Axenic promastigote forms of Leishmania (Viannia) Lainsoni as an alternative source for Leishmania antigen production

ARTICLE *in* JOURNAL OF PARASITOLOGY · JULY 2005

Impact Factor: 1.23 · DOI: 10.1645/GE-396R1 · Source: PubMed

6 AUTHORS, INCLUDING:

Márcio Sobreira Silva Araújo
Fundação Oswaldo Cruz
44 PUBLICATIONS **299** CITATIONS
SEE PROFILE

Reginaldo Brazil
Fundação Oswaldo Cruz
177 PUBLICATIONS **1,472** CITATIONS
SEE PROFILE

J. Parasitol., 91(3), 2005, pp. 551–556

AXENIC PROMASTIGOTE FORMS OF *LEISHMANIA* (*VIANNIA*) *LAINSONI* AS AN ALTERNATIVE SOURCE FOR *LEISHMANIA* ANTIGEN PRODUCTION

José R. Corrêa, Sidney G. Santos*, Márcio S. Araújo†, Cibele Baptista‡, Maurilio J. Soares, and Reginaldo P. Brazil§

Lab. Biologia Celular de Microrganismos, Departamento de Ultra-estrutura e Biologia Celular, Instituto Oswaldo Cruz/FIOCRUZ, Av. Brasil 4365, 21040-900 Rio de Janeiro, RJ, Brazil. *e-mail:correa@ioc.fiocruz.br*

ABSTRACT: The present study demonstrates that axenic cultures of *Leishmania* (*Viannia*) *lainsoni* produce larger cell masses in NNN-LIT medium, as well as higher amounts of total proteins in cell extracts, than *Leishmania* (*Leishmania*) *amazonensis*. Antigenicity of *L.* (V.) *lainsoni* whole promastigotes is similar to that of *L.* (*L.*) *amazonensis*, as demonstrated by an indirect immunofluorescence diagnostic test using sera from human patients and dogs infected with visceral leishmaniasis. Infectivity of the *L.* (*V.*) *lainsoni* strain used in the present work was demonstrated by the detection by transmission-electron microscopy of tissue amastigotes in skin lesion samples from an experimentally infected hamster. Incubation of lesion fragments in NNN-LIT medium allowed us to obtain promastigote forms, which could be cultivated successfully in vitro. Isoenzyme analysis of such promastigotes confirmed the parasite strain as *L.* (*V.*) *lainsoni*, as compared to other *Leishmania* reference strains. Our data indicate that *L.* (*V.*) *lainsoni* is a useful alternative source for antigen production as well for use in assays that depend on large cell volumes of *Leishmania* spp. parasites.

Flagellate protozoa of the *Leishmania* spp. (Kinetoplastida: Trypanosomatidae) are etiological agents of infections known collectively as leishmaniasis. This disease affects humans and other mammals and has diverse clinical manifestations (cutaneous, mucocutaneous, and visceral) that depend on the parasite species and the immunological status of the vertebrate host (Liew and O'Donnel, 1993; Noronha et al., 1996; Handman, 1999). In the New World, *Leishmania* spp. parasites belonging to the *Viannia* subgenus are responsible for cutaneous and mucocutaneous forms of leishmaniasis, presenting considerable diversity and intraspecific genetic variation (Cupolillo et al., 1995, 2001).

Leishmania (*Viannia*) *lainsoni* is a recently recognized species that was first isolated from infected humans in the state of Pará, Brazil (Silveira et al., 1987). It produces a cutaneous form of the disease with self-limiting nodules or small ulcers, with scarce tissue amastigotes at the lesion site and without posterior nasopharyngeal infections. Because the Brazilian Amazon region possesses a high number of wild bacteria and fungi species, secondary infections may occur if leishmanial lesions remain open for long periods, even after adequate medical treatment (Silveira et al., 1987). *Leishmania* (*V.*) *lainsoni* can be distinguished from *L.* (*V.*) *braziliensis* by the presence of elongated tissue amastigotes with a large kinetoplast and from *Leishmania* (*Leishmania*) *amazonensis* and *L.* (*V.*) *braziliensis* by isoenzyme profiles (Lucas et al., 1994; Eresh et al., 1995).

Leishmania (*V.*) *lainsoni* is widely distributed in the Brazilian Amazon but also has been isolated from humans in the sub-Andean region of Peru and Bolivia (Lucas et al., 1994, 1998; Martinez et al., 2001; Bastrenta et al., 2002). This protozoan has a strong impact on local populations, because the sand fly vector (*Lutzomyia ubiquitalis*) is anthropophylic when humans invade the forest environment. Furthermore, the insect vectors present massive infections with this parasite, and the identified mammal reservoir (*Agouti paca*) is widespread in all of the Amazon region (Silveira, Shaw et al., 1991; Silveira, Souza et al., 1991).

Several laboratory techniques involving biological and/or molecular approaches have been employed using extracts of *Leishmania* spp. promastigotes, all of which depend on large numbers of parasites produced in axenic culture under laboratory conditions (Dwyer, 1972; Ozbilgin et al., 1995; Limoncu et al., 1997). These procedures include purification of antigens for diagnostic tests, production of vaccines, and experimental studies of parasite infectivity in animal models or cell cultures (Mora et al., 1999; Späth and Beverley, 2001). *Leishmania* (*L.*) *amazonensis* currently is the preferred species among the New World *Leishmania* because of its ease of maintenance during in vitro culture and the high yield of promastigote forms obtained in axenic media (Mora et al., 1999).

When analyzing the biological behavior in vitro of *L.* (*V.*) *lainsoni* in several axenic culture media, we observed the production of large numbers of cells in a short period of time. The ease of maintaining this species in the laboratory led us to compare its antigenicity, growth curve, and protein mass with those of *L.* (*L.*) *amazonensis*. Furthermore, we present here some morphological data regarding *L.* (*V.*) *lainsoni* tissue amastigotes in an experimental hamster skin lesion as well as axenic promastigotes obtained from the same skin sample.

