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Vaccine-Induced Immunity against Cutaneous Leishmaniasis in BALB/c Mice

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Partially purified antigens, derived from *Leishmania infantum* or *L. major* promastigotes and isolated under reducing conditions, were used to immunize BALB/c mice. Three subcutaneous injections of the 64- to 97-kilodalton preparation in conjunction with muramyl dipeptide conferred long-lasting immunity against *L. mexicana* subsp. *mexicana* and *L. major* infection; they led to the development of antibodies neutralizing the infectiousness of promastigotes, induced specific delayed-hypersensitivity reactions, and generated populations of peritoneal macrophages capable of killing amastigotes. Vaccination resulted in no harmful effects, since these antigens neither exacerbated preexisting *Leishmania* infection nor impeded the formation of antibodies to other antigens administered concomitantly.

In humans, prophylactic immunization against the cutaneous leishmaniases has remained, with perhaps one exception (2), unrewarding (9). In laboratory animals, nonviable vaccines have until recently given questionable results (3, 21, 29, 31, 34); irradiated or heat-killed promastigotes have conferred protection when administrated by intravenous (i.v.) injections, whereas subcutaneous (s.c.) administration was counterproductive in terms of host resistance (19, 22, 35).

We found that hybridomas able to neutralize the infectivity of *Leishmania* promastigotes were also able to recognize three separate parasite antigens common to six species (23). Having separated the lysates of *Leishmania infantum*, by polyacrylamide gel electrophoresis, into six fractions according to molecular weight, we found that three of them had immunoprophylactic attributes when injected i.v. into C57BL/6 and BALB/c mice (24, 26). The immunological properties of these antigenic preparations in BALB/c mice were studied more closely. Our results demonstrate that s.c. immunization with these fractions, especially the one whose major component has a molecular mass of 67 kilodaltons (kDa), induces resistance against *L. mexicana* subsp. *mexicana* and *L. major* infection and neither alters the course of preexisting infection nor interfers with the humoral immune response to another antigen.

MATERIALS AND METHODS

Animals and parasites. Female BALB/c mice were obtained from an inbred colony maintained at the Institut de Recherches sur le Cancer, Centre National de la Recherche Scientifique, Villejuif, France. They were used between 8 and 14 weeks of age. *L. infantum* MCAN/GR/82/LEM497, *L. major* MRHO/SU/59 Neal P, and *L. mexicana* subsp. *mexicana* MNYC/BZ/62/M379 were maintained by continuous passages in BALB/c mice. For the preparation of promastigotes, the parasites were grown at 25°C in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum (HIFBS), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). In all experiments, promastigotes were used after one to three passages in vitro.

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Preparation of antigens. Approximately 1.5×10^9 *L. infantum* promastigotes, collected at the beginning of the stationary phase, were washed twice in cold phosphate-buffered saline and lysed for 10 min at 2°C in 2 ml of buffer containing 0.5% (vol/vol) Nonidet P-40, 1% (wt/vol) sodium dodecyl sulfate (SDS), 100 U of aprotinin per ml, 2 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride. The extract was sonicated three times for 30 s at 0°C. Insoluble material was removed by centrifugation at 2°C, and the supernatant, made up to a final concentration of 0.06 M Tris hydrochloride (pH 6.8), 2% (vol/vol) glycerol, and 5% (vol/vol) 2-mercaptoethanol, was electrophoresed on SDS-polyacrylamide gel electrophoresis slab gels (17). The gels were sliced into six strips by following molecular weight markers run in parallel. The proteins, electroeluted from each fragmented polyacrylamide gel strip, were dialyzed against cold deionized water containing 0.1% SDS, 100 U of aprotinin per ml, and 0.1 mM phenylmethylsulfonyl fluoride and were immediately lyophilized. The six fractions were defined by their apparent molecular mass ranges: LiF1, above 94 kDa; LiF2, 94 to 67 kDa; LiF3, 67 to 40 kDa; LiF4, 40 to 30 kDa; LiF5, 30 to 20 kDa; and LiF6, below 20 kDa. A 94- to 67-kDa fraction derived from *L. major* promastigotes was prepared in the same way and was designated LmF2. Protein concentrations were determined with the BCA protein assay reagent (Pierce Chemical Co., Rockford, Ill.).

