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Characterization of an Antigen from *Leishmania amazonensis* Amastigotes Able To Elicit Protective Responses in a Murine Model

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Lymphoproliferative responses to an antigen from *Leishmania amazonensis* amastigotes with an apparent molecular mass of 30 kDa, termed p30, were evaluated with BALB/c mice. The p30 antigen was purified after separation of parasite extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electroelution. Lymphoproliferative responses to p30 were obtained by subcutaneous immunization of animals with *L. amazonensis* amastigote extracts, and maximal stimulation indices were observed at an antigen concentration of 5 μg/ml. Induction of lymphoproliferation by p30 is stage specific, and no differences in the responses to this antigen between mice susceptible and resistant to *L. amazonensis* were detected. The predominant T cells characterized in the lymphocyte cultures were $CD4^+$. Lymphokine analysis of the supernatants from these cultures indicated that Th1 is the subset involved in the lymphoproliferative responses to the antigen. BALB/c mice immunized with p30 and challenged with *L. amazonensis* amastigotes showed a very low level of infection, indicating a protective role for p30 and a correlation between Th1 and protection. Further biochemical characterization studies showed that this antigen presents cysteine proteinase activity.

Parasites of the genus *Leishmania* present two forms in their life cycle, promastigotes, which multiply in the midgut of the sandfly vector, and amastigotes, the obligate intracellular forms which live within macrophage phagolysosomes from the vertebrate host. Species of *Leishmania* cause a spectrum of human disease such as cutaneous, mucocutaneous, and visceral leishmaniasis. The *Leishmania mexicana* complex comprises species which are distributed in a wide range throughout the New World, extending from southern Texas, in the United States, to southern Brazil (28). These species can cause a form of cutaneous leishmaniasis characterized by large, histocytoma-like lesions extremely rich in parasites.

It is a consensus now that the immune cellular response plays a central role in leishmaniasis, and the current hypothesis for the murine model of the cutaneous form of the disease is that $CD4^+$ Th cells are involved in both protection and progression of the disease. The protective T cell belongs to the Th1 subset and secretes gamma interferon (IFN-γ) and interleukin-2 (IL-2), whereas the disease-enhancing cell belongs to the Th2 subset and secretes IL-4, IL-5, and IL-10 (6, 22, 46).

An important issue in leishmaniasis is characterization of *Leishmania* antigens capable of selectively activating Th1 cells in order to obtain protection. The ability of several purified parasite antigens to prevent the development of cutaneous lesions in both susceptible and resistant mouse strains has been examined elsewhere (7, 20, 42, 44, 47). Most of the work performed in protection studies of leishmaniasis has used the promastigote form of *Leishmania* as antigenic source, whereas the evaluation of protection conferred by amastigote antigens is still rare (24, 25, 49, 55).

Studies in our laboratory have focused mainly on the identification of antigens from *Leishmania amazonensis* amastigotes implicated in cellular immune responses in the murine model (3, 18, 51). The present work describes a 30-kDa antigen able to induce a protective response against infection in susceptible mice. Biochemical characterization of this antigen showed that p30 presents cysteine proteinase activity.

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MATERIALS AND METHODS

Mice and parasites. Female BALB/c mice 8 to 12 weeks old were obtained from breeding stock maintained at the Division of Immunology of the University of São Paulo, São Paulo, S.P., Brazil. The *L. amazonensis* strain used, MHOM/BR/73/M2269, was kindly provided by J. J. Shaw, Instituto Evandro Chagas, Belém, Pará, Brazil, and maintained as amastigotes by inoculation into footpads of golden hamsters every 4 to 6 weeks. Amastigote suspensions (AM) were prepared by homogenization of excised lesions, disruption by four passages through 22-gauge needles, and centrifugation 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 1640. The suspension was kept under agitation for 4 h at room temperature and centrifuged at 250 × *g* for 10 min. The final pellet contained purified amastigotes which were essentially free of contamination by other cells (3).

Immunization protocols. Two schedules of immunization were used. Animals received one subcutaneous dose of 10^7 *L. amazonensis* amastigotes isolated from hamster footpads, disrupted by sonication, and mixed with complete Freund's adjuvant (CFA), or they were immunized with CFA plus 25 μg of the 30-kDa antigen purified as described below. Ten to fourteen days after immunization, spleen and lymph node cells from the immunized mice were isolated for use in the lymphoproliferation assays.

T-cell screening. The procedure was used as previously described (1, 17). About 100 μg of total extracts of 10^9 *L. amazonensis* amastigotes in sample buffer were subjected to sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were transferred at 200 mA for 12 h in transfer buffer (10 mM Na_2HPO_4 plus 20% methanol) to nitrocellulose membrane (NC) (0.1 μm; Gibco BRL, Gaithersburg, Md.). Blots were cut into horizontal 5-mm strips, each one containing a compound of a known molecular mass range as calculated from a standard curve obtained with Pharmacia protein low-molecular-mass markers (15- to 94-kDa range) (Pharmacia Biotech, Uppsala, Sweden). Each strip was sliced and solubilized in a glass tube with 1 ml of dimethyl sulfoxide, precipitated with an equal volume of 50 mM carbonate-bicarbonate buffer (pH 9.6), washed three times with RPMI 1640, and stored at −20°C in the same medium used in the lymphoproliferation assays (17).

Electroelution of proteins from SDS-PAGE gels. After identification of proteins which induce lymphoproliferative responses by T-cell screening, parasite extracts were subjected to SDS-PAGE, and proteins were localized in gels by their molecular weight and eluted by electrophoresis in transfer buffer (0.025 M Tris, 0.192 M glycine in 20% methanol) at 200 mA for 12 h. After elution, the

proteins were dialyzed, lyophilized, resuspended in phosphate-buffered saline (PBS), and filtered through 0.22-μm-pore-size membranes for further utilization in lymphocyte cultures. Protein was quantified by a Coomassie blue reagent assay (Pierce, Rockford, Ill.).

