Leishmania pifanoi Amastigote Antigens Protect Mice against Cutaneous Leishmaniasis

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In the search for a leishmaniasis vaccine, extensive studies have been carried out with promastigote (insect stage) molecules. Information in this regard on amastigote (mammalian host stage) molecules is limited. To investigate host immune responses to *Leishmania* amastigote antigens, we purified three stage-specific antigens (A2, P4, and P8) from in vitro-cultivated amastigotes of *Leishmania pifanoi* by using immunoaffinity chromatography. We found that with *Corynebacterium parvum* as an adjuvant, three intraperitoneal injections of 5 µg of P4 or P8 antigen provided partial to complete protection of BALB/c mice challenged with 10⁵ to 10⁷ *L. pifanoi* promastigotes. These immunized mice developed significantly smaller or no lesions and exhibited a 39- to 1.6 × 10⁵-fold reduction of lesion parasite burden after 15 to 20 weeks of infection. In addition, P8 immunization resulted in complete protection against *L. amazonensis* infection of CBA/J mice and partial protection of BALB/c mice, suggesting that this antigen provided cross-species protection of mice with different H-2 haplotypes. At different stages during infection, vaccinated mice exhibited profound proliferative responses to parasite antigens and increased levels of gamma interferon production, suggesting that a Th1 cell-mediated immune response is associated with the resistance in these mice. Taken together, the data in this report indicate the vaccine potential of amastigote-derived antigens.

Protozoan parasites of the genus *Leishmania* are associated with a broad spectrum of diseases, ranging from simple cutaneous to visceral leishmaniasis. *Leishmania pifanoi*, a member of the *L. mexicana* complex, causes both simple and diffuse cutaneous leishmaniasis. The latter form of the disease is characterized by large histiocytoma-like nodules that contain masses of heavily parasitized macrophages (8) and by a parasite-specific impairment of the cell-mediated immune response (9, 33). Patients with diffuse cutaneous leishmaniasis are generally resistant to current forms of chemotherapy (6).

Leishmania spp. are dimorphic obligate intracellular parasites. Flagellated promastigotes replicate and differentiate in the gut of the sandfly vector and are transmitted to a vertebrate when the sandfly takes a blood meal. Survival of the parasite within a vertebrate host is dependent on successful entry into a macrophage and transformation into the amastigote form. Nonmotile amastigotes live and multiply within the phagolysosome of host macrophages; continued infection involves rephagocytosis of amastigotes released by lysed macrophages. For most *Leishmania* species, the promastigote form can be readily cultivated in cell-free media at room temperature (23 to 27°C) (14). Thus, this stage of the parasite has been extensively studied. The procedures for isolation of amastigotes, either from lesions or by in vitro cultivation in macrophages and macrophage-like cell lines, are usually time-consuming and produce low yields of amastigotes contaminated with host components. However, the availability of axenic cultures of several *Leishmania* strains (5, 11, 13, 29) allows detailed studies of biological and immunologic functions of amastigote proteins. In the present study, we employed axenically cultured *L. pifanoi* amastigotes that have been demonstrated to be similar,

if not identical, to intracellular amastigotes by several criteria (29, 30, 32, 34).

Studies of animal models (20, 25, 42) and humans (2, 28) have indicated that development of a vaccine against leishmaniasis, especially the cutaneous forms of the disease, is feasible. In general, two approaches have been taken to generate immunoprophylactic preparations. One approach is induction of protection by using whole parasites, either attenuated, killed, or disrupted (17, 21). The other approach is subcellular fractionation of the parasites with the aim of identifying, isolating, and inducing protection with purified antigens (4, 15, 27). Appreciable levels of protection against cutaneous leishmaniasis have been achieved in genetically susceptible mice after immunization with promastigote surface membrane antigens, such as lipophosphoglycan, gp63, and GP46/M-2 (7, 16, 23, 36, 49), with soluble promastigote antigens (42, 43), or with defined gp63 epitopes (19, 50). It has been documented that lipophosphoglycan and gp63 also play an important role in parasite binding and invasion of macrophages (37). In contrast to the promastigote *Leishmania* form, relatively little is known about the mechanisms of amastigote adhesion and survival within the degradative milieu of macrophage phagolysosomes (26). Although amastigote-specific lipophosphoglycans and proteoglycans have been identified on the surface of different *Leishmania* species (3, 38, 48), the role of these structures in parasite-host interactions and in host immune reactivity remains poorly understood (44). In the search for immunogens that invoke potent host-protective responses, we have focused on molecules that are preferentially expressed in the amastigote stage, the primary form responsible for disease in mammalian hosts. Since the curative response requires a relatively long time to develop, it is likely that the antigens responsible for induction of self-healing in cutaneous leishmaniasis are synthesized by amastigotes (35). In this study, we evaluated the efficacy of immunization of BALB/c mice with three purified *L. pifanoi* amastigote proteins. This strain of mice is exceedingly susceptible to infection with *L. pifanoi* and *L. amazonensis* and

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thus can be used as a rigorous model for demonstrating protection. The data presented here indicate the vaccine potential of amastigote-derived antigens.

