and Dyer 1976; Schlein et al. 1992). The promastigote stages can be easily cultivated in different types of media (Hendricks et al. 1978; Hart et al. 1981; Jaffe et al. 1984) and are frequently used in scientific studies. In contrast, the difficulty in obtaining large numbers of amastigotes, free from host cell contaminants, has hampered the investigation of their metabolic, biochemical and biological properties (Chang 1980; Hart et al. 1981). Moreover, a study on macrophage receptors for *Leishmania* demonstrated the presence of host immunoglobulins on the surface of lesion-derived amastigotes (Peters et al. 1995), something that would particularly hinder their use in biological and immunological studies.

The first successful long-term propagation of amastigote-like forms of *Leishmania*, namely *Leishmania pifanoi*, in a cell-free medium was reported by Pan (1984). Since then, attempts at the cultivation of amastigotes in cell free media have been carried out by many authors (reviewed in Bates 1993; Pan et al. 1993). Some reports have focused on the in vitro transformation of promastigotes to amastigote-like forms in response to elevated temperature (Hendricks 1978; Hunter et al. 1982; Leon et al. 1995), whereas few authors have described serial axenic cultivation of the intracellular stage (Pan 1984; Al-Bashir et al. 1992; Hodgkinson et al. 1996). The axenic cultivation of amastigotes of *Leishmania mexicana* has been more effective than that of other species of *Leishmania* (Bates 1993). Some authors have reported *Leishmania donovani* amastigote transformation in axenic cultures (Al-Bashir et al. 1992; Saar et al. 1998), whereas there is only one study on the differentiation to axenic amastigotes of *Leishmania chagasi*, another visceral species (Leon et al. 1995).

In spite of these reports on the axenic cultivation of *Leishmania* amastigotes, there still remain controversies about the reproducibility and/or timing of parasite transformations. In this paper, a simple and quick method of obtaining up to 4×10^7 axenic amastigotes/ml

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of culture is described, providing large amounts of pure and viable parasites. This method may obviate the need for using experimental laboratory animals to obtain amastigotes and the laborious procedures for intracellular parasite purification.

Materials and methods

Parasites and culture

The *Leishmania* species used were isolated from human patients and identified as *Leishmania amazonensis* (MHOM/BR88/BA-125 Leila strain), *Leishmania braziliensis* (MHOM/BR/3456) and *Leishmania chagasi* (MHOM/BR2000/Merivaldo2 strain). They were kept by passaging in golden hamsters. The cultures were grown at 22°C in Schneider's *Drosophila* medium (Sigma, St. Louis, Mo., USA), pH 7.2, supplemented with either 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, Utah, USA) for *L. amazonensis* or 20% FCS for *L. braziliensis* and *L. chagasi*. The promastigotes used to initialize amastigote axenic cultures were maintained in cultures for no more than ten passages. Different culture conditions for promastigote transformation in relation to: pH (5.4, 6.4 and 7.2), temperature (32°, 34°, 35° and 37°C) and FCS concentration (2.5%, 5%, 10% and 20%), were tested. To determine growth curves, parasite cultures were initiated at a concentration of 5×10^6 promastigotes/ml in 25 cm^2 tissue culture flasks containing 5 ml of medium. Cell density was estimated using a haemocytometer. To disrupt the aggregates which were formed mainly in *L. braziliensis* cultures, parasites were passed through a 25-gauge needle before counting. Intracellular amastigotes were isolated from footpad lesions of infected hamsters and purified using a Percoll (Sigma) gradient, as previously reported (Chang 1980).

Morphological assessment

To evaluate the morphology of transformed parasites, axenic amastigotes were harvested from cultures and either smeared onto slides for Giemsa staining or spun down in Eppendorf tubes for electron microscopy. The supernatant was discarded and the pellets were fixed for 1 h at 4°C with 2% glutaradehyde. Parasites were pelleted, post-fixed with 1% osmium tetroxide for 30 min at room temperature, dehydrated in ethanol-propylene oxide series and embedded in Spurr resin. After polymerization, ultrathin sections were collected in copper grids, stained with uranyl acetate and lead citrate, and examined on a Zeiss EM109 electron microscope at 50 kV.