Lymphoproliferation assays. The lymphocyte cultures were obtained from spleen and inguinal lymph nodes of normal and immunized BALB/c mice. The cells were isolated and counted, and after evaluation of cell viability by trypan blue exclusion, 2.5×10^5 cells per well were cultured in RPMI 1640 containing 20 mM $NaHCO_3$, 10 mM HEPES, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 5 mM sodium pyruvate, 100 μM nonessential amino acid solution, and 0.5% normal mouse serum; this was designated complete medium (CM).

Antigens tested in these experiments were either those coupled to nitrocellulose for T-cell screening or the proteins eluted from gels, as described above. Extracts from *L. amazonensis* amastigotes were also tested in the lymphocyte cultures: 2×10^9 amastigotes were resuspended in 1 ml of PBS (pH 7.2), lysed by sonication, and centrifuged at $200 \times g$ for 2 min, and the supernatant was sterilized by filtration (0.22-μm-pore-size filter) and dissolved in CM before use in the lymphocyte cultures (AM).

Lymphocyte cultures were incubated for 96 h at 37°C in a humidified chamber containing 5% CO_2. Cells were pulsed with 1 μCi of [^{3}H]thymidine ([^{3}H]TdR; 86 Ci/mmol; Amersham International, Amersham, England) per well 18 h before they were harvested on glass fiber paper. [^{3}H]TdR uptake was measured in a β-scintillation counter (1600TR, TRI Carb; Packard, Meriden, Conn.).

Characterization of T-cell population induced by p30 antigen. In order to characterize the T-cell population involved in the lymphoproliferative responses, the lymph node cultures were stimulated with p30 at 2.5 μg/ml, and at the same time, either 25 μg of monoclonal antibody (MAb) anti-CD4 (clone H129.19; rat immunoglobulin G2a [IgG2a]; Gibco BRL) per ml or 25 μg of anti-CD8 (clone 53-6.7; rat IgG2a; Gibco BRL) per ml was added. An irrelevant antibody, rat IgG at 25 μg/ml, was also used as a control.

Lymphokine assays. (i) IFN-γ, IL-4, IL-5, and IL-10. Supernatants from 10^7 lymph node cells per ml maintained for 24 to 30 h in the presence of 2.5 μg of p30 per ml were used for lymphokine dosages. Levels in the supernatants were determined by a double-sandwich enzyme-linked immunosorbent assay (ELISA). Supernatants from lymph node cells cultured in CM were used as a negative control for lymphokine dosages. Costar 96-well vinyl assay plates were coated overnight at 4°C with specific MAb directed to each lymphokine tested: XMG 1.2 (anti-IFN-γ), 11B11 (anti-IL-4), TRFK4 (anti-IL-5), and SXC and A5 (anti-IL-10) were used at 0.1, 0.25, 0.05, and 0.2 μg/well, respectively. After washing in 0.05% Tween 20–PBS (PBS/T) and blocking with PBS/T containing 10% fetal calf serum for 60 min at 37°C, 100 μl of supernatants was added to wells. Standard curves were generated with recombinant murine IFN-γ, IL-4, IL-5, and IL-10. After 3 h at 37°C, plates were washed with PBS/T, and a second antibody specific to each lymphokine was added: rabbit polyclonal anti-IFN-γ (Sigma, St. Louis, Mo.), NIP BVD, anti-IL-4; and biotinylated TRFK-5 and SXC-1, anti-IL-5 and anti-IL-10, respectively. After 60 min at 37°C, the plates were washed with PBS/T and enzyme conjugates were added (peroxidase-conjugated goat anti-rabbit IgG for IFN-γ and IL-4 and streptavidin-peroxidase for IL-5 and IL-10) for 30 min followed by incubation with 4-chloro-1-naphthol and H_2O_2. Plates were read at 405 and 490 nm with a Titertek Multiscan ELISA Microreader. Concentrations from the supernatants higher than minimal values obtained from the respective standards were considered positive. The minimal values for lymphokines analyzed are shown in Table 2.

(ii) IL-2. Detection of this lymphokine was carried out by using 30-h supernatants from lymph node cells stimulated by p30 with a murine tumor-specific cytotoxic T-cell line (CTLL) responsive to IL-2 (15). About 10^4 cells per well were cultured in the presence or absence of MAb anti-IL-2 (Genzyme Diagnostics, Cambridge, Mass.) and supernatants. Supernatant from BALB/c spleen cells stimulated with 5 μg of concanavalin A (ConA) (T-cell growth factor) was used as a positive control. Cell growth was evaluated by [^{3}H]TdR incorporation by CTLL after 24 h at 37°C in a humidified chamber containing 5% CO_2. A pulse of 0.5 μg/well ([^{3}H]TdR; 86 Ci/mmol; Amersham International) was done 6 h before the cell harvesting.

Active immunization. p30 purified by electroelution as described above was used to immunize 8- to 10-week-old female BALB/c mice. Animals received one dose of 25 μg of p30 in CFA by the subcutaneous route in the footpad or the base of the tail. Control represents BALB/c mice which received PBS in CFA. After 20 days, animals were challenged with 10^7 *L. amazonensis* amastigotes in PBS in the hind footpad. At various times after infection, mice were subjected to foot measurement by a dial caliper. In addition, the parasite load was evaluated by Giemsa-stained (Merck) imprinting preparations of the foot lesion. Five fields and 500 mononuclear cells were counted, and the amastigote-to-mononuclear cell ratio was calculated (13). Another parameter evaluated was the parasite visceralization, which is a frequent occurrence in *L. amazonensis*-infected BALB/c mice. Thus, after the last foot measurement, cells from lymph nodes and spleen were isolated and cultured in Novy, Nicolle, and MacNeal's medium-brain heart infusion containing 5% fetal calf serum at 24°C for 5 days and observed by optical microscopy to assess promastigote load.