MATERIALS AND METHODS

Leishmania parasites. Two *Leishmania* strains were used. *L. pifanoi* MHOM/VE/60/Ltrod was maintained at 31°C in an amastigote form in simplified F-29 medium containing 20% fetal bovine serum (GIBCO, Grand Island, N.Y.) as previously reported (30). Parasite pathogenicity was maintained by transforming parasites from amastigote forms (cultured at 31°C) into promastigote forms (cultured at 23°C) and by regular passage through mice. *L. amazonensis* MHOM/BR/77/LT0016 was grown at 23°C in complete Schneider's *Drosophila* medium supplemented with 20% fetal bovine serum. Parasite pathogenicity was maintained by regular passage through mice. Low-passage promastigotes were harvested in the late-log phase, washed with phosphate-buffered saline (PBS), and used for infection. To prepare amastigote lysates for proliferation assays, parasites were washed with PBS and adjusted to 10^7 organisms per ml in PBS. After five freeze-and-thaw cycles, antigens were kept at -70°C until use.

Monoclonal antibodies (MAbs). The preparation and characterization of MAbs A-2 (C-2F9-F8), P-2 (XCII-3D8-D8), P-4 (CXXV-4C5-H6), and P-8 (CXVI-3G11-C10) against *L. pifanoi* amastigotes have been reported previously (31, 47). These antibodies appear to react selectively with the amastigote stage and have been previously shown to cross-react with *L. amazonensis* amastigotes. Purified MAbs bound to CNBr-activated Sepharose 2B (Pharmacia, Piscataway, N.J.) were used as affinity columns for protein purification.

***L. pifanoi* amastigote protein purification.** *L. pifanoi* amastigotes, cultured in roller bottles at 31°C, were washed with PBS and resuspended in lysis buffer containing 20 mM Tris-HCl (pH 7.3)-40 mM NaCl-10 mM EDTA-1 mM phenylmethylsulfonyl fluoride-1 mM iodoacetamide-1 mM 1,10-phenanthroline. Parasites were disrupted by nitrogen cavitation (Parr Instrument Co., Moline, Ill.) at 1,800 lb/in² for 20 min on ice, and the homogenate was subfractionated by differential centrifugation. Cell membrane was collected from the pellet after centrifugation at $40,000 \times g$ for 30 min. To purify P4 proteins, the membrane fraction was solubilized with lysis buffer containing 1% sodium deoxycholate (BDH Chemicals Ltd., Poole, England) at 4°C for 8 to 12 h. The mixture was centrifuged at $40,000 \times g$ for 30 min; the supernatant was diluted with column wash buffer (20 mM Tris-HCl [pH 7.3], 40 mM NaCl, 10 mM EDTA) and then loaded onto a P4 affinity column. After extensive washing, bound proteins were eluted with 0.1 M Na₂CO₃ (pH 11.4) containing 0.05% decanoyl-N-methylglucamid (MEGA-10; Boehringer, Mannheim, Germany) and then immediately neutralized with 1 M Tris-HCl (pH 6.0). To purify P8 proteins, the membrane fraction was solubilized with PBS containing 1% MEGA-10 at 4°C overnight. From 10 detergents examined (each at a 1:1 ratio of detergent to protein), MEGA-10 was selected on the basis of a competitive radioimmune assessment of antigen solubilization. The mixture was diluted with PBS and then loaded onto a P8 affinity column. After extensive washing with PBS containing 0.05% MEGA-10, bound proteins were eluted with 50 mM triethanolamine (pH 11.5) containing 0.15 M NaCl and 0.05% MEGA-10 and then neutralized with 1 M Tris-HCl (pH 6.0). The A2 cysteine proteinase was purified from the $40,000 \times g$ supernatant fraction of cell lysates as previously reported (12). Fractions eluted from affinity columns were initially assessed for protein by UV A_{280} and then evaluated for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis followed by Coomassie brilliant blue staining. Peak fractions were pooled, concentrated approximately 10- to 15-fold with Centricon-10 microconcentrators (Amicon, Danvers, Mass.), and stored at -70°C. The amount of protein used for immunization was quantitated by amino acid analysis at the Yale University School of Medicine Protein Facility. The antigenic activity of each preparation was estimated by immunoblot analysis.

Immunoblot analysis. Crude *L. pifanoi* amastigote lysates, membrane preparations, or affinity column-purified proteins were resolved by SDS-12.5% PAGE and electrophoretically transferred to Immobilon P transfer membranes (Millipore Corp., Bedford, Mass.). After nonspecific binding was blocked with PBS containing 5% nonfat dry milk, the filters were probed with diluted MAbs or sera from immunized mice. The filters were washed and incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (1:750; Bio-Rad, Melville, N.Y.). Antigenic bands were visualized by development with a 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt)-nitroblue tetrazolium phosphatase substrate (KPI, Inc., Gaithersburg, Md.). The specificity of the immune response of immunized mice was assessed by immunoblot analysis employing pooled preimmune sera and sera collected 2 weeks after the final immunization (1:100 dilution with PBS containing 5% nonfat dry milk).

Immunization of mice. Female BALB/c (*H-2^d*) mice (Charles River, Wilmington, Mass.), five per group, were immunized by three intraperitoneal (i.p.) injections of 5 µg of individual *L. pifanoi* amastigote proteins together with 100 µg of the adjuvant *Corynebacterium parvum* (Wellcome Research Laboratories, Research Triangle Park, N.C.). Injections were given at biweekly intervals. Mice receiving PBS (used to dilute purified proteins) or 100 µg of adjuvant alone served as controls. Four weeks after the last immunization, mice were challenged in the right hind foot with 10^5 to 10^7 late-log-phase promastigotes of *L. pifanoi*.

