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Identification and purification of immunogenic proteins from nonliving promastigote polyvalent *Leishmania* vaccine (Leishvacin®)

Identificação e purificação de proteínas imunogênicas da vacina polivalente de promastigotas mortas de *Leishmania* (Leishvacin®)

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Abstract *Immunogenic proteins from nonliving promastigote polyvalent* Leishmania *vaccine against American tegumentary leishmaniasis (Leishvacin®), produced by Biobrás (Biochemistry of Brazil), Montes Claros, State of Minas Gerais, Brazil, were identified and purified by polyacrylamide electrophoresis gel and electroelution. C57BL/10 mice were vaccinated with proteins with estimated molecular weights of 42, 46, 63, 66, 73, 87, 97, and 160kDa in three doses of 30µg of each protein at 15-day intervals combined with 250µg of* Corynebacterium parvum *followed by a challenge infection with 10^5 infective promastigotes from* Leishmania (Leishmania) amazonensis*. The ability of these proteins to induce immune response and protection was analyzed. No statistical difference was observed in the level of IFN-γ induced by proteins in vaccinated groups in comparison with control groups. Six months after challenge infection, protection levels of 28.57; 42.86; 57.14; 42.86; 42.86, 57.14; 42.86 and 57.14% were demonstrated for each purified protein.*

Key-words*: Tegumentary leishmaniasis. Immunogenic proteins. Leishvacin®.*

Resumo *Proteínas imunogênicas da vacina polivalente de promastigotas mortas de leishmanias (Leishvacin®) produzida pela Biobrás – Bioquímica do Brasil, Montes Claros, Minas Gerais, Brasil foram identificadas e purificadas por eletroforese em gel de poliacrilamida e eletroeluição. Camundongos C57BL/10 foram vacinados com proteínas de pesos moleculares estimados em 42, 46, 63, 66, 73, 87, 97 e 160kDa em três doses de 30µg de cada proteína combinada com 250µg de* Corynebacterium parvum *em intervalos de 15 dias e desafiados com uma infecção desafio de 10^5 promastigotas infectantes de* Leishmania (Leishmania) amazonensis *na base da cauda. Foram avaliadas a habilidade dessas proteínas em induzir resposta imune e proteção dos animais vacinados após a infecção desafio. Nenhuma diferença estatística foi observada nos níveis de IFN-γ nos grupos vacinados em comparação ao grupo controle. Proteção de 28,57; 42,86; 57,14; 42,86; 42,86, 57,14; 42,86; 57,14% foi demonstrado para cada proteína.*

Palavras-chaves: *Leishmaniose tegumentar. Proteínas imunogênicas. Leishvacin®.*

American tegumentary leishmaniasis (ATL) is a disease caused by different species of *Leishmania*, which constitutes an important public health problem in Latin America[9]. The disease affects people living in a variety of ecological settings, including primary rain forest as well as agricultural and urban areas. Even without considering under-reporting and the large number of incorrect diagnoses, the estimated prevalence is high and incidence of American cutaneous leishmaniasis has increased as a consequence of environmental changes and difficulties in controlling the vectors and reservoirs.

Protective immunity against leishmaniasis is largely mediated by T cells[3 7 14 15]. The nature of the host immune response to different *Leishmania* infections is one of the main factors controlling the outcome of the disease. In mice, susceptibility and resistance to *L. major* have been correlated with selective stimulation of the CD4+ cell subsets, Th1 and Th2, and the type of cytokines that they produce[4 31]. In general, a protective role is associated with the cells of the Th1 subset that secrete IL-2 and IFN-γ, whereas the expansion of cells of Th2 subset, which

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produce IL-4 and IL-10, exacerbates disease[12 29 30 31 32]. Similarly to the *L. major* in Balb/c[30], treatment of Balb/c mice with anti-IL-4 monoclonal antibodies abrogates its susceptibility to *L. amazonensis*[6]. In addition, *in vitro* and *in vivo* studies indicate that IL-12[1 6] and tumor necrosis factor a (TNF-α) are also crucial to the establishment of resistance against experimental leishmaniasis[33].

Efforts to develop a vaccine to control ATL have been made by several laboratories. Different vaccine compositions including crude and purified *Leishmania* components have been tested in animal models as well as in human vaccination trials. In Brazil, the development of a non-living promastigote vaccine against ATL was initiated by Pessoa's group in the decade of 1940[25 26], followed by the first publication by Mayrink's group in 1979[19]. Significant progresses were made in several clinical trials demonstrating the immunogenicity of the vaccine, such as: (a) induction of a delayed-type hypersensitivity, as demonstrated by a positive skin test[19]; (b) no appreciable side effects[21]; (c) conversion of delayed-type hypersensitivity with a statistically significant level of protection compared with non