

Specific immunization of mice against *Leishmania mexicana amazonensis* using solubilized promastigotes

M. BARRAL-NETTO, S. G. REED*†, M. SADIGURSKY & G. SONNENFELD†

*Centro de Pesquisas Goncalo Moniz (Fundacao Oswaldo Cruz and Universidade Federal da Bahia) Project Cornell-Bahia, Salvador, Bahia, Brazil, *Cornell University Medical College, New York, NY, and †Department of Microbiology and Immunology, School of Medicine, University of Louisville, Louisville, KY*

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SUMMARY

Successful immunization of highly susceptible BALB/c mice against progressive infection by *Leishmania mexicana amazonensis*, using whole solubilized promastigotes was achieved. The best immunization schedule consisted of three weekly injections of 5×10^7 parasite equivalents. Intravenous was superior to intraperitoneal or subcutaneous immunization. Protection persisted for up to 2 months after immunization, and beneficial effects could be observed in long-term follow-up (24 weeks after infection). Immunized mice exhibited marked reduction in primary lesion size, as well as reduction of the number of parasites in the spleen, and developed less metastases. High titres of specific anti-*L. m. amazonensis* IgG antibodies resulted from immunization, but titres did not correlate with protection. Groups with widely differing pre-infection antibody titres were equally protected, and similar antibody titres resulted in different levels of protection. Immunization alone did not induce significant serum interferon-gamma levels and specific delayed-type hypersensitivity (DTH) reactions, but resulted in the persistence of positive (DTH) reactions after infection, at a time when infected control animals had suppressed responses. Resistance to leishmaniasis appears to depend on cell mediated immune mechanisms, and the possibility of immunization with a solubilized antigen without adjuvant is intriguing and opens new perspectives in this area.

Keywords leishmaniasis vaccine BALB/c mice solubilized antigens

INTRODUCTION

The leishmanial diseases constitute important world-wide public health problems, and current efforts to control them are insufficient. In the Americas, leishmanial infections extend from the southern United States to Argentina, and typically occur in poor rural settings where medical facilities are poor or lacking.

Immunization programmes may be important in the control of cutaneous leishmanial infections because: (1) natural or experimental infections appear to provide lasting immunity against reinfection (Guirgues, 1971; Preston & Dumonde, 1976); (2) disease occurs in restricted and identifiable populations; (3) present chemotherapeutic agents, including pentavalent antimonials or amphotericin B, are toxic; and (4) insect vectors are often inaccessible to insecticide control.

Correspondence: Dr M. Barral-Netto, Hospital Prof. Edgard Santos, Rua Joao das Botas S/N, 40.000 Salvador, Bahia, Brazil.

† Present Address: Issaquah Health Research Institute, 1595 Gilman Boulevard, Issaquah, WA 98027, USA.

Some attempts at immunization of man against leishmanial infection have been primarily directed at inducing a controlled self-healing cutaneous lesion with *L. tropica* (reviewed by Greenblatt, 1980). Approaches using living or irradiated parasites to achieve immunity are considered of unacceptably high risk when dealing with *Leishmania* of high virulence such as many of those found in the Americas. Immunization with ultrasonicated promastigotes (Preston & Dumonde, 1976) or antigen-antibody complexes (Handman *et al.*, 1977) has been achieved in genetically resistant animals. Partial protection of highly susceptible BALB/c mice against *L. tropica* infection has been obtained by intraperitoneal injection of frozen-thawed infected macrophages plus *Corynebacterium parvum* as adjuvant (Mitchell, Curtis & Handman, 1981). Recently the same group reported protection of BALB/c mice using crude frozen-thawed promastigotes given intraperitoneally with *C. parvum* (Mitchell & Handman, 1983). Protection of BALB/c mice against fatal infection with *L. tropica* has been achieved using whole irradiated promastigotes (Howard *et al.*, 1982).

Leishmania m. amazonensis is an important cause of cutaneous and metastatic disease of humans in Central and South America. It has also been identified as a valuable agent for reproducing the spectrum of leishmanial diseases in mice. The BALB/c strain is exceedingly susceptible and develops uncontrolled metastatic disease (Barral *et al.*, 1983; Andrade *et al.*, 1984) and thus is a rigorous model for demonstrating protection.