Production and purification of MAb 2E5D3. The hybridoma producing MAb 2E5D3 (anti-p30) was obtained as previously described (27, 50). Briefly, BALB/c mice were immunized with p30 purified by electroelution. The immunization schedule included a subcutaneous dose of 5 μg of the antigen with CFA followed by one intraperitoneal injection of 5 μg of p30 with incomplete Freund's adjuvant and one intravenous injection of 5 μg of p30 in PBS. The interval between each dose was 1 week. Spleen cells from immunized mice were fused with SP2/0-Ag14 cells. The hybridoma supernatants were screened by ELISA by using p30 purified by electroelution. Cloned hybridoma cells secreting MAb 2E5D3 were injected by the intraperitoneal route into BALB/c mice to induce ascites formation. Purification of 2E5D3 from ascitic fluid was carried out in a protein A-Sepharose column.

Immunoprecipitation and biosynthetic labeling of parasites. *L. amazonensis* amastigotes were radiolabeled with ^{125}I by the Iodogen method (14). Alternatively, parasites were radiolabeled with L-[^{35}S]methionine, by biosynthetic incorporation. The radiolabeled cells (4×10^8) were solubilized for 10 min at room temperature with 0.5% Nonidet P-40 (400 μl) in PBS containing proteolytic inhibitors and centrifuged at $1,400 \times g$ for 5 min. Radiolabeled supernatant (100 μl) was incubated overnight at 4°C with ascitic fluids (5 μl), and the immune complexes were incubated with a suspension of 10% *Staphylococcus aureus* Cowan 1 (100 μl) for 1 h at room temperature (26). Immune complexes bound to *S. aureus* were washed several times with 0.1% Nonidet P-40 and 0.1% Nonidet P-40–0.05% SDS in PBS, boiled in SDS-PAGE sample buffer, and subjected to SDS-PAGE on 10% polyacrylamide gels. Gels were fixed and stained; after destaining, they were dried and exposed to X-Omat K films (Kodak) at −70°C.

Western blotting. Western blotting was carried out as described elsewhere (52). After electrophoresis, proteins from amastigotes or promastigotes were transferred to NC for at least 8 h at 200 mA. After blocking of the sheets with 0.5% powdered skim milk in PBS, NC was incubated with MAb 2E5D3, washed, incubated with peroxidase-conjugated secondary antibody, and developed with diaminobenzidine and H_2O_2.

Electron microscopy immunocytochemical studies. Footpad lesions isolated from *L. amazonensis*-infected hamsters were excised, fixed in 2% glutaraldehyde, freeze substituted in 100% ethanol containing 0.5% uranyl acetate, and embedded in Lowicryl K4M at a low temperature. Immunogold labeling with MAb 2E5D3 and protein A-gold (15 nm) was carried out in ultrathin sections.

Purification of p30 and proteinase activity detection. Purification of p30 was performed by immunoaffinity chromatography employing 2E5D3 covalently coupled to CNBr-activated Sepharose 2B (Pharmacia Biotech). Washed amastigotes (5×10^9) were resuspended in lysis buffer (50 mM Tris [pH 7.4] containing 0.5% Nonidet P-40; 1 mM $MgCl_2$; 0.5 mM EGTA; 10 μg of leupeptin, aprotinin, and antipain per ml; and 2 mM phenylmethylsulfonyl fluoride). Disruption of parasites was completed after sonication (Ultrasonic processor; Heat Systems, Farmingdale, N.Y.). Cell lysate was centrifuged at $1,400 \times g$ for 5 min, and the supernatant was incubated with Sepharose coupled to 2E5D3 for 2 h under agitation at 4°C. The resin was then washed extensively with PBS. p30 was eluted from the affinity resin with 0.1 M glycine buffer, pH 2.8. Eluates were neutralized with 1 M Tris, pH 9.0. Protein concentrations were estimated by Coomassie blue assay or by UV adsorption at 280 nm. Protein fractions were pooled and concentrated (Speed Vac VR-I; Heto, Allerød, Denmark).

Proteolytic activity of p30 was determined by zymography employing unheated and nonreduced immunoaffinity-purified p30 resolved on 10% acrylamide gels containing 0.1% gelatin (Gibco) by low-voltage (50 V) electrophoresis (43). Proteinase activity was detected after 1 h of incubation, under agitation, in 0.1 M sodium acetate buffer (pH 5.0) containing 2.5% Triton X-100; 2 h of incubation in the same buffer in the absence of Triton X-100; and Coomassie blue staining. Molecular weight markers (Pharmacia LKB) were visible on the background of stained gelatin when used in a fivefold excess.

Statistical analysis. Student's *t* test and one-way analysis of variance were used to evaluate the significance of the data with SigmaStat software (Jandel Corp., San Rafael, Calif.) ($P < 0.01$).

RESULTS

T-cell screening. In order to establish the optimum conditions for lymphocyte proliferation induced by *L. amazonensis* amastigote antigens, BALB/c mice were immunized with AM by either the intravenous, the intraperitoneal, or the subcutaneous route with different extract concentrations. Immunization through the intravenous route induced low lymphoproliferative responses at all antigen concentrations tested, whereas at increased doses intraperitoneal immunization resulted in enhanced responses and the subcutaneous route led to the highest degree of lymphoproliferation (data not shown). Once the optimum conditions for BALB/c lymphoproliferative responses were determined, *L. amazonensis* amastigote antigens were first analyzed by T-cell screening. It was observed that several antigens induced lymphoproliferative responses in BALB/c mice (Fig. 1). The antigen with an apparent molecular mass of 30 kDa was able to elicit marked lymphoproliferation.

