Testing of Four *Leishmania* Vaccine Candidates in a Mouse Model of Infection with *Leishmania* (Viannia) *braziliensis*, the Main Causative Agent of Cutaneous Leishmaniasis in the New World\(^7\)

G. Salay,\(^1,2\) M. L. Dorta,\(^1,3\) N. M. Santos,\(^1,2\) R. A. Mortara,\(^2\) C. Brodsky,\(^4,5\) C. I. Oliveira,\(^4\) C. L. Barbiéri,\(^2\) and M. M. Rodrigues\(^{1,2*}\)

Centro Interdisciplinar de Terapia Gênica, Universidade Federal de São Paulo–Escola Paulista de Medicina, Rua Mirassol, 207, São Paulo, SP 04044-010, Brazil;\(^1\) Disciplina de Parasitologia–Departamento de Microbiologia, Immunologia e Parasitologia, Universidade Federal de São Paulo–Escola Paulista de Medicina, Rua Botucatu, 862, 6º Andar, São Paulo, SP 04023-062, Brazil;\(^2\) Disciplina de Imunologia–Departamento de Microbiologia Imunonologia e Parasitologia, Universidade Federal de Goiânia, Goiânia, Brazil;\(^3\) Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121, Salvador, BA 40295-001, Brazil;\(^4\) and Departamento de Biointeração, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Av. Reitor Miguel Calmon, S/N, Salvador, BA 40110-160, Brazil\(^5\).

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We evaluated whether four recombinant antigens previously used for vaccination against experimental infection with *Leishmania* (*Leishmania*) *major* could also induce protective immunity against a challenge with *Leishmania* (Viannia) *braziliensis*, the species responsible for 90% of the 28,712 annual cases of cutaneous and mucocutaneous leishmaniasis recorded in Brazil during the year of 2004. Initially, we isolated the homolog genes encoding four *L. (V.) braziliensis* antigens: (i) homologue of receptor for activated C kinase, (ii) thiol-specific antioxidant, (iii) *Leishmania* elongation and initiation factor, and (iv) *L. (L.) major* stress-inducible protein 1. At the deduced amino acid level, all four open reading frames had a high degree of identity with the previously described genes of *L. (L.) major* being expressed on promastigotes and amastigotes of *L. (V.) braziliensis*. These genes were inserted into the vector pcDNA3 or expressed as bacterial recombinant proteins. After immunization with recombinant plasmids or proteins, BALB/c mice generated specific antibody or cell-mediated immune responses (gamma interferon production). After an intradermal challenge with *L. (V.) braziliensis* infective promastigotes, no significant reduction on the lesions was detected. We conclude that the protective immunity afforded by these four vaccine candidates against experimental cutaneous leishmaniasis caused by *L. (L.) major* could not be reproduced against a challenge with *L. (V.) braziliensis*. Although negative, we consider our results important since they suggest that studies aimed at the development of an effective vaccine against *L. (V.) braziliensis*, the main causative agent of cutaneous leishmaniasis in the New World, should be redirected toward distinct antigens or different vaccination strategies.

The protozoan parasite *Leishmania* (Viannia) *braziliensis* commonly causes localized cutaneous leishmaniasis (CL); however, a chronic mucosal leishmaniasis (ML) may develop in some infected individuals, with severe and progressive manifestations (reviewed in reference 11). At present, this species accounts for more than 90% of the 28,712 annual cases of CL and ML recorded in Brazil in 2004 (7). Although chemotherapies for CL and ML do exist, there are several limitations: (i) drug treatment is rarely affordable by those who need them, (ii) drug treatment requires daily injections of the drug for weeks, (iii) drug treatment can be associated with side effects, and (iv) drug resistance is becoming an increasing problem (13, 24; reviewed in references 3 and 32). To make matters worse, control of CL and ML is problematic due to the sylvatic nature of both vectors and reservoirs, making insecticide spraying and the elimination of reservoirs particularly difficult (26). Due to these difficulties, in the long run, the development of an effective vaccine may help both the prevention and the treatment of CL and ML caused by *L. (V.) braziliensis*.

Most studies conducted thus far in vaccination against CL used genes and/or antigens isolated and characterized from *Leishmania* (Leishmania) *major* or *L. (L.) amazonensis* (8, 31; reviewed in references 9 and 21). Among the leading candidates to the development of a vaccine against CL, there are homologues of the receptor for activated C kinase protein (LACK or p36), *Leishmania* elongation and initiation factor (LeIF), *L. (L.) major* stress inducible protein 1 (LmSTI1), and thiol-specific antioxidant (TSA) from *L. (L.) major* (5, 6, 10, 14, 15, 28, and 30).