MATERIALS AND METHODS

Parasites

Culture promastigote forms of *L.* (*V.*) *lainsoni* (MHOM/BR/81/M6426), *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2903), *L.* (*V.*) *guyianensis* (MHOM/BR/75/M4147), *L.* (*L.*) *amazonensis* (IFLA/BR/67/PH8), and *L.* (*L.*) *chagasi* (MHOM/BR/74/PP75) were grown at 25 C in 100-ml Erlenmeyer flasks containing 5 ml of NNN medium as solid phase and 5 ml of LIT medium as liquid phase (NNN-LIT medium). The LIT medium was enriched with 10% inactivated fetal bovine serum and contained 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 40 μg/ml of gentamicin. Passages were made at 5-day intervals by transferring 250-μl aliquots of the liquid medium to new Erlenmeyer flasks containing fresh NNN-LIT medium.

Received 11 May 2004; revised 31 August 2004; 8 September 2004; accepted 22 September 2004.

* Lab. de Leishmanioses, Centro de Pesquisas René Rachou/FIOCRUZ, Av. Augusto de Lima, 1715, 30190-002 Belo Horizonte, MG, Brazil.
† Lab. Entomologia Médica, Centro de Pesquisas René Rachou/FIOCRUZ, Av. Augusto de Lima, 1715, 30190-002 Belo Horizonte, MG, Brazil.
‡ Lab. Leishmanioses. Hospital Evandro Chagas/FIOCRUZ, 21045-900 Rio de Janeiro, RJ, Brazil.
§ Lab. Bioquímica, Fisiologia e Imunologia de Insetos, Instituto Oswaldo Cruz/FIOCRUZ, Av. Brasil 4365, 21040-900 Rio de Janeiro, RJ, Brazil.

Characterization of *L.* (*V.*) *lainsoni* by isoenzyme electrophoresis

The identity of the *L.* (*V.*) *lainsoni* strain used in our experiments was assessed using isoenzyme electrophoresis by comparison with the original *L.* (*V.*) *lainsoni* (MHOM/BR/81/M6426) strain and with 2 other *Leishmania* spp. reference strains: *L.* (*L.*) *chagasi* (MHOM/BR/74/PP75), and *L.* (*V.*) *guyianensis* (MHOM/BR/75/M4147). Isoenzymatic characterization was performed in 1% agarose gels for 8 enzyme loci (Grimaldi et al., 1991; Franco et al., 1996): 6PGDH (6-phosphogluconate dehydrogenase, EC.1.1.1.43), GPI (phosphoglucose isomerase, EC.5.3.1.9), NH (nucleoside hydrolase, EC. 3.2.2.1), G6PDH (glucose-6-phosphate dehydrogenase, EC.1.1.1.49), ME (malic enzyme, EC.1.1.1.40), IDHNADP (isocitrate dehydrogenase, EC.1.1.1.42), MDH (malate dehydrogenase, EC.1.1.1.37), and PGM (phosphoglucomutase, EC.2.7.5.1).

Infectivity test and ultrastructure of *L.* (*V.*) *lainsoni* amastigotes

To evaluate the infectivity of the *L.* (*V.*) *lainsoni* strain used in our experiments, 14-day-old culture parasites (stationary phase) grown in NNN-LIT medium were collected and inoculated (1×10^5 cells/ml) in the hind foot of a hamster. After 45 days, the animal was killed, and a sample of the hind-foot skin was obtained at the lesion site. Lesion fragments were minced in phosphate-buffered saline (PBS; pH 7.2) and inoculated into culture tubes containing NNN-LIT medium. After 6 days at 25 C, culture aliquots were examined by light microscopy to verify the presence of promastigote forms.

Some lesion fragments also were processed for transmission-electron microscopy to detect tissue amastigotes. Briefly, the fragments were fixed for 2 hr with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), postfixed for 2 hr with 1% osmium tetroxide/0.8% potassium ferrycianide/5 mM calcium chloride in 0.1 M cacodylate buffer, dehydrated in acetone series, and embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, Pennsylvania). Stained ultrathin sections (70 nm in thickness) were observed in a Zeiss EM10C (Oberkochen, Germany).

Growth curves

Four-day-old culture forms of *L.* (*V.*) *lainsoni*, *L.* (*V.*) .) *ziliensis*, and *L.* (*L.*) *amazonensis* were inoculated at a concentration of 1×10^6 cells/ml in 5 ml of NNN-LIT medium supplemented with 10% fetal bovine serum and antibiotics. Growth curves were obtained by daily counting in a Neubauer chamber, in triplicate, until the cell number in the cultures was similar to that in the initial inoculum.

Four-day-old culture forms of *L.* (*V.*) *lainsoni* also were grown (inoculum 1×10^6 cells/ml) in the following liquid media: LIT, MEM (Sigma Chemical Co., St. Louis, Missouri), α-MEM (Sigma), D-MEM (Sigma), and RPMI 1640 (Sigma). All media were supplemented with 10% fetal bovine serum and antibiotics. Growth curves were obtained by daily counting in a Neubauer chamber, in triplicate, until the cell number in the cultures was similar to that in the initial inoculum.

Indirect immunofluorescence antibody test

Thirty serum samples from patients with suspected clinical cases of visceral leishmaniasis were used together with 30 serum samples from visceral leishmaniasis in symptomatic dogs. All sera were obtained from the serum bank of the Laboratório de Leishmanioses, Centro de Pesquisas René Rachou/FIOCRUZ, Belo Horizonte, MG, Brazil.

