

## PROTECTION OF C57BL/10 MICE BY VACCINATION WITH ASSOCIATION OF PURIFIED PROTEINS FROM *Leishmania (Leishmania) amazonensis*

Ana Mariela MORA, Wilson MAYRINK, Roberto Teodoro DA COSTA, Carlos Alberto DA COSTA, Odair GENARO & Evaldo NASCIMENTO

### SUMMARY

In the past few years, induction of protective immunity to cutaneous leishmaniasis has been attempted by many researchers using a variety of antigenic preparations, such as living promastigotes or promastigote extracts, partially purified, or defined proteins. In this study, eleven proteins from *Leishmania (Leishmania) amazonensis* (LLa) with estimated molecular mass ranging from 97 to 13.5kDa were isolated by polyacrylamide gel electrophoresis and electro-elution. The proteins were associated as vaccine in different preparations with gp63 and BCG (Bacilli Calmette-Guérin). The antigenicity of these vaccines was measured by their ability to induce the production of IFN- $\gamma$  by lymphocyte from subjects vaccinated with Leishvacin<sup>®</sup>. The immunogenicity was evaluated in vaccinated mice. C57BL/10 mice were vaccinated with three doses of each vaccine consisting of 30  $\mu$ g of each protein at 15 days interval. One hundred  $\mu$ g of live BCG was only used in the first dose. Seven days after the last dose, they received a first challenge infection with 10<sup>5</sup> infective promastigotes and four months later, a second challenge was done. Two months after the second challenge, 42.86% of protection was obtained in the group of mice vaccinated with association of proteins of gp63+46+22kDa, gp63+13.5+25+42kDa, gp63+46+42kDa, gp63+66kDa, and gp63+97kDa; 57.14% of protection was demonstrated with gp63+46+97+13.5kDa, gp63+46+97kDa, gp63+46+33kDa, and 71.43% protection for gp63 plus all proteins. The vaccine of gp63+46+40kDa did not protect the mice, despite the good specific stimulation of lymphocytes (LSI = 7.60) and 10.77UI/ml of IFN- $\gamma$  production. When crude extract of *L. (L.) amazonensis* was used with BCG a 57.14% of protection was found after the first challenge and 28.57% after the second, the same result was observed for gp63 along with BCG. The data obtained with the vaccines can suggest that the future vaccine probably have to contain, except the 40kDa, a cocktail of proteins that would protect mice against cutaneous leishmaniasis.

**KEYWORDS:** *Leishmania*; Proteins; Mice; Vaccine.

### INTRODUCTION

*Leishmania (Leishmania) amazonensis* is a parasitic protozoon that causes localized cutaneous lesions and in some cases, diffuse cutaneous lesions (LAINSON, 1983). Cutaneous lesions develop in one or two months as a hard nodule that, later, develop into an open lesion that secretes serous liquid, and secondary infection becomes established in most of the cases. Diffuse lesions are characterized by large histiocytoma-like nodules disseminated in the skin containing numerous parasites and by deficient cellular immunity, probably due the immunosuppression (CONVIT *et al.*, 1993; PETERSEN *et al.*, 1982; SHAW & LAINSON, 1975).

The induction of protective immunity against cutaneous leishmaniasis is an important strategy for disease control. In the past, experiments to protect mice against cutaneous leishmaniasis have been conducted using parasite extract (BARRAL-NETO *et al.*, 1987; FROMMEL *et al.*, 1988; LIEW *et al.*, 1987; MITCHELL *et al.*, 1985; MODDABER, 1989;

SCOTT *et al.*, 1987) and a single antigen such as gp63 (RUSSELL & ALEXANDER, 1988) or lipophosphoglycan (HANDMAN & MITCHELL, 1985).

NASCIMENTO *et al.* (1990) reported the 90% correlation between *Leishmania* skin test and induction of in vitro cellular immune response by PBL from vaccinated subjects with Leishvacin<sup>®</sup> 5 or Leishvacin<sup>®</sup> 6 (vaccines with 5 or 6 *Leishmania* stocks). First attempts to identify the protective antigens from Leishvacin<sup>®</sup> by immunoprecipitation with homologous sera, revealed the presence of 8 major immunogenic components of the Leishvacin<sup>®</sup> with estimated masses ranging from 160 to 13.5kDa including gp63.