Based on these promising prospects, the present study was designed to test whether four recombinant antigens previously used for vaccination against experimental infection with *L. (L.) major* (LACK, LmSTI1, LeIF, and TSA) could also generate protective immunity against an intradermal (i.d.) challenge with *L. (V.) braziliensis*. We considered this question very important because, as mentioned above, this species is responsible for most cases of CL and ML in Brazil and in the New World.

\(^*\) Corresponding author. Mailing address: CINTERGEN–Centro Interdisciplinar de Terapia Gênica, UNIFESP–EPM, Rua Mirassol, 207, São Paulo, SP 04044-010, Brazil. Phone: 55-11-5084-8807. Fax: 55-11-5574-5071. E-mail: mmrodrigues@ecb.epm.br.

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MATERIALS AND METHODS
Parasites and mice. L. (V.) braziliensis (MHOM/BR/1975/M2903 and MHOM/BR/01/BA788) and L. (L.) major (Friedlin strain) promastigotes were generated at 26°C in 199 medium (Life Technologies) supplemented with 40 mM HEPES, 0.1 mM adenine, 2 mM l-glutamine, 5 mg of hemin/ml (in 50% triethanolamine), 100 U of penicillin/ml, 100 mg of streptomycin/ml, and 10% heat-inactivated fetal bovine serum (all from Life Technologies). To L. (V.) braziliensis promastigote culture was added 2% of sterile human male urine. The parasites were isolated from stationary phase in culture (5 to 6 days old). L. (V.) braziliensis amastigotes were obtained from cultured mouse bone marrow–derived macrophages (1). Amastigote suspensions of L. (V.) braziliensis were prepared by disruption of infected macrophages scraped from the flask 48 h after infection with promastigotes. These cells were ruptured through a 22-gauge needle and were centri- fuged at 250 × g for 10 min; the resulting supernatant was centrifuged at 1,400 × g for 10 min, and the pellet was resuspended in RPMI. The DNA was purified with GeneClean II kit (Bio 101). The products were purified from pMOSBlue vector by treatment with the specific restriction enzymes (2). Amastigotes suspensions of L. (V.) braziliensis were fixed for 40 min in 3.5% paraformaldehyde–PBS and washed three times with cold PBS, and 10 μl was injected into the ear dermis of each mouse. The infection was assessed at 3 weeks postinfection by counting the number of amastigotes per 2 × 2 mm square area (20).

To perform the indirect immunofluorescence assay (IIA), BALB/c mice were challenged with Trypanosoma cruzi (20). Briefly, cultured promastigote forms of T. cruzi were added to a culture of mouse peritoneal cells. After 48 h, the culture medium was replaced by fresh medium, and the cell culture was incubated for an additional 24 h. The cell culture was then washed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at 4°C. The coverslips were then washed three times with PBS and permeabilized with 0.1% saponin (BDH; Amersham, United Kingdom) in 0.2× phosphate-buffered saline (PBS). Coverslips were then washed three times with PBS. Slides containing promastigotes or amastigote-infected macrophages were blocked with PBS containing 2% gelatin and 0.1% NaN3 and then incubated with different sera from mice immunized with recombinant proteins (LACK, TSA, LbSTI, or LeIF) diluted to appropriate dilutions in 10% FBS/PBS. The slides were then incubated with an appropriate secondary antibody (1:200 dilutions in 10% FBS/PBS). The slides were then washed three times with PBS and incubated with DAPI (1 μg/ml) for 10 min. Coverslips were then mounted using Vectorshield mounting medium (Vector Laboratories) and analyzed using a Nikon TE2000 microscope.

H. sapiens sera. To test for reactivity of sera from healthy volunteers, we used sera from five volunteers, each from a different country. Human sera were inactivated at 56°C for 30 min and then centrifuged at 2,000 × g for 10 min. The supernatants were collected and stored at −20°C until use.

Immunizations. To perform the indirect immunofluorescence assay (IIA), BALB/c mice were injected in the hind footpad (subcutaneously) with 10 μg of each purified recombinant protein (His6-LACK, His6-TSA, His6-LbSTI, and His6-LeIF) in Freund complete adjuvant (CFA). After 2 weeks the mice were injected at a location 1 cm distal to the ramp with 10 μg of each recombinant protein with incomplete Freund adjuvant (IFA). Mice were bled with 7 days after the first dose and 10 days after the last dose. The plasma fraction was obtained and stored at −20°C.