Vaccination and determination of resistance. In a first set of experiments, groups of 10 mice were immunized with (per mouse) 15 μg (protein equivalent) of each fraction plus 100 μg of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP; Laboratoires Choay, Paris, France). Control groups received either eluates from gels which had not been loaded with *Leishmania* extracts or MDP alone. Three s.c. injections in the rump were given at 4-week intervals. Mice were infected 1 month after completion of the immunization protocol. As a challenge, 10^3 *L. mexicana* subsp. *mexicana* promastigotes, collected after 8 days of culture and suspended in 0.1 ml, were injected intradermally (i.d.) at the base of the tail. The grading of the lesions was made according to the score system described by Handman and Mitchell (11). Four mice in the protected group, immunized with LiF2, LiF5, and LiF6, were killed 4 months after infection; impressions of

their spleens, livers, and bone marrow were examined after Giemsa staining. At 5 months after their first challenge, the remaining six mice in the three protected groups were reinfected under the same conditions.

All further immunizations with LiF2 and LmF2 plus MDP were carried out by s.c. injection three times at 2-week intervals. The challenge was undertaken 21 to 30 days after the third immunization. The dose of LiF2 and the number of parasites in the infective inoculum varied. A group of 10 mice were immunized simultaneously with LmF2 and an anti-influenza vaccine (fractionated anti-A/New Jersey/76 vaccine [Institut Pasteur, Paris, France], 1 μg of hemagglutinin per mouse and per injection), controls receiving the antiviral vaccine along with MDP. Lastly, a group of eight mice was immunized with three 100-μg portions of unfractionated lysate together with MDP.

Immunization of infected mice. At 40 days after an i.d. inoculation of 10^4 *L. major* promastigotes, a group of 10 mice (mean score lesion, 0) received four weekly s.c. injections of 15 μg of LiF2 plus 100 μg of MDP. An identical group underwent the same treatment 70 days after the initial inoculation (mean score lesion, 1.9). Mice in the control group were given MDP alone.

Assays for antileishmania antibodies. Measurements of antibody levels were performed by indirect immunofluorescence with acetone-fixed *L. infantum* promastigotes or amastigotes (27). The neutralizing properties of the immune sera were evaluated by their ability to protect, in a Winn-type assay system (1), BALB/c mice against a challenge with *L. major* promastigotes. Briefly, 10^3 promastigotes in the early stationary growth phase were incubated with 50 μl of heat-inactivated serum for 30 min and then injected i.d. at the root of the tail of each BALB/c mouse. All tests were carried out with serum samples drawn either before or 3 weeks after the last immunization procedure.

Measurement of anti-influenza antibodies. Antibody titers were determined by a standard hemagglutination microtechnique with 4 hemagglutinin units of antigen (15).

Determination of cell-mediated immunity. Delayed-type hypersensitivity (DTH) was assayed by the footpad swelling method. Immunized and control mice were injected in the top of the left hind footpad with 50 μl of Formalin-fixed *L. infantum* promastigotes (2×10^8 parasites per ml) (20), and the increase in footpad thickness was measured 24 and 48 h later with a dial gauge caliper (Schnelltaster; Kröplin AG, Federal Republic of Germany). The increase in footpad thickness recorded after 24 h was corrected by subtracting the increase caused by Formalin-fixed parasites in control mice.

In vitro culture of peritoneal cells. At 2 to 3 weeks after the last immunization or injection of MDP, resident peritoneal cells of noninfected mice were collected by lavage of the unstimulated peritoneal cavity with RPMI 1640 medium buffered with 25 mM HEPES. After three washes, cells were plated in 35-mm plastic petri dishes (Becton Dickinson France, Grenuble, France). After 2 h of incubation at 37°C in 5% CO_2–95% air, nonadhering cells were removed by rinsing with cold medium. Prior to infection, the adhering cells were cultured in buffered RPMI 1640 medium supplemented with 10% HIFBS plus antibiotics for 40 h to allow for adequate spreading.

Infection of cultured cells and assessment of amastigote viability. After three rinses and renewal of the RPMI 1640 medium-HIFBS, the cultured cells were exposed to *L. major* promastigotes at a ratio of two parasites per cell and incubated at 37°C in 5% CO_2–95% air. After periods of infection of 4, 24, and 48 h, the macrophage monolayers were washed, fixed for 30 min in 1% glutaraldehyde, and subjected to Giemsa staining. The level of infection in these cultures was calculated by counting over 800 macrophages in random microscopic fields in two separate culture dishes.

