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Enhancement of Experimental Cutaneous Leishmaniasis by *Leishmania* Molecules Is Dependent on Interleukin-4, Serine Protease/Esterase Activity, and Parasite and Host Genetic Backgrounds▽

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Most inbred strains of mice, like the BALB/c strain, are susceptible to *Leishmania amazonensis* infections and resistant to *Leishmania braziliensis* infections. This parasite-related difference could result from the activity of an *L. amazonensis*-specific virulence factor. In agreement with this hypothesis, it is shown here that the intravenous injection of BALB/c mice with *L. amazonensis* amastigote extract (*La*E) but not the *L. braziliensis* extract confers susceptibility to *L. braziliensis* infection. This effect was associated with high circulating levels of IgG1 anti-*L. amazonensis* antibodies and with an increase in interleukin-4 (IL-4) production and a decrease in gamma interferon production by draining lymph node cells. Moreover, the effect was absent in IL-4-knockout mice. The biological activity in the *La*E was not mediated by amphiphilic molecules and was inhibited by pretreatment of the extract with irreversible serine protease inhibitors. These findings indicate that the *La*E contains a virulence-related factor that (i) enhances the *Leishmania* infection by promoting Th2-type immune responses, (ii) is not one of the immunomodulatory *Leishmania* molecules described so far, and (iii) is either a serine protease or has an effect that depends on that protease activity. In addition to being *Leishmania* species specific, the infection-enhancing activity was also shown to depend on the host genetic makeup, as *La*E injections did not affect the susceptibility of C57BL/6 mice to *L. braziliensis* infection. The identification of *Leishmania* molecules with infection-enhancing activity could be important for the development of a vaccine, since the up- or downmodulation of the immune response against a virulence factor could well contribute to controlling the infection.

Leishmaniasis is expanding both in the number of reported human cases and in the area and number of regions in which it is endemic (3, 4). Infection by *Leishmania* may either be asymptomatic or give rise to different forms of tegumentary and visceral diseases, depending on the *Leishmania* species and/or on the genetic/immunologic status of the host (21). Localized cutaneous leishmaniasis is the most common manifestation of *Leishmania* infection in the Americas. In Brazil, it is most often caused by *Leishmania braziliensis* and is less frequently caused by *Leishmania amazonensis* (16). It usually consists of skin lesions that self-heal within a few months (localized cutaneous leishmaniasis) (12). In a small percentage of cases, however, it may evolve either into disseminated leishmaniasis, which is characterized by a large number of acneiform, papular, nodular, and ulcerated lesions (9, 35), or, usually when it is caused by *L. braziliensis*, into mucosal leishmaniasis, which is marked by mutilating nasal and/or oral lesions (12) and an intense Th1-dependent inflammatory reaction (11). Diffuse cutaneous leishmaniasis, on the other hand, is mostly associated with *L. amazonensis* infection in Brazil and is characterized by (i) disseminated, nonulcerating nodular skin lesions which resemble those of lepromatous leprosy, (ii) refractoriness to treatment, and (iii) a specific lack of detectable anti-*Leishmania* Th1-type immune responses (12, 22).

The study of murine experimental models of leishmaniasis has contributed to the clarification of the role played by $CD4^+$ T-cell subpopulations in host susceptibility and resistance to *Leishmania* (19, 30) and, indeed, to the understanding of the immune response in general (8). As with humans, different species of *Leishmania* cause different murine diseases, depending also on the genetics of the mouse. Most inbred strains of mice, such as the syngeneic BALB/c strain, are susceptible to *L. amazonensis* infection, developing chronic nonhealing skin lesions and having a predominantly Th2-type, ineffective immune response (13). In contrast, *L. braziliensis* usually induces only a transient cutaneous disease in BALB/c mice and in most other mouse strains (10). The relative resistance of BALB/c mice to *L. braziliensis*-induced disease has been attributed to the activity of the gamma interferon (IFN-γ) produced during a Th1-type immune response. This Th1 immune response would not be downmodulated by the low levels of interleukin-4 (IL-4) observed to be produced (10).

Since in both mice and some humans (those who develop diffuse cutaneous leishmaniasis) *L. amazonensis* causes infections with much higher parasite loads than those in infections caused by *L. braziliensis*, leading to progressive diseases asso-

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ciated with a reduced or absent parasite-specific Th1 immune response (10, 12, 13), it is possible that the two *Leishmania* species differ, in that *L. amazonensis*, but not *L. braziliensis*, produces one or more immunoregulatory virulence factors. The identification of these putative virulence factors may well be translated into therapeutic or prophylactic procedures to deal with severe cases of *L. amazonensis*-induced disease. Moreover, most functional investigations on *Leishmania*-derived proteins have focused on molecules from the promastigote stage of the parasite, notwithstanding the fact that amastigotes are the forms that spread the infection in the vertebrate host (6). In this work, the existence of an *L. amazonensis*-specific amastigote factor that both increases the parasite burden and changes the nature of the immune response in *L. braziliensis*-infected mice is demonstrated. Moreover, the infection-enhancing activity was shown to depend on the presence of a parasite-derived serine protease activity and of host IL-4 and on the host genetic background.