The host immune response to *Leishmania* infections is the main factor that controls the outcome of the disease (LIEW *et al.*, 1987; LOCKSLEY & LOUIS, 1992; SCOTT, 1989). In mice, susceptibility and resistance to *Leishmania major* have been correlated with the preferential stimulation of the different CD4<sup>+</sup>T cell subsets, Th1 and Th2, and the

type of cytokines that they produce. Protection is associated with cells that secrete interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ) (LOCKSLEY & LOUIS, 1992; BRETSCHER *et al.*, 1992; SCOTT, 1991), whereas the expansion of the Th2 cells that produce IL-4 and IL-10 exacerbates the disease (CHATELAIN *et al.*, 1992; HEINZEL *et al.*, 1989; SADICK *et al.*, 1990).

A Th1 response is detected in patients with active cutaneous or mucocutaneous leishmaniasis and a predominant Th2 response occurs in patients with the diffuse form of the disease (CÁCERES-DITTMAR *et al.*, 1993; PIRMEZ *et al.*, 1993). CD8<sup>+</sup> T cells are also able to produce IFN- $\gamma$  and evidences have been raised on the protective role played by these cells (CHAN, 1993; DA CRUZ *et al.*, 1993; MULLER *et al.*, 1991; NASCIMENTO *et al.*, 1990).

Other cytokines such as IL-12 (AFONSO *et al.*, 1994; SYPEK *et al.*, 1993) and the tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (STENGER *et al.*, 1994; TITUS *et al.*, 1989) are also crucial to the establishment of a protective response in experimental leishmaniasis whereas an increased expression of transforming growth factor  $\beta$  (TGF- $\beta$ ) is associated with susceptibility of infection (BARRAL-NETO *et al.*, 1993).

CARDOSO *et al.* (submitted) have previously purified the proteins of 42, 46, 63, 66, 73, 87, 97, and 160kDa from Leishvacin® (MAYRINK *et al.*, 1979). In the vaccination protocol each mouse received three doses of 30 $\mu$ g of each protein plus *Corynebacterium parvum*, as adjuvant, at 15 day intervals. Six months after challenge infection with *L. (L.) amazonensis* the levels of protection ranged from 57.1% to 20%.

The aim of the present study was to identify immunogenicity of association of proteins from *L. (L.) amazonensis* through their abilities to induce IFN- $\gamma$  synthesis, stimulation of lymphocyte proliferation and also to protect C57BL/10 mice against infection by *L. (L.) amazonensis*.

## MATERIAL AND METHODS

**Parasite stock.** The stock of *L. (L.) amazonensis* (IFLA/BR/67/PH8) was maintained in the Department of Parasitology, Institute of Biological Sciences of the Federal University of Minas Gerais, Brazil, by continuous passages in hamster. Infective promastigotes and crude extract were obtained by isolation of parasites from hamsters lesions following inoculation in NNN/LIT biphasic medium. Cultures were maintained at 23 °C for 12 days.

**Antigen preparation.** Promastigotes from stationary phase were grown in scaled up culture at 23 °C for seven days using a bottle with acellular LIT (CAMARGO, 1964). The parasites (10<sup>8</sup> promastigotes per tube) were pelleted by centrifugation in 1200g for 10 min at 4 °C. The pellet of promastigotes was washed three times in PBS, and maintained at -85 °C until use.

**Protein purification.** The pellet of promastigotes was treated by an addition of 500  $\mu$ L of buffer [1 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2mM EDTA, 1mM phenylmethylsulfonyl fluoride, 10 mM 1,10-phenanthroline], and were vigorously mixed. One ml of sample buffer (125M Tris-HCl, 4% SDS, 10% 2 – mercaptoethanol, 20% glycerol) was added, mixed and boiled for 10 min. The crude extract was loaded in 10% polyacrylamide gel, as described previously by NASCIMENTO

*et al.* (1990). After electrophoresis, protein bands ranging from 13.5 to 97 kDa were visualized. The bands were excised from the gel, and submitted to electro-elution, followed by dialysis against PBS pH 7.2. The dialysis bags were transferred to Petri dishes and cover by sucrose to concentrate the protein. Another dialysis, in the same conditions, was performed to remove residual sucrose. Protein concentrations were determined according LOWRY *et al.* (1951). The proteins were associated with gp63 for in vitro studies and for vaccination experiments.