We undertook a series of experiments to determine whether solubilized *L. m. amazonensis* promastigotes could protect mice against progressive cutaneous leishmanial infection. The present report demonstrates that BALB/c mice can be successfully immunized with solubilized antigen, and that this protection is associated with the persistence of delayed type hypersensitivity.

MATERIAL AND METHODS

Animals. Inbred female BALB/c mice were obtained from the Fiocruz colony, and were used at 10 to 12 weeks of age.

Parasites. For both infection and antigen preparation the 'Josefa' strain (MHOM/BR/76/Josefa) was used. This strain has been characterized as *L. m. amazonensis* by isoenzymic patterns and by a panel of monoclonal antibodies (courtesy of Dr Gabriel Grimaldi; Fiocruz, Rio de Janeiro), as well as by kDNA restriction analysis (courtesy of Dr Peter Jackson, Walter Reed Army Institute of Research, Washington DC), as described (Andrade *et al.*, 1984). The parasite is maintained in our laboratory by subcutaneous passage in BALB/c mice.

Antigen preparation. Stationary-phase promastigotes cultivated in liver infusion tryptose medium supplemented with 5% fetal bovine serum (LIT-FBS), were washed three times (1800 g, 20 min, 4°C) in phosphate-buffered saline with 2% glucose (PBS-G), and resuspended in 'lysing buffer' (50 mM Tris pH 7.7; 0.12 mol/l NaCl; 0.5% NP-40; 0.25% sodium deoxycholate; 5 TIU/ml of aprotinin and 5 mM EDTA) at a concentration of 10^9 parasites/ml. Following incubation for 10 min at room temperature, under occasional mixing, the material was submitted to dialysis against 500 volumes of PBS at 4°C. Concentrated material was expressed as 'parasite equivalent per ml' (p.e./ml), as a function of initial number of promastigotes and final volume of the preparation. Ten million promastigotes yielded approximately 13 µg protein upon solubilization.

Irradiation of parasites. LIT-FBS grown promastigotes were washed three times in PBS-G and adjusted to 10^8 /ml. Immediately before use, parasites were dispensed at 2 ml volumes in plastic Petri dishes (Falcon, 60 × 15 mm) and placed 3–4 cm from a 30 W u.v. source for 15 min. A sample of each batch was removed for evaluating viability by 10 day-cultivation in LIT-FBS. The irradiation procedure resulted in a complete loss of viability as determined by growth in culture.

Immunization and infection. Mice were injected i.v., i.p., or s.c. (in the shaved rump) with one to three weekly doses of solubilized antigen or irradiated parasites, in different concentrations (1 to 5×10^7 p.e./ml) in volumes of 0.2 ml. One week or 2 months after the last immunizing injection, mice were challenged s.c., into one of the hind foot-pads, with 5×10^6 , or 5×10^7 or 5×10^8 , viable promastigotes in 0.025 ml. Foot-pad thickness was measured with a dial gauge micrometer caliper (C. Starret, Athol, MA). Differences between the infected foot-pads and the contralateral

uninfected one, were expressed as 'lesion size' in millimeters. Results were expressed as group means \pm s.e.m.

Quantification of viable parasites in the spleen. At different periods of infection, spleens of immunized or control animals were aseptically removed, minced, and single cell suspensions were cultured in RPMI 1640 over NNN agar slants. Quantification of viable organisms was performed as previously described (Barral *et al.*, 1983).

Assay for anti-L. mexicana amazonensis antibody titres. An enzyme-linked immunosorbent assay (ELISA) was performed with promastigote antigen. Plates were sensitized with antigen at a concentration of 10 μ g/ml in pH 9.6 carbonate-bicarbonate buffer, at 0.1 ml per well, for 3 h at 37°C and at 4°C until the time of use. Before the reaction, the plates were washed three times with PBS containing 0.5% Tween 20 (PBS-tween). Serial dilutions of sera were incubated for 1 h at 37°C, followed by three washings with PBS-tween. Peroxidase-conjugated rabbit anti-mouse IgG (Sigma, St Louis, MO), diluted 1:1000 in PBS + 10% newborn bovine serum, was incubated for 1 h at 37°C. The plates were washed and substrate solution (0.04% ortho-phenylenediamine, 0.012% hydrogen peroxide in pH 5.0 citrate-phosphate buffer) was added. Following incubation for 30 min the reaction was stopped by adding 16 mol/l sulphuric acid (0.025 ml/well), and read in a Titertek Multiskan spectrophotometer (Flow Laboratories, Ayrshire, Scotland, UK) at 495 nm.