MATERIALS AND METHODS

Mice. Specific-pathogen-free, 8- to 12-week-old, male, wild-type, or IL-$4^{-/-}$ BALB/c mice and wild-type C57BL/6 mice were maintained at the animal facilities of the Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Salvador, Brazil, and were provided with rodent diet and water *ad libitum*. All procedures were approved and conducted according to the institutional Committee for Animal Care and Utilization.

Parasites, parasite extracts, and extract fractions. The *L. amazonensis* MHOM/Br87/Ba125 and *L. braziliensis* MHOM/Br/3456 strains were used. Their infectivities were maintained by regular inoculations of promastigotes into susceptible BALB/c mice and golden hamsters, respectively. Promastigotes, derived from tissue amastigotes, were cultured at 23°C in Schneider's medium (Sigma Chemical Co., St. Louis, MO), pH 7.2, supplemented with 50 μg/ml of gentamicin and 10% heat-inactivated fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY) for *L. amazonensis* or 20% FBS for *L. braziliensis*. *L. amazonensis* and *L. braziliensis* axenic amastigotes were obtained by the differentiation of promastigotes in axenic cultures, as described elsewhere (34). The amastigotes were washed three times in ice-cold sterile saline, resuspended in saline, and lysed by exposure to ultrasound (10 1-min, 300-W pulses, with 30-s intervals in between, on ice; Sonifier cell disruptor; Branson Sonic Power Company, Danbury, CT). The lysates were centrifuged at 16,000 × *g* for 10 min at 4°C, and the supernatants were filtered on membranes with 0.22-μm-diameter pores (Millipore, São Paulo, Brazil) and immediately stored at −70°C in aliquots. In this report, these filtered saline supernatants are called *L. braziliensis* extract and *L. amazonensis* extract (*La*E). They were shown to be free of bacterial endotoxin by the *Limulus* amebocyte enzyme assay (Biowhittaker, Walkersville, MD), and their protein content was determined by the method of Lowry et al. (18). Extracts from stationary-phase promastigotes were prepared in the same manner. Aliquots of the prepared *La*E were supplemented with (i) a mixture of three serine protease inhibitors (5.7 mM *N*-*p*-tosyl-L-phenylalanyl chloromethyl ketone [TPCK], 5.4 mM Nα-tosyl-L-lysine chloromethyl ketone hydrochloride [TLCK], and 5.9 mM 4-nitrophenyl 4-guanidinobenzoate hydrochloride [NPGB]) and a protease inhibitor with both serine and cysteine protease activities (10 mM phenylmethylsulfonyl fluoride [PMSF; Sigma Chemical Co.]), (ii) a mixture of two serine protease inhibitors (5.7 mM TPCK and 5.4 mM TLCK), (iii) a cysteine protease inhibitor (5 mM iodoacetamide; Sigma Chemical Co.), or (iv) a mixture of two serine protease inhibitors and a cysteine protease inhibitor (5.7 mM TPCK, 5.4 mM TLCK, and 5 mM iodoacetamide). A hydrophilic fraction was prepared by depleting the *La*E of amphiphilic and hydrophobic molecules by precipitation at 37°C with Triton X-114 (2). Saline was equally treated with Triton X-114 to be used as the control vehicle. The precipitated amphiphilic and hydrophobic fractions were extensively dialyzed against 0.15 M saline containing 0.1% (vol/vol) Brij 35 (Sigma Chemical Co.) for removal of the Triton X-114.

Treatment of mice with *Leishmania* extracts and extract fractions. Each animal from groups of 5 to 10 mice received four 0.2-ml intravenous injections of (i) *La*E, (ii) *L. braziliensis* extract, (iii) amphiphilic or hydrophilic fractions of *La*E, or (iv) *La*E supplemented with protease inhibitors. The injections were separated by an interval of 2 weeks, and each contained 200 μg of protein. Control groups consisted of mice that received four 0.2-ml injections of saline, Triton X-114-treated saline, or protease inhibitor-supplemented saline.

Murine model of cutaneous leishmaniasis. *L. braziliensis* promastigotes (10^7), obtained from stationary-phase culture, were subcutaneously inoculated into one of the hind footpads of BALB/c or C57BL/6 mice 1 week after the first injection of the extract or extract fractions. Lesion size was monitored with a digital caliper and estimated by subtracting the thickness of the uninfected footpad from the thickness of the infected footpad. Parasite loads in the footpads were estimated by limiting dilution (17). Briefly, the infected footpads were macerated in Schneider's medium and centrifuged at 50 × *g* for 10 min, at 4°C. The supernatants were recentrifuged at 1,540 × *g* for 10 min at 4°C, and the pellets were resuspended in Schneider's medium supplemented with 50 μg · ml^{-1} gentamicin and 20% FBS. The suspension was serially diluted in 10-fold dilutions and distributed in triplicate in 96-well culture plates. The number of viable parasites in each footpad was determined from the reciprocal of the highest dilution at which promastigotes could be detected after 7 days at 23°C and was expressed as the number of parasites per lesion.