Leishmania (*V.*) *lainsoni* antigen was prepared from axenic promastigotes collected on the 12th day of cultivation (stationary phase of growth) in NNN-LIT medium. The cells were centrifuged at 4 C for 10 min at 2,500 *g*, the supernatant discarded, and the pellet washed 3 times with 1% bovine serum albumin (BSA). The pellet was then fixed for 30 min with 4% paraformaldehyde in PBS, washed with 1% BSA, and resuspended in 10 ml of sterile PBS. The cell number was adjusted to $3–4 \times 10^6$ cells/ml, and the samples were stored at 4 C until use. For comparison, we have used the *Leishmania* sp. promastigote antigen from the commercially available indirect immunofluorescence antibody test (IFAT) kit produced and distributed by Bio-Manguinhos (FIOCRUZ, Rio de Janeiro, Brazil).

For IFAT, 10 μl of antigen were placed in circles of 12-well immunofluorescence slides and left to dry overnight at room temperature. Ten μl of serum samples (diluted 1:40 to 1:640) were then placed in the slide circles, and the reaction mixture was incubated for 30 min at 37 C. The slides were then washed twice with PBS and once with distilled water and then air-dried at room temperature. A fluorescein isothiocyanate–conjugated secondary antibody (obtained from the Bio-Manguinhos IFAT kit) was diluted 1:100 in PBS and added to the incubation mixture together with 0.1% (v/v) Evans blue. After incubation for 30 min at 37 C, the slides were washed twice in PBS and once in distilled water and then air-dried at room temperature. Ten μl of PBS-buffered glycerin were added to each slide circle, which was then covered with a coverslip. The samples were identified by numbers and observed by fluorescence microscopy.

A qualified technician with no previous knowledge of the samples made the observations. The test was performed in triplicate to minimize errors. The results obtained with the 2 antigens (*L.* (*V.*) *lainsoni* and Biomanguinhos kit) were then compared with the McNemar's chi-square test using the EPI-INFO software (Dean et al., 1994).

Quantification of membrane and soluble proteins

Axenic cultures of *L.* (*V.*) *lainsoni* and *L.* (*L.*) *amazonensis* were initiated at the same day and time with an inoculum of 1×10^6 cells/ml in 6 ml of LIT medium. The parasites were transferred every 5 days to larger volumes until a final volume of 80 ml was obtained for each sample. This volume was centrifuged at 4 C for 10 min at 4,000 *g*, the supernatant discarded, and the pellets washed 3 times with PBS. At the end of this process, the wet weight of the samples was adjusted to 0.310 g each. This cell mass was then diluted in 300 μl of PBS.

The cell masses were disrupted by freezing in liquid nitrogen and thawing at 40 C in a water bath. Thereafter, the cells were homogenized with a Dounce homogenizer (Wilmad-Lab Glass, Buena, New Jersey) and further disrupted by sonication for 15 min with a Branson Sonicator (model 450; Branson Ultrasonic Corporation, Laredo, Texas) operated at 100 W, 22.5 kHz, and 70% of power. Aliquots of 10 μl were examined by light microscopy to determine the extent of cell disruption. The samples were then diluted in sucrose buffer (0.25 M sucrose, 10 mM Tris-HEPES [pH 7.4], 1% [v/v] aprotinin, 1 mM dithiothreitol, 0.167 mM pepstatin A, and 0.167 mM leupeptin) and centrifuged at 1,600 *g* for 15 min to remove cell debris. The resulting supernatant was centrifuged for 1 hr at 30,000 *g* to obtain cytoplasmic (supernatant) and membrane (pellet) fractions. Both fractions were frozen and stored at −70 C until use.

Protein concentration was determined in cytosolic and membrane fractions by the Lowry method (Ho et al., 1983). Protein concentration was analyzed in an ELISA reader using 5 replicates for each sample. Protein standards were albumin solutions at 1 and 10 mg/ml. Analysis of the results was performed by the GraphPad Instat™ software (GraphPad Software, San Diego, CA) to determine the similarity between both species for each sample (membrane and soluble proteins).

RESULTS

Biochemical characterization of the *L.* (*V.*) *lainsoni* strain

The *L.* (*V.*) *lainsoni* strain (MHOM/BR/M6426) used in our experiments was obtained previously from the Hospital Evandro Chagas (Belém, PA, Brazil) and maintained frozen in the *Leishmania* Bank of the Laboratório de Leishmanioses of the Centro de Pesquisas René Rachou/FIOCRUZ (CPqRR, Belo Horizonte, MG, Brazil). To certify the identity of this strain, its isoenzymatic banding pattern was compared with those obtained from the original type strain (the first isolate of the reference strain *L.* (*V.*) *lainsoni* M6426) kept in hamsters and from the reference strains of *L.* (*V.*) *guyianensis* and *L.* (*L.*) *chagasi*.

Analysis of the electrophoresis gels for all tested isoenzymes demonstrated that the *L.* (*V.*) *lainsoni* strain used in our experiments presented the same banding pattern as the reference strain *L.* (*V.*) *lainsoni* (MHOM/BR/81/M6426) (Fig. 1). Both *L.* (*V.*) *lainsoni* strains showed distinct banding patterns when compared to the other reference *Leishmania* sp. tested in the same gel (Fig. 1).