Western blot analysis

Lysates of promastigotes, amastigotes from lesions and axenic amastigotes were subjected to 10% polyacrylamide gel electrophoresis (Laemmli 1970). Lysates from different forms of the same *Leishmania* species were run in the same gel and a total of 10^7 lysed parasites of each *Leishmania* stage was loaded per lane. The separated proteins were analyzed by Western blot as described elsewhere (Balanco et al. 1998), utilizing an 1:200 dilution of a pool of five sera from mice chronically infected with *L. amazonensis*.

Functional studies

The viability and functional status of amastigote-like forms were tested by assessing their ability to revert back to promastigotes and to infect mice. Reversion to promastigotes was accomplished by washing the parasites three times in 0.15 M phosphate-buffered saline (pH 7.2) and transferring them to Schneider's medium with 10–20% FCS, pH 7.2, at 22°C. To evaluate infectivity, axenic amastigotes and promastigotes of *L. braziliensis* and *L. amazonensis* were inoculated into BALB/c mouse hind footpads. The BALB/c mice were obtained from the Gonçalo Moniz Research Center animal facilities, and were maintained under specific pathogen-free conditions, with balanced mouse food and water ad libitum. The infection experiments were conducted in accordance with the Oswaldo Cruz Foundation guidelines for experimentation with animals.

Results

Cultivation of amastigote-like forms

The first aim of this study was to monitor parasite growth and transformation in axenic cultures in order to establish the best conditions for obtaining amastigote-like forms. Starting with a 100% promastigote population and using Schneider's medium, cultures were subjected to variations in pH, temperature and concentration of FCS to obtain the maximal rate of transformation for each *Leishmania* species. For *L. braziliensis*, a high proportion (98%) of amastigote-like forms were observed in 3-day cultures supplemented with 20% FCS, at pH 5.4 and carried out at 34°C (Fig. 1a). *L. amazonensis* parasites had similar levels of differentiation into amastigote-like forms (97%) by the 7th to 10th days of culture in Schneider's medium with 5% FCS, pH 5.4, at 32°C (Fig. 1b). For *L. chagasi*, 90% of the promastigotes transformed into amastigote-like forms after 13 days of axenic culture in Schneider's with 20% FCS, pH 7.2 and at 35°C (Fig. 1c). Table 1 summarizes the best culture conditions for obtaining amastigote-like forms for each *Leishmania* species.

Morphology under light and electron microscopy

Optical microscopy of Giemsa-stained amastigote-like forms of the three *Leishmania* species studied revealed oval or pyriform cells lacking a free flagellum. Dividing amastigotes with two nuclei and two kinetoplasts could readily be observed in *L. amazonensis*, less frequently in *L. braziliensis* and even less frequently in *L. chagasi* cultures (not shown). The non-motile, oval forms of *L. braziliensis* from the 3rd day of axenic culture also had an amastigote morphology at the ultrastrutural level, e.g. a non-emergent flagellum inside the flagellar pocket (Fig. 2).

Viability of axenic amastigotes

Axenic amastigotes of all three *Leishmania* species were able to revert back to promastigotes when cultured under suitable conditions. The proportion transforming was high, as shown by the absence or low numbers of either amastigotes or dead cells in *L. braziliensis* (Fig. 3) and *L. amazonensis* cultures (less than 10%), and in *L. chagasi* cultures (less than 30%, mostly due to the

presence of dead cells/cell debris). In addition, BALB/c mice could be infected in the hind footpads both with axenic amastigote-derived promastigotes or axenic amastigotes, as *L. braziliensis* and *L. amazonensis* could be isolated 2–3 months later from skin lesions and from draining lymph nodes of infected mice. The development of lesions in BALB/c mice infected with *L. amazonensis* axenic amastigotes was similar to that observed in promastigote-infected mice (Fig. 4). In fact, two of the three mice infected with axenic amastigotes developed lesions