For the preparation of cell suspensions, the dishes were washed with cold RPMI 1640 medium with no serum added and incubated on ice for 30 min, and the cells were gently removed with a rubber policeman. A total of 1,000 ± 250 detached macrophages were transferred into 45-ml culture flasks (Becton Dickinson), each containing 7 ml of RPMI 1640 medium–15% HIFBS and kept at 25°C to assess the potential of the amastigotes to differentiate into promastigotes. The development of promastigotes was examined after 12 days of culture.

TABLE 1. Immunologic data for BALB/c mice immunized with *L. Infantum*-derived preparations

Antigen	Antibody activity[a]		DTH (mm^{-2})[b]	Resistance to infection at[c]:	
	IFAT titer[d]	Neutralization (Winn-type assay)[e]		30 days p.i.	150 days p.i.
LiF1	1:400	1/10	ND[f]	1/10	ND
LiF2	1:200	10/10	48 ± 10	10/10	6/6
LiF3	1:200	2/10	45 ± 8	3/10	ND
LiF4	1:300	3/10	ND	2/10	ND
LiF5	1:600	10/10	50 ± 6	10/10	4/6
LiF6	1:800	10/10	27 ± 9	10/10	4/5[g]
MDP alone	1:20	0/10[h]	2 ± 3	0/10[i]	0/6[j]

[a] Sera were studied 21 days after the third immunization.
[b] DTH was determined in nonchallenged mice 24 days after the last antigen injection.
[c] Number resistant/number studied. p.i., Postimmunization.
[d] Median value of titers. IFAT, Indirect immunofluorescence.
[e] Number neutralized/number studied.
[f] ND, Not determined.
[g] One mouse died from a cause unrelated to *Leishmania* infection.
[h] The onset of cutaneous lesions was 58 ± 7 days. Furthermore, none of 12 randomly chosen preimmunization sera abrogated *L. major* infection in the Winn-type assay.
[i] All mice revealed infection by day 67.
[j] A new group of mice was used as the control in this experiment. The DTH reaction measured in control mice 10 weeks after infection with *L. major* was 69 ± 8 mm^{-2}.

RESULTS

Formation of antileishmania antibodies. The s.c. immunization with the six *L. infantum*-derived fractions led to the formation of antibodies which reacted with acetone-fixed promastigotes and amastigotes. Antibodies neutralizing the virulence of *L. major*, as assessed by the Winn-type assay, were produced in all mice given LiF2, LiF5, or LiF6, since no lesions had been detected at day 120. The protective action conveyed by the antisera to the LiF1, LiF3, and LiF4 fractions was not significant. Sera of nonimmune mice afforded no protection, and signs of infection occurred from day 50 onwards (mean, day 58 ± 7 days) (Table 1).

Resistance to infection. All mice in the three groups immunized with 15 μg of LiF2, LiF5, or LiF6 were resistant to a challenge of 10^3 *L. mexicana* subsp. *mexicana* promastigotes. At day 120 after infection, no amastigotes were detected on spleen, liver, or bone marrow smears. In control animals, this inoculum determined, from week 7 on, the formation of lesions progressing to typical punched-out ulcers.

Mice vaccinated with LiF2 resisted a second challenge given 5 months after completion of the immunization proce-