To test whether purified *L. pifanoi* amastigote proteins would provide cross-species protection, groups of five BALB/c mice were immunized by following the immunization protocol indicated above. Four weeks after the last immunization, mice were challenged in the right hind foot with either 10^3 or 10^5 late-log-phase promastigotes of *L. amazonensis*. In a separate experiment, seven female CBA/J (*H-2^k*) mice (Jackson Laboratory, Bar Harbor, Maine) were immunized by three i.p. injections of 5 µg of purified P8 together with 100 µg of *C. parvum*. Injections were given at 1-week intervals. Mice receiving PBS or 100 µg of adjuvant alone served as controls. Approximately 5 weeks after the last immunization, mice were challenged in the right hind foot with 7×10^4 late-log-phase promastigotes of *L. amazonensis*.

Evaluation of infection. The course of infection was monitored by measuring the increase in footpad thickness, compared with the uninfected left footpad, with a dial gauge caliper. At the times designated in Results, mice were sacrificed to estimate the parasite burden in the footpad and draining lymph node tissues by following procedures reported previously, with some modifications (46). Briefly, 10-fold serial dilutions of footpad tissue homogenates (in equal weight of tissue) or single-cell suspensions of the draining lymph nodes (in equal cell number) were made in complete Schneider's medium. Aliquots of 100 µl of each serial dilution were added to 16 replicate wells in 96-well plates (Corning Glass Works, Corning, N.Y.). The cultures were incubated at 23°C for 10 days and scored with an inverted microscope. It has been noted that *L. pifanoi* Ltrod grows more rapidly in the amastigote form than in the promastigote form (unpublished observations) and that transformation from amastigotes into promastigotes can be readily induced and observed 48 h after changing the culture temperature from 31 to 23°C (29). Thus, to maximize sensitivity of detection, serial dilutions of footpad tissue homogenates (made in complete F29 medium) from mice challenged with *L. pifanoi* were incubated at 31°C for 6 days before transferring cultures to 23°C. Recovery of viable *L. pifanoi* promastigotes was determined by daily examination for 5 days after cultures were transferred to 23°C. A well was considered to be positive for parasite growth when motile organisms were observed. Data were analyzed by using a modified computer program originally designed by Taswell (45).

T-cell proliferation assays. Six weeks after the last immunization (day 0) and at 10, 30, 50, and 90 days postinfection, two mice each from both the *C. parvum* control group and the immunized group with P4 plus *C. parvum* were sacrificed. Spleen cells from two mice were collected and pooled for lymphocyte proliferation assays. Single-cell suspensions were prepared in Iscove's modified Dulbecco's medium (IMDM) (materials used in spleen cell culture were from GIBCO unless otherwise specified) supplemented with 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, Mo.), 50 µg of gentamicin per ml 100 U of penicillin per ml, and 100 µg of streptomycin sulfate per ml. Cell suspensions were enriched for T cells by passage over nylon-wool columns (Polysciences, Inc., Warrington, Pa.). Fluorescence-activated cell sorter analyses using phycoerythrin-conjugated hamster anti-mouse MAb CD3 (PharMingen, San Diego, Calif.) and fluorescein-conjugated rat anti-mouse Ly-5 (B220) (Caltag, South San Francisco, Calif.) indicated no significant difference in terms of T- and B-cell populations between mice immunized with P4 plus *C. parvum* and those given *C. parvum* alone (data not shown). To measure the responses of T cells to leishmanial antigens, 2×10^5 enriched T cells (100 µl) together with 10^5 irradiated (3,000 rads of ¹³⁷Cs) syngeneic spleen cells (100 µl) were dispensed into 96-well, flat-bottom tissue culture plates in triplicate. Fifty-microliter volumes of twofold serial dilutions of either purified P4 antigen (20 µg/ml) or amastigote lysates (10^6 parasite equivalents per ml) were then added. In certain cases, cells were stimulated with 5 µg of concanavalin A per ml or 10 µg of lipopolysaccharide per ml. The wells were cultured with or without antigen for 3 days at 37°C in an atmosphere of 5% CO₂. After 3 days, 1 µCi of [³H]thymidine (New England Nuclear, Boston, Mass.) was added to each well and the mixture was incubated overnight. Cells were collected on filters with a harvester (PHD Cell Harvester; Cambridge Technology, Inc.), and [³H]thymidine incorporation was determined with a liquid scintillation counter.

Cytokine enzyme-linked immunosorbent assays (ELISAs). Before the addition of [³H]thymidine, 75 µl of cell culture supernatant per well was harvested and kept at -20°C until cytokine measurements. Gamma interferon (IFN-γ) levels were measured as previously described (23). Two-site interleukin 4 (IL-4) or IL-2 ELISAs were conducted with 15 µg of protein G-purified MAb BVD4-1D11 (American Type Culture Collection, Rockville, Md.) per ml and biotin-labeled anti-mouse IL-4 MAb BVD6-24G2 (PharMingen) or with 1 µg of anti-mouse IL-2 MAb JES6-1A12 (PharMingen) per ml and biotin-labeled anti-mouse IL-2 MAb JES6-5H4 (PharMingen). Briefly, plates were coated with the first MAb at 4°C overnight. After washing with PBS-Tween and blocking with PBS-5% fetal calf-serum; diluted culture supernatants were incubated at 37°C for 1 h or at 4°C overnight. After washing, the plates were incubated with 1 µg of a secondary MAb per ml at 37°C for 45 min and ELISAs were developed by reaction of streptavidin-alkaline phosphatase with a *p*-nitrophenol phosphate substrate (Pierce, Rockford, Ill.). Plates were analyzed on an ELISA reader at 405 nm. The specific cytokine concentrations were calculated on the basis of standard curves of recombinant cytokines.