DNA immunization was performed as described earlier (4), using the recombinant DNA plasmids either alone or together. Empty pcDNA3 plasmid was used as a control. Both tibial anterioris muscles were injected with 3.5 μg of cardiotin (Sigma). Five days later, 50 μg of plasmid DNA was injected intramuscularly at the same sites as for the cardiotin injection (a total of 100 μg of plasmid DNA per mouse). The subsequent doses consisted of the same amount of plasmid DNA injected 2 and 4 weeks after the first immunizing dose.

The protocol for immunization with the recombinant proteins was performed by the intraperitoneal administration of 25 μg of each purified protein in the presence of CpG ODN 1826 (10 μg/dose/mouse; Coley Pharmaceuticals, Welle- sley, MA) and 2% liposomes (50 μg of liposomes/ml; Sigma). The total volume of each injection was 100 μl. The animals were bled with 7 days after the first dose and 10 days after the last dose. The plasma fraction was obtained and stored at −20°C.

The immunization protocol before the i.d. challenge consisted of two doses of the recombinant DNA plasmids followed by one or two doses of the purified recombinant proteins. At 2 weeks after the last dose, mice were challenged with promastigotes of L. (V.) braziliensis. BALB/c mice were challenged i.d. as described previously (20). Briefly, cultured promastigotes forms of L. (V.) braziliensis (MHOM/BR/01/BA788) were adjusted to a concentration of 107 parasites/ml in PBS, and 10 μl was injected in the ear dermis of each mouse. The infection was monitored for 9 to 10 weeks, and lesion formation was measured once a week with a direct caliper.

III. Macrophages (105 cells/coverslip) were infected with stationary promas- tigotes of L. (V.) braziliensis (at a 10:1 parasite/cell ratio); 48 h later the coverslips were used in IIA. Promastigotes of L. (V.) braziliensis or infected macrophages were fixed for 40 min in 3.5% paraformaldehyde–PBS and washed three times with PBS, and the solution was adjusted to a concentration of 107 parasites/ml per ml. Portions (10 μl) of the parasite suspension were layered onto round, coated coverslips and allowed to stand overnight at room temperature. Coverslips were then washed three times with PBS and the solution was adjusted to a concentration of 107 parasites/ml per ml. Portions (10 μl) of the parasite suspension were layered onto round, coated coverslips and allowed to stand overnight at room temperature. Coverslips were then washed three times with PBS and the solution was adjusted to a concentration of 107 parasites/ml per ml. Portions (10 μl) of the parasite suspension were layered onto round, coated coverslips and allowed to stand overnight at room temperature. Coverslips were then washed three times with PBS and the solution was adjusted to a concentration of 107 parasites/ml per ml. Portions (10 μl) of the parasite suspension were layered onto round, coated coverslips and allowed to stand overnight at room temperature.
TABLE 1. Comparison of the predicted amino acids sequences of the genes lack, lbsti1, lef, and tsa cloned from L. (V.) braziliensis or other species of Leishmania

| Gene       | L. (V.) braziliensis* | L. (L.) major | L. (L.) amazonensis | L. (L.) chagasi | L. (L.) donovani |
|------------|----------------------|---------------|---------------------|----------------|-----------------|
| lack       | 43, 58, 99, 115, 148, 183, 231, 237, 254, 256, 277 | 31, 43, 58, 99, 148, 183, 231, 237, 254, 256, 277 | 43, 58, 99, 148, 183, 231, 237, 254, 256, 277 | 43, 48, 99, 183, 231, 237, 254, 256, 277 |
| tsa        | NA                   | 8, 14, 18, 32, 56, 58, 63, 64, 70, 74, 75, 78, 91, 99, 101, 111, 113, 115, 120, 122, 123, 136, 138, 144, 152, 181, 182, 188, 189, 191, 192 | NA | 5, 8, 10, 18, 33, 35, 58, 61, 62, 63, 64, 70, 74, 75, 78, 91, 99, 101, 111, 113, 120, 123, 136, 138, 139, 144, 181, 182, 188, 189, 191, 192 |
| lef        | 363                  | 2, 4, 6, 7, 125, 149, 384 | NA                   | NA | 61, 69, 100, 106, 158–220*, 251, 271, 287, 310, 312, 316, 379 |
| sti1       | NA                   | 60, 68, 98, 158–220*, 250 | 254, 270, 310, 314, 530 | NA | NA |

* The positions of amino acid residue substitutions are based on the predicted amino acid sequences obtained by CLUSTAL W alignments of the lack, tsa, lbsti1, and lef genes from different species of Leishmania. + predicted amino acids between positions 158 and 220 of sti1 (for details, see Fig. 1).