Antibody activity and cytokine measurements. For determination of IgG1 and IgG2a anti-*Leishmania* antibodies, 96-well microtiter plate wells were coated by overnight incubation at 4°C with 100 μl of *La*E (containing 10 μg of protein · ml^{-1}) diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6. The plates were blocked by incubation for 1 h at room temperature with 150 μl of 0.15 M phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20 (PBS-T) and 10% FBS. Serum samples were diluted 1:1,000 in PBS-T with 5% FBS and incubated for 1 h at room temperature. Bound antibodies were detected by incubation with biotinylated rat anti-mouse IgG1 or IgG2a (Pharmingen, Minneapolis, MN) for 1 h, followed by incubation with avidin-conjugated peroxidase for 30 min, at room temperature. The reaction was developed in 100 μl of 50 mM citrate-phosphate buffer, pH 5.2, containing 1.1 μM *o*-phenylenediamine and 0.015 volume of H_2O_2, and stopped by the addition of 25 μl of 4 M H_2SO_4. The absorbance at 490 nm was determined in an enzyme-linked immunosorbent assay microtiter plate reader. For cytokine measurements, 24-well culture plate wells were coated overnight at 4°C with 100 μl of 50 μg · ml^{-1} anti-CD3 (R&D Bioscience, San Diego, CA). Draining lymph node cells (5 × 10^6 · ml^{-1}) in 500 μl of RPMI 1640 medium containing 10% FBS, 50 μg · ml^{-1} gentamicin, 2 mM glutamine, and 2 mM sodium pyruvate, in the presence or absence of *La*E (40 μg of protein · ml^{-1}), were cultured at 37°C and 5% CO_2 for 48 h. IFN-γ and IL-4 in culture supernatants were assayed with commercially available kits, following the manufacturer's instructions (Pharmingen, San Diego, CA).

Statistical analyses. Due to the relatively small number of animals per experimental group (5 to 10), the distribution of the data was assumed to be non-Gaussian. Comparisons between two experimental groups were performed using Mann-Whitney's U test. Comparisons among more than two experimental groups were performed by the Kruskal-Wallis test, followed by Dunn's posttest. Results were considered significant when the value of *P* was ≤0.05.

RESULTS

***L. amazonensis*, but not *L. braziliensis*, amastigote extract enhances *Leishmania* infection in BALB/c mice.** As described in the literature, *L. amazonensis* promastigotes were highly infective for BALB/c mice, whereas *L. braziliensis* promastigotes caused only self-healing lesions in mice of the same strain (Fig. 1A). *L. braziliensis*-infected BALB/c mice that received four biweekly *La*E intravenous injections, starting 1 week before being infected with 10^7 stationary-phase *L. braziliensis* promastigotes in the footpad, had significantly ($P < 0.05$) larger footpad lesions than animals injected with *L. braziliensis* extract or with saline, in which the disease tended to subside. This difference was observed from the second week after the beginning of the infection onwards but was more evident from the fifth week after the beginning of the infection (Fig. 1A). In the particular experiment whose results are shown in Fig. 1, the *L. braziliensis* extract increased the lesion size only in the second week postinfection. This transient increase in lesion size was not seen in the other four experiments.

There were approximately 1,000-fold more parasites in the

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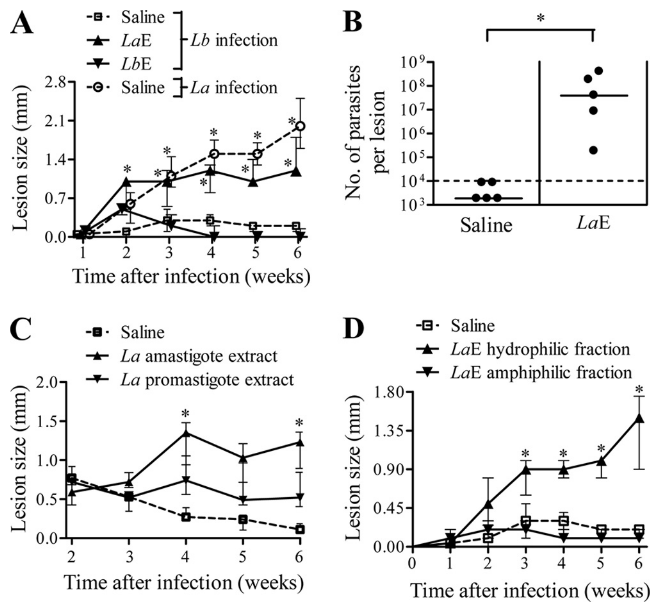

FIG. 1. Injections of *Leishmania amazonensis* extract or its hydrophilic and amphiphilic fractions but not *Leishmania braziliensis* extract increased lesion sizes and parasite burdens of BALB/c mice infected in the footpad with 10^7 *Leishmania braziliensis* stationary-phase promastigotes. Each mouse received four biweekly intravenous injections of the extracts or their fractions, starting 1 week before the infection, as detailed in the Materials and Methods. (A) Mice were infected with *L. amazonensis* (*La*) or with *L. braziliensis* (*Lb*). The *L. braziliensis*-infected mice were treated with *La*E, *L. braziliensis* extract (*Lb*E), or saline. Data are representative of those from five independent experiments. (B) Parasite burden, assessed by limiting dilution 6 weeks after infection with *L. braziliensis*, in the footpads of mice treated with *La*E or saline in the experiment that produced the results depicted in panel A. Each circle represents the result obtained from an individual animal, the horizontal continuous lines indicate the medians of the results, and the horizontal broken line indicates the upper limit of the 75th percentile of the saline-treated group. (C) Mice were infected with *L. braziliensis* and injected with *L. amazonensis* axenic amastigote extract, *L. amazonensis* stationary-phase promastigote extract, or saline. The result is representative of those from two experiments. (D) Mice were infected with *L. braziliensis* and treated with hydrophilic or amphiphilic fractions of *L. amazonensis* extract or with the vehicle, i.e., Triton X-114-treated saline. In panels A, C, and D, each point represents the median lesion size from five mice and the vertical bars correspond to the interquartile interval. $*$, $P < 0.05$ (in relation to the saline group).