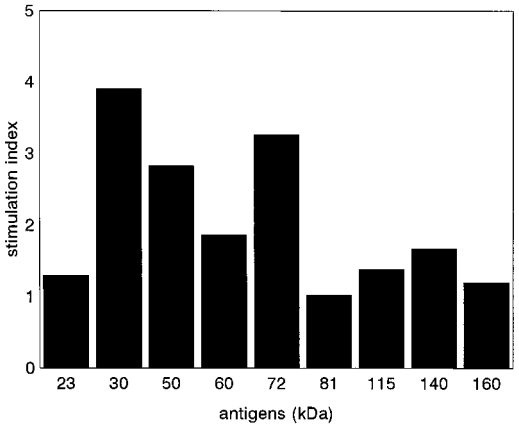

FIG. 1. Evaluation of lymphoproliferative responses of BALB/c mice to antigens from *L. amazonensis* amastigotes by T-cell screening. The proteins used in the lymphoproliferative assays were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose. They are represented on the abscissa by their apparent molecular masses (in kilodaltons). Results represent the stimulation indices determined by dividing the mean counts per minute of cells stimulated by the antigens in triplicate by the mean counts per minute of control (lymphocytes cultured in the presence of NC strips without antigen, which presented values of 11,000 cpm).

Due to the abundance of p30 after electrophoresis separation (Fig. 2, lane B), as well as the difficulty of working concomitantly with several antigens, p30 was chosen for the subsequent experiments.

Effect of antigen concentration on lymphoproliferative responses. After the screening of parasite antigens, the next step was the purification of p30 by electroelution. The parasite extract was subjected to SDS-PAGE, and after the localization of p30, gel strips containing the antigen were electroeluted. Figure 2 shows the profile of *L. amazonensis* amastigote extract and purified p30 subjected to SDS-PAGE. BALB/c mice were immunized with 25 μg of p30 subcutaneously, and 15 days after, lymph nodes were isolated. Lymphoproliferation assays were carried out, and the concentration of p30 which induced maximal lymphoproliferation indices was 5.0 μg/ml (data not shown).

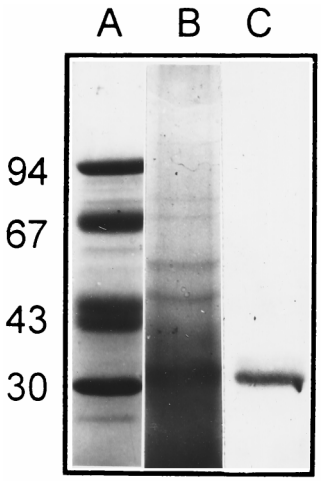

FIG. 2. Profiles of *L. amazonensis* amastigote extract (lane B) and p30 (lane C) separated by SDS-PAGE followed by Coomassie blue staining. Lane A represents low-molecular-mass markers. Numbers at left indicate apparent molecular masses in kilodaltons.

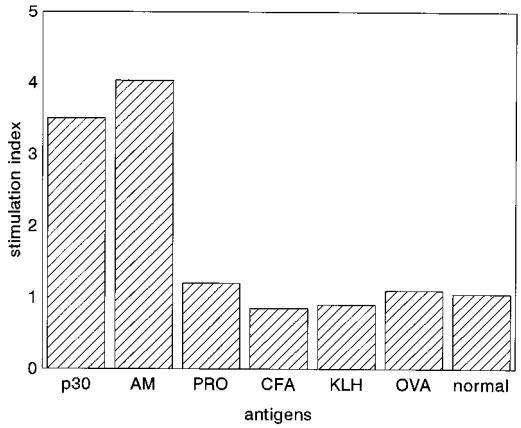

FIG. 3. Lymphoproliferative responses to p30 of BALB/c mice immunized with different antigens. The antigens used for animal immunization are indicated on the abscissa. Results represent the stimulation indices determined by dividing the mean counts per minute of cells stimulated with p30 in triplicate by the mean counts per minute of medium controls. PRO, *L. amazonensis* promastigotes.

Specificity of p30. In order to demonstrate the specificity of p30, lymphoproliferation tests in the presence of 5 μg of p30 per ml were performed with lymph node cells from BALB/c mice subjected to different schedules of immunization: *L. amazonensis* amastigote extract, *L. amazonensis* promastigote extract, PBS plus CFA, hemocyanin (keyhole limpet hemocyanin [KLH]), and ovalbumin (OVA). Cells from these animals proliferated in the presence of ConA, whereas p30 did not stimulate lymph node cells from mice immunized with KLH, OVA, and CFA. In contrast, these cells were stimulated by the homologous antigens (Fig. 3).

Lymph node cells isolated from animals immunized with *L. amazonensis* promastigotes were also not stimulated by p30, indicating that the lymphoproliferative responses to p30 are amastigote specific.

Lymphoproliferative responses to p30 from different strains of inbred mice immunized with *L. amazonensis* amastigotes. The goal of these experiments was to compare the patterns of lymphoproliferative responses to p30 between susceptible (BALB/c) and resistant (C57BL/6 and C3H/HeJ) mouse strains. Lymph node cells from three mouse strains previously immunized with *L. amazonensis* amastigotes were cultured in the presence of different concentrations of p30. Statistical analysis showed that the differences in the mean values among the three groups are not significant and permitted us to conclude that the three strains presented very similar lymphoproliferative responses to p30 (data not shown). The lymphoproliferative responses to p30 were also determined for BALB/c and C57BL/6 mice infected with *L. amazonensis* (45 days after infection). Results from these experiments showed that the lymphoproliferative responses induced by p30 are not significantly different between the two infected mouse strains (stimulation indices obtained from BALB/c and C57BL/6 infected mice were 5.5 and 5.2, respectively).

Characterization of T-cell population involved in lymphoproliferative responses to p30. The T-cell set induced by p30 was characterized by adding to the lymphocyte cultures MAbs directed to $CD4^+$ (clone H129.19) and to $CD8^+$ (clone 53-6.7). Eighty-eight percent inhibition was observed in the presence of anti-CD4. In contrast, lymphoproliferative responses to p30 were inhibited by 25% in the presence of both anti-CD8 and rat IgG (Table 1). These results clearly indicated that $CD4^+$ is the population induced by p30.