FIG. 1. Purification of the A2, P4, and P8 proteins from *L. pifanoi* amastigotes. Coomassie blue-stained SDS-12.5% PAGE of whole-parasite lysates (lane 1), soluble proteins (lane 2), and membrane-enriched preparations solubilized with sodium deoxycholate (lane 3) or Mega-10 (lane 4) is shown. Immunoaffinity-purified A2, P4, and P8 are shown in lanes 5 to 7. MW, molecular mass.

RESULTS

Purification of *L. pifanoi* amastigote proteins. To purify *L. pifanoi* amastigote proteins, methods including differential centrifugation, detergent solubilization, and immunoaffinity chromatography were performed (see Materials and Methods). Purified A2 cysteine proteinase showed a major band at 27 kDa and minor bands between 29 and 37 kDa (Fig. 1, lane 5), corresponding to precursor forms of the proteinase. The gene encoding A2 and the relationship of high-molecular-weight precursors to the mature proteinase has been reported previously (47). On SDS-PAGE, purified P4 appeared as a doublet of proteins with estimated molecular masses of 33 and 35 kDa (Fig. 1, lane 6). Tryptic peptide mapping of protein bands recovered from SDS-PAGE and pulse-chase analysis of metabolically labeled axenic *L. pifanoi* amastigotes suggested that the 35-kDa protein is likely to be the precursor of the 33-kDa protein (unpublished data). A more diffuse pattern was observed in purified P8, with three groups of bands at molecular masses near 30, 43, and 100 kDa (Fig. 1, lane 7). The precise biochemical nature of P8 is unclear; however, evidence suggests that P8 contains a core protein with a molecular mass of 30 kDa and it is closely complexed or associated with carbohydrates and/or phosphoglycan components (data not shown). Therefore, high-molecular-weight bands seen in P8 could be the result of posttranslational modifications of the protein and/or aggregation of modified proteins. Similar high-molecular mass components were evident on Western blot (immunoblot) analysis of whole amastigotes and the P8 MAb in previous studies (30, 31). The antigenic activities of the three affinity-purified proteins were confirmed by immunoblot analyses with specific MAbs (data not shown).

Protection of BALB/c mice against *L. pifanoi* infection. BALB/c mice were immunized with either antigen A2, P4, or P8 together with *C. parvum* as described in Materials and Methods. The mice were then challenged with either 10^5 , 10^6 , or 10^7 infective *L. pifanoi* promastigotes. As shown in Fig. 2, mice given PBS or adjuvant alone developed a progressive

FIG. 2. Evaluation of protection against infection with *L. pifanoi* in A2-, P4-, and P8-immunized BALB/c mice. Mice (five per group) were immunized i.p. three times at biweekly intervals with purified antigens together with the adjuvant *C. parvum* as described in Materials and Methods. Control groups were immunized similarly either with PBS (○) or with *C. parvum* (□) alone. Eight weeks after the initial immunization, the mice were challenged with either 10^5 , 10^6 , or 10^7 late-log-phase promastigotes of *L. pifanoi* in the right hind foot. Infection was monitored by footpad measurements which are expressed as a ratio of the size of the infected right foot to that of the uninfected left foot. The data shown are average values for each group. Symbols: ●, A2 plus *C. parvum*; ▲, P4 plus *C. parvum*; ■, P8 plus *C. parvum*.

TABLE 1. Numbers of parasites in footpads of control and antigen-immunized mice after challenge with different doses of *L. pifanoi*^a

No. of parasites in challenge dose	Time of testing (wpi)	Immunization group	No. of parasites (10 ³)/footpad
10 ⁵	20	PBS	791,490.0
		<i>C. parvum</i>	47,946.0
		A2 + <i>C. parvum</i>	94.3
		P4 + <i>C. parvum</i>	1.0
		P8 + <i>C. parvum</i>	4.7
10 ⁶	17	PBS	3,193.2
		<i>C. parvum</i>	27,774.9
		A2 + <i>C. parvum</i>	23,042.5
		P4 + <i>C. parvum</i>	19.4
		P8 + <i>C. parvum</i>	0.0
10 ⁷	16	PBS	14,047.4
		<i>C. parvum</i>	16,770.0
		A2 + <i>C. parvum</i>	103,267.0
		P4 + <i>C. parvum</i>	359.0
		P8 + <i>C. parvum</i>	0.1

^a BALB/c mice (five per group) were immunized with purified *L. pifanoi* amastigote antigens together with *C. parvum* and then challenged with the indicated doses of *L. pifanoi* promastigotes by footpad injection (see Materials and Methods). Mice given PBS or adjuvant alone were used as controls. At the indicated times postinfection, footpads were quantitatively assessed for the parasites with a limiting-dilution assay. The number of parasites per footpad was obtained by multiplying the number of parasites per gram of foot tissue by the average weight (in grams) of the footpads.