The L. (V.) braziliensis lack and lef genes had already been cloned from different parasitic strains. NA, gene sequence not available in GenBank.

RESULTS

Identification and characterization of the homolog genes lack, lbsti1, lef, and tsa from L. (V.) braziliensis. The deduced amino acid sequences of the different genes isolated from L. (V.) braziliensis were compared to previously described genes of Leishmania. The deduced amino acid sequence of the lack ORF was almost identical to the published sequence of L. (V.) braziliensis, being different in only 11 amino acids, four of them with conservatives modifications (Table 1). The previously described immunodominant T-cell epitope recognized by CD4+ T cells from H-2d mice (amino acids 156 to 173) was invariant (19).

The tsa gene cloned from L. (V.) braziliensis had not been previously published. Comparing the complete deduced amino acid sequence from L. (V.) braziliensis to the L. (L.) major sequence (35), we observed 83% of identity with 32 amino acid substitutions, with 17 being conservative. Also, the loss of two amino acid residues was observed in the L. (V.) braziliensis sequence (Fig. 1).

The lef gene cloned from L. (V.) braziliensis had the amino- and the carboxy-terminal regions highly conserved compared to the same gene from L. (L.) major, with only a few substitutions (Table 1) (34, 36). Interestingly, within the region comprising the amino acids 158 to 220 there are several amino acid modifications. In this region, there are 30 different amino acids, with seven conservative modifications. Also, the loss of two amino acid residues was observed in the L. (V.) braziliensis sequence (Fig. 1).

The lack ORF cloned from L. (V.) braziliensis had the amino- and the carboxy-terminal regions highly conserved compared to the same gene from L. (L.) major, with only a few substitutions (Table 1) (34, 36). Interestingly, within the region comprising the amino acids 158 to 220 there are several amino acid modifications. In this region, there are 30 different amino acids, with seven conservative modifications. Also, the loss of two amino acid residues was observed in the L. (V.) braziliensis sequence (Fig. 1).
motifs described previously as characteristic of the DEAD box family of RNA helicases (29).

Expression of lack-, tsa-, Lbsti-, and leif-coding ORFs during the L. (V.) braziliensis life cycle. In order to characterize the transcripts expressed by the different forms of L. (V.) braziliensis, Northern blotting was performed with total RNA that was hybridized with the coding ORFs of lack, tsa, Lbsti, leif, and \( \alpha \)-tubulin (positive control) (12). As shown in Fig. 2, both life stages of L. (V.) braziliensis and promastigotes of L. (L.) major (positive control) displayed bands corresponding to transcript sizes of approximately 1.5 to 2.0 kb (lack, Fig. 2A), 2.0 kb (tsa, Fig. 2B), 2.5 to 3.0 kb (leif, Fig. 2C), and 2.5 kb (lbsti1, Fig. 2D). The hybridization was specific for the parasite RNA, since the RNA from uninfected macrophages failed to hybridize with lack, tsa, Lbsti, and leif probes. All RNA samples from the different forms of the parasite and control uninfected macrophages hybridized with \( \alpha \)-tubulin probe used as positive controls (Fig. 2E).

IIA for detection of LACK, TSA, LbSTI1, and LeIF antigens on amastigote and promastigote forms of L. (V.) braziliensis. The coding ORFs of lack, tsa, Lbsti, or leif were subcloned in the pET22(+) vector, and the recombinant protein was expressed and purified as described in Materials and Methods. Figure 3 shows the SDS-PAGE analyses of the purified recombinant proteins. Each protein migrated as a single band (except the LeIF protein) and according to the expected molecular mass. To determine whether these four antigens were in fact expressed by promastigotes and amastigotes of L. (V.) braziliensis, we generated specific polyclonal antibodies to the different antigens. By IIA, it was possible to determine that promastigote forms of L. (V.) braziliensis (MHOM/BR/1975/M2903 strain) were specifically recognized by antibodies from mice immunized with each recombinant protein (Fig. 4). Antibody recognition was specific to the recombinant proteins because sera from mice immunized with CFA or IFA alone failed to react with promastigotes (Fig. 4J). Immunoblot analysis of the parasite extract confirmed that each antigen was recognized by the specific immune sera raised by mouse recombinant protein immunization (Fig. 4M).