Assay for delayed-type hypersensitivity. *Leishmania mexicana amazonensis* promastigotes cultured in LIT + FCS were used as antigen source, and the footpad swelling test was performed as described previously (Barral *et al.*, 1983). Mice received 50 μ g protein of leishmanial antigen solution in a volume of 0.025 ml into the ventral aspect of the hind footpad. Measurements were performed with a dial gauge micrometer as indicated above. Results are presented as the thickness measured 24 h after antigen injection minus the thickness before injection in millimeters. A group of uninfected BALB/c mice was injected in the footpad with the antigen for determination of non-specific reaction. Skin tests were considered positive when the mean of footpad thickness increase above that of normal mice was beyond the 95% confidence limit.

Statistical analysis. Groups means, at each time point, were compared by one-way analysis of variance. When only two groups were compared, we used Student's *t*-test. Tests for comparison of antibody titres were performed with log transformed values. Frequency of metastases between immunized and control animals were analysed by the χ^2 test.

Interferon assay. Interferon (IFN) antiviral activity of samples was determined by means of a plaque reduction assay on mouse L-929 cells using the Indiana strain of vesicular stomatitis virus as the target virus (Hanna, Merigan & Jawets, 1966). The IFN titre corresponded to the reciprocal of the greatest dilution of test sample that reduced virus plaques by 50%. One IFN antiviral unit in this assay was equivalent to 0.88 NIH 6-002-904-511 mouse reference units.

RESULTS

Route of immunization. Groups of 6 BALB/c mice were immunized i.v., i.p., or s.c. with three doses of 2×10^7 p.e. of solubilized promastigotes per animal dose, at weekly intervals. One week after immunization the animals were challenged with 5×10^6 viable promastigotes of *L. m. amazonensis*. Figure 1 shows that mice immunized i.v. had much smaller lesions than any other group ($P < 0.02$ by analysis of variance at 8 or 10 weeks), differing at a level statistically significant ($P < 0.01$) even from mice immunized i.p. On the other hand mice immunized by s.c. or i.p. routes did not differ significantly from unimmunized controls.

Comparison between solubilized antigen and irradiated parasites. Figure 2 compares the evolution of lesion size in groups of 10 BALB/c mice infected with 5×10^6 viable promastigotes of *L. m. amazonensis* without prior immunization or 1 week after i.v. immunization with three doses of 2×10^7 p.e. of solubilized antigen per animal per dose or with a similar protocol using irradiated parasites, at weekly intervals. From the fourth week values for both immunized groups were different from unimmunized animals (*F* values varying from 6.61 to 14.19 with $P < 0.01$ or < 0.005). There were no statistically significant differences at any time point between groups immunized with solubilized antigen or irradiated parasites.

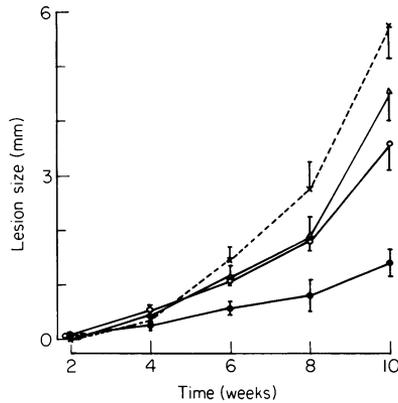


Fig. 1. Time course of lesion development in BALB/c mice infected with 5×10^6 promastigotes of *L. m. amazonensis*, unimmunized (\times), or previously immunized with 3 doses of 2×10^7 solubilized *L. m. amazonensis* promastigotes s.c. (Δ), i.p. (\circ) or i.v. (\bullet). Points in all figures represent mean of determination in six mice, and s.e.m.