TABLE 1. Characterization of T-cell population involved in lymphoproliferative responses to p30[a]

Antigen	[^{3}H]TdR incorporation (10^3 cpm)	% Inhibition
p30	36.40 (±1.30)	
p30 plus anti-CD4	4.37 (±0.16)[b]	88
p30 plus anti-CD8	27.30 (±0.70)[b]	25
p30 plus rat IgG	26.57 (±1.20)[b]	27

[a] Proliferation of lymph node cells from BALB/c mice immunized with *L. amazonensis* amastigotes was induced by p30 in the presence and absence of MAbs anti-CD4 and anti-CD8. After 96 h, cells were harvested and the [^{3}H]TdR incorporation was measured. Results represent the mean counts per minute of triplicates subtracted from the mean counts per minute of controls ± standard deviations. Controls were performed with lymphocytes cultured in the absence of antigen and maintained in CM, CM plus anti-CD4, CM plus anti-CD8, and CM plus rat IgG and presented background values of 5,200, 2,000, 2,700, and 4,700 cpm, respectively.

[b] $P < 0.01$ compared to T cells cultivated with antigens in the absence of the MAbs.

Lymphokine analysis. The characterization of the lymphocyte population induced by p30 as $CD4^+$ led us to determine the subset of $CD4^+$ involved in these responses. Thus, IL-4, IL-5, IL-10, and IFN-γ were assayed by ELISA in the supernatants from lymphocytes cultured in the presence of p30, *L. amazonensis* amastigote extract, and ConA. IL-2 was evaluated by adding supernatants to CTLL cultures. Table 2 shows data from these analyses.

High levels of lymphoproliferation were observed in the presence of p30, *L. amazonensis* amastigote extract, and ConA. IL-4 and IL-5 were not detected in the supernatants tested, and only very low levels of IL-10 were found. Cultures from the three groups produced significant levels of IFN-γ and IL-2, indicating that the production of these lymphokines correlates directly with proliferation responses and that Th1 is the subset induced by p30.

Infection of BALB/c mice challenged with *L. amazonensis* amastigotes after active immunization with p30. The possible protective role of p30 was analyzed with BALB/c mice previously immunized with p30 and challenged with *L. amazonensis* amastigotes. Figure 4 shows that animals immunized with p30 presented foot lesions significantly smaller than those of control (animals which received PBS in CFA) until 40 days after infection. In the following days, there was a small increase of the foot diameter from p30-immunized mice compared to that from noninfected animals (normal).

TABLE 2. Lymphokine production by BALB/c lymphocytes stimulated with p30[a]

Antigen	IL-2[b] (10^3 cpm)	IFN-γ[c] (ng/ml)	IL-4[c] (U/ml)	IL-5[c] (pg/ml)	IL-10[c] (U/ml)
CM	7.439	<1.562	<0.156	<1.875	3.9
p30	18.511	11.3	<0.156	<1.875	6.0
AM	16.870	6.0	<0.156	<1.875	4.2
ConA	56.055	>50.0	<0.156	<1.875	6.6

[a] Lymphocytes from BALB/c mice immunized with *L. amazonensis* amastigotes were cultivated in the presence of indicated antigens or ConA. Supernatants were obtained after 30 h of cultivation, and lymphokines were measured in an ELISA (IFN-γ, IL-4, IL-5, and IL-10) or by growth of a CTLL lineage (IL-2), as described in Materials and Methods.

[b] Supernatants were used at a 1:4 dilution. Results represent the means of triplicate wells, and standard deviations did not exceed 10% of the values. [^{3}H]TdR incorporation was also measured in CTLL cultured in supernatants from lymphocytes maintained in the presence of CM, p30, *L. amazonensis* AM, and ConA plus MAb anti-IL-2 and presented values of 4,864, 4,273, 10,662, and 14,098 cpm, respectively.

[c] Minimal values considered from lymphokine dosages were as follows: IFN-γ, 1.562 ng/ml; IL-4, 0.156 U/ml; IL-5, 1.875 pg/ml; and IL-10, 3.9 U/ml. The data shown are means of triplicate wells, and standard deviations did not exceed 10% of the values.

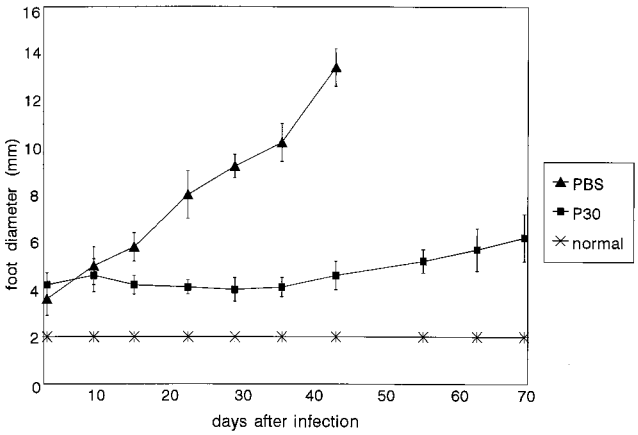

FIG. 4. BALB/c mice immunized with 25 μg (vol/vol) of p30 emulsified in CFA by the subcutaneous route in the footpad and after 20 days challenged with 10^7 *L. amazonensis* amastigotes. Symbols for the p30-immunized mice and controls are shown at right. Results represent the means of four mice ± standard deviations. P is <0.01 compared to control animals.