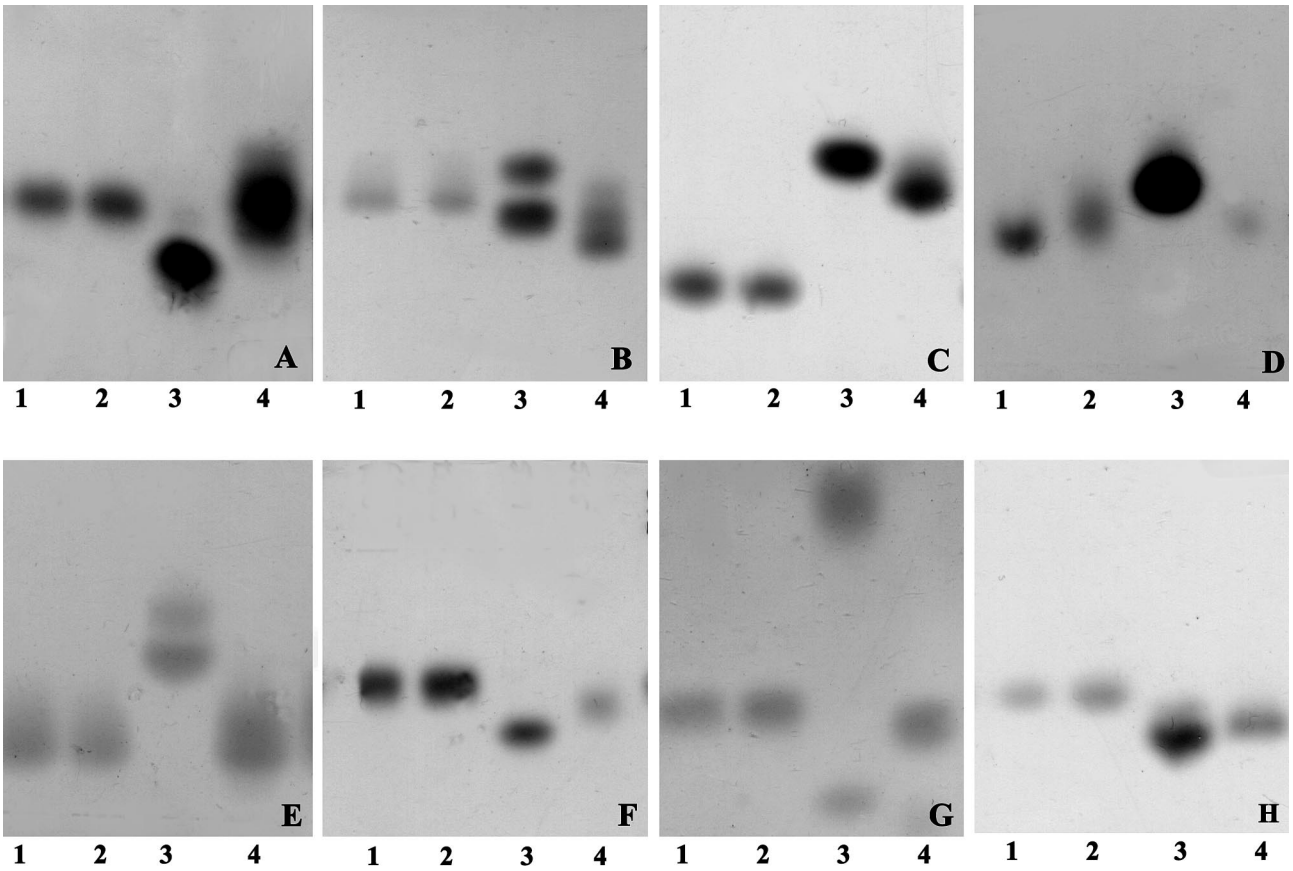

FIGURE 1. Isoenzyme profile in agarose gels for the following enzyme loci: (**A**) 6PGH, (**B**) G6PH, (**C**) GPI, (**D**) IDH, (**E**) MDH, (**F**) ME, (**G**) NH, and (**H**) PGM. Lane 1: *Leishmania* (*Viannia*) *lainsoni* (MHOM/BR/81/M6426), strain used in this work; lane 2: *L.* (*V.*) *lainsoni* (MHOM/BR/81/M6426), reference strain, the first isolate; lane 3: *Leishmania* (*Leishmania*) *chagasi* (MHOM/BR/74/PP75); lane 4: *Leishmania* (*Viannia*) *guyianensis* (MHOM/BR/75/M4147).

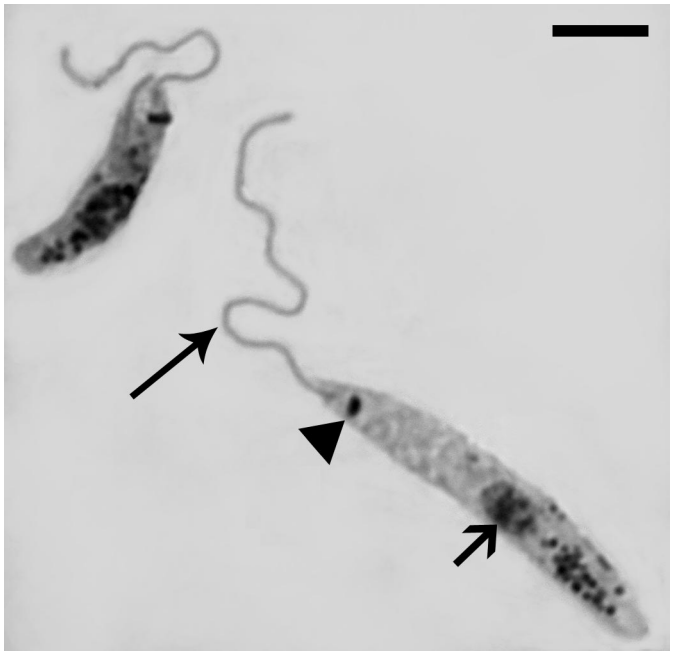

FIGURE 2. Light microscopy of Giemsa-stained *Leishmania* (*Viannia*) *lainsoni* promastigotes. Nucleus (short arrow), kinetoplast (arrowhead), and flagellum (long arrow) are indicated. Bar = 5 μm.

Obtaining promastigote forms from hamster skin lesion and fine structure of tissue amastigotes

When tissue samples from a skin lesion of an infected hamster were inoculated into NNN-LIT medium, flagellates could be observed after 6 days of incubation at 25 C. Observation of Giemsa-stained cells by light microscopy showed promastigote forms with a long body, a centrally located nucleus, and a kinetoplast at the anterior end (Fig. 2).