**Induction and assay for gamma interferon.** To determine if each purified protein was able to induce the synthesis of IFN- $\gamma$ , peripheral blood leukocytes (PBL) from seven vaccinated subjects with Leishvacin® were separated by Ficoll/Hypaque gradient centrifugation as described by NASCIMENTO *et al.* (1990). The IFN- $\gamma$  response was assayed in triplicate culture. A total of 20  $\mu$ g of antigen per ml of crude extract or association of proteins was used for PBL stimulation. The culture was incubated for three days at 37 °C in a 5% CO<sub>2</sub> incubator. After this time, the supernatant of each stimulated culture was pooled and the level of IFN- $\gamma$  measured by ELISA following the instructions of the manufacture (Holland Biotechnology CO). The results were expressed in UI/ml.

**Vaccination of mice.** Female inbred C57BL/10 mice 8 to 12 weeks old were obtained from animal facilities at Federal University of Minas Gerais, Minas Gerais State, Brazil. Each group of seven mice was vaccinated subcutaneously according to FERNANDES *et al.*, (1997) into the left footpad with three doses of combined proteins with 30  $\mu$ g each at 15 days intervals with BCG (Bacilli Calmette-Guérin – Fundação Athaulpho de Paiva, Rio de Janeiro, Brazil) as an adjuvant, in the first dose. Group of control animals received 100  $\mu$ g of live BCG, 100  $\mu$ l of PBS (phosphate buffer saline) or 100  $\mu$ g of Leishvacin® in 100  $\mu$ l of PBS, respectively, following the same immunization scheme. Seven days after the last vaccination, each animal was challenged with 10<sup>5</sup> promastigotes from *L. (L.) amazonensis*.

**Challenge infection.** Infective promastigotes were obtained as described in “Parasite stock”. After 12 days of cultivation at 23 °C the promastigotes were counted in a Neubauer chamber. Vaccinated mice or control groups received the first challenge of 10<sup>5</sup> infective promastigotes seven days after the last dose of the vaccine. The injection was done in the left footpad. The second challenge was done in the same place 143 days after the first one. The development of lesions was monitored at 15 days interval, during 203 days after the first challenge.

**Lymphocyte stimulation index (LSI).** Three months after the second challenge the animals were sacrificed and the spleens were transferred to Petri dishes containing RPMI 1640 (Gibco, USA) medium. The spleens were homogenized and the cells were counted in a Neubauer chamber. For vaccinated and control C57BL/10 mice 0.5 x 10<sup>6</sup> cells/well were used. The cells were stimulated with each association of proteins in the concentration as used for IFN- $\gamma$  assay. They were maintained at 37 °C in a 5% CO<sub>2</sub> incubator for 5 days. Five micrograms per ml of PHA was used as mitogen. The cells were then pulsed with 0.5 $\mu$ Ci of [<sup>3</sup>H] thymidine (specific activity, 6.7 Ci/mMol: Dupont, NEN Research Products, Boston, Mass, USA) per culture per 18 hours, harvested, and processed for the scintillation counter (BetaRack, Pharmacia, Sweden). The magnitude of proliferation was determined by calculating a Lymphocyte Stimulation Index (LSI) for each vaccinated subject by using the following formula: LSI = the average of mean counts per minute of triplicate cultures –

machine background/average of mean triplicate unstimulated cultures – machine background (NASCIMENTO *et al.*, 1990).

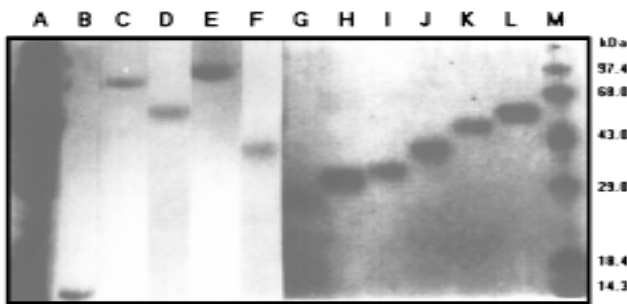
**Vaccine efficacy.** Clinical observations of the animals and lesion development were evaluated during 203 days after the first and second challenge infections at seven day interval. Lesion measurements were done at 15 days intervals with a micrometer (Mitutoyo do Brasil). The results were expressed as percentages of protection [% protection = (number of animals without lesion / number of animals of the group) x 100]. The animals were sacrificed and smear from footpad skin was Giemsa stained for the presence of parasites. For histopathological examinations a biopsy was taken at the site of the infection, biopsy fragments were fixed in 10% formalin, washed in water for 4 hours, dehydrated and embedded in paraffin, cut (3-4 µm thick) and stained with hematoxylin and eosin for optical microscope examination (TAFURI *et al.*, 1996).