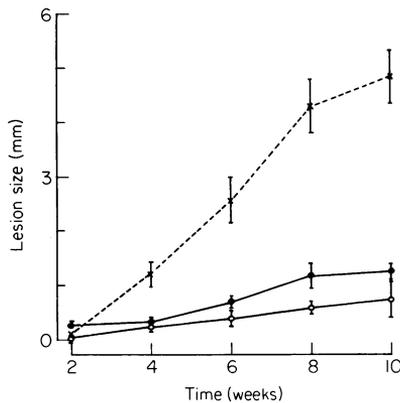


Fig. 2. Evolution of lesion size of BALB/c mice, following infection by 5×10^6 promastigotes of *L. m. amazonensis*, in groups of 10 animals unimmunized (\times) or immunized with three doses of 2×10^7 irradiated (\circ) or solubilized (\bullet) promastigotes of *L. m. amazonensis*.

Influence of antigen concentration. Protection of immunized mice was a function of the concentration of antigen, expressed as parasite equivalents per dose, as illustrated in Fig. 3. Mice immunized with three doses of 1×10^7 p.e./animal/dose had mean lesion sizes not statistically different from unimmunized animals. One the other hand animals immunized with three doses of 2×10^7 or 5×10^7 p.e./animal/dose had lesions of similar sizes which were smaller than those of control mice at 4 to 10 weeks after infection. Immunization with three doses of antigen was consistently better than with a single dose.

Duration of protection. Follow-up of immunized mice after infection showed the persistence of protection for at least 24 weeks (Fig. 4). At the end of the observation period there was still a 70% reduction of lesion size in the immunized animals as compared to unimmunized controls. After this length of time, no further growth in lesion size occurred in immunized animals (data not shown). In other experiments, mice were infected 2 months after the immunization. In these mice, the achieved protection was similar to that observed in animals infected 1 week after immunization.

Influence of the size of inoculum in the challenge infection. The degree of protection conferred by solubilized promastigotes at three doses of 5×10^7 p.e./animal/dose was a function of the number of parasites used in the challenge infection (Fig. 5). The smallest inoculum (5×10^5 viable

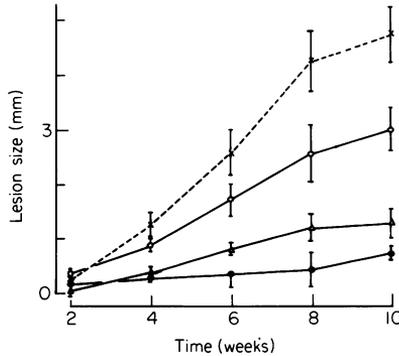


Fig. 3. Time course of lesion development in BALB/c mice, infected with 5×10^6 promastigotes of *L. m. amazonensis*, unimmunized (\times) or previously immunized with three doses (at weekly intervals) of solubilized promastigotes at concentrations of 1×10^7 (O); 2×10^7 (Δ) or 5×10^7 (\bullet) parasite-equivalents per dose.

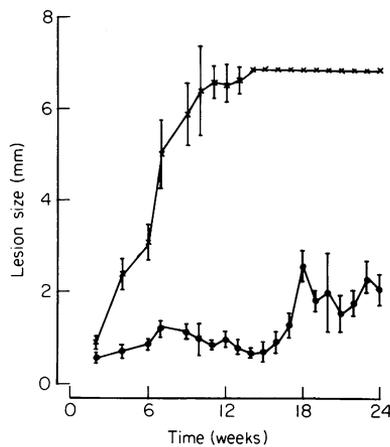


Fig. 4. Long-term evolution of lesion size of BALB/c mice infected with 5×10^6 promastigotes of *L. m. amazonensis* without previous immunization (\times) or immunized i.v. with three doses of 5×10^7 solubilized parasite equivalents of *L. m. amazonensis*. (\bullet).

promastigotes) was associated with significant protection (the differences between immunized vs control animals from 6 to 10 weeks reached a level of $P < 0.05$). The standard inoculum of 5×10^6 viable promastigotes was also associated with significant protection (differences between immunized \times controls from 6 to 10 weeks, $P < 0.01$), whereas the highest parasite inoculum (5×10^7 viable promastigotes) overwhelmed the protective effect of immunization.

Dissemination of parasites. Immunization with solubilized promastigotes was effective in protecting mice against dissemination of parasites. Table 1 shows that immunized mice had significantly fewer parasites in the spleen at 10 weeks after infection than did unimmunized controls. Immunized animals also developed fewer metastatic lesions by 26 weeks after infection, evaluated both as frequency of metastases and as number of lesions per group.