infection in the infected foot at all challenge doses. Immunization with the A2 cysteine proteinase plus the adjuvant resulted in a delayed onset of lesion development when mice were challenged with 10⁵ parasites. However, this antigen failed to protect mice challenged with the larger doses of parasites. In contrast to the results found with the A2 proteinase, mice immunized with the membrane-derived P4 antigen plus adjuvant developed significantly smaller lesions at all three challenge doses. In mice immunized with the P8 antigen plus adjuvant, no measurable lesion was detected after a challenge with up to 10⁶ to 10⁷ parasites at 15 to 17 weeks postinfection (wpi).

To further assess the level of protection, mice were sacrificed at 16 to 20 wpi to measure parasite burdens in the lesions, draining lymph nodes, and spleen by using limiting-dilution assays. Mice infected with *L. pifanoi* did not show metastatic spread of parasites to the spleen or draining lymph nodes after infection with either 10⁵ or 10⁶ parasites. However, live parasites were recovered in the draining lymph nodes in one of five mice given PBS alone and three of five mice immunized with A2 proteinase plus the adjuvant after infection with 10⁷ *L. pifanoi* parasites (data not shown). No parasites were found in the draining lymph nodes of mice immunized with *C. parvum* plus the P4 or P8 antigen and infected with 10⁷ *L. pifanoi* parasites. As summarized in Table 1, no or very low numbers of parasites were recovered in mice immunized with P8 plus *C. parvum* and challenged with different doses of parasites. Immunization with P4 plus *C. parvum* resulted in a 7.9 × 10⁵-fold reduction of lesion parasites compared with PBS control mice after 20 weeks of infection with 10⁵ parasites. When P4-immunized mice were challenged with 10⁶ and 10⁷ parasites, there was a 39- to 164-fold reduction of parasite burden after 16 to 17 weeks of infection, indicating partial protection of these mice. A detailed analysis of lesion parasite burdens during the

course of infection, using 12 mice immunized with P4 plus *C. parvum* and challenged with 10⁶ *L. pifanoi* promastigotes, revealed that antigen immunization resulted in a 10-fold reduction of parasites per footpad on days 30 and 50 and an 870-fold reduction on day 90 compared with adjuvant control mice (data not shown). These results indicated that protection was evident during an early stage of infection and inhibition of parasite growth in the immunized mice was most significant at a later stage of infection. In agreement with lesion size measurements (Fig. 2), immunization with the A2 proteinase reduced lesion parasite burdens only in mice challenged with 10⁵ parasites (Table 1). Taken together, these observations indicated that immunization with *L. pifanoi* amastigote-derived P4 and P8 antigens in conjugation with *C. parvum* could result in significant, and in some cases complete, protection against *L. pifanoi* infection.

Generation of antibody response to immunization. The antibody response to vaccination was examined by immunoblot analysis. Preimmune sera and sera from PBS or adjuvant control mice did not show significant binding to *L. pifanoi* amastigote lysates, although background or nonspecific binding to components with high molecular masses was noted (Fig. 3, lanes 1 to 3). The sizes of molecules recognized by immunized sera corresponded to those of affinity-purified antigens, with some variations (compare Fig. 3, lanes 4 and 5, to Fig. 1, lanes 5 to 7). Mice immunized with the A2 proteinase showed an antibody response to molecules of about 27 and 37 kDa (Fig. 3, lane 4), corresponding to the mature proteinase and its precursor form, respectively. Sera from P4-immunized mice recognized two strong bands at 33 and 38 kDa (Fig. 3, lane 5). P8-immunized mouse sera recognized two distinctive bands at 30 and 36 kDa and a third, diffuse component ranging from 50 to 80 kDa (Fig. 3, lane 6). Similar patterns were observed on immunoblot analysis employing the P8 MAb (31; data not shown). The 50- to 80-kDa components appeared to be relatively minor components on an SDS-PAGE gel of the purified P8 antigen (Fig. 1, lane 7).

Examination of T-cell responses in immunized mice. Resistance to *Leishmania* infection is believed to be associated with

FIG. 3. Immunoblot of sera from mice before and after immunization. *L. pifanoi* amastigote lysates (lanes 1 to 4) and membrane-enriched fractions (lanes 5 to 6) were separated by SDS-12.5% PAGE, blotted onto nitrocellulose, and reacted with sera as described in Materials and Methods. Shown are the reactions with pooled preimmune mouse sera (lane 1) and sera collected 2 weeks after the final booster immunization from mice given PBS (lane 2) or *C. parvum* alone (lane 3) or immunized with purified A2, P4, or P8 together with *C. parvum* (lanes 4 to 6). MW, molecular mass.