**RESULTS**

The proteins of estimated molecular mass of 13.5, 22, 25, 33, 40, 42, 46, 63, 66, 85, and 97kDa were purified from *L. (L.) amazonensis* using SDS-PAGE and electro-elution (Figure 1), and were associated as vaccines as shown in Table 1.

In order to know if each association of proteins is able to induce the synthesis of IFN-γ, peripheral blood leukocytes (PBL) from vaccinated subjects with Leishvacin® (NASCIMENTO *et al.*, 1990) was evaluated. The results showed that the levels of IFN-γ in the supernatant of cultures were not statistically significant (p>0.05) ranging from 19.14UI/ml to 10.08UI/ml. Levels of IFN-γ obtained with the vaccines gp63+46+97kDa, gp63+46+22kDa, gp63+46+97+13.5kDa, gp63+46+33kDa, gp63+63+23.5+25+42kDa, gp63+66kDa, gp63+97+85kDa, gp63 plus all proteins were always higher in comparison with 13.32UI/ml obtained with the vaccine *L. L. amazonensis* crude extract. Using gp63+46+13.5kDa, gp63+46+40kDa, gp63+46+42kDa, gp63+97kDa, gp63 the levels of IFN-γ were bellow those obtained with *L. (L.) amazonensis* crude extract and above those observed for BCG (Table 1).

The vaccine gp63+46+13.5kDa, gp63+46+22kDa, gp63+85+97kDa, and gp63 showed similar LSI in comparison with 10.80 of LSI obtained



**Fig. 1** - Proteins in 10% polyacrylamide gel electrophoresis (SDS-PAGE) purified from *Leishmania (Leishmania) amazonensis*. A - crude extract of *L. (L.) amazonensis* (LLa), B - protein of 13.5 kDa, C – 85 kDa, D – 66 kDa, E – 97 kDa, F - 22 kDa, G – 25 kDa, H – 33 kDa, I – 40 kDa, J – 42 kDa, K - 46 kDa, L – gp63, M - molecular weight markers in kDa.

**Table 1**  
Gamma interferon (IFN-γ) production by human peripheral blood leukocytes from vaccinated subjects and lymphocyte stimulation index (LSI) from vaccinated C57BL/10 mice by vaccine stimulation

Group of 7 mice	Vaccine components (kDa)	IFN-γ (U/ml)	LSI (Ratio)
1	gp63+46+97+BCG	15.54	8.00
2	gp63+46+13.5+BCG	11.23	10.00
3	gp63+46+22+BCG	16.80	10.40
4	gp63+46+97+13.5+BCG	13.56	9.60
5	gp63+46+33+BCG	19.14	11.70
6	gp63+46+40+BCG	10.77	7.60
7	gp63+46+42+BCG	12.55	9.00
8	gp63+13.5+25+42+BCG	13.81	7.80
9	gp63+66+BCG	14.00	7.20
10	gp63+97+BCG	11.23	8.30
11	gp63+85+97+BCG	15.82	10.10
12	gp63+all proteins+BCG	14.61	8.50
13	gp63+BCG	10.08	10.20
14	LLa+BCG	13.32	10.80
15	BCG	9.16	5.10
16	PBS	5.11	1.50

LLa: crude extract of *L. (L.) amazonensis*. 11 proteins:13.5, 22, 25, 33, 40, 42, 46, 63, 66, 85, 97 kDa, BCG: Bacillus Calmett-Guérin. LSI – average of lymphocyte stimulation index. PHA stimulation was 62486 cpm.