After the last foot measurement, mice were sacrificed, and sizes of spleen were compared among the three groups. Animals immunized with p30 showed normal-size spleens comparable to those from noninfected mice and markedly smaller than spleens from nonimmunized animals (data not shown). Cultures from lymph node and spleen from both control and p30-immunized mice maintained in axenic medium presented promastigotes after 7 days at 24°C, although the number of parasites was about 10 times lower in mice immunized with p30 compared to control. Foot lesion burden as evaluated by imprinting preparation showed a 21.90 ± 11.25 ratio of amastigotes to mononuclear cells in control versus 2.04 ± 0.21 in p30-immunized mice. These results showed that p30 is able to induce partial but significant protection in mice against infection with *L. amazonensis* amastigotes.

Characterization and subcellular localization of p30 by use of MAb. In order to characterize MAb 2E5D3, immunoblotting tests and immunoprecipitation of ^{125}I- and galactose oxidase-sodium boro[^{3}H]hydride-labeled amastigotes were performed. Both surface labelings showed negative results while Western blotting experiments showed that the MAb reacted strongly with p30 from *L. amazonensis* amastigotes and promastigotes (Fig. 5). Electrophoretic separation of *L. amazonensis* amastigote extracts and purified p30 followed by Schiff staining indicated that this protein is glycosylated. In order to verify whether the 2E5D3-reactive epitope is a carbohydrate, the periodate oxidation test was performed (57) and showed that the ability of p30 to react with MAb 2E5D3 was not abolished after periodate treatment (data not shown).

The subcellular localization of p30 was demonstrated by immunocytochemistry at electron microscopy level after incubation of ultrathin sections of foot lesions from *L. amazonensis*-infected hamsters with MAb 2E5D3 and protein A-gold labeling. It is observable that gold particles are predominantly distributed in structures very similar to megasomes (Fig. 6). The presence of these numerous lysosome-like organelles in the amastigote is a characteristic of species belonging to the *L. mexicana* complex, and association of cysteine proteinase activity with these organelles has been demonstrated in amastigotes from these species (41). The megasomal localization of

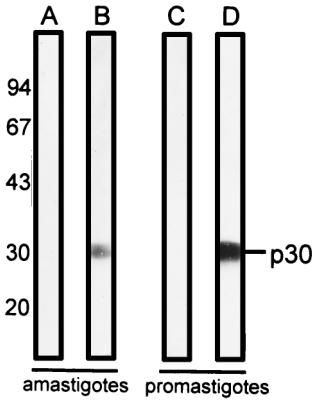

FIG. 5. Immunoblot analysis of MAb 2E5D3-reactive antigen of *L. amazonensis* amastigotes and promastigotes. A total of 1×10^8 amastigotes or 2×10^7 promastigotes were subjected to SDS-PAGE and Western blotting. MAb 2E5D3 was used at a 1:500 dilution (lanes B and D). Ascitic fluid from SP2/0-Ag14 was used as a negative control (lanes A and C). Standard molecular masses are represented on the left (in kilodaltons).

p30 led us to investigate the possible proteolytic activity of the antigen.

Proteolytic activity of p30. The antigen obtained with the MAb 2E5D3 immunoaffinity resin was subjected to SDS-PAGE with gelatin-coupled gels. Most of the proteolytic activity of the antigen migrates as a 32- to 35-kDa band (Fig. 7). The proteolytic activity detected at a higher molecular mass (70 to 110 kDa) can be due to self-association of the proteinase and/or precursor molecules which remain aggregated because of the mild conditions used (no heating or reduction of the sample) for gel separation. The proteolytic activity of the antigen was completely inhibited after SDS-PAGE separation and incubation of gelatin-coupled gels in the presence of antipain or *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane (E64) (data not shown).

DISCUSSION

Few studies of the identification of amastigote antigens implicated in protection in leishmaniasis have been performed (24, 25, 49, 55). However, the interaction between the intracellular forms of the parasite and the vertebrate cells, leading to immunological host responses, certainly plays a crucial role in the evolution of the disease. The present work describes the identification of *L. amazonensis* amastigote antigens involved in proliferative responses in inbred mice and focuses on one of them further characterized as a cysteine proteinase. Firstly, the optimum conditions of animal immunization were established. In *Leishmania*-susceptible mice, it has been demonstrated that the administration of irradiated parasites, soluble parasite extract, and lipophosphoglycan and/or gp63-containing liposomes by the intraperitoneal and intravenous routes confers protection on the host against reinfection, while administration through the subcutaneous route leads to progression of the disease (30, 44). In contrast, BALB/c mice were protected against infection by *Leishmania* species after subcutaneous immunization with antigens from *Leishmania infantum* promastigotes purified by electroelution (35). More recent evidence has shown that the site of infection also influences susceptibility and expression of immune response to *Leishmania major* (36). Our data favor the subcutaneous route and emphasize the importance of the pathway in immunization studies.

Among several amastigote antigens screened, p30 was chosen since it was one of the best inducers of T-cell responses in BALB/c mice (Fig. 1). This antigen was eluted from gels to be used in soluble form for lymphocyte assays, and the optimum conditions of lymphoproliferation induced by p30 were determined regarding the antigen concentration and specificity.

Nonspecific lymphocyte activation has been described with a peptide derived from a *Trypanosoma cruzi* surface antigen. It was demonstrated that this peptide coupled to a carrier is able to stimulate lymph node cells from mice immunized with CFA, KLH, or tetanus toxoid (38). In our system, the specificity of p30 was analyzed in comparison to that of other proteins, such as KLH and OVA, as well as to that of the adjuvant used in immunization schedules and mouse proteins. Our data permitted us to conclude that the induction of lymphoproliferation by p30 is specific and that no cross-reaction occurs with either mouse antigens or the other antigens tested (Fig. 3).

The stage specificity of p30 for T-cell responses was also demonstrated since lymphocytes from animals immunized with *L. amazonensis* promastigotes do not proliferate in the presence of p30 (Fig. 3). However, further characterization of p30 by reaction with MAb 2E5D3 showed that this antigen shares B-cell epitopes with p30 from promastigotes. These data are in agreement with those from the literature which have demonstrated that some major surface molecules are common to both forms (10, 33, 54), although they may present different T-cell epitopes.