FIG. 4. Cellular immune responses of P4-immunized mice to *L. pifanoi* antigens. BALB/c mice were immunized with either purified P4 antigen plus *C. parvum* or with *C. parvum* alone as described in Materials and Methods. Six weeks after the last immunization, two mice in each group were sacrificed. Splenic T cells from adjuvant control mice (----) and from P4-immunized mice (—) were enriched with nylon-wool columns. T-cell proliferative response to twofold serial dilutions of either purified P4 antigen (20 µg/ml) (▲) or *L. pifanoi* amastigote lysates (10^6 parasite equivalents per ml) (○) was detected by [3 H]thymidine incorporation after 3 days in culture. The data shown represent the stimulation index, which was calculated by dividing mean counts per minute of experimental samples by mean counts per minute of medium controls.

cell-mediated immunity (22). Thus, the capacity of spleen cells from P4 vaccinated mice to respond to parasite antigens was measured by lymphocyte proliferation and cytokine production by using T-cell-enriched splenic lymphocytes. Subsequent to the infection with 10^6 *L. pifanoi* promastigotes, the T cell-enriched populations from control mice failed to respond to either purified P4 or *L. pifanoi* lysates; the stimulation indexes were near or below 2.0. In contrast, T cells from P4-immunized mice exhibited significant response to parasite lysates, as well as to purified P4, after immunization and at 10 days after infection (Fig. 4). Nonresponsiveness in adjuvant control mice was not due to a marked change of T- and B-cell populations, as judged by flow cytometric analysis (data not shown). This nonresponsiveness is parasite specific, since splenic T cells from these mice exhibited proliferative responses to concanavalin A and lipopolysaccharide comparable to those of cells from P4-immunized mice (data not shown).

To understand whether the protection in immunized mice correlated with a specific pattern of lymphokine production, culture supernatants from proliferation assays were collected for measurement of IFN- γ , IL-4, and IL-2 levels with specific ELISAs. While T cells from P4-immunized mice produced significant levels of IFN- γ in response to purified antigen or parasite lysates at various times after infection, there was no measurable level of IFN- γ in T cells from adjuvant control mice (Table 2). In terms of IL-2 production, there was no major difference between control and P4-immunized mice, since in both groups approximately 3 to 4 U of IL-2 per ml was detected on day 10 and 18 to 22 U/ml was detected on day 90 postinfection. When T cells were stimulated with purified P4 or parasite lysates for 3 days, no IL-4 was detected in cells from either control or P4-immunized mice at any stage during infection.

Cross-species protection from *L. amazonensis*. Since the MAbs used in protein purification recognize amastigote components of both *L. pifanoi* and *L. amazonensis* (31), the possi-

bility that purified *L. pifanoi* amastigote proteins can protect BALB/c mice against infection with *L. amazonensis* was investigated. Mice immunized i.p. with the A2 cysteine proteinase, P4, or P8 were challenged with 10^3 or 10^5 *L. amazonensis* parasites. Similar to the results found in *L. pifanoi* infection, the A2 proteinase was ineffective in protecting against *L. amazonensis* infection, as judged by both lesion size measurement (Fig. 5) and parasite burden (Table 3). Immunization with P4 did not appear to inhibit lesion development (Fig. 5), although a slight lesion parasite number reduction in these mice was noted compared with control mice (Table 3). In contrast, P8-immunized mice developed smaller lesions after a challenge with 10^3 or 10^5 *L. amazonensis* parasites (Fig. 5). Consistent with these observations, these mice displayed a 17- to 70-fold reduction of lesion parasites in comparison with control mice (Table 3). These results indicated that P8 could provide partial protection of BALB/c mice against *L. amazonensis* infection.

The potential of P8 as a leishmanial vaccine candidate was further tested with CBA/J mice. This strain of mice is susceptible to *L. amazonensis* infection but relatively resistant to this infection in comparison with the BALB/c strain of mice (7). After three i.p. injections of P8 together with *C. parvum*, seven mice were challenged with 7×10^4 late-log-phase *L. amazonensis* promastigotes. The course of infection was monitored by lesion size measurement. At 2, 6, and 15 wpi, two mice from each group were sacrificed to determine the parasite burdens within the lesions. As shown in Table 4, significant reduction of parasite growth in P8-immunized mice was observed at 6 wpi compared with PBS and adjuvant control mice, at a time when the lesions in control mice had not significantly developed. At 15 wpi, mice remaining in the PBS and adjuvant control groups (3) developed large lesions, whereas all P8-immunized mice (3) showed no sign of lesion development. Limiting-dilution analyses confirmed the complete protection of P8-immunized mice (Table 4).

DISCUSSION

In the present study, we purified a soluble protein (A2) and two membrane-associated proteins (P4 and P8) from axenically cultured amastigotes of *L. pifanoi* and investigated the

TABLE 2. IFN- γ levels in splenic T cells from P4-immunized mice after infection with 10^6 *L. pifanoi* parasites^a

Immunization group	Time of testing (dpi)	IFN- γ level (ng/ml) ^b		
		PromAg	AmasAg	Purified P4
P4 + <i>C. parvum</i>	0	ND ^c	10.28	ND
	10	<1	11.21	13.36
	30	15.90	27.41	19.06
	90	4.36	12.55	13.41
<i>C. parvum</i>	0	ND	<1	ND
	10	<1	<1	<1
	30	<1	<1	<1
	90	<1	<1	<1

^a Groups of two mice were sacrificed approximately 6 weeks after the last immunization (day 0 of infection) and at the indicated days postinfection (dpi).