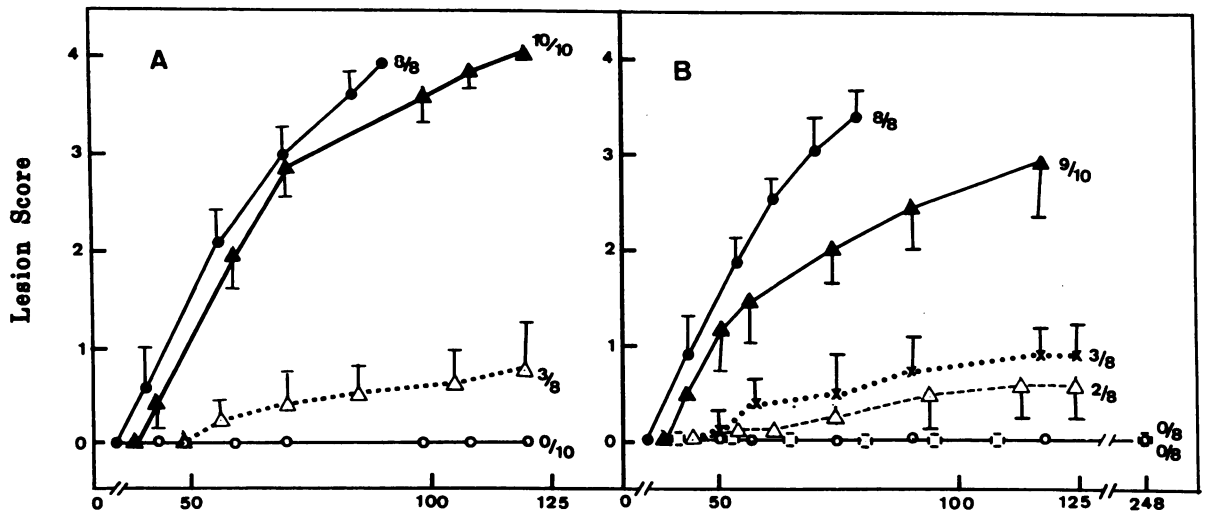

FIG. 1. Parasite load and species and rate of protection. (A) Development of lesions in BALB/c mice challenged with 1×10^4 (○, ●) or 5×10^4 (△, ▲) live *L. mexicana* subsp. *mexicana* promastigotes. Open symbols: mice immunized three times with 15 μg of LiF2 plus MDP; solid symbols: mice given MDP alone. (B) Development of lesions in mice infected with 1×10^4 (○, □, △, ×) or 5×10^4 (△, ●) *L major* promastigotes. ○, △, mice injected three times with 15 μg of LiF2; ×, mice injected three times with 7.5 μg of LiF2 plus MDP; □, mice immunized three times with 15 μg of LmF2. ●, ▲, mice given MDP alone. Data are expressed as lesion score ± standard error of the mean. Scoring system: 1, local swelling; 2, lesion < 5 mm in diameter; 3, lesion 5 to 10 mm; 4, lesion > 10 mm or systematic lesions (11). The number at the right of each curve is the number of mice infected/number of mice per group.

dure, again with 10^3 *L. mexicana* promastigotes. Those treated with LiF5 and LiF6 expressed partial resistance after renewed challenge (Table 1).

The further studies undertaken with mice immunized three times with 15 μg of LiF2 showed that full protection was achieved against inocula of 10^4 *L. mexicana* and *L. major* promastigotes. Infection with 5×10^4 promastigotes resulted in partial protection with a delay in the onset of lesions (Fig. 1). Protection against *L. major* was similarly imparted by the LmF2 vaccine. The susceptibility of mice injected with MDP without antigen and challenged after a 3-week interval with 5×10^3 *L. mexicana* promastigotes did not differ from the susceptibility of those having received eluates from unloaded gels alone. Similarly, the course of infection was not significantly modified in mice immunized with whole lysate and MDP.

Combined immunization of LmF2 and an anti-influenza vaccine hardly modified the immune response against either antigen, the titers of the anti-influenza antibodies, however, being less consistent in the group receiving the two vaccines (Table 2).

Vaccine administration to infected mice. Infected mice given four injections of LiF2, commencing either 10 days before or 25 days after the onset of cutaneous lesions, had an unremarkable progression of the disease; 112 days after infection their mean lesion scores were 2.9 ± 0.7 and 3.2 ± 0.8, respectively, and that of the untreated control was 3.3 ± 0.7.

Acquisition of cell-mediated immunity. By 24 h DTH reactions developed in 9 of the 10 mice in the group which received LiF2 and in all animals injected with LiF3, LiF5, and LiF6 (Table 1). At 4 months after immunization with LiF2, and in the absence of an infectious challenge, the DTH response waned (8 ± 10^{-2} mm). The traces of SDS, present in the antigen preparations, prevented their use in i.d. reactions and lymphocyte proliferation response assays.

Parasiticidal activities of peritoneal macrophages. At 4 h after infection with *L. major* promastigotes, 31% of macrophages isolated from mice immunized with LiF2 contained intracellular parasites. The proportion of infected cells obtained from mice given LiF3 or MDP alone (controls) was 45% and 38%, respectively. After 24 h of culture, the proportion of parasitized macrophages remained stable in the monolayers derived from the LiF2-immunized animals, and when studied 48 h after infection, amastigotes could be found in only 17% of these mononuclear phagocytes. By contrast, the proportion of parasitized macrophages increased in the cells obtained from mice sensitized with LiF3: 73% at 24 h and 75% at 48 h after infection (Table 3).