The lymphoproliferative responses to p30 of BALB/c, C57BL/6, and C3H/HeJ mice were very similar at all antigen concentrations used. Although there are some controversies about the susceptibility of C57BL/6 and C3H mice to *L. amazonensis* which can be associated with the parasite strains and/or uncloned populations used in infection experiments (4, 5, 9, 19, 32), a resistant profile of C57BL/6 and C3H/HeJ mice to the *L. amazonensis* strain used in the present work could be demonstrated (16). The data shown in this paper indicate that there are no differences between *Leishmania*-susceptible and -resistant strains in regard to p30. The in vitro stimulation of spleen and lymph node cells by homologous antigens has been compared between BALB/c and C57BL/6 mice infected with *L. major* (31). It was demonstrated that, although only lymph node cells from resistant mice produced significant concentrations of IFN-γ, BALB/c cells pretreated with sublethal irradiation or MAb GK 1.5 were able to secrete IFN-γ in vitro after stimulation with *L. major* antigens. These results indicated that the BALB/c susceptibility is not due to the absence of T cells able to respond to *Leishmania* antigens which induce IFN-γ secretion. Our results corroborate these findings since p30 was similarly recognized by the three mouse strains.

The lymphocyte population stimulated by p30 is $CD4^+$, as demonstrated by the marked inhibition of lymphoproliferation induced by p30 in the presence of MAb anti-CD4. The inhibition levels obtained with anti-CD8 and rat IgG were very similar (25 and 27%, respectively), indicating that the reduction exerted by anti-CD8 is nonspecific (Table 1). $CD4^+$ lymphocytes have been involved in immune responses to *Leishmania* (34, 39), and several pieces of evidence indicate the participation of two $CD4^+$ subsets in these responses, Th1 and Th2, related to resistance and susceptibility, respectively (6, 21, 22, 29, 31, 45). In our work, the pattern of lymphokines secreted in the supernatants of lymphocyte cultures stimulated by p30 was IFN-γ and IL-2, while IL-4, IL-5, and IL-10 were absent (Table 2), indicating that Th1 is the subset implicated in these lymphoproliferative responses. Correlation of Th1 with resistance and Th2 with susceptibility has also been observed in protection studies involving antigens purified from *Leishmania* promastigotes. BALB/c mice can be immunized and protected against *L. major* infection by using an antigenic preparation

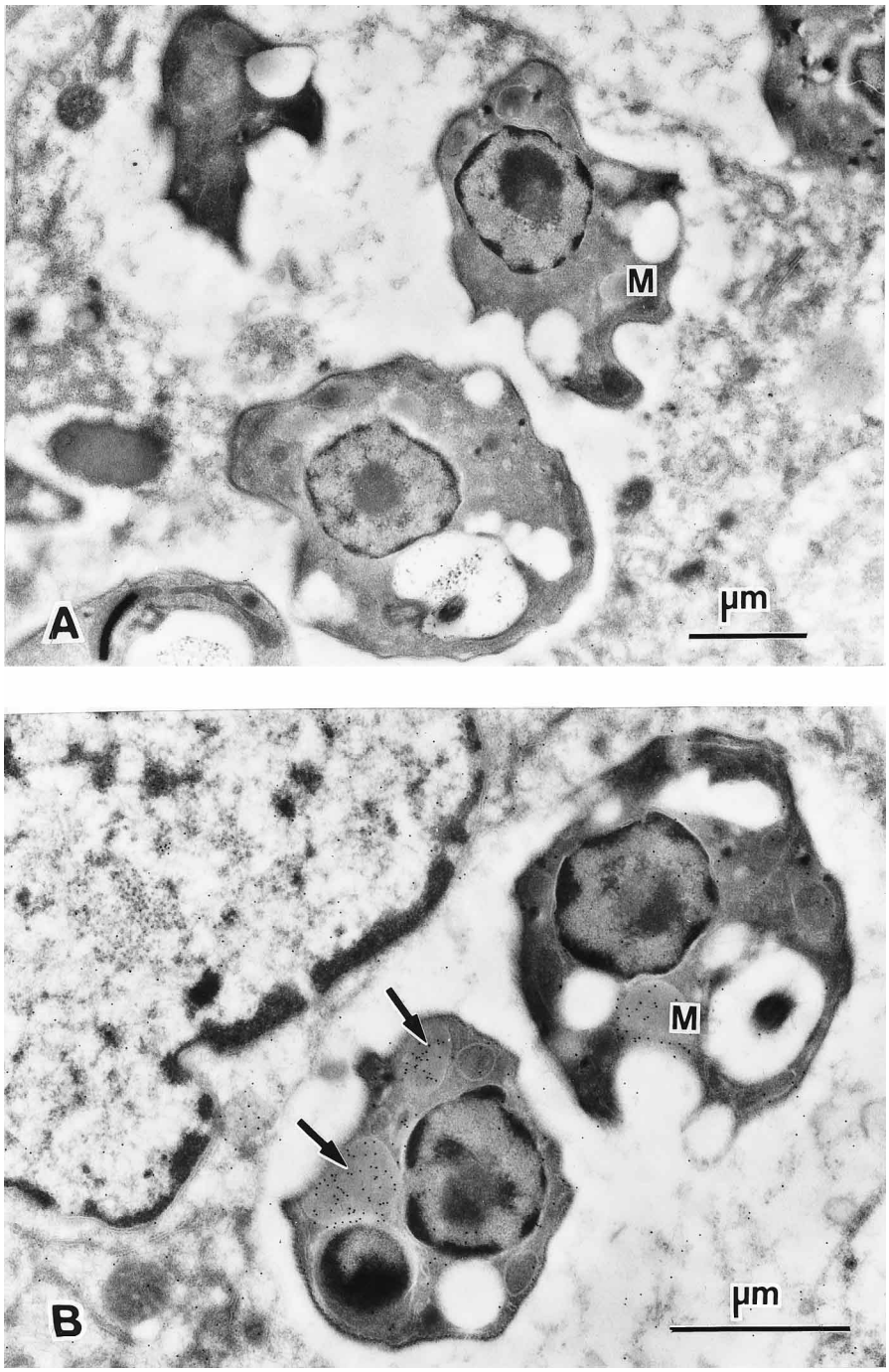

FIG. 6. Immunocytochemistry in foot lesions from *L. amazonensis*-infected hamsters. Ultrathin sections were incubated with normal mouse serum (A) or 2E5D3 MAb (B) followed by protein A-gold labeling. Gold particles are indicated by arrows. M, megasomes.

