

A *dhfr-ts*⁻ *Leishmania major* Knockout Mutant Cross-protects against *Leishmania amazonensis*

PST Veras/⁺, CI Brodskyn^{**}, FMP Balestieri^{***}, LAR de Freitas, APS Ramos, ARP Queiroz^{**}, A Barral^{**}, SM Beverley^{****}, M Barral-Netto^{*}

Laboratório de Patologia e Biologia Celular *Laboratório de Imuno-regulação e Microbiologia, Centro de Pesquisas Gonçalo Moniz-Fiocruz, Rua Valdemar Falcão 121, 40295-001 Salvador, BA, Brasil **Serviço de Imunologia, Hosp. Univ. Prof. Edgard Santos, UFBA, Salvador, BA, Brasil ***Laboratório de Tecnologia Farmacêutica, Departamento de Fisiologia e Patologia, UFPB, João Pessoa, PB, Brasil ****Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110, USA

E10-5A3 is a dhfr-ts⁻ *Leishmania major* double knockout auxotrophic shown previously to induce substantial protection against virulent *L. major* infection in both genetically susceptible and resistant mice. We investigated the capacity of *dhfr-ts*⁻ to protect against heterologous infection by *L. amazonensis*. The degree of protection was evaluated by immunization of BALB/c or C57BL/6 mice with E10-5A3, followed by *L. amazonensis* challenge. Whether immunized by subcutaneous (SC) or intravenous (IV) inoculation, susceptible and resistant mice displayed a partial degree of protection against challenge with virulent *L. amazonensis*. SC-immunized BALB/c mice developed lesions 40 to 65% smaller than non immunized mice, while IV immunization led to protection ranging from 40 to 75% in four out of six experiments compared to non immunized animals. The resistant C57BL/6 mice displayed comparable degrees of protection, 57% by SC and 49% by IV immunization. Results are encouraging as it has been previously difficult to obtain protection by SC vaccination against *Leishmania*, the preferred route for human immunization.

Key words: auxotrophic *Leishmania major* - *Leishmania amazonensis* - immunization - cross-protection

Leishmaniasis is an endemic disease in several parts of the World, including South and Central America, Africa and Asia. At least 400 million people are under the risk of infection, with an incidence of 600 thousand new cases of leishmaniasis per year and a prevalence of 12 million infected individuals (Desjeux 1992). People residing in endemic areas exhibit different clinical manifestations of leishmaniasis, determined by the species of infecting parasite and also by host factors.

Resistance and recovery to all forms of leishmaniasis has been linked to cell-mediated immune responses (Belosevic et al. 1989). Patients with New World cutaneous leishmaniasis (CL) and

muco-cutaneous leishmaniasis (ML) develop *in vitro* lymphocyte proliferative responses with abundant IFN- γ (interferon- γ) production (Carvalho et al. 1985b), which may be induced by early IL-12 (interleukin 12) production. On the other hand, susceptibility is linked to a Th2 (T helper 2) type lymphocyte stimulation, with production of IL-4 (interleukin 4) (Bogdan et al. 1996, Romagnani 1996, Launois et al. 1997). Individuals with diffuse cutaneous leishmaniasis (DCL) or visceral leishmaniasis (VL) mount a non-protective Th2 response (Carvalho et al. 1985a, Romagnani 1996).

For many reasons, it is important to develop a vaccine against leishmaniasis, and several lines of evidence suggest that this should be possible (Modabber 1990, Grimaldi Jr 1995, Handman 1997). Indigenous residents of highly endemic zones of zoonotic CL usually suffer the disease only once, and its severity is less than that occurring in non-indigenous visitors. Individuals from endemic areas have protected themselves from multiple lesions on the face by infecting their arms with virulent parasites, a practice known as leishmanization. Live vaccination has been used previously in several countries. However, since virulent organisms were used, vaccination-acquired leishmaniasis frequently proved more troublesome than the natu-

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⁺Corresponding author. Fax: 55-71-3564292. E-mail: pveras@svn.com.br.