Skin lesion fragments analyzed by transmission-electron microscopy showed several amastigote forms, either free in the intercellular space (Fig. 3A) or inside host-cell vacuoles (Fig. 3B). The amastigotes presented a round shape, a large nucleus with condensed peripheral chromatin and a round nucleolus, and the typical trypanosomatid organelles, such as acidocalcisomes, glycosomes, and a bar-shaped kinetoplast (Fig. 3A). No megasomes were observed.

Comparative growth curve in NNN-LIT medium

Growth of *L.* (*V.*) *lainsoni* in NNN-LIT medium was compared to that of representative *Leishmania* spp. strains of the *Leishmania* and *Viannia* subgenera. Maximal growth for *L.* (*V.*) *lainsoni* was obtained after 8–9 days of cultivation, with approximately 5–6 $\times$ 10^7 cells/ml. Growth of *L.* (*L.*) *amazonensis* was not so intense, and the number of cells was lower through-

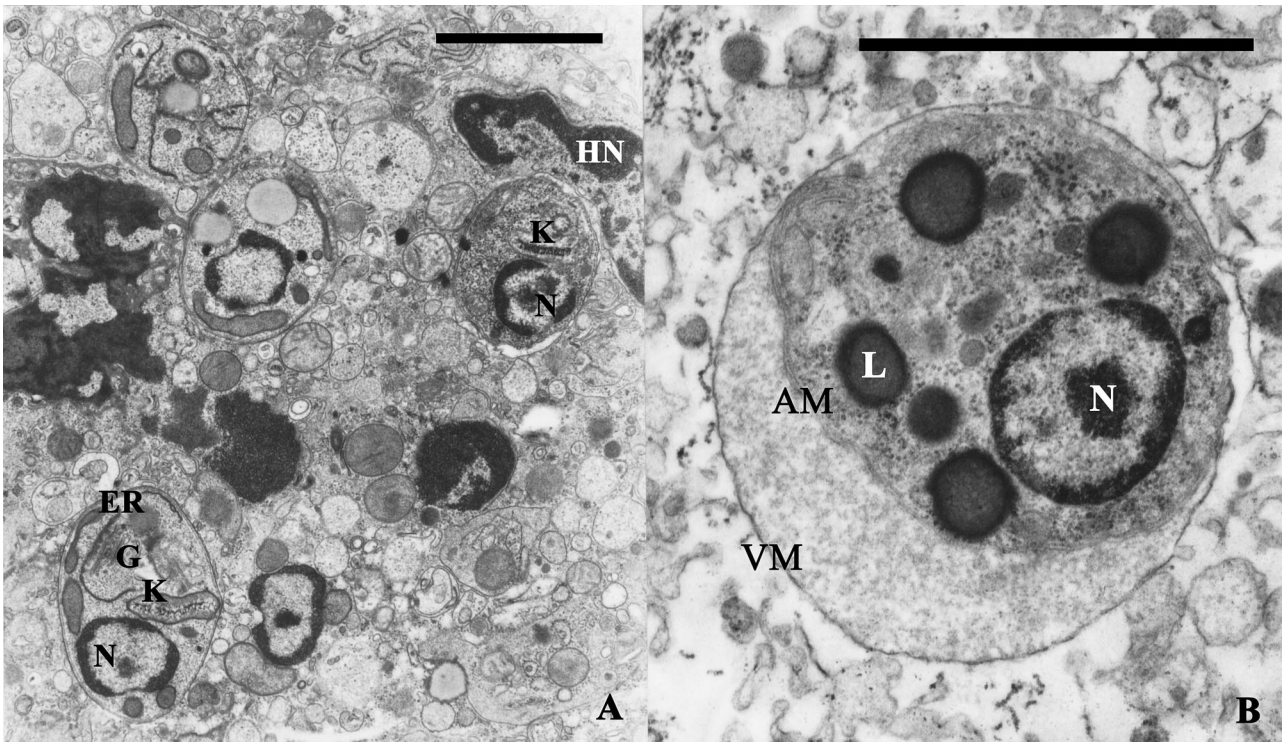

FIGURE 3. Transmission-electron microscopy of tissue amastigotes of *Leishmania* (*Viannia*) *lainsoni*. **A.** Intracellular amastigote form. ER, endoplasmic reticulum; G, Golgi complex; HN, host cell nucleus; K, kinetoplast; N, nucleus. Bar = 2 μm. **B.** Extracellular amastigote still inside a parasitophorous vacuole. AM, amastigote membrane; L, lipid inclusion; N, nucleus; VM, parasitophorous vacuole membrane. Bar = 2 μm.

out the whole growth period, with the same phase intervals as *L*. (*V*.) *lainsoni* (Fig. 4A). Maximal number of cells (3–4 × 10^7 cells/ml) was obtained at day 9. The growth curve of *L*. (*L*.) *braziliensis* was more moderate in terms of cell number than that obtained with the 2 other *Leishmania* spp. parasites (Fig. 4A).

Growth curve profiles of *L*. (*V*.) *lainsoni* in different culture media

Because superior growth of *L*. (*V*.) *lainsoni* was obtained in LIT-NNN medium, we tested whether similar growth could be obtained in other liquid media. Axenic cultivation of *L*. (*V*.) *lainsoni* in LIT, MEM, α-MEM, D-MEM, and RPMI also resulted in excellent growth, with similar growth profiles in all tested media. Typical markers for the growth curve in LIT medium were (1) increased cell division rate from the 4th to the 9th day, generating a high-sloped exponential phase; (2) a peak in cell number after 8–9 days (5–6 × 10^7 cells/ml); (3) a lag phase of approximately 4 days; and (4) an extensive decline phase. The total growth curve for this species was completed in 22 days (Fig. 4B).

Growth curves of *L*. (*V*.) *lainsoni* in MEM, α-MEM, and D-MEM media presented slight variations, but the high number of cells obtained and the general growth profiles were similar (Fig. 4B). A difference was observed with the RPMI-1640 medium: The duration of the phases was approximately the same, but the number of cells was lower throughout the growth curve (Fig. 4B).