^b Splenic T cells from *C. parvum* control mice and mice immunized with P4 plus *C. parvum* were enriched with nylon-wool columns and stimulated with either *L. pifanoi* amastigote (AmasAg) or promastigote (PromAg) lysates (2.5×10^6 parasites per ml) or with purified P4 (5 µg/ml) for 3 days. Supernatants were collected, and IFN- γ measurements were performed by ELISA as described in Materials and Methods. In the absence of an antigen, the IFN- γ level was <1 ng/ml. The data shown are means of three duplicate wells.

^c ND, not determined.

FIG. 5. Cross-species protection against infection with *L. amazonensis* in P8-immunized BALB/c mice. Mice (five per group) were immunized i.p. three times at biweekly intervals with purified antigens together with *C. parvum* as described in Materials and Methods. Control groups were immunized similarly either with PBS (○) or with *C. parvum* (□) alone. Eight weeks after the initial immunization, the mice were challenged with either 10^3 or 10^5 late-log-phase promastigotes of *L. amazonensis* in the right hind foot. Infection was monitored by footpad measurements. The data shown represent average lesion size ratios for each group. Symbols: ●, A2 plus *C. parvum*; ▲, P4 plus *C. parvum*; ■, P8 plus *C. parvum*.

potential of these antigens to elicit a protective immune response in BALB/c and CBA/J mice against challenges with infective promastigotes of *L. pifanoi* and *L. amazonensis*. Our previous studies have demonstrated that these proteins are preferentially expressed in the amastigote stage of the parasites (30, 31). We have shown here that these proteins are associated with different levels of immunoprotection, ranging from partial to complete. This work indicates the vaccine potential of amastigote-derived immunogens in murine cutaneous leishmaniasis.

We demonstrated that immunization with the P8 antigen together with *C. parvum* as an adjuvant induced significant, reproducible protection in BALB/c mice against infection with 10^5 to 10^7 *L. pifanoi* promastigotes (Fig. 2 and Table 1). These vaccinated mice showed no major sign of lesion development at 15 to 20 wpi. Lesion parasite burden analyses further confirmed these results, in that no live parasites were recovered from P8-immunized mice challenged with 10^6 parasites, suggesting complete protection. More important for a potential vaccine candidate, the P8 antigen provided partial protection

TABLE 3. Numbers of parasites in footpads of control and immunized mice after infection with different doses of *L. amazonensis*^a

No. of parasites in challenge dose	Time of testing (wpi)	Immunization group	No. of parasites/footpad
10^3	21	PBS	$>1.40 \times 10^{10}$
		<i>C. parvum</i>	1.30×10^8
		A2 + <i>C. parvum</i>	$>1.61 \times 10^{10}$
		P4 + <i>C. parvum</i>	4.00×10^7
		P8 + <i>C. parvum</i>	7.66×10^6
10^5	16	PBS	7.52×10^8
		<i>C. parvum</i>	5.91×10^7
		A2 + <i>C. parvum</i>	2.21×10^9
		P4 + <i>C. parvum</i>	1.16×10^6
		P8 + <i>C. parvum</i>	8.38×10^5

^a BALB/c mice (five per group) were immunized with either antigen A2, P4, or P8 together with *C. parvum* and then challenged with the indicated doses of *L. amazonensis* by footpad injection (see Materials and Methods). Mice given PBS or adjuvant alone were used as controls. At the indicated times postinfection, the footpads of all of the mice were assessed for the presence of parasites with a limiting-dilution assay. The number of parasites per footpad was obtained by multiplying the number of parasites per gram of foot tissue by the average weight (in grams) of the footpads.

of BALB/c mice (Fig. 5 and Table 3) and complete protection of CBA/J mice (Table 4) against *L. amazonensis* infection, indicating that P8 can induce efficient cross-protection in mice with different *H-2* haplotypes. Appreciable protection against infection with *L. major* and *L. amazonensis* has been achieved in genetically susceptible mice with defined promastigote antigens (19, 36, 49, 50). Direct comparison of the efficacy of P8 with that of other promastigote immunogens is difficult because of the different *Leishmania* species employed, the complexity of factors and variables involved in these vaccine studies, and the lack of a standardized measurement of infection (such as parasite burden in the lesion) in most of the studies. Our initial comparative study of the P8 antigen with GP46/M-2, a protective immunogen purified from *L. amazonensis*

TABLE 4. Lesion size and parasite numbers in control and P8-immunized CBA/J mice after infection with 7×10^4 *L. amazonensis* parasites^a

Time of testing (wpi)	Immunization group	No. of parasites/footpad	Mean lesion size ratio (right foot/left foot) $\pm$ SE
2	PBS	26.0	ND ^b
	<i>C. parvum</i>	22.3	ND
	P8 + <i>C. parvum</i>	1.0	ND
6	PBS	1.6×10^6	1.08 ± 0.03
	<i>C. parvum</i>	4.8×10^6	1.08 ± 0.03
	P8 + <i>C. parvum</i>	1.0	1.05 ± 0.01
15	PBS	3.7×10^{13}	1.79 ± 0.06
	<i>C. parvum</i>	3.4×10^{12}	1.86 ± 0.14
	P8 + <i>C. parvum</i>	0	1.00 ± 0.01

^a CBA/J mice (seven per group) were immunized with either P8 plus *C. parvum* or with PBS or adjuvant alone and then infected with 7×10^4 *L. amazonensis* parasites by footpad injection (see Materials and Methods). At the indicated times postinfection, the footpads of two mice in each group were assessed for the presence of parasites with a limiting-dilution assay. The number of parasites per footpad was obtained by multiplying the number of parasites per gram of footpad tissue by the average weight (in grams) of the footpads.