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rally-acquired disease. In addition, *Leishmania* is an easy parasite to manipulate and maintain in axenic culture. Several studies using experimental animals showed a protective effect in response to vaccination (reviewed in Barral-Netto & Barral 1987, Modabber 1990, Connel et al. 1993).

The protection obtained by using live parasites has led to a search for attenuated parasites for immunization against leishmaniasis (Handman et al. 1983, Mitchell et al. 1984, Marchand et al. 1987, Kimsey et al. 1993) as the use of attenuated parasites could induce protection without producing pathology. Although the defect(s) of these lines is unknown, a defined auxotrophic line has been produced by targeted deletion of an essential metabolic gene, *DHFR-TS* (encoding the bifunctional dihydrofolate reductase-thymidylate synthase) in *L. major* (Cruz & Beverley 1990, Cruz et al. 1991). This line did not cause disease even in highly susceptible mouse strains, and vaccination conferred substantial and specific protective immunity in BALB/c, C57BL/6 and CBA mice challenged one week later with virulent *L. major* (Titus et al. 1995).

Studies with cross-protection are very rare (Alexander & Phillips 1978, Howard et al. 1981, 1982), but it has already been demonstrated protection of BALB/c mice infected with *L. tropica*, a causative agent of Old World CL, against New World CL agents (*L. panamensis*, *L. mexicana* and *L. amazonensis*). In addition works of cross-protection using primates are controversial (Lainson & Shaw 1977, Barral-Netto & Barral 1987). In the present report, we evaluated the ability of the *L. major dhfr-ts*⁻ mutant E10-5A3 to confer protection of mice against heterologous infection by *L. amazonensis*, a parasite which induces several different forms of leishmaniasis in humans (Barral et al. 1991).

MATERIALS AND METHODS

Animals - Inbred BALB/c and C57BL/6 were obtained from the Central Animal Facilities of Fiocruz (Rio de Janeiro, BR) and used beginning at eight weeks of age. Mice were maintained at Centro de Pesquisas Gonçalo Moniz (CPqGM), with water and balanced commercial ration *ad libitum*.

Parasites - The *dhfr-ts*⁻ *L. major* (strain E10-5A3) (Cruz et al. 1991) was maintained in M199 medium, supplemented with 10 µg/ml Thymidine, 10 mM Adenine, 0.25% Hemin, 20 mM HEPES and 20% Fetal Calf Serum (FCS, Hyclone). *L. amazonensis*, strain MHOM/BR-88/BA-125, was maintained in Schneider's medium supplemented with 10% FCS. *dhfr-ts*⁻ and *L. amazonensis* stationary phase promastigotes were washed and suspended in saline for animal inoculation.

Immunization and challenge - Groups of susceptible mice (n = 5) were immunized subcutaneously in the right footpad, or intravenously, with *dhfr-ts*⁻ *L. major* suspension in saline containing 10⁴, 10⁶ or 10⁸ E10-5A3 promastigotes in 20 µl for SC or 200 µl for IV vaccination and challenged one week later with 10⁶ stationary phase *L. amazonensis* promastigotes in the right footpad. The control group (n = 5) received an equal volume of phosphate-buffered saline (PBS). As previously described (Titus et al. 1995), mice were challenged one week later by SC inoculation in the right hind footpad with stationary phase *L. amazonensis* promastigotes (10⁶ or 5x10⁶ parasites in 20 µl). The course of infection was monitored by measuring the footpad thickness using a dial gauge caliper. Graphs show the lesion size, expressed as the difference between the infected and the contralateral uninfected footpads. Text results are expressed as percentage of protection or the relative reduction of lesion size, compared to lesion from control, non-immunized mice.