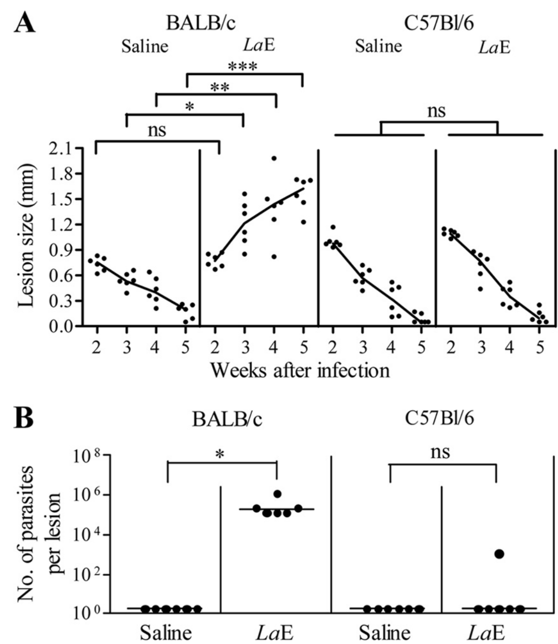

FIG. 2. Injections of *La*E do not affect the lesion sizes or parasite burdens in C57BL/6 mice infected in the footpad with 10^7 *Leishmania braziliensis* stationary-phase promastigotes. Each BALB/c or C57BL/6 mouse received four biweekly intravenous injections of *La*E or saline, starting 1 week before the infection, as detailed in the Materials and Methods. Each circle represents the result obtained from an individual animal. (A) Lesion sizes. The curves are defined by the median values obtained in groups of five or six mice. (B) Parasite burden, as assessed by limiting dilution 5 weeks after infection with *L. braziliensis*, in the footpads of the mice of the experiment that produced the results depicted in panel A. The horizontal lines indicate the medians of the results. $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$; ns, nonsignificant.

infected footpads of *La*E-treated BALB/c mice than in the footpads of control, saline-treated mice ($P < 0.0001$; Fig. 1B).

The injection of extracts prepared from *L. amazonensis* promastigotes at the stationary phase of growth, although apparently interfering with the healing of the footpad lesions caused by *L. braziliensis*, did not have a statistically significant ($P > 0.05$) effect on the size of those lesions (Fig. 1C).

The *Leishmania* infection-enhancing activity of *La*E in BALB/c mice is mediated by hydrophilic molecules. There was a significant increase ($P < 0.05$) in lesion sizes of BALB/c mice infected with *L. braziliensis* and injected with an *La*E hydrophilic fraction, prepared by depletion of amphiphilic molecules, compared with those of a control group that was injected with Triton X-114-treated saline (Fig. 1C). No differences in lesion sizes were found between mice injected with an *La*E amphiphilic fraction and those of the control group (Fig. 1D).

***L. amazonensis* amastigote extract does not enhance *Leishmania* infection in C57BL/6 mice.** The injection of *La*E, following the same protocol that clearly promoted *L. braziliensis* infection in BALB/c mice, did not have any effect on lesion sizes or tissue parasitism in *L. braziliensis*-infected C57BL/6 mice (Fig. 2).

Protease inhibitors reduced the experimental leishmaniasis-promoting activity of *La*E. The addition of two different mixtures of serine and/or cysteine protease inhibitors (PMSF, TPCK, TLCK, and NPGB, Fig. 3A; TPCK, TLCK, and iodoacetamide, Fig. 3C) or of two specific (27) serine protease inhibitors (TPCK and TLCK, Fig. 3C) to the *La*E significantly decreased the ability of the extract to promote *L. braziliensis*-induced lesions in BALB/c mice. Animals that were treated with cysteine protease inhibitor (iodoacetamide)-supplemented *La*E in general had lesions that were smaller than those of the animals treated with nonsupplemented *La*E (Fig. 3C). The difference in lesion size between these two groups of mice, however, was not statistically significant.

Mice injected with protease inhibitor-supplemented saline had the same sizes of lesions as mice injected with nonsupplemented saline (data not shown).