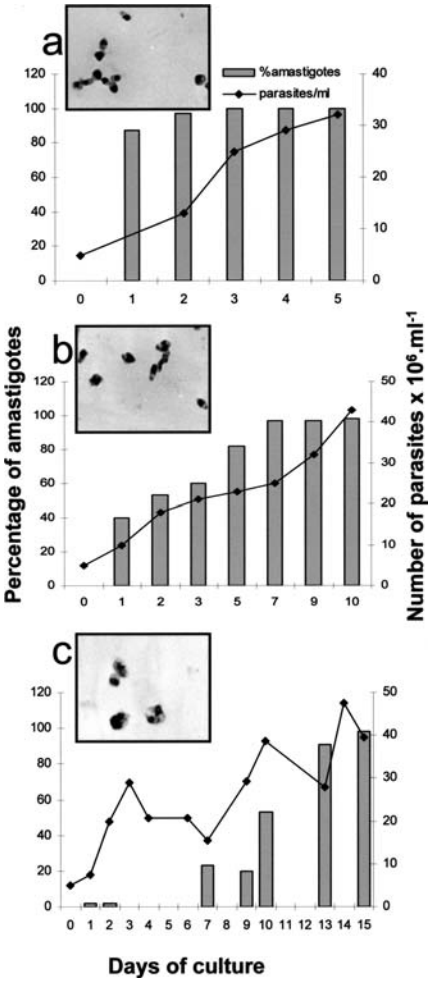

Fig. 1. In vitro growth of *Leishmania* amastigotes in axenic cultures: **a** *L. braziliensis*, **b** *L. amazonensis* and **c** *L. chagasi*. Cultures were initiated with 5×10^6 promastigotes/ml and kept under the specific conditions described in the Materials and methods section. *Lozenges* represent the number of parasites as determined by counting in a haemocytometer, and *bars* represent the percentages of amastigote-like forms. *Insets* show the typical oval- or elliptical-shape axenic amastigotes of each *Leishmania* species (Giemsa,×4,800)

Table 1. Axenic culture conditions, in Schneider's medium, for the transformation of *Leishmania* promastigotes into amastigote-like organisms

Species	Temperature	pH	FCS	Days of culture	Percent amastigotes
L. braziliensis	34°C	5.4	20%	3	98%
L. amazonensis	32°C	5.4	5%	7	97%
L. chagasi	35°C	7.2	20%	13	90%

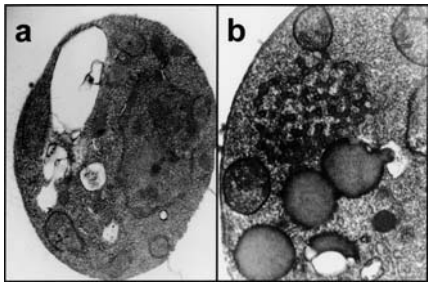

Fig. 2a, b. Electron micrograph of *L. braziliensis* amastigote-like forms from axenic cultures. *L. braziliensis* promastigotes were cultivated in Schneider's medium with 20% of FCS, pH 5.4 and at 34°C. After 3 days, parasites were harvested from cultures and fixed with glutaraldehyde. Note the non-emergent flagellum inside the flagellar pocket (**a** ×34,800) and lipids droplets in cytoplasm (**b** ×41,500)

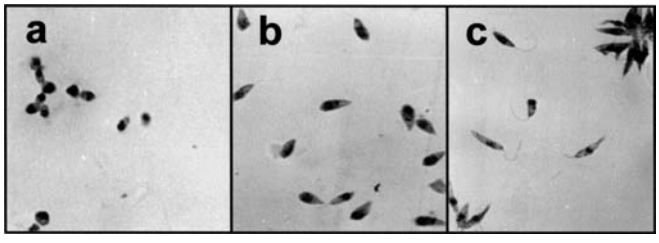

Fig. 3a–c. *L. braziliensis* amastigote-like forms revert to promastigotes. Axenic amastigotes of *L. braziliensis* were subjected to suitable conditions for transformation to promastigotes and monitored daily by optical microscopy after staining. **a** Axenic amastigotes in Schneider's with 20% FCS at pH 5.4 and 34°C; **b** 3-day culture and **c** 4-day culture in Schneider's medium with 20% FCS at pH 7.2 and 22°C (Giemsa, ×4,800)