RESULTS

We asked whether the *dhfr-ts*⁻ strain of *L. major* was able to protect against heterologous challenge with promastigotes of *L. amazonensis*. We used the same protocol of immunization with which we previously obtained high degree of specific protection against homologous *L. major* challenge infection. Groups of susceptible BALB/c mice (n = 5) were immunized subcutaneously with 10⁴, 10⁶ or 10⁸ E10-5A3 promastigotes, and challenged one week later with 10⁶ stationary phase *L. amazonensis* promastigotes in the right footpad. Control animals received the same volume of saline. The group immunized with 10⁸ *dhfr-ts*⁻ parasites displayed smaller lesions compared to the non-immunized animals (p<0.001) or the groups immunized with 10⁴ (p<0.01) or 10⁶ (p<0.05) E10-5A3 (Fig. 1a). In four experiments, SC immunization conferred better protection between the 7th and the 9th weeks post challenge infection. Protection ranged between 40 and 65%. Overall, the degree of protection found was somewhat less than that observed previously by homologous challenge with virulent *L. major* (Titus et al. 1995).

Intravenous immunization of BALB/c mice was also evaluated. Animals were immunized with the doses of 10⁴, 10⁶ or 10⁸ E10-5A3 stationary phase promastigotes each. One week later, the immunized mice were challenged with 5 x 10⁶ *L. amazonensis* stationary phase promastigotes, 5-fold more parasites used to challenge animals immunized by SC route. Protection of IV BALB/c immunized mice ranged between 12 and 75%, but in four out of six experiments lesions were 40 to 75%

smaller than control animals. Mice immunized with 10^6 dhfr-ts^r promastigotes displayed greater protection ($p < 0.05$). Animals vaccinated with 10^4 auxotrophic promastigotes showed in two experiments a worst course of infection with lesions 20 to 50% larger than to those from control animals (Fig. 1b).

The effect of two doses was compared to a single dose in both SC (Fig. 2) and IV (data not shown) routes of immunization. BALB/c mice were vaccinated with a SC dose of 10^8 promastigotes, a dose, which conferred a higher degree of protection against *L. amazonensis* challenge. E10-5A3 promastigotes were inoculated three or one week or both three and one week before challenge. At five to six weeks after infection double-immunized animals displayed lesions 65%

smaller when compared to non-immunized animals ($p < 0.001$). Levels of protection in mice immunized once or twice doses were similar ($p > 0.05$). In this experiment, compared to non immunized animals, lesions of those immunized three weeks before infection were 57 to 33% smaller ($p < 0.05$) and of those immunized one week before infection were 42 to 45% ($p < 0.01$) smaller (Fig. 2).

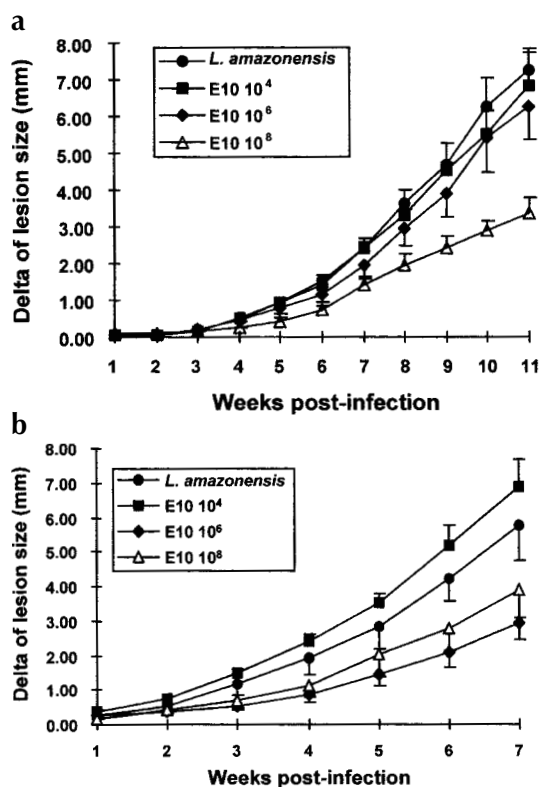


Fig. 1: E10-5A3 immunized BALB/c mice showed partial protection from *Leishmania amazonensis* challenge infection. Animals were vaccinated by (a) subcutaneous (SC) or (b) intravenous (IV) injection of 10^4 , 10^6 or 10^8 stationary phase promastigotes of auxotrophic *L. major* and one week later, were challenged with 10^6 (a) or 5×10^6 (b) stationary phase promastigotes of *L. amazonensis* in one hind footpad. Results represent the differences in lesion size of test animals contrasted with size of the contralateral footpad that received only saline instead of immunogen agent. Each point represents the mean of measurements done in five animals \pm SEM. The results shown are representative of four experiments for SC and seven experiments for IV immunization.