In three experiments, lesion parasitism in the mice that were injected with protease inhibitor-supplemented *La*E was assessed by limiting dilution. Although the differences observed in relation to the results for the control, untreated *La*E-in-

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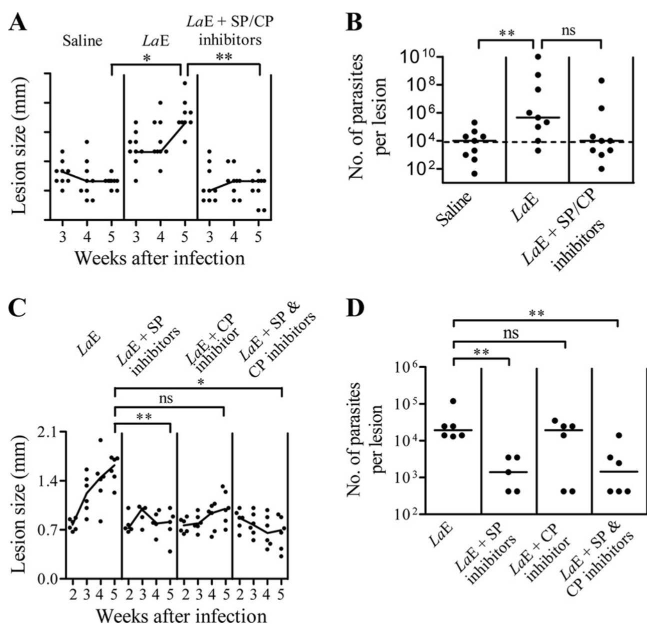

FIG. 3. Protease inhibitors reduce the infection-enhancing activity of *L. amazonensis* amastigote extract in BALB/c mice infected in the footpad with 10^7 stationary-phase *L. braziliensis* promastigotes. Mice were treated with four biweekly injections of saline, *L. amazonensis* amastigote extract, or *L. amazonensis* amastigote extract supplemented with protease inhibitors, starting 1 week before the infection, as detailed in the Materials and Methods. (A) Lesion sizes in the footpads of mice treated with *La*E, saline, or *La*E supplemented with a mixture of serine and/or cysteine protease inhibitors (TPCK, TLCK, NPGB, and PMSF; *La*E + SP/CP inhibitors). The data are representative of those from five independent experiments. (B) Parasite burden, as assessed by limiting dilution 5 weeks after infection with *L. braziliensis*, in the experiment that produced the results depicted in panel A. The horizontal broken line indicates the upper limit of the 75th percentile of the saline-treated group. (C) Lesion sizes in the footpads of mice treated with *La*E, saline, *La*E supplemented with a mixture of two serine protease inhibitors (TPCK and TLCK; *La*E + SP inhibitors), *La*E supplemented with a cysteine protease inhibitor (iodoacetamide; *La*E + CP inhibitor), and *La*E supplemented with a mixture of TPCK, TLCK, and iodoacetamide (*La*E + SP & CP inhibitors). (D) Parasite burden 5 weeks after infection with *L. braziliensis* in the experiment that produced the results depicted in panel C. In panels A and C, the curves are defined by the median values obtained in groups of five to nine mice; in panels B and D, the horizontal lines indicate the median values of the group results. *, $P < 0.01$; **, $P < 0.05$; ns, nonsignificant.

jected mice were not statistically significant in two of the three experiments, the lesions of the majority (6 out of 9) of these mice contained relatively few parasites (fewer parasites than the 75th percentile of the parasite numbers in lesions from the control, saline-injected mice; Fig. 3B). This contrasted with what was observed in the positive-control groups, composed of nonsupplemented *La*E-injected mice, of these experiments (8 out of 9 of those mice had more parasites per lesion than the 75th percentile of the saline-injected mice; Fig. 3B). In a third experiment, the mice that were injected with *La*E that had been supplemented with a mixture of two serine protease inhibitors or with a mixture of serine and cysteine protease inhibitors had significantly fewer parasites in their lesions than the control mice that were injected with nonsupplemented *La*E (Fig. 3D). No statistically significant difference in parasite

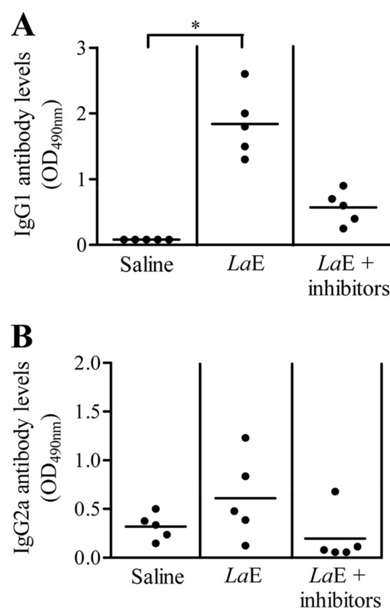

FIG. 4. Levels of anti-*L. amazonensis* extract antibodies in the sera of BALB/c mice infected in the footpad with 10^7 stationary-phase *L. braziliensis* promastigotes. IgG1 (A) and IgG2a (B) antibodies were measured in sera, prepared from blood collected 6 weeks after the infection, of mice that had been treated with four biweekly injections of saline, *La*E, or *La*E supplemented with a mixture of serine/cysteine protease inhibitors (*La*E + inhibitors), starting 1 week before the infection, as detailed in the Materials and Methods. The horizontal lines indicate the median value of each group of five animals. *, $P < 0.001$.

numbers per lesion was found between the groups of mice injected with cysteine protease inhibitor-supplemented *La*E and those injected with nonsupplemented *La*E in this third experiment (Fig. 3D).