Specific antibody responses. Immunization induced elevated antileishmania IgG antibody titres, but such response did not correlate with protection (Table 2). Despite highly different antibody titres, between animals immunized i.v. with three doses of irradiated parasites or 5×10^7 solubilized promastigotes/dose, protection in these two groups was similar. Mice immunized with 10^7 or 5×10^7 solubilized promastigotes/dose had similar antibody titres but significantly different degrees of

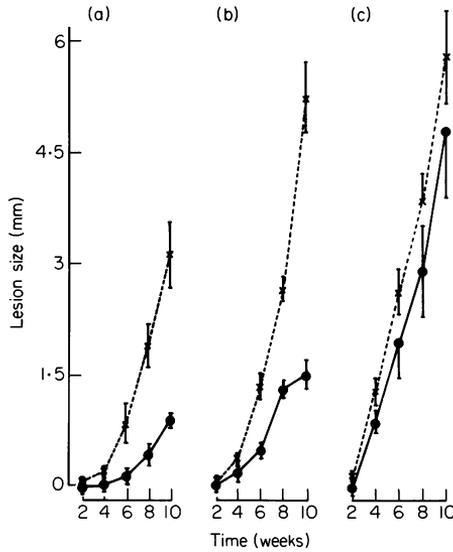


Fig. 5. The effect of different challenge inocula on lesion development in infected BALB/c mice, unimmunized (---) or previously immunized (—) as described in Fig. 1. Challenge doses of 5×10^5 (A), 5×10^6 (B), or 5×10^7 (C) viable promastigotes were injected 1 week after the last immunizing dose.

Table 1. Parameters of parasite dissemination after infection with *L. m. amazonensis* on BALB/c mice immunized i.v. with solubilized antigen or unimmunized controls

	Total no. of parasites in the spleen* (mean log \pm s.e.m.)	Frequency of metastases†	No. of metastases/group
Control	3.18 \pm 0.75	10/10	102
Immunized	1.33 \pm 0.98‡	2/10‡	2‡

* Comparison at 10 weeks after infection ($n=3$).

† Comparison at 26 weeks after infection.

‡ Control vs immunized $P < 0.01$.

Table 2. Comparison of pre-infection specific antibody titres and protection against *L. m. amazonensis* infection in BALB/c mice immunized with irradiated promastigotes or solubilized parasites

Immunization*	Pre-infection anti-leishmania IgG ELISA titres (geo. mean)	Mean percent reduction in lesion size† (10 weeks after infection)
Irradiated parasites (i.v.)	2350	84.2
Solubilized parasites (i.v.)		
1×10^7 p.e./dose	195	38.5
5×10^7 p.e./dose	306	74.6
Solubilized parasites (5×10^7 p.e./dose)		
i.p.	8038	40.8
i.v.	1200	67.4

* Mice were given three doses at weekly intervals.

† Compared to unimmunized controls in each case.

* Parasite equivalents

Table 3. Time course of DTH responses in infected BALB/c mice immunized with solubilized promastigotes* or in unimmunized controls

Weeks	Unimmunized	Immunized	<i>P</i> <
	$\bar{x} \pm \text{s.e.m. (mm} \times 10^{-2})$	$\bar{x} \pm \text{s.e.m. (mm} \times 10^{-2})$	
0	3.00 \pm 1.77	4.44 \pm 1.92	NS
1	1.28 \pm 1.28	0.67 \pm 0.68	NS
7	17.10 \pm 3.46	27.44 \pm 7.86	NS
10	10.80 \pm 3.37	22.80 \pm 4.29	0.05
13	12.40 \pm 7.34	41.28 \pm 6.69	0.02

* Mice were immunized i.v. with 10^7 p.e. in three weekly doses and infected s.c. with 5×10^6 parasites in the right hind footpad 1 week after the last immunizing dose; five mice/group. NS, Not significant, *P* > 0.05.

protection. Moreover, use of solubilized promastigotes injected i.p. induced antibody titres much higher than did i.v. immunization, although the latter conferred better protection.