**Table 2**  
Efficacy of vaccines constituted with combination of purified proteins from *Leishmania (Leishmania) amazonensis* to protect C57BL/10 mice after two challenge infections with homologous 10<sup>5</sup> infective promastigotes

Group of 7 mice	Vaccine components (kDa)	Efficacy (%)	
		143 days after first challenge	60 days after second challenge
1	gp63+46+97+BCG	57.14	57.14
2	gp63+46+13.5+BCG	57.14	28.57
3	gp63+46+22+BCG	57.14	42.86
4	gp63+46+97+13.5+BCG	57.14	57.14
5	gp63+46+33+BCG	57.14	57.14
6	gp63+46+40+BCG	00.00	00.00
7	gp63+46+42+BCG	42.86	42.86
8	gp63+13.5+25+42+BCG	42.86	42.86
9	gp63+66+BCG	42.86	42.86
10	gp63+97+BCG	42.86	42.86
11	gp63+85+97+BCG	57.14	28.57
12	gp63+all proteins+BCG	71.43	71.43
13	gp63+BCG	57.14	28.57
14	LLa+BCG	57.14	28.57
15	BCG	14.29	00.00
16	PBS	00.00	00.00

LLa: crude extract of *L. (L.) amazonensis*. 11 proteins:13.5, 22, 25, 33, 40, 42, 46, 63, 66, 85, 97 kDa, BCG: Bacillus Calmett-Guérin.

with *L. (L.) amazonensis* crude extract. Indeed, the vaccine of gp63+46+33kDa showed higher index of T cell stimulation (Table 1).

The lesions start to grow one month after first challenge. The mice were considered protected four months after the second challenge. After two months the presence of lesions was not observed in all vaccinated mice (Figure 2). The presence of parasites in the footpad lesions was evaluated by histopathological studies under microscope examination in serial cut of the tissue. Parasites were not found in most animals. Intracellular and extracellular amastigotes were found in all mice belonging to the control groups (data not shown).

The vaccination of C57BL/10 mice with these vaccines resulted in different levels of protection (Table 2). But, surprising results were

observed with the vaccine of gp63+46+40kDa that did not protect the mice (Figure 2), despite good lymphocyte proliferation (LSI = 7.60) and 10.77UI/ml of IFN- $\gamma$  production. Mice injected with BCG alone or with PBS developed progressive infections in the footpad. The group injected with BCG 14.29% of the mice did not develop lesions following the first challenge, but all became infected after the second challenge (Figure 2A).

The efficacy of the vaccines to protect C57BL/10 mice can be observed in Table 2. The lowest protection (28.57%) was obtained using the vaccine gp63+46+13.5kDa, gp63+85+97kDa, gp63kDa or LLa. Good protection (42.86%) was obtained with gp63+46+22kDa, gp63+46+42kDa, gp63+13.5+25+42kDa, gp66+66kDa, and gp63+97kDa, but very good protection (57.14%) was obtained using