Treatment of *L. braziliensis*-infected BALB/c mice with *La*E increased IgG1 antibody levels and changed the pattern of the cytokines produced by anti-CD3-stimulated lymph node cells. Anti-*La*E IgG1 antibody levels were significantly higher in the group of mice treated with nonsupplemented *La*E than in the group injected with saline ($P < 0.001$; Fig. 4A) at 5 weeks after being infected with *L. braziliensis*. No anti-*La*E IgG1 antibody could be detected in sera from control, *L.* braziliensis-infected, saline-treated mice (Fig. 4A). The levels of anti-*La*E IgG2a antibody did not differ statistically among the groups of mice (Fig. 4B).

The infection-enhancing effect of the extract was associated with a significantly higher level of production of IL-4 (Fig. 5A) and a significantly lower level of production of IFN-γ (Fig. 5B) by anti-CD3-stimulated draining lymph node cells.

***La*E did not enhance *L. braziliensis* infection in IL-4-knockout mice.** The intravenous injection of *La*E did not increase the lesion size (Fig. 6A) or the number of intralesion parasites (Fig. 6B) 6 weeks after the infection of IL-$4^{-/-}$ BALB/c mice with *L. braziliensis*.

DISCUSSION

As shown here, *L. amazonensis* differs from *L. braziliensis* in terms of producing one or more hydrophilic molecules that

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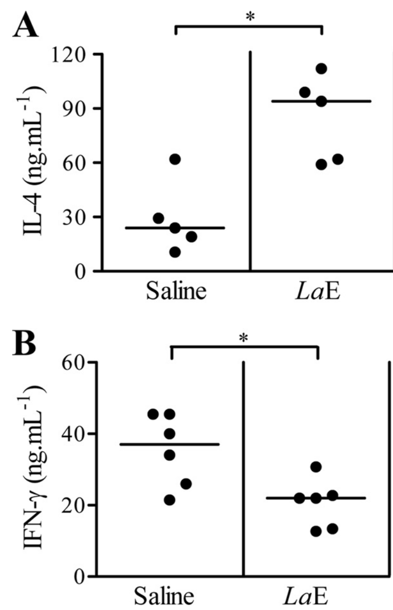

FIG. 5. Amounts of cytokines produced by draining lymph nodes cells of BALB/c mice infected in the footpad with 10^7 stationary-phase *L. braziliensis* promastigotes. The mice were treated with four biweekly injections of saline or *La*E, starting 1 week before the infection. The lymph node cells, collected 1 week after the last saline or extract injection (i.e., 5 weeks after the infection), were *in vitro* stimulated with anti-CD3, and their supernatants were assayed for IL-4 (A) and IFN-γ (B), as detailed in the Materials and Methods. The horizontal lines indicate the median value of each group of five or six animals. *, $P < 0.05$.

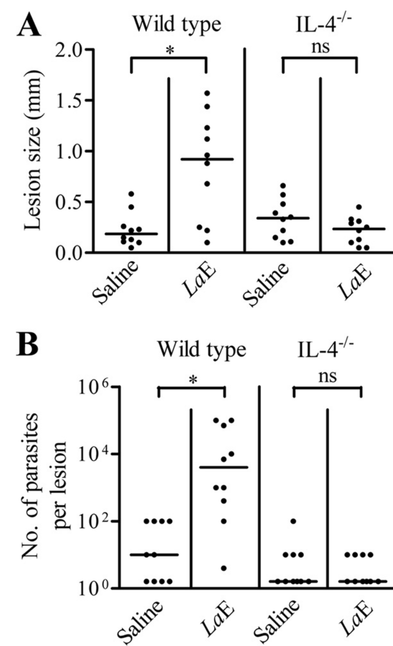

FIG. 6. Injections of *L. amazonensis* extract do not affect lesion sizes or parasite burden of IL-4$^{-/-}$ BALB/c mice infected in the footpad with 10^7 *L. braziliensis* stationary-phase promastigotes, determined 6 weeks after infection. (A) Lesion sizes; (B) parasite burdens. The mice received four biweekly intravenous injections of *La*E or saline, starting 1 week before the infection, as detailed in the Materials and Methods. The horizontal lines indicate the median value of each group of 10 animals, and the data are representative of those from two independent experiments. *, $P < 0.05$; ns, nonsignificant.

promote *L. braziliensis* infection in an otherwise relatively resistant host. This is in accordance with the observation that the immunization of BALB/c mice with a whole *L. amazonensis* extract but not with an *L. braziliensis* extract or with a lipophosphoglycan (LPG)-depleted *L. amazonensis* extract promoted an increased susceptibility to *L. amazonensis* infection (24, 25). In that work, however, the enhancing effect was shown in a homologous situation (*L. amazonensis* extract into *L. amazonensis*-infected animals), and it was not clarified whether or not the *L. braziliensis* extract would also potentiate the infection in a correspondingly homologous situation (*L. braziliensis* extract into *L. braziliensis*-infected animals). Moreover, whereas the enhancing effect in that homologous system was attributed to LPG (25), in the present work the disease-enhancing activity was present in the hydrophilic fraction, a fact that rules out the participation of LPG and other amphiphilic molecules, such as glycosphingolipids (33) and bacterial lipopolysaccharides (2). However, one cannot completely exclude the possibility that the solubilized amphiphilic fraction was also biologically active, since the possibility that the detergent-based extraction altered a putatively active membrane-bound amphiphilic molecule cannot be ruled out.