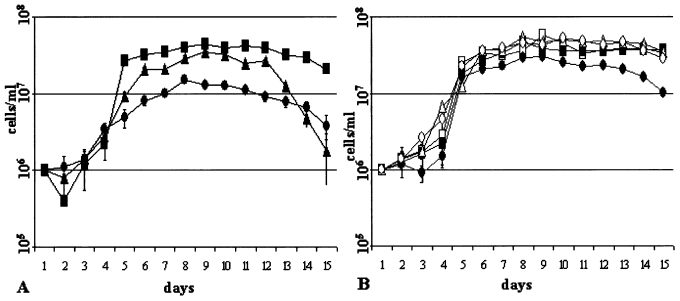

FIGURE 4. **A.** Growth curves of *Leishmania* (*V*.) *lainsoni* (■), *Leishmania* (*L*.) *amazonensis* (▲), and *Leishmania* (*V*.) *braziliensis* (●) at 26 C in NNN-LIT medium. **B.** Growth curves of *Leishmania* (*V*.) *lainsoni* at 26 C in LIT (□), MEM (○), α-MEM (Δ), D-MEM (■), and RPMI (●) culture media.

Indirect immunofluorescence assay

Leishmania (*V*.) *lainsoni* antigen probed by an indirect immunofluorescence test against 30 human serum samples resulted in 18 positive and 12 negative sera, compared with 16 positive and 14 negative sera obtained with the Biomanguinhos kit. When tested against 30 canine serum samples, the *L*. (*V*.) *lainsoni* antigen showed 25 positive and 5 negative results, compared with 23 positive and 7 negative results obtained with the Biomanguinhos kit. Analysis of these results with the EPI-INFO software to determine the concordance coefficient between the samples resulted in ''fair'' (K = 0.595) and ''good'' (K = 0.793) results for the human and canine sera, respectively. A posteriori clinical analysis of the 4 discrepant positive test

results in human sera (2 cases) and canine sera (2 cases) demonstrated that these individuals had leishmaniasis. Reactivity of their sera with our experimental *L.* (*V.*) *lainsoni* antigen showed titers of 1:40 (considered to be low or doubtful infection). The experimental antigen from *L.* (*V.*) *lainsoni* did not present false-positive results in the immunoassay test.

Protein concentration

Quantification of total and soluble protein content by the Folin phenol reagent method (Lowry et al., 1951) demonstrated that 0.310 g of wet cell mass provided a higher amount of proteins for axenic promastigotes of *L.* (*V.*) *lainsoni* compared to *L.* (*L.*) *amazonensis*, for both total proteins (2.498 ± 0.002 and 2.331 ± 0.001 mg/ml, respectively) and soluble proteins (1.86 ± 0.002 and 1.243 ± 0.003 mg/ml, respectively).

DISCUSSION

The banding pattern by isoenzyme electrophoresis for the *L.* (*V.*) *lainsoni* (MHOM/BR/81/M6426) strain used in our experiments was identical to that obtained with the original reference strain of *L.* (*V.*) *lainsoni* (MHOM/BR/81/M6426) maintained at the Hospital Evandro Chagas. The biochemical data demonstrated that we used a bona fide *L.* (*V.*) *lainsoni* strain in our experiments.

Heteroxenic trypanosomatids usually lose their infectivity when maintained for long periods in axenic media, as demonstrated previously for *Trypanosoma cruzi* (Menezes, 1968) and several *Leishmania* sp. (Nolan and Herman, 1985). Parasites of the *Leishmania* spp. also lose their ability to infect macrophages and to transform into the amastigote form in vitro. Although competent to bind to the macrophage cell membrane, the parasites are not able to survive and divide inside phagosomes, being eliminated through the microbicidal action of the host cells (Handman, 1983; Nolan and Herman, 1985; Becker and Jaffe, 1997). Our studies, however, demonstrated that *L.* (*V.*) *lainsoni*, even though maintained for a long time in axenic conditions, did not lose its capacity to infect a mammal host, inducing the formation of a skin lesion from which promastigote forms could be obtained by incubating lesion samples in NNN-LIT medium. The ultrastructural data allowed us to demonstrate the presence of tissue amastigotes in the skin lesion with the typical morphological characteristics of the trypanosomatid protozoa. No megasomes were found in the lesion amastigote forms, which was expected, because these organelles typically are found only in New World species belonging to the subgenus *Leishmania* (Coombs et al., 1986; Duboise et al., 1994).

Ease of cultivation and maintenance in the laboratory are factors that contribute to the choice of *L.* (*L .*) *mazonensis* as a primary source of *Leishmania* sp. antigen for various assays in Brazil. Thus, we selected this species as a model for comparison with *L.* (*V.*) *lainsoni*. We also evaluated the growth of *L.* (*V.*) *braziliensis* cultures to determine whether *L.* (*V.*) *lainsoni* growth was similar to that of a typical species of subgenus *Viannia*. Comparison of the growth curves for these 3 species demonstrated that *L.* (*V.*) *lainsoni* offers an alternative to *L.* (*L.*) *amazonensis* as an antigen source, because it could be grown easily in the laboratory conditions and, considering its high rate of cell division, produced a larger cell mass in all tested axenic media.

The antigenicity of *L.* (*V.*) *lainsoni*, as demonstrated by IFAT, suggests its usefulness for antigen production in the laboratory. Several assays to induce immunity against cutaneous leishmaniasis in humans were carried out with parasite extracts obtained from murine models (Mitchell et al., 1985; Barral-Neto and Barral, 1987; Frommel et al., 1988; Moddaber, 1989) or with purified antigens: glycoprotein gp63 (Russel and Alexander, 1988), lipophosphoglycans (Handman and Mitchell, 1985), or even an association between glycoproteins, total proteins, and the immunomodulator BCG (Lagrange, 1977; Mora et al., 1999). Because gp63 and LPG are surface antigens common to all species of *Leishmania* and the present forms to induce immunity go through the production of antigens from promastigote forms in axenic media (River et al., 1999; Brittingham et al., 1999), *L.* (*V.*) *lainsoni* possesses useful characteristics for this kind of application.