The leishmanicidal activity expressed by the macrophages of mice immunized with LiF2 was further demonstrated by the strong reduction in parasite survival, which became patent after 48 h of culture. When transferred to an axenic medium and kept at 25°C, the amastigotes derived from macrophages cultured for 48 h showed a different ability to transform into promastigotes and replicate over a 12-day period: 41 parasites per μl (range, 0 to 300) for the LiF2 series and 2.3×10^3 and 1.85×10^3 parasites per μl (range, 1.5×10^3 to 3.2×10^3) for the LiF3 and control series,

TABLE 2. Combined immunization of BALB/c mice with LmF2 and anti-influenza vaccines

Group	Vaccine	No. susceptible/ total no. (score)[a]	Anti-influenza antibody titers[b]
A	LmF2 + MDP	0/8	0 ± 0
B	LmF2 + anti-influenza + MDP	2/8 (0.4 ± 0.6)	3.53 ± 1.67
C	Anti-influenza + MDP	7/8 (2.8 ± 1.5)	4.19 ± 0.35

[a] Infectious challenge with 10^4 *L. major* promastigotes; the score determined 101 days after infection.

[b] Mean ± standard deviation of $\log_2$ transformed reciprocal titers measured from serum samples collected 24 days after the end of the immunization schedule.

TABLE 3. Resistance to infection and macrophages parasiticidal activity

Duration (h) of coculture (37°C)	% of infected macrophages[a] ± SEM in:			No. of promastigotes/μl ± SEM after 12 days in[b]:		
	Control mice	LiF2-immunized mice	LiF3-immunized mice	Control mice	LiF2-immunized mice	LiF3-immunized mice
4	38 ± 11	31 ± 13	45 ± 12	850 ± 226	500 ± 130	700 ± 260
24	73 ± 13	30 ± 14	73 ± 9	2,000 ± 400	420 ± 190	2,140 ± 297
48	78 ± 11	17 ± 8[c]	75 ± 16	1,850 ± 280	41 ± 102	2,300 ± 600

[a] Results of three series of experiments run in duplicate. Variations within separate experiments were <20% of the mean.
[b] At the end of cocultures at 37°C, the petri dishes were washed, and 1,000 ± 250 detached macrophages were placed at 25°C into fresh RPMI 1640–15% HIFBS for 12 days. Results from three experiments are shown for each macrophage population.
[c] $P < 0.001$ with respect to control or LiF3-immunized groups (t paired-samples test).

respectively ($P < 0.01$ by the Mann-Whitney rank test). The amastigotes harvested 4 and 24 h after infection permitted the differentiation into promastigotes in all cases; the counts did not differ significantly among the three series (Table 3).

DISCUSSION

Immunization i.v. of self-healing C57BL/6 and vulnerable BALB/c mice with three partially purified *L. infantum*-derived preparations induced host protection against infection brought about by *L. mexicana* subsp. *mexicana* and *L. major* (24, 26). In the work described in this report, we verified that these vaccines maintained their immunoprophylactic properties when administered s.c. to the highly susceptible BALB/c mice, together with a synthetic water-soluble adjuvant, a muramyl dipeptide. Indeed, we observed that mice immunized with the 94- to 67-kDa fraction produced antibodies neutralizing the infectiousness of promastigotes, developed delayed hypersensitivity to Formalin-fixed promastigotes, and formed a parasiticidally active macrophage population. These mice became resistant to promastigote challenges which, in matched controls, provoked chronic infection.

The antibody activity promoting killing of promastigotes was documented in the Winn-type assay: antibodies produced against LiF2, LiF5, and LiF6 prevented infection against an inoculum of 10^3 *L. mexicana* subsp. *mexicana* promastigotes. In the same experimental model, a few murine antileishmania monoclonal antibodies were found to abolish the infectivity of *L. mexicana* and *L. major* promastigotes (1, 7, 10, 23); likewise, inhibition of parasite growth in macrophage cultures has been achieved by exposing promastigotes to monoclonal antibodies prior to infection of macrophage monolayers (10). More recently, we observed that canine antibodies raised against LiF2 retarded significantly, in the Winn-type assay, the onset of lesions in BALB/c mice infected with *L. major* promastigotes (25; B. W. Ogunkolade, I. Vouldoukis, D. Frommel, B. Davoust, A. Rhodes-Feuillette, and L. Monjour, Vet. Parasitol., in press). In preliminary analyses, murine and canine anti-LiF2 sera collected before infectious challenge immunoprecipitated mainly a ^{35}S-metabolically-labeled 28-kDa parasite antigen. As demonstrated by immunogold staining, murine anti-LiF2 antibodies recognized determinants expressed in small clusters on the surface membranes of *L. infantum* promastigotes (B. W. Ogunkolade, D.Sc. thesis, University of Paris VII, Paris, France, 1987).