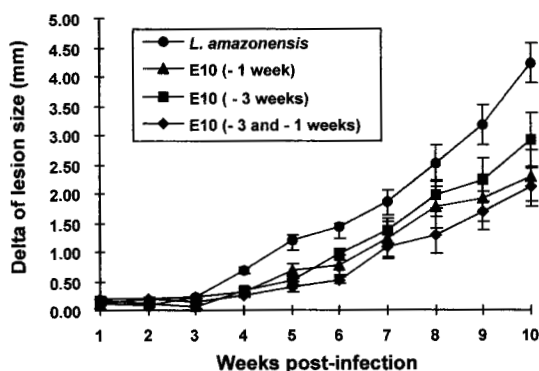


Fig. 2: BALB/C mice double-immunized with E10-5A3. Animals were vaccinated subcutaneously with 10^8 stationary phase promastigotes of auxotrophic *Leishmania major* three weeks and again one week before *L. amazonensis* (10^6) challenge infection. Each point represents the mean of measurements done in five animals \pm SEM. The results shown are similar to another experiment by IV immunization.

C57BL/6 more closely resembles human CL as they undergo clinical apparent cure after lesion development following *L. amazonensis* infection. Groups of C57BL/6 mice ($n = 5$) were vaccinated and challenged similarly to BALB/c mice. C57BL/6 mice immunized subcutaneously with 10^8 E10-5A3 promastigotes showed consistently higher protection against *L. amazonensis* infection compared to BALB/c mice. Subcutaneously immunized animals (Fig. 3a) displayed 50 to 65% protection compared to control unvaccinated mice ($p < 0.05$). Intravenously immunized C57BL/6 mice showed protection ranging from 40 to 70% when compared to control mice ($p < 0.05$). The 10^6 IV dose of auxotrophic promastigotes conferred the strongest protection of C57BL/6 mice against *L. amazonensis* infection (Fig. 3b). In general, the degree of protection obtained in these *L. amazonensis* partial resistant mice (Barral et al. 1983) was comparable or better than those obtained previously by homologous challenge with *L. major* (Titus et al. 1995). However, C57BL/6 mice were probably not sterilized and parasites did not were cleared at the site of infection, as immunization with E10-5A3 induced a partial protection against *L. amazonensis*.