from this parasite which induces Th1 proliferation and high levels of IFN-γ (47, 48). Protection conferred on these animals is attributed to destruction of parasites by macrophages activated by IFN-γ. In our system, IFN-γ was produced by mouse lymphocytes stimulated by p30, and the next step was to establish a possible correlation between p30 and protection. Thus, susceptible BALB/c mice were immunized with p30 and challenged with *L. amazonensis*. These animals showed significantly lower levels of infection compared to control animals (without previous immunization) (Fig. 4).

The evaluation of parasite burden from foot lesions and spleens constitutes an important parameter to be considered in active immunization experiments. Data on changes in foot lesions can be misleading because the decrease in lesion size depends primarily on the resolution of inflammation and not only on the reduction in the number of viable parasites. It has been reported that the acquired resistance of mice to *Leishmania tropica* results in the elimination of the parasites from the primary lesion. Nevertheless, some viable parasites were still found persisting within the lymph node for at least 6

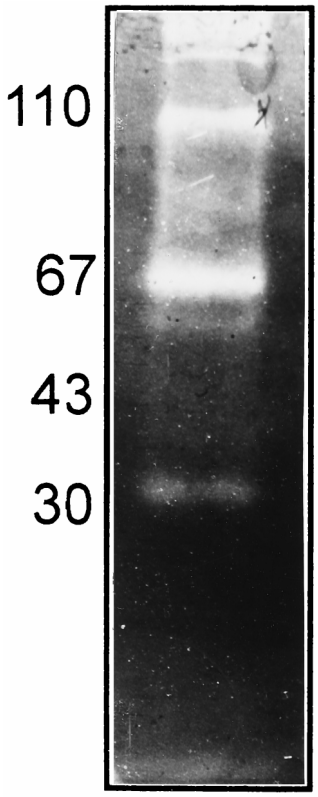

FIG. 7. Proteinase activity of p30 from *L. amazonensis* amastigotes. The antigen was isolated by MAb 2E5D3 immunoaffinity chromatography and separated by SDS-PAGE on a 10% acrylamide gel containing 0.1% gelatin under nonreducing conditions. Numbers at left indicate apparent molecular masses in kilodaltons.

months, in spite of resistance to reinfection (23). In our active immunization assays, p30-immunized mice presented foot lesions very similar in size to those from noninfected animals. In addition, these animals showed a significantly lower parasite burden in foot lesions compared to that from control mice. Although parasites were also isolated from spleens of all animals tested, the promastigote load was 10 times smaller in p30-immunized mice in relation to control animals without immunization. One hypothesis to explain the late reduction of protection observed (40 days after challenge) would be the growth of those parasites able to escape from the immunological responses induced by p30. It is also important to point out in our work the nature and number of parasites used to challenge p30-immunized mice: animals were infected with the amastigote form of parasites, which is much more infective than the promastigote form (8), and high challenge doses were used (10^7 parasites).

Characterization of p30 by use of MAb directed to this antigen showed that p30 presents proteolytic activity. Cysteine proteinases with an apparent molecular mass of around 30 kDa have been described in the amastigote form of *Leishmania pifanoi* and other species of the *L. mexicana* complex (2, 11, 37, 40). These enzymes have been associated with large organelles called megasomes which are unique to amastigotes of the *L. mexicana* complex (41). The apparent molecular weight, subcellular localization, and proteolytic activity of the antigen after immunoaffinity purification, as well as the loss of this activity by specific inhibitors, strongly indicated that p30 from *L. amazonensis* amastigotes is a cysteine proteinase. The capacity of this enzyme to induce lymphoproliferative responses in mice has been reported elsewhere (56). A recombinant cysteine proteinase obtained from *Escherichia coli* expressing a gene encoding one member of the *L. mexicana* amastigote cysteine proteinases was used to immunize mice and obtain a specific T-cell line from the Th1 phenotype (56). Our data corroborate these results. Although a different species was used in the present work, cysteine proteinase activity mainly associated with megasomes has also been detected in *L. amazonensis* amastigotes (12) and the genes encoding these cysteine proteinases have been described for other *Leishmania* species (53).

Regarding the protection conferred by p30 (Fig. 4), our results are in disagreement with recent data from the literature which showed little or no protection of BALB/c mice immunized with *L. pifanoi* cysteine proteinase and challenged with *L. pifanoi* or *L. amazonensis* (49). One possible explanation for this discrepancy is the different conditions used in the immunization protocols related to immunization route, adjuvant, antigen doses, and parasites used for challenge. Another important factor to be considered is the antigenic source. In spite of the similarities of cysteine proteinases present in species belonging to the *L. mexicana* complex in relation to proteolytic activity and cellular distribution, differences in the expression of T-cell epitopes could be responsible for the differences in cellular immune responses induced by these enzymes. Cloning and sequencing of the gene encoding p30 are currently in progress in order to define the immunodominant epitopes of this antigen.

Our data indicate a correlation between Th1 and resistance in BALB/c mice immunized with p30 and provide a basis for future studies directed toward the use of the native and recombinant forms of this antigen in protection schedules against *L. amazonensis* infection.

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