^b ND, not determined.

promastigotes (7, 23), suggested that the level of protection elicited in P8-immunized CBA/J mice was significantly better than that in GP46/M-2-immunized mice (unpublished data). Since P8 can stimulate a lymphocyte proliferative response in cutaneous leishmaniasis patients infected with *L. braziliensis* (10a), the potential of this immunogen as a human vaccine candidate seems feasible. This is the first report of protective immunogens from amastigotes in murine cutaneous leishmaniasis. Our initial protein chemistry analysis and studies of subcellular localization with MAbs revealed that P8 is a protein complex located on the surface and in the flagellar pocket region of amastigotes (data not shown). It remains to be determined how these molecules might be involved in the various parasite-macrophage interactions that occur within infected cells.

Similar to P8, the P4 antigen was purified from solubilized membrane preparations of *L. pifanoi* amastigotes (Fig. 1). Immunization with the P4 antigen together with *C. parvum* significantly protected BALB/c mice against *L. pifanoi* infection at challenge doses ranging from 10^5 to 10^7 parasites. These mice showed a delay in onset of the disease and a significant reduction of lesion parasite burden, especially at the 10^5 -parasite inoculum (Fig. 3 and Table 1). When stimulated with parasite antigens, T cells from these immunized mice displayed strong proliferative responses before challenge (Fig. 4) and at early stages during infection compared with cells from control mice (data not shown). It has been suggested that natural killer cells are a source of IFN- γ at an early stage of *Leishmania* infection (40). We found that T cells from protected mice produced a high level of IFN- γ at both early and late stages of infection (Table 2), suggesting that a Th1-mediated immune response was associated with resistance in these mice. Additional evidence from cell depletion and/or cell transfer experiments is necessary to define the precise mechanism(s) involved in protection. We have previously shown that the MAb used in P4 antigen purification cross-reacts with *L. amazonensis* amastigotes (31). In addition, there is evidence that the P4 mRNA is expressed in *L. amazonensis* amastigotes (43a). Surprisingly, we found that isolated *L. pifanoi* P4 protein failed to provide comparable protection of BALB/c mice against *L. amazonensis* infection (Fig. 5), although a slight reduction in parasite burden was observed (Table 4). The reasons for this result remain unclear. It is possible that the immunization protocol used in this study is not sufficient to provide cross-species protection and that the P4 antigen in these two species of parasites is not the same. It is well documented that the extent of protection in murine models of cutaneous leishmaniasis can be determined by several factors, including mouse strain, adjuvant, immunogen concentration, and parasite dose (4, 15, 17, 24, 36). Therefore, it remains to be determined whether a higher concentration of the P4 immunogen is required to achieve complete protection against *L. pifanoi* and *L. amazonensis* infections. In addition, it will be of interest to examine whether long-lasting protection can be generated when the P4 antigen is administered together with IFN- γ or IL-12, two cytokines that have been successfully used as adjuvants in vaccination of BALB/c mice against *L. major* (1, 41).

We have previously reported that A2 is an abundant (accounting for 1% of the total amastigote proteins), *L. pifanoi* amastigote-specific cysteine proteinase encoded by the Lpcys2 gene family (47). Although the major proteinase is located in the megasomes, a low level of mature proteinase is associated with the flagellar pocket and plasma membrane of amastigotes, and there is evidence that the proteinase is secreted (12). Thus, its relative abundance and accessibility, together with its antigenicity in immunized mice (Fig. 3 and unpublished data),

have led to investigation of whether this proteinase can protect mice against *Leishmania* infection. Surprisingly, we found that immunization with the A2 proteinase provided little to no protection of BALB/c mice against infection with 10^5 to 10^7 *L. pifanoi* parasites (Fig. 3 and Table 1). In fact, immunization with this proteinase enhanced parasite growth in the footpads and draining lymph nodes of mice infected with *L. amazonensis* (Table 3 and unpublished data). The molecular basis of the enhanced parasite growth in these mice was not explored in the current study, but it could perhaps reflect the ability of this proteinase to promote tissue damage by proteolysis, as suggested by detection of parasite cysteine proteinases in lesions of mice infected with *L. mexicana* amastigotes (18). Obviously, the A2 proteinase is not a potential vaccine candidate, although cysteine proteinases from *Leishmania* spp. and *Trypanosoma cruzi* have been proposed as targets for antiparasitic drugs (10, 30, 39).

In summary, we have demonstrated the purification of three distinct parasite antigens from axenic cultivated *L. pifanoi* amastigotes and have shown that these antigens are associated with different levels of immunoprotection against murine cutaneous leishmaniasis. This work represents the initial step in identifying amastigote vaccine immunogens. Although there is evidence that a Th1 cell-mediated immune response is associated with the resistance in these protected mice, additional evidence from cell depletion and/or cell transfer experiments is necessary to define the precise mechanism(s) involved in protection. Characterization of the functional parameters of T cells from immunized mice is under way. In addition, research efforts are ongoing to define the nature of immunodominant epitopes of P4 and P8 and the genes encoding them. Information in this regard is of critical importance for future leishmanial vaccine development.

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