DTH responses. Immunization of BALB/c mice with solubilized promastigotes failed to induce an anti-leishmania DTH response but was associated with persistence of positive DTH after infection (Table 3). Early in infection, responses in immunized animals did not differ significantly from those of unimmunized controls. At 10 or 13 weeks, the DTH responses of immunized animals remained strongly positive, whereas unimmunized animals had markedly diminished responses.

IFN production. Infection of mice with *L. m. amazonensis* resulted in intermittent production of serum IFN (44-85 units) early in the course of infection (up to 4 h after infection). Immunization of mice by any of the protocols described above did not result in production of detectable levels of serum IFN.

DISCUSSION

In this report we show that solubilized parasite antigen can effectively protect highly susceptible BALB/c mice against *L. m. amazonensis* infection, without the need for adjuvant or the use of intact parasites. This is particularly intriguing considering that resistance against cutaneous leishmaniasis is thought to depend mainly on cell-mediated immune mechanisms (Pretson & Dumonde, 1976; Bryceson *et al.*, 1970; 1972; Hale & Howard, 1981), classically induced most efficiently by the use of live immunization or with adjuvants.

Although we do not have an explanation for the higher efficacy of i.v. immunization, this observation confirms previous findings of Howard *et al.*, (1982) in the protection of BALB/c mice against *L. tropica* infection. Although these authors used gamma-irradiated parasites to immunize, we found that u.v. irradiated parasites are equally effective. The superiority of i.v. over i.p. or s.c. injections may explain the weak protection obtained in other systems of immunization against leishmaniasis (Preston & Dumonde, 1976; Handman *et al.*, 1977; Mitchell & Handman, 1983).

The immunization schedule induced extremely elevated anti-leishmania IgG antibody titres, much higher than those attained during the early phases of infection in unimmunized mice. These titres, however, did not seem to correlate with protection. Despite the lethal effect of serum against culture promastigotes (Pearson & Steigbigel, 1980; Mosser & Edelson, 1984) or phlebotime-grown promastigotes (Barral-Netto *et al.*, unpublished), high titres of antibody are generally not correlated with protection in leishmaniasis (Hale & Howard, 1981). Additionally, passive transfer of large amounts of serum obtained from immunized (and protected) BALB/c mice had no effect in protecting the recipients (Howard *et al.*, 1984).

Resistance against cutaneous leishmaniasis has been correlated with CMI, as evaluated by DTH

(Preston & Dumonde, 1976; Detolla, Scott & Farrell, 1984). Several studies demonstrated that early in infection, BALB/c mice exhibit a DTH response similar to the resistant strains but the susceptible strains show a diminishing response as infection progresses (Barral *et al.*, 1983; Andrade *et al.*, 1984; Perez, Arredondo & Gonzalez, 1978; Howard, Hale & Liew, 1980). Although immunization by itself did not induce a DTH response, immunized mice showed a persistence of CMI response throughout the infection. Protection in the absence of cutaneous DTH could be due to entrapment of DTH effector cells (Milon *et al.*, 1983). Another explanation could be the induction of specific suppressor cells or of a tolerant state. This seems unlikely however, since immunized animals developed cutaneous DTH response following challenge infection. It is also noteworthy that adoptive transfer of T cells, capable of inducing strong DTH reactivity to *L. tropica* in BALB/c mice, resulted in exacerbation of the disease (Titus *et al.*, 1984) emphasizing the dissociation between DTH response and protection.

It has been postulated that IFN and γ -IFN in particular, could have some immunoregulatory effects and influence the course of *Leishmania* infections (Murray, Rubin & Rothermel, 1983). In the current study, serum IFN was intermittently produced early on during the course of *L. m. amazonensis* infection of mice, but no serum IFN was produced in immunized mice. It is possible that during immunization, IFN could have been produced in the spleen or lymph nodes at levels that would not have been detectable in serum. Such IFN could still have had immunoregulatory effects and could have contributed to the protection that was observed. Alternatively, IFN may not be involved in the protection observed.

The data presented in this study are similar to those published by Howard *et al.*, (1982) except that a different parasite and u.v. irradiation were used. However, in addition to confirming these previous studies, we have shown that intact parasites are not required for effective immunization. These non-viable parasite preparations were not capable of inducing complete protection, because lesions did not disappear completely in immunized mice. However, they were effective in inducing inhibition of lesions development and death in BALB/c mice.

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