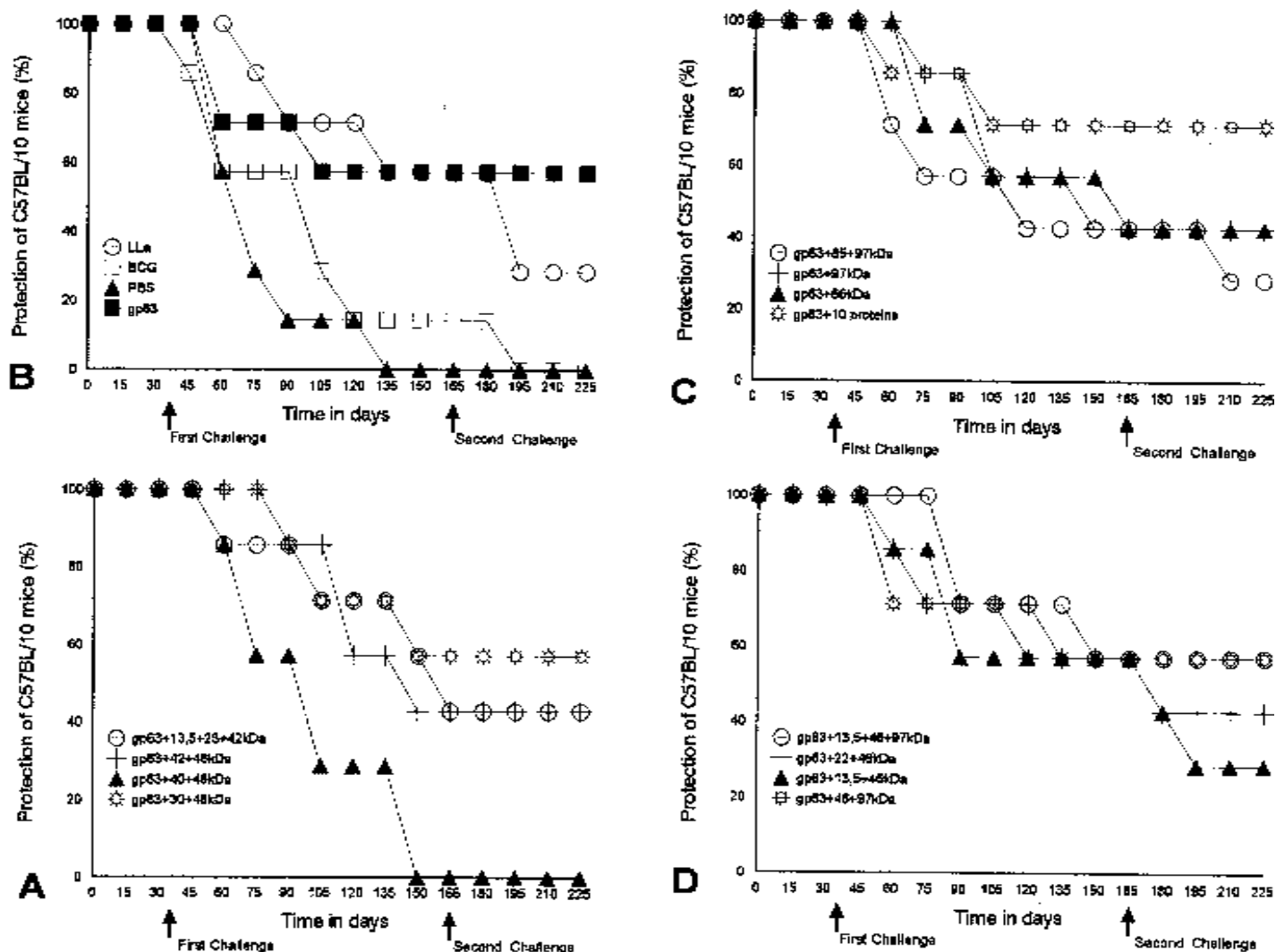


Fig. 2 - Efficacy of the combination of proteins purified from *Leishmania (Leishmania) amazonensis* (LLa) to protect C57BL/10 mice against two challenge infections with  $10^5$  homologous infective promastigotes. (A, B, C, D). Each point represents the mean plus standard deviations per group of seven animals during experimental period. BCG – Bacilli Calmette-Guérin. The results are expressed as percentages of protection (see Material and Methods).

gp63+46+97kDa, gp63+13.5+85+97kDa or gp63+46+33kDa. The best protection (71.43%) was obtained using the vaccine constituted by gp63 plus all proteins and BCG (Table 2).

## DISCUSSION

In experimental studies using animal models or in human trials, it is clear that resolution of the lesions and resistance to *Leishmania* infections require the induction of effective cell-mediated immunity able to activate efficiently the macrophages to kill the parasite. T cells and INF- $\gamma$  production are mainstays of this protective response (SCOTT *et al.*, 1987; CHAMPSI & McMAHON-PRATT, 1988; CÁCERES-DITTMAR *et al.*, 1993; CHAN, 1993). Antigens inducing this type of response, especially in human primed T cells can be considered potential vaccine candidates. Therefore, the INF- $\gamma$  production by human primed T cells, and lymphocyte proliferation of immunized mice can provide a reliable screening of such antigens.

Reports from different laboratories have shown that purified protein and recombinant *Leishmania* antigens, such as the gp46, gp63 and 33kDa, can induce partial protection against *Leishmania* infection in animal models (CHAMPSI & McMAHON-PRATT, 1988; RUSSELL & ALEXANDER, 1988; BUTTON & McMASTER, 1988; FERNANDES *et al.*, 1997).