The development by 24 h of a skin hypersensitivity reaction occurred in mice immunized with protective and nonprotective antigens. Thus, a positive DTH reaction does not mirror vaccine-induced resistance, just as in natural and experimental infections in which strong DTH reactions may coexist with nonhealing leishmaniasis (12). More noteworthy, an i.d. injection of 10^7 killed promastigotes into mice immunized with LiF2, undertaken 15 days prior to infectious challenge, did not abate prophylactic immunity, in contrast to the situation with mice made resistant by i.v. injections of irradiated promastigotes (19). It appears likely that the immunologic status elicited by the LiF2 antigens and MDP is circumventing the generation of suppressor cells which, following s.c. injection of nonviable *L. major* parasites into BALB/c mice, facilitate the development of cutaneous leishmaniasis (20, 35).

For the assessment of leishmanicidal activity displayed by macrophages from immune mice, we used resident peritoneal cells cultured for 40 h prior to parasite exposure (16). These plastic-adherent cell populations contain a limited number of T lymphocytes. During the first hour of culture, no difference in parasite uptake into immune or normal cells was discernible. Throughout the next 20 h, macrophages from mice immune to LiF2 showed a partial resistance to infection, yet the intracellular amastigotes maintained their viability and replication potential. After 48 h, however, most macrophages from protected mice had eliminated the amastigotes, and the remaining parasites had a significantly reduced capacity to differentiate and grow at 25°C. Thus the parasiticidal activity displayed by mononuclear phagocytes from mice vaccinated with LiF2 appears to be expressed with a lag period of some 24 h; a finding in accordance with the sequence reported for *Leishmania* killing by lymphokine-activated macrophages (30). Cells collected from mice immunized with LiF3 did not impede *Leishmania* infection and multiplication, thereby illustrating a restriction of parasiticidal activity to cells of mice given host-protective antigen, and in disagreement with the positive DTH skin test. The findings that immunization with distinct parasite fractions led to a dissociation of these two cell-mediated responses underline the singularity of the epitopic structures involved in triggering the host-protective mechanisms.

For s.c. immunization we selected MDP as adjuvant. Indeed, we observed that in dogs, which are natural hosts of *Leishmania* spp., MDP and its nonpyrogenic derivative murabutide (18, 36) were the best potentiators of the immune response to LiF2 (Ogunkolade et al., in press). It remains to be determined whether an increase in the amount of antigen used for immunization confers protection against an inoculum of 10^6 promastigotes, as achieved after i.v. vaccination with irradiated promastigotes (14) or after intramuscular injections of a soluble promastigote extract together with *Corynebacterium parvum* (32). Repeated injections of LiF2, or LmF2 plus MDP, appeared innocuous and were harmless, although inoperative, in mice already infected. Besides that, no depression in antibody response against another common vaccine occurred following a combined immunization with

LmF2 and anti-influenza vaccines associated with MDP. Immunoprophylaxis developing after injections of *L. infantum*-derived fractions was not species restricted: similar cross-protections have been observed after i.v. immunization with irradiated promastigotes (13).

Immunity elicited by the LiF2 vaccine is not stage specific, and it encompasses both the promastigote, whose virulence is destroyed, and the amastigote, whose replication cycle is interrupted. In our view, anti-LiF2 antibodies provide a first line of defense against invading extracellular organisms, and the T-cell immune response supplies a second front of protection against the intracellular parasite, by promoting cytocidal activities of the macrophages (4, 28). The interconnections existing between these two effector systems require further investigation, in particular the temporal extent, at the level of the macrophage, of effective LiF2-induced cell-mediated resistance. Indeed, within a few weeks in the absence of renewed challenge, DTH was waning, whereas the serum expressed a more prolonged promastigote-neutralizing activity.

An as yet incomplete molecular characterization of the four proteins identified in LiF2 and LmF2 by SDS-polyacrylamide gel electrophoresis prevents comparison with the *Leishmania* antigens purified to homogeneity (5, 6, 8, 11). Lately, Scott et al. isolated from *L. major* promastigotes a soluble fraction which actively stimulates protective immunity (33). The molecular weight range of these protein antigens includes that of the LiF6 fraction, which we also found to induce resistance.

The present study demonstrates that three fractions, isolated under reducing conditions from *Leishmania* promastigotes and injected s.c. with MDP as adjuvant, provide protection against experimental leishmaniasis. The relevance of these antigen preparations, injected either separately or together, for immunoprophylaxis in natural hosts of *Leishmania* parasites is under investigation.

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