In the work described here, the *La*E was prepared from axenic amastigotes. Despite the fact that axenic amastigotes are not completely identical to lesion-derived amastigotes in terms of protein makeup (34) and mRNA expression pattern (28), their extract promoted the *Leishmania* infection to a greater degree than the promastigote extract.

Quite interestingly, adding protease inhibitors to the extract inhibited its ability to increase the size of the footpad lesions. This is in agreement with previously reported data showing that *Leishmania mexicana* parasites that had been genetically modified so as to lack a cysteine protease (which has been shown to promote Th2 immune responses) (26) are less virulent than the wild-type parasites and that the decreased virulence is associated with the development of a Th1 immune response (5). In addition to the cysteine protease, it has recently been reported that a serine protease of *Leishmania donovani* metacyclic promastigotes has been indirectly associated with parasite virulence (7). In the present work, the addition of specific serine protease inhibitors (27) to the *La*E decreased its infection-enhancing activity, as measured both by lesion size and by lesion parasitism. The addition of a specific cysteine protease inhibitor (iodoacetamide), on the other hand, did not produce a statistically significant effect on the *La*E biological activity. The results described above allow one to conclude that a serine protease activity plays a role in the infection-promoting activity of *La*E, whereas the role played by a cysteine protease in *La*E, if any, is less evident. Additional experiments should be done with cysteine protease inhibitors, however, since it has been reported that a parasite-derived cysteine protease is associated with inhibition of protective immunity in an experimental infection model that employed a *Leishmania* species (*L. mexicana*) of the same subgenus (*Leishmania*) as that of *L. amazonensis* (5, 26). Moreover, the addition of the protease inhibitors did not always completely re-

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verse the lesion-enhancing activity of the *La*E, as it can be seen by comparing the data shown in Fig. 2A (for the saline-treated BALB/c group) with the data shown in Fig. 3C (for the groups labeled *La*E + SP inhibitors and *La*E + SP & CP inhibitors). This observation would suggest that, in addition to a protease, another virulence factor(s) is present in the *La*E. On the other hand, despite the fact that the addition of protease inhibitors to the *La*E clearly reduced its effect on the sizes of the lesions, the number of parasites per lesion did not change in a statistically significant manner in two out of the three experiments performed. This discrepancy could be accounted for if the *La*E injections affected two different phenomena: parasite growth and inflammation. *La*E injections may enhance parasite growth by interfering with the development of an effective immune response. In addition, it may also promote inflammation, possibly with a contribution from the resulting increased number of parasites, therefore leading to an increase in lesion sizes. The observed effect of protease inhibitors could be explained by their addition inhibiting more intensely the inflammatory activity of *La*E than its parasite growth-promoting activity.

On the basis of the results described above, it could be expected that the intravenous injection of the protease inhibitors alone, without *La*E, could have an effect on the development of a *Leishmania* infection. However, the intravenous injection of protease inhibitors alone in the same amounts and following the same injection protocol used in the mice injected with protease inhibitor-treated *La*E did not affect the infection of BALB/c mice by *L. braziliensis* (data not shown) or by *L. amazonensis* (V. M. G. Silva et al., unpublished results).

It has been shown that *La*E induces T-cell anergy *in vitro* in a partially protease-dependent process (23). Anergy, however, does not seem to occur in the *in vivo* model used in the present work, since the injection of *La*E induced the production of higher levels of IgG1 antibody and of IL-4 than those observed in saline-injected animals. Another possible candidate to mediate the infection-enhancing activity would be the *Leishmania* homologue of receptors for activated C kinase (LACK) antigen, which promotes IL-4 production by $V\beta4V\alpha8CD4^+$ T cells (29). The infection-promoting activity of LACK, however, seems to depend on its being the target of an immune response and not on the presence of enzymatic activity (15).

The mechanism by which the protease inhibitors affect the infection-enhancing activity of the *La*E is not clear. TLCK and TCPK, in addition to inhibiting serine proteases, are often regarded as serine esterase inhibitors (31). The infection-enhancing molecule either could be a molecule with protease/esterase activity or could become activated by its hydrolyzation by a protease/esterase. The protease inhibitors used in the present work are irreversible, so that the timing of treating the extract with the protease inhibitors, before they are injected into the animals, could discriminate between the two possibilities. In fact, the protease inhibitors were added to extracts that have been under preparation (i.e., subjected to ultrasound treatment, centrifugation, and filtration) for up to 40 min at 4°C, so that time was unintentionally allowed for the possible activation of a putative provirulence factor by the protease.