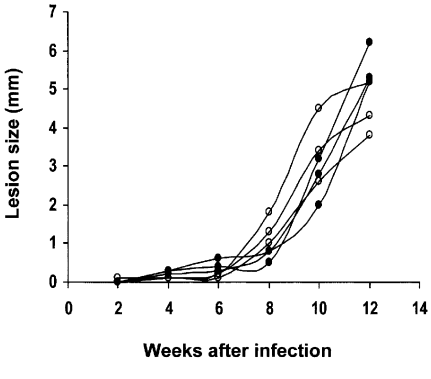

Fig. 4. Infectivity of axenic amastigotes. Groups of three BALB/c mice were infected in hind footpads with either 10^5 axenic amastigotes (*closed circles*) or 10^5 promastigotes (*open circles*) of *Leishmania amazonensis*. Lesion sizes were calculated by subtracting the widths of uninfected from infected footpads. Each curve corresponds to data obtained from an individual animal. Data are representative of two experiments

slightly larger than those observed in mice infected with promastigotes (Fig. 4).

Stability of axenic amastigotes in culture

The non-motile and ovoid shaped forms of *L. amazonensis* proliferated in culture and could be inoculated into fresh medium and expanded at least twice. After 3 days, no promastigotes could be detected in *L. braziliensis* axenic cultures. However, the parasites demonstrated morphological signs of cell injury and started to die by the 5th day of culture. By the 15th day of culture, most of the *L. chagasi* axenic amastigotes were morphologically unhealthy (they had an irregular cytoplasm and a fragmented nucleus).

Recognition of *Leishmania* proteins by antiserum

The anti-*L. amazonensis* serum recognized a 46 kDa protein in *L. amazonensis* amastigotes (axenic and lesion-derived), but not in promastigotes (Fig. 5). At least three bands corresponding to proteins with apparent molecular weights larger than 84 kDa were observed in axenic, but not in lesion *L. amazonensis* amastigotes. Faint bands between 28 and 32 kDa were revealed only in lesion-derived *L. amazonensis* amastigotes, whereas a single band of around 30 kDa appeared in both axenic amastigotes and promastigotes. A 65 kDa protein was strongly recognized in *L. braziliensis* promastigotes and very weakly in axenic amastigotes and bands of 113 and 117 kDa were present only in promastigotes.

Discussion

In this report, the conditions for the production of large numbers of axenic amastigotes of *L. amazonensis, L. braziliensis* and *L. chagasi* using Schneider's medium are described. The morphological and ultrastructural characteristics of the axenic amastigotes were very similar to those of lesion amastigotes and their propagation was neither time-consuming nor technically demanding. The amastigote-like forms were viable and expressed stage-specific antigens.

Some authors have accomplished the serial cultivation of *Leishmania* axenic amastigotes in cell-free medium with a complex composition, including a mixture of nucleotides and vitamins (Pan 1984), or with different protein sources and rabbit blood lysate (Al-Bashir et al. 1992). The diversity of reagents in the prepared media may impede the reproducibility of the results in different laboratories. In fact, we have carefully tested the RBLM medium for axenic amastigotes, as described elsewhere (Al-Bashir et al. 1992), without success for any of the three *Leishmania* species studied (data not shown).

Specific requirements for temperature, pH and concentration of FCS were observed for the three species of *Leishmania* studied, indicating that culture conditions for amastigote differentiation in cell-free media is species dependent . For example, a relatively low pH (5.4) in the medium was required for the transformation of *L. braziliensis* and *L. amazonensis*, but not of *L. chagasi*. At pH 5.4, the maximum rate of promastigote transformation of this species was 26% (4-day culture) and the cells died by the 6th day of culture (data not shown). This was in contrast to 90% transformation at pH 7.2 (13-day culture) under the same conditions. These specific requirements demonstrate differences in pH and temperature sensitivities among *Leishmania* species.