Subsequently, we performed IIA analysis with macrophages infected with L. (V.) braziliensis (MHOM/BR/1975/M2903 strain) as substrates. Amastigotes were specifically recognized by antibodies from mice immunized with each recombinant

!!TABLE!!

| ORF  | L. (L.) major promastigotes | L. (V.) braziliensis promastigotes | L. (V.) braziliensis amastigotes | Macrophages |
|------|-----------------------------|----------------------------------|-------------------------------|-------------|
| lack | -                           | 1                                | 3                             | 4           |
| tsa  | 1                           | 2                                | 3                             | 4           |
| leif | 1                           | 2                                | 3                             | 4           |
| lbsti1 | -                           | 1                                | 3                             | 4           |

FIG. 2. RNA expression of lack, tsa, leif, or Lbsti1 genes from L. (V.) braziliensis. Northern blot analysis was performed with lack (A), tsa (B), leif (C), Lbsti (D), and \( \alpha \)-tubulin (E) (12) ORFs as probes. Each lane contains 10 \( \mu \)g of total RNA of promastigotes of L. (L.) major (lane 1, positive control), promastigotes (lane 2) or amastigotes (lane 3) of L. (V.) braziliensis, or uninfected cultured macrophages (lane 4, negative control)/lane.
Antibody recognition was specific to the recombinant proteins because sera from mice immunized with CFA alone failed to react with control macrophages (data not shown) and infected macrophages (Fig. 5R). In order to visualize the amastigotes clearly, these infected macrophages were costained with sera from humans infected with L. (V.) braziliensis.

Immune response generated by immunization with recombinant antigens from L. (V.) braziliensis. To characterize the immune response generated by different immunization strategies using the L. (V.) braziliensis antigens, we immunized mice with plasmids encoding each antigen or the respective recombinant proteins. Recombinant proteins were administered in the presence of CpG ODN 1826 and alum.

In BALB/c mice immunized with each plasmid DNA individually or a combination of all plasmids we only observed protein (Fig. 5).
specific antibodies to the LbSTI1 antigen. This immune response was present in mice immunized with the *lbsti1* gene or all of the genes together (Fig. 6A). In both cases, a predominance of IgG2a over IgG1 was observed. The ratios of IgG1 to IgG2a were 0.93 and 0.76 in mice immunized with the *lbsti1* gene or all genes together, respectively. These immune sera were also tested by IIA. We observed that sera from DNA vaccinated mice failed to recognize promastigotes (data not shown).

In BALB/c mice immunized with each recombinant protein individually or a combination of all of them we detected high titers of antibodies to the different antigens in each group of immunized mice (Fig. 6B). The ratios of IgG1 to IgG2a for each mouse group were as follows: 1.3, LACK; 0.92, TSA; 1.18, LeIF; 1.45, LbSTI1; and 1.48, all antigens. These immune sera were also tested by IIA. We observed that sera from DNA vaccinated mice failed to recognize promastigotes (data not shown).

In BALB/c mice immunized with each recombinant protein individually or a combination of all of them we detected high titers of antibodies to the different antigens in each group of immunized mice (Fig. 6B). The ratios of IgG1 to IgG2a for each mouse group were as follows: 1.3, LACK; 0.92, TSA; 1.18, LeIF; 1.45, LbSTI1; and 1.48, all antigens. These immune sera were also tested by IIA. We observed that sera from DNA vaccinated mice strongly recognized promastigotes (data not shown).

We also measured the IFN-γ produced by spleen cells from mice immunized with the different recombinant antigens as plasmid DNA or recombinant proteins. Upon in vitro restimulation with recombinant proteins, we detected antigen-specific IFN-γ production in cultured spleen cells from most groups of mice immunized with the distinct antigen either as plasmid DNA or recombinant protein (Fig. 6B and D). However, after antigenic stimulation in vitro, spleen cells from mice immunized with the *tsa* gene or the LbSTI1 recombinant protein failed to produce levels of IFN-γ that were significantly different from those of control cultures (Fig. 7A and C). The antigen-specific IFN-γ production was dependent on the immunization with the *Leishmania* antigens since spleen cells from control mice immunized with pcDNA3 (Fig. 7A) or CpG ODN 1826 and alum (Fig. 7C) failed to secrete this cytokine in the presence of the different recombinant antigens.