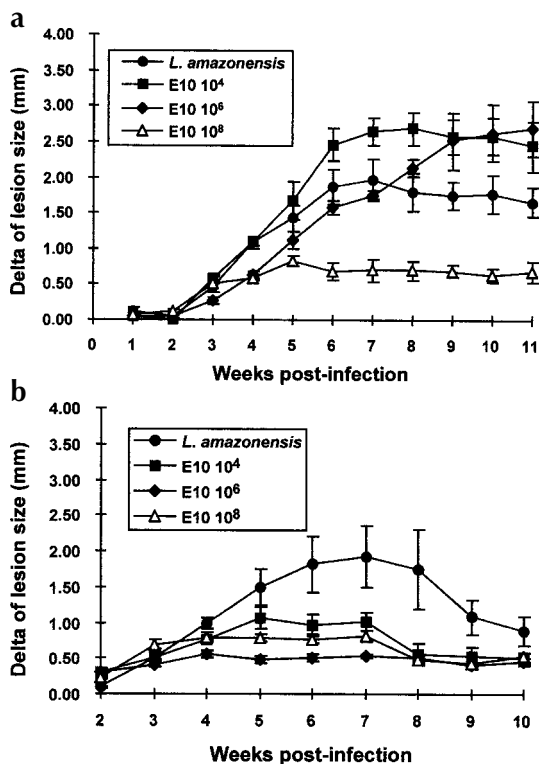


Fig. 3: E10-5A3 immunized C57BL/6 mice displayed partial protection from *Leishmania amazonensis* challenge infection. As in previous experiments, animals were vaccinated by (a) subcutaneous (SC) or (b) intravenous (IV) injection of 10^4 , 10^6 or 10^8 stationary phase promastigotes of auxotrophic *L. major* and one week later, were challenged with 10^6 (a) or 5×10^6 (b) stationary phase promastigotes of *L. amazonensis* in one hind footpad. Results represent the differences in lesion size of test animals contrasted with size of the contralateral footpad that received only saline instead of immunogen agent. Each point shows the mean of measurements done in five animals \pm SEM. The results shown are representative of two experiments for SC and three experiments for IV immunization.

DISCUSSION

Large scale human trials have demonstrated that protective immunity can result from prior controlled induction of disease with a virulent parasite. These data suggested that infections with *Leishmania* species that produce benign self-healing lesions can be linked to a solid protective immunity (Barral-Netto & Barral 1987, Modabber 1990, Grimaldi Jr 1995, Handman 1997). However, infection with virulent parasites in order to induce immunity is not an acceptable practice anymore, and workers have turned to the use of attenuated parasites instead (Rivier et al. 1993, Titus et al. 1995).

In this report we have studied a genetically defined attenuated *L. major* vaccine line, gener-

ated by homologous gene targeting of the essential DHFR-TS locus. These *dhfr-ts*⁻ parasites are auxotrophic for thymidine, and incapable of causing disease in genetically susceptible mice (Titus et al. 1995). It has been previously demonstrated that vaccination with a *dhfr-ts*⁻ auxotrophic stationary phase or metacyclic promastigotes but not parasite lysate led to high levels of protection against homologous virulent *L. major* challenge infection, in both susceptible and resistant strains of mice (Titus et al. 1995). Although challenge was performed one week after E10-5A3 infection, we have demonstrated that the response was specific to parasites as lymphocytes proliferated only in response to mitogen or parasite stimuli and not to unrelated antigens (Titus et al. 1995). In this report we used the same vaccination protocol (Titus et al. 1995) and demonstrated that this auxotrophic mutant is also able to confer protection in both BALB/c or C57BL/6 mice against a heterologous challenge with *L. amazonensis* infection.

In our experiments the direct comparison of protection by SC and IV routes is slightly complicated as we used 10^6 *L. amazonensis* promastigotes to challenge mice immunized by SC route instead of 5×10^6 parasites to animals immunized intravenously. Protection can be achieved through SC immunization, but the level of protection found after IV immunization appears to be greater in the 10^6 immunized animals. These results are encouraging as there are relatively few reports that demonstrate protection against *Leishmania* infection by SC vaccination. Non-viable *L. major* or *L. amazonensis*, when administered subcutaneously, showed no effect, although they were protective by the IV route (Howard et al. 1981, Barral et al. 1991). However, Rivier et al. (1993) demonstrated that radioattenuated *L. major* induced a protective SC immunization in resistant CBA mice against challenge infections by both homologous avirulent and heterologous virulent strains of *L. major*. Ideally, a vaccine against *Leishmania* would protect both susceptible and resistant individuals, when administered by SC or IM (intramuscular) inoculation routes (instead of IV or intraperitoneal routes).

An important consideration in vaccination is protection against challenge by heterologous strains or species of parasites. This is particularly important in South America, where many different strains and/or species of parasite occur close together. It is therefore encouraging that a reasonable degree of protection against *L. amazonensis* was obtained in our studies by vaccination with *L. major*. Together our results suggest that in human trials cross-protection could be achieved between parasites from Old and New Worlds (Alexander & Phillips 1978, Alexander & Blackwell 1983). Pos-

sibly, a stronger degree of protection would be observed if a *dhfr-ts* strain of *L. amazonensis* had been used as an immunogen. The degree of protection could also perhaps be enhanced by further genetic manipulations of this auxotrophic mutant or by the use of adjuvants (Afonso et al. 1994). Taken together our results indicate that the use of E10-5A3 or other attenuated parasites for protection against New World leishmaniasis is promising and deserves further study.

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