Cutaneous and mucocutaneous leishmaniasis in South America are mainly caused by *L. braziliensis* (Grimaldi et al. 1989). This species differs from others by the presence of small numbers of amastigotes in the lesions, hindering their isolation from tissue. Indeed, this very paucity of *L. braziliensis* amastigotes in skin lesions prevented their purification in large enough numbers to allow their comparison with axenic culture-derived amastigotes in the present study. Therefore, obtaining extracellular *L. braziliensis* amastigotes forms would facilitate a number of biochemical and immunological studies on this parasite. Eperon and McMahon-Pratt (1989) have previously reported the transformation of *L. braziliensis* promastigotes to amastigotes in Schneider's medium at 28°C, without indicating the culture pH. In their study, an acclimatization period and a slow increase in temperature was necessary. Balanco and collaborators (1998) have also obtained *L. braziliensis* amastigotes in axenic cultures using a different medium (UM-54) at 34°C and pH 6.3. With the 2°C increments in temperature, 1 week was required to obtain 95% of amastigote-like forms in culture. As described herein, a rapid and high proportion (98%) of differentiation of *L. braziliensis* promastigotes into amastigote-like forms was observed, without changes in pH or temperature during cultivation.

The culture conditions to obtain axenic amastigotes of *L. amazonensis* described here are an adaptation of those reported by Hodgkinson and collaborators (1996). In order to obtain stable amastigote-like populations, these authors increased the temperature by 2°C intervals, followed by decreases of 0.5 pH units down to pH 5.0 and then of 0.2 pH units until pH 4.6 was reached. In the study described here, *L. amazonensis* differentiation was gradual and took at least 7 days to reach maximal levels at pH 5.4, without any changes in culture conditions.

In order to obtain *L. chagasi* amastigote forms, the cultivation of promastigotes at 37°C and/or at an acidic pH was first tested. None of these conditions led to parasite transformation or growth. It was necessary to decrease the temperature to 35°C and increase the pH to 7.2 in order to stimulate differentiation which took at least 13 days to reach maximal levels. Dividing amasti-

gote-like forms were easily observed in *L. braziliensis* and *L. amazonensis*, but very infrequently in *L. chagasi* axenic cultures. This fact, taken together with the relatively long time (13 days) needed for *L. chagasi* promastigote differentiation, with a lower proportion (maximal of 90%) of axenic amastigotes in comparison with *L. amazonensis* (97%) and *L. braziliensis* (98%) axenic amastigote cultures, and the presence of more cell debris in amastigote-derived promastigote cultures, may indicate that *L. chagasi* is more fastidious than the other two *Leishmania* species in terms of its requirements for in vitro stage transformation.

The good condition of the axenic culture-derived amastigotes was evaluated by four different criteria. First, all three *Leishmania* species amastigotes had a regular, smooth morphological appearance. Second, *L. amazonensis* amastigotes could be subcultured, leading to a progressive increase in the homogeneity of the amastigote-like population, with the absence of promastigotes. Third, the axenic amastigotes of all three species were able to revert back to promastigotes. Fourth, *L. braziliensis* and *L. amazonensis* axenic amastigotes kept their viability and infectivity to mice by the time (3 and 10 days, respectively) of the maximal ratio of transformation in culture, without signs of cellular death.