Immunization with *L. (V.) braziliensis* antigens failed to elicit protective immunity against experimental infection in BALB/c mice. To determine whether immunization with any of these genes or antigens alone or in combination could elicit protective immunity against experimental infection, BALB/c mice were immunized twice with plasmid DNA, followed by one or two doses of recombinant protein in the presence CpG ODN 1826 and alum. The immune sera of these mice were tested by IIA. We observed that sera from vaccinated mice recognized promastigotes (data not shown).

Two weeks after the last dose, mice were then challenged with infective promastigotes of *L. (V.) braziliensis* (MHOM/BR/01/BA788 strain) in the dermis in both ears. We monitored the nodule formation for 9 or 10 weeks. We did not observe a

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**FIG. 5.** IIA recognition of amastigotes of *L. (V.) braziliensis* by immune sera from mice immunized with recombinant proteins. Mouse bone marrow macrophages infected with amastigotes of *L. (V.) braziliensis* were incubated with mouse antibodies specific to LACK (A to D), TSA (E to H), LeIF (I to L), or LbSTI1 (M to P). Additional slides were stained with negative (Q to T) or positive (U to X) control sera. All slides were costained with a pool of sera from humans infected with ML and with DAPI. Secondary antibodies to mouse IgG or human IgG were labeled with rhodamine (red fluorescence) or anti-FITC (green fluorescence), respectively. The images were captured by using Nomarski differential interference contrast (A, E, I, M, Q, and U) or fluorescence for rhodamine (B, F, J, N, R, and V) or FITC (C, G, R, O, S, and W). Fluorescence images for rhodamine, FITC, and DAPI (blue) were merged in panels D, H, L, P, T, and X. Magnification, ×1,000. Negative control sera were obtained from mice immunized with CFA or IFA alone.
protective immune response as expressed by a significant reduction in the lesion size in immunized mice compared to the control group that received control plasmid followed by adjuvant (Fig. 8).

**DISCUSSION**

The present study was designed to evaluate the vaccination potential of four *L. (V.) braziliensis* genes or antigens (LACK, LbSTI1, LeIF, and TSA) in a newly developed experimental model of infection (20). These four genes or antigens were selected based on previous successful vaccination against experimental infection in BALB/c mice with *L. (L.) major* (reviewed in references 9 and 21). Initially, we cloned these four genes from genomic DNA of *L. (V.) braziliensis*. Deduced amino acid sequence analysis of each one of these genes showed that they are quite conserved. Nevertheless, *lbsti1* ORF presented a clear polymorphism compared to *L. (L.) major* and *L. (L.) donovani* ORFs in the region spanning amino acids 158 to 220 (Fig. 1). The significance of this polymorphism should be further evaluated in the future.

Subsequently, we characterized whether these genes or antigens were expressed on the different forms of *L. (V.) braziliensis*. We provided evidence that all four genes or antigens were expressed on promastigote and amastigote forms. Based on the fact that these antigens were expressed in the intracellular forms of *L. (V.) braziliensis*, we performed immunization studies with either recombinant plasmids or bacterial recombinant proteins. We found that plasmid immunization failed to induce an antibody response except after immunization with the gene *lbsti1*. Nevertheless, IFN-γ-producing spleen cells were generated after individual immunization with three of the
C57BL/6 mice with the different plasmids individually or all challenge.

any significant reduction in the lesion size up to 10 weeks after infective promastigotes, we were unable to observe braziliensis ogous prime-boost immunization with these genes or antigens (33). In spite of the immune responses induced by a heterol-
celled protocol consisting of priming with plasmid DNA fol-
tioned protective immunity capable of significantly reduce the lesion size after a footpad challenge with L. (L.) major (data not shown). Similarly, Melby et al. (16) showed that vaccination with the lack gene induced potent immune responses but failed to provide protective immunity against an experimental challenge with L. (L.) donovani (visceral leishmaniasis). Based on that finding, we considered that the biological differences between L. (L.) major, L. (V.) braziliensis, and L. (L.) donovani may account for the inability to elicit protective immune responses against a challenge with the last two.

In summary, although our results are essentially negative in terms of the induction of protective immunity, we consider it important because it suggests that the research efforts aimed at the development of a recombinant vaccine against L. (V.) braziliensis should be redirected toward different parasite antigens or different vaccination strategies.

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