In this report, we demonstrated that at least five (13.5, 33, 46, 63 and 97kDa) out of eleven proteins from *L. (L.) amazonensis* had the ability to stimulate lymphocytes in C57BL/10 vaccinated mice, induce INF- $\gamma$  production of PBL from Leishvacin<sup>®</sup> vaccinated subjects (Table 1), and also partially protected mice against two challenge infections by *L. (L.) amazonensis* (Table 2). As previously observed, gp63 is one of the major components of Leishvacin<sup>®</sup> (NASCIMENTO *et al.*, 1990), and by all of the parameters analyzed, it elicited responses equivalent to *L. (L.) amazonensis* crude extract, and induced 57.14% of protection of mice after first challenge and 28.57% after the second one.

In contrast, the association of gp63+46+40kDa was not able to induce protective immunity in vaccinated mice, but was able to stimulate lymphocyte proliferation with LSI of 7.60 and 10.77U/ml of INF- $\gamma$  production. These data suggest that the 40kDa maybe is an immunosuppressive protein and with inhibitory effect on T cell proliferation, resulting in an absence of immunity against this infection. If this is true, the vaccine that stimulates higher levels of INF- $\gamma$  and LSI probably indicate a great immunological ability to protect mice (Table 1, 2, Figure 2A).

The proteins of 22, 25, 42, and 66kDa stimulated INF- $\gamma$  production and stimulated mice T cells (Table 1), but only 42.86% of protection as observed against the second infective challenge (Table 2). The greatest protection (71.43%) was obtained using the vaccine constituted by all proteins. In addition, a correlation was observed between synthesis of INF- $\gamma$ , lymphocyte proliferation and protection (Table 1, 2).

Finally, the present results with different associations of *L. (L.) amazonensis* proteins and BCG strongly suggest that an efficient vaccine against cutaneous leishmaniasis, will have to contain a cocktail of immunogenic proteins.

## RESUMO

### Proteção de camundongos C57BL/10 vacinados por vacinas contituidas pelas combinações de proteínas purificadas de *Leishmania (Leishmania) amazonensis*

A indução de imunidade no homem contra a leishmaniose cutânea tem sido estudada por vários pesquisadores usando uma grande variedade de preparações antigênicas, como: promastigotas vivas ou atenuadas, extratos de promastigotas, antígenos parcialmente purificados e proteínas puras. Neste trabalho foram isoladas 11 proteínas de *L. (L.) amazonensis* com pesos moleculares variando de 13.5 a 97 kDa por eletroforese em gel de poliacrilamida e por eletroeluição. Estas proteínas foram combinadas em diferentes preparações vacinais com gp63 e BCG. As vacinas foram avaliadas *in vitro* quanto à capacidade de estimular linfócitos de pessoas vacinadas com Leishvacin<sup>®</sup> a produzirem INF- $\gamma$  e a estimularem a proliferação de linfócitos de camundongos vacinados. Assim, camundongos C57BL/10 foram vacinados em intervalos de 15 dias com três doses de cada vacina contendo 30  $\mu$ g de cada proteína. 100  $\mu$ g de BCG foram usados somente na primeira dose. Sete dias após a última dose os animais receberam a primeira infecção desafiado com 10<sup>5</sup> promastigotas infectantes e um segundo desafio foi administrado 143 dias após, com o mesmo número de parasitas. Sessenta dias após o segundo desafio, proteções de 42,86% foram obtidas com as vacinas constituídas de gp63+46+22kDa, gp63+13.5+25+42kDa, gp63+46+42kDa, gp63+66kDa e gp63+97kDa; 57,14% de proteção foi obtido com a vacina gp63+46+97kDa, gp63+46+97+13.5kDa, gp63+46+33kDa, e 71,43% com a vacina constituída de gp63 mais todas as proteínas. Em contraste, a vacina gp63+46+33kDa não induziu proteção nos camundongos vacinados, indicando que possivelmente a proteína de 40kDa induziu a uma atividade imunossupressora da resposta imunoprotetora. Estes resultados sugerem que uma futura vacina contra a leishmaniose cutânea deverá conter, excluindo-se a proteína de 40kDa, um coquetel de proteínas imunogênicas indutoras de proteção de camundongos contra a leishmaniose cutânea.

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