Despite their homogeneous morphology, *L. amazonensis* axenic amastigotes displayed a Western blot profile intermediate between promastigotes and intracellular amastigotes. A protein of 46 kDa from *L. amazonensis*, recognized by serum from infected BALB/c mice, was present only in the axenic and lesion amastigotes. This protein may correspond to a 46 kDa protein that was detected in *Leishmania infantum* axenic amastigotes using sera from human beings and dogs with visceral leishmaniasis (Cibrelus 1999), to a 45 kDa band which was recognized in *L. donovani* using a monoclonal antibody specific to the A-2 multi-gene family of proteins, expressed only in amastigotes (Saar et al. 1998) and/or to a 45 kDa protein of *L. pifanoi* detected with a monoclonal antibody in both axenic and intracellular amastigotes by Pan and collaborators (1993). *L. amazonensis* proteins with apparent molecular weights of 23 and 30 kDa were recognized in both axenic promastigotes and axenic amastigotes, a fact that may be explained by the presence in amastigote axenic cultures of intermediate forms expressing some proteins of the promastigote stage. In this study, proteins around 30 kDa were recognized by antiserum in all three morphological forms. Colmenares and collaborators (2001) have identified a *L. pifanoi* 31 kDa protein, recognized by an amastigote-specific monoclonal antibody, as a cystein proteinase associated with glycolipids. Either the 30 kDa seen in axenic amastigotes or the 32 kDa protein seen in lesion-derived amastigotes could be that cystein proteinase.

The different methods applied for the characterization of *Leishmania* molecules, such as Western blot and radioimmunoprecipitation using monoclonal antibodies

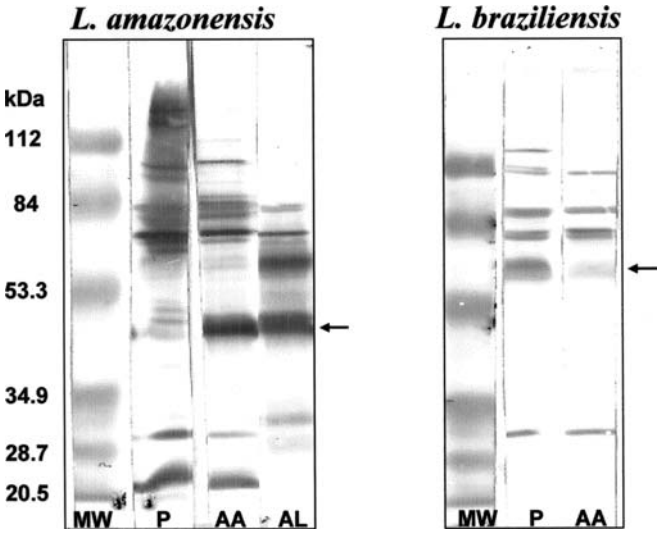

Fig. 5. Reactivity of *L. amazonensis* and *L. braziliensis* antigens with polyclonal antibodies. Lysates of promastigotes (*P*), axenic amastigotes (*AA*) and amastigotes from lesions (*AL*) were analysed by Western blot using sera from *Leishmania*-infected BALB/c mice. The positions of molecular weight (*MW*) standards are indicated on the left. *Arrows* indicate the high expression of a 46 kDa protein in *L. amazonensis* amastigotes and the under expression of a 65 kDa protein in *L. braziliensis* axenic amastigotes

(Hodgkinson et al. 1996; Pan and McMahon-Pratt 1988), surface radioiodination (Balanco et al. 1997) or Western blot using polyclonal anti-*L. amazonensis* sera (this report) may account for the distinct group of molecules demonstrated in different studies.

Only *L. braziliensis* axenic amastigotes and promastigotes were compared in terms of recognition patterns by specific antibodies (Fig. 5b) due to the difficulties in obtaining intracellular amastigotes from the pad lesions of infected hamsters. A protein of 65 kDa, which was recognized by specific antibodies more intensely in promastigotes, could indeed be the gp65, and its down-regulation in axenic amastigotes shown here is consistent with previously reported data (Kweider et al. 1989). A reduction in intensity of a 117 kDa protein in promastigote-derived amastigotes is also consistent with its down-regulation at this stage.

Axenic amastigotes may be useful for the study of molecules selectively expressed by them and by intracellular amastigotes. The availability of a pure amastigote preparation from different *Leishmania* species would also facilitate studies aimed at identifying the *Leishmania* molecules involved in the modulation of chronic infection and/or of potential immunotherapeutic or immunoprophylatic value, as promastigotes enter into contact with the host only for very short periods.

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