Based on data for protein quantification in *L.* (*V.*) *lainsoni*, together with the high number of cells produced in vitro, it appears that crude production of proteins can be obtained with *L.* (*V.*) *lainsoni* that is greater and less expensive than that obtained with *L.* (*L.*) *amazonensis*, the current choice for this purpose in Brazil (Mora et al., 1999). Further studies with a purified *L.* (*V.*) *lainsoni* surface glycoprotein fraction are underway in our laboratory to evaluate its protective action as well as its potential use in an intradermic diagnostic test in a murine model.

ACKNOWLEDGMENTS

The authors would like to thank Fernando Tobias Silveira (Instituto Evandro Chagas, Belém, PA, Brazil) for kindly furnishing the *L.* (*V.*) *lainsoni* (MHOM/BR/81/M6426) strain as well as the *Leishmania* Bank of the Laboratório de Leishmanioses of the Hospital Evandro Chagas (IPEC/FIOCRUZ, Rio de Janeiro, RJ, Brazil) for kindly furnishing the *L.* (*L.*) *chagasi* (MHOM/BR/74/PP75) and *L.* (*V.*) *guyianensis* (MHOM/BR/75/M4147) strains. The authors also thank Dr. Paulo Filemon Paolucci Pimenta (Laboratório de Entomologia Médica, Centro de Pesquisas René Rachou/FIOCRUZ, Belo Horizonte, MG, Brazil) for providing support for the protein quantification and Meire Closel (Laboratório de Imunopatologia, Centro de Pesquisas René Rachou/FIOCRUZ, Belo Horizonte, MG, Brazil) for helpful analysis of the protein concentration. This work was supported by CNPq, FIOCRUZ, and PAPES-III/FIOCRUZ.

LITERATURE CITED

BARRAL-NETTO, M., AND A. BARRAL. 1987. Specific immunization in cutaneous leishmaniasis. Memórias do Instituto Oswaldo Cruz **82:** 293–302.

BASTRENTA, B., R. BUITRAGO, F. VARGAS, F. LE PONT, M. TORREZ, M. FLORES, N. MITA, AND S. F. BRENIÈRE. 2002. First evidence of transmission of *Leishmania* (*Viannia*) *lainsoni* in a sub-Andean region of Bolivia. Acta Tropica **83:** 249–253.

BECKER, S., AND C. L. JAFFE. 1997. Effect of protein kinase inhibitors on the growth, morphology, and infectivity of *Leishmania* promastigotes. Parasitology Research **83:** 273–80.

BRITTINGHAM, A., G. CHEN, B. S. MCGWIRE, K. P. CHANG, AND D. M. MOSSER. 1999. Interaction of *Leishmania* gp63 with cellular receptor for fibronectin. Infection and Immunity **67:** 4477–4484.

COOMBS, G. H., L. TETLEY, V. A. MOSS, AND K. VICKERMAN. 1986. Three dimensional structure of the *Leishmania* amastigote as revealed by computer-aided reconstruction from serial sections. Parasitology **92:** 13–23.

CUPOLILLO, E., F. AGUIAR ALVES, L. R. BRAHIM, M. F. NAIFF, L. O. PEREIRA, M. P. OLIVEIRA-NETO, A. FALQUETO, AND G. GRIMALDI. 2001. Recent advances in the taxonomy of the New World leishmanial parasites. Medical Microbiology and Immunology **190:** 57–60.

———, G. GRIMALDI, H. MOMEN, AND S. M. BERVELEY. 1995. Intergenic region typing (IRT): A rapid molecular approach to the characterization and evolution of *Leishmania*. Molecular and Biochemical Parasitology **73:** 144–55.

DEAN, A. G., J. A. DEAN, D. COULOMBIER, K. A. BRENDEL, D. C. SMITH, AND A. H. BURTON. 1994. EPI-INFO, Version 6: A word-processing, database, and statistics program for epidemiology on microcomputers. Center for Disease Control and Prevention, Atlanta, Georgia.

DUBOISE, S. M., M. A. VANNIER-SANTOS, D. COSTA-PINTO, L. RIVAS, A. A. PAN, Y. TRAUB-CSEKO, W. DE SOUZA, AND D. MCMAHON-PRATT. 1994. The biosynthesis, processing, and immunolocalization of *Leishmania pifanoi* amastigote cysteine proteinases. Molecular and Biochemical Parasitology **68:** 119–32.

DWYER, D. M. 1972. A monophasic medium for cultivating *Leishmania donovani* in large numbers. Journal of Parasitology **58:** 847–848.

ERESH, S., H. L. MAARTEN, M. H. L. DE BRUIJN, J. A. MENDOZA-LEON, AND D. C. BARKER. 1995. *Leishmania* (*Viannia*) *lainsoni* occupies a unique niche within the subgenus *Viannia*. Transactions of the Royal Society of Tropical Medicine and Hygiene **89:** 231–236.

FRANCO, A. M. R., H. MOMEN, R. D. NAIFF, C. F. S. MOREIRA, M. P. DEANE, AND G. GRIMALDI, JR. 1996. Enzyme polymorphism in *Endotrypanum* and numerical analysis of isoenzyme data. Parasitology **113:** 39–48.

FROMMEL, D., B. W. OGUNKOLADE, I. VOULDOUKIS, AND L. MOUNJOUR. 1988. Vaccine induced immunity against cutaneous leishmaniasis in Balb/c mice. Infection and Immunity **56:** 843–848.

GRIMALDI, JR., G., H. MOMEN, R. D. NAIFF, D. MCMAHON-PRATT, AND T. B. BARRETT. 1991. Characterization and classification of leishmanial parasites from humans, wild mammals, and sandflies in the Amazon Region, Brazil. American Journal of Tropical Medicine and Hygiene **44:** 645–661.