Since IgG1 and IgG2a antibody isotypes are associated in the mouse with Th2 and Th1 immune responses, respectively (32), the present data on the IgG subclasses of the anti-*Leishmania* antibody indicate that the administration of *La*E to BALB/c mice changed an otherwise polarized Th1 response to a mixed Th2-Th1 response. Moreover, the animals that received protease inhibitor-free extract and that had the most intense cutaneous disease were the ones that produced the highest level of anti-*Leishmania* IgG1 antibody. This finding indicates that the injection of the extract promoted the development of a Th2 immune response, which could account for the progression of the infection. This hypothesis is supported by two different findings. First, anti-CD3-stimulated draining lymph node cells from the *La*E-injected animals produced higher levels of IL-4 and lower levels of IFN-γ than the levels found in the saline-injected animals. Although specific lymphocyte responses to *Leishmania* antigens were not investigated in the present work, the anti-CD3 stimulation of cells from the draining lymph nodes reveals the functional phenotype of the T cells that are present in the local immune response environment. It may be worthwhile mentioning that the draining lymph nodes of the *L. braziliensis*-infected, *La*E-injected mice were markedly enlarged in comparison with the draining lymph nodes of the *L. braziliensis*-infected, saline-injected mice (a more than 400% difference in draining lymph node volumes between the two groups of mice was observed; data not shown). Second, the *La*E did not enhance the infection in IL-$4^{-/-}$ animals. This last result apparently conflicts with a previous report that IL-$4^{-/-}$ C57BL/6 mice are fully susceptible to *L. amazonensis* infection, indicating that IL-4 does not contribute to the development of the infection in that mouse strain (14). However, the susceptibility of C57BL/6 mice to *L. amazonensis* has been ascribed to an IL-4-independent reduction of IL-12 receptor expression (14). It is not unlikely that different mechanisms confer susceptibility to *Leishmania* infection either in the same or in different mouse strains, and as under any redundant condition, interference with a particular mechanism (e.g., the induction of increased production of IL-4 in C57BL/6 mice by *L. amazonensis* molecules, which would not interfere with the reduction of IL-12 receptor) might not affect the final outcome of the infection. This last assumption was tested in the present work, and, indeed, *La*E injections did not interfere with *Leishmania* infection in C57BL/6 mice. The infection-enhancing molecule studied in the present work, therefore, in addition to being *Leishmania* species specific, also depends on the host genetic background. This last observation is consistent with the epidemiology of diffuse cutaneous leishmaniasis, which affects only a minority of the patients with *L. amazonensis*-induced disease.

Another interesting indication that BALB/c mice tend to produce Th1 immune responses when they are infected with *L. braziliensis* comes from the finding that the control *L. braziliensis*-infected mice, which were not injected with *La*E, produced only cross-reactive anti-*L. amazonensis* antibodies of the IgG2a isotype and not of the IgG1 isotype (Fig. 4, saline group), a fact that is consistent with their production of IFN-γ. The results described here demonstrate that *La*E promotes *Leishmania* infection in BALB/c mice in an IL-4-dependent manner; i.e., in the absence of IL-4, it had no effect on the infection. Although an increased amount of IL-4, inhibiting the development of a potentially protective Th1-dependent immune response, could explain the present results, one cannot exclude the possibility that the *La*E could redundantly enhance infection through

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more than one mechanism, such as by also inducing the production of Treg cell-, macrophage-, and/or dendritic cell-related cytokines, such as IL-10 and transforming growth factor β (TGF-β).

The experimental model for investigating *Leishmania* virulence factor that was used in the present work involves the injection of *Leishmania* extract in the intravenous compartment. It is known that the route of antigen entry into an animal may affect the immune response outcome. In fact, it has been shown that the intravenous inoculation of irradiated *Leishmania major* leads to protection of BALB/c mice against the subsequent challenge with untreated parasites (1). Quite the opposite was observed, however, in the experiments described here: the intravenous injection of *La*E led to promotion rather than control of the infection. On the other hand, the animal model that was used in the present experiments was a model of relative resistance to infection (injection of *L. braziliensis* into BALB/c mice), which is not an ideal model to look for the protection that could theoretically arise from the intravenous injection of *L. braziliensis* extract rather than *L. amazonensis* extract into the mice. The injection of *La*E by the intradermal route, following a protocol otherwise identical to the one used in the present work, also promoted *L. braziliensis* infection (Silva et al., unpublished data).

Another question raised by the findings reported here is whether molecules in the *La*E would function as an immunogen, i.e., whether they would act by eliciting Th2 or T-regulatory immune responses to *L. amazonensis* antigens shared with *L. braziliensis*. An alternative hypothesis is that the biologically active molecules in the *La*E act nonspecifically, biasing the immune system toward Th2 or Treg immune responses against any antigen that would be introduced into the animal during a certain period. The two hypotheses could be discriminated by studying the effect of the transfer of known subpopulations of T lymphocytes from *L. braziliensis*-infected, *La*E-injected mice on the development of *L. braziliensis* infection in naïve recipient mice. Regardless of which one is the right explanation, it is clear that *L. amazonensis* synthesizes molecules that preferentially induce Th2 immune responses, as shown here, or stimulates Treg cells, as shown elsewhere (24). The identification of these molecules could be important for the development of a vaccine, since an immune response against a virulence factor has an increased chance of being protective. Of course, if it is used in a vaccine, the antigen would not be injected intravenously, as was done in the present work (which aimed at studying the biological activities of *L. amazonensis* molecules), but would be administered by an appropriate, more immunogenic route and would be associated with an effective adjuvant that could change a putative permissive immune response to an efficacious one. In addition, a soluble parasite protein or a synthetic polypeptide/recombinant protein based on that protein that biases the immune system to mount Th2 immune responses could perhaps be employed in the control of diseases associated with Th1 or Th17 immune responses, such as mucocutaneous leishmaniasis and most autoimmune diseases.

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We declare that we have no competing interests.

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