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+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.

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, histocyto-ma-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).

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, Belem, 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 107 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 107 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 Na2HPO4, 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) (Pharma-cia 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

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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 at 254 nm.

Electrophoretic characterization of amastigote antigens. L. amazonensis amastigotes were radiolabeled with 35S by the Iodogen method (14). Alternatively, parasites were radiolabeled with 3H[3H]methionine, by biosynthetic incorporation in the lymphocyte cultures. 2 × 109 amastigotes were resuspended in 1 ml of PBS (pH 7.2), lysed by sonication, and centrifuged at 200 × 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 (AMASTIGOTES 2053).

Lymphoproliferative responses of BALB/c mice to 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; Gibco BRL) or 25 μg of antibody (anti-IL-2) were used as a control. Standard curves were generated with recombinant murine IFN-γ, IL-4, IL-5, IL-7, and IL-10. After 60 min at 37°C, the 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.), NIH BVD, anti-IL-4; and biotinylated TRFK-5 and SXC and A5 (anti-IL-5) and SXC-1 and A5 (anti-IL-10), respectively. The cell cultures were stimulated with p30 purified for 10 min at 37°C, 100 μl of supernatant was added to wells. The resin was then washed extensively with PBS. Protein was quantified by a Coomassie blue reagent assay at 254 nm. After washing, the cell cultures were stimulated with p30 for 24 to 30 h in the presence of 2.5 μg of p30 per ml for 24 h at 37°C in a humidified chamber containing 5% CO2.

Electron microscopy immunocytochemical studies. Footpad lesions isolated from L. amazonensis-infected hamsters were fixed, embedded in 2% glutaraldehyde, freeze substituted in 10% 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.

Production and purification of MAb 2E5D3. The hybridoma supernatants were screened by ELISA by using p30 purified from infected footpad tissue. Protein was quantified by a Coomassie blue reagent assay at 254 nm.

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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).

**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 MAb directed to CD4<sup>+</sup> (clone H129.19) and to CD8<sup>+</sup> (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<sup>+</sup> is the population induced by p30.
The subset of CD4+ presence of p30, L. amazonensis by adding supernatants to CTLL cultures. Table 2 shows data 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, IL-5, and IL-10 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 challenge. Supernatants were used at a 1:4 dilution. Results represent the means of four mice ± standard deviations. P < 0.01 compared to T cells cultured with antigens in the absence of the MAb.

**TABLE 1. Characterization of T-cell population involved in lymphoproliferative responses to p30**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>[3H]Tdr incorporation (10^3 cpm)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>p30</td>
<td>36.40 (±1.30)</td>
<td></td>
</tr>
<tr>
<td>p30 plus anti-CD4</td>
<td>4.37 (±0.16)</td>
<td>88</td>
</tr>
<tr>
<td>p30 plus anti-CD8</td>
<td>27.30 (±0.70)</td>
<td>25</td>
</tr>
<tr>
<td>p30 plus rat IgG</td>
<td>26.57 (±1.20)</td>
<td>27</td>
</tr>
</tbody>
</table>

* Proliferation of lymph node cells from BALB/c mice immunized with L. amazonensis amastigotes was induced by p30 in the presence and absence of MAb anti-CD4 and anti-CD8. After 96 h, cells were harvested and the [3H]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.

**TABLE 2. Lymphokine production by BALB/c lymphocytes stimulated with p30**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>IL-2* (10^3 cpm)</th>
<th>IFN-γ (ng/ml)</th>
<th>IL-4 (U/ml)</th>
<th>IL-5 (pg/ml)</th>
<th>IL-10 (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>7.439</td>
<td>&lt;1.562</td>
<td>&lt;0.156</td>
<td>&lt;1.875</td>
<td>3.9</td>
</tr>
<tr>
<td>p30</td>
<td>18.511</td>
<td>11.3</td>
<td>&lt;0.156</td>
<td>&lt;1.875</td>
<td>6.0</td>
</tr>
<tr>
<td>AM</td>
<td>16.870</td>
<td>6.0</td>
<td>&lt;0.156</td>
<td>&lt;1.875</td>
<td>4.2</td>
</tr>
<tr>
<td>ConA</td>
<td>56.055</td>
<td>&gt;50.0</td>
<td>&lt;0.156</td>
<td>&lt;1.875</td>
<td>6.6</td>
</tr>
</tbody>
</table>

* 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 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 < 0.01 compared to control animals.

Infection. In the following days, there was a small increase of the foot diameter from p30-immunized mice compared to that of noninfected animals (normal).

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 animals 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 [35S]methionine and galactose oxidase-sodium borohydride-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 infection.
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 an-tigen was completely inhibited after SDS-PAGE separation.

FIG. 5. Immunoblot analysis of MAb 2E5D3-reactive antigen of *L. amazonensis* amastigotes and promastigotes. A total of 1 × 10^7 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).

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**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 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 antigen was completely inhibited after SDS-PAGE separation.

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 MAAb anti-CD4. The inhibitory 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...
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 \textit{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 \textit{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
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 "memory" 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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