HANDMAN, E. 1983. Association of serum proteins with cultured Leishmania. Parasite Immunology **5:** 109–12.

———. 1999. Cell biology of *Leishmania*. Advances in Parasitology **44:** 1–39.

———, AND G. F. MITCHELL. 1985. Immunization with *Leishmania* receptors for macrophages protects mice against cutaneous leishmaniasis. Proceedings of the National Academy of Sciences of the United States of America **82:** 5910–5914.

HO, M., J. LEEUWENBURG, G. M. BUGUA, A. WAMACHI, AND A. VOLLER. 1983. An enzyme-linked immunosorbent assay (ELISA) for field diagnosis of visceral leishmaniasis. American Journal of Tropical Medicine and Hygiene **32:** 943–946.

LAGRANGE, P. H. 1977. Comparative studies of different strains of BCG vaccine in mice: T-cell dependent immune responses. Developments in Biological Standardization **38:** 223–229.

LIEW, F. Y., AND C. A. O'DONNEL. 1993. Immunology of Leishmaniasis. Advances in Parasitology **32:** 161–259.

LIMONCU, M. E., I. C. BALCIOGLU, K. YERELI, Y. OZBEL, AND A. OZBILGIN. 1997. A new experimental in vitro culture medium for cultivation of *Leishmania* species. Journal of Clinical Microbiology **35:** 2430–2431.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry **193:** 265–275.

LUCAS, C. M., E. D. FRANKE, M. I. CACHAY, A. TEJADA, D. CARRIZALES, AND R. D. KREUTZER. 1994. *Leishmania* (*Viannia*) *lainsoni*: First isolation in Peru. American Journal of Tropical Medicine and Hygiene **51:** 533–537.

———, ———, ———, ———, M. E. CRUZ, R. D. KRUTZER, D. C. BARKER, S. H. MCCANN, AND D. M. WATTS. 1998. Geographic distribution and clinical description of leishmaniasis cases in Peru. American Journal of Tropical Medicine and Hygiene **59:** 312–317.

MARTINEZ, E., F. LE PONT, S. MOLLINEDO, AND E. CUPOLILLO. 2001. A first case of cutaneous leishmaniasis due to *Leishmania* (*Viannia*) *lainsoni* in Bolivia. Transactions of the Royal Society of Tropical Medicine and Hygiene **95:** 375–377.

MENEZES, H. 1968. Histological lesions in vaccinated mice with a no virulent strain of *Trypanosoma cruzi*. Revista Brasileira de Medicina **25:** 160–165.

MITCHELL, G., E. HANDMAN, AND H. SPITHILL. 1985. Examination of variable in the vaccination of mice against cutaneous leishmaniasis using living avirulent cloned lines and killed promastigotes of *Leishmania major*. International Journal for Parasitology **15:** 667–684.

MODDABER, F. 1989. Experiences with vaccines against cutaneous leishmaniasis of men and mice. Parasitology **98:** 549–560.

MORA, A. M., W. MAYRINK, R. T. DA COSTA, C. A. DA COSTA, O. GENARO, AND E. NASCIMENTO. 1999. Protection of C57BL/10 mice by vaccination with association of purified proteins from *Leishmania* (*Leishmania*) *amazonensis*. Revista do Instituto de Medicina Tropical de São Paulo **41:** 243–248.

NOLAN T. J., AND R. HERMAN. 1985. Effects of long-term in vitro cultivation on *Leishmania donovani* promastigotes. Journal of Protozoology **32:** 70–75.

NORONHA, F. S., F. J. RAMALHO-PINTO, AND M. F. HORTA. 1996. Cytolytic activity in the genus *Leishmania*: involvement of a putative pore-forming protein. Infection and Immunity **64:** 3975–3982.

OZBILGIN, A., Y. OZBEL, M. Z. ALKAN, M. ATAMBAY, AND M. A. OZCEL. 1995. Cultivation of *Leishmania* sp. in nutrient broth. Journal of the Egyptian Society of Parasitology **25:** 437–441.

RIVER, D., P. BOVAY, R. SHAH, S. DIDISHEIM, AND J. MAUEL. 1999. Vaccination against *Leishmania major* in CBA mouse model of infection: Role of adjuvants and mechanism of protection. Parasite Immunology **21:** 461–473.

RUSSELL, D., AND J. ALEXANDER. 1988. Effective immunization against cutaneous leishmaniasis with defined membrane antigens reconstituted into liposomes. Journal of Immunology **140:** 1272–1279.

SILVEIRA, F. T., J. J. SHAW, R. R. BRAGA, AND E. ISHIKAWA. 1987. Dermal leishmaniasis in the Amazon region of Brazil: *Leishmania* (*Viannia*) *lainsoni* sp. n., a new parasite from the state of Pará. Memórias do Instituto Oswaldo Cruz **82:** 289–291.

———, ———, ———, ———, AND A. A. SOUZA. 1991. Leishmaniose cutânea na Amazônia: Isolamento de *Leishmania* (*Viannia*) *lainsoni* do roedor *Agouti paca* (Rodentia: Dasyproctidae), no Estado do Pará, Brasil. Revista do Instituto de Medicina Tropical de São Paulo **26:** 272–275.

———, A. A. A. SOUZA, R. LAINSON, J. J. SHAW, R. R. BRAGA, AND E. A. ISHIKAWA. 1991. Cutaneous leishmaniasis in the Amazon region: Natural infection of the sandfly *Lutzomyia ubiquitalis* (Psychodidae: Phlebotominae) by *Leishmania* (*Viannia*) *lainsoni* in Pará state, Brazil. Memórias do Instituto Oswaldo Cruz **86**: 127–130.

SPÄTH, G. F., AND S. M. BEVERLEY. 2001. A lipophosphoglycan-independent method for isolation of infective *Leishmania* metacyclic promastigotes by density gradient centrifugation. Experimental Parasitology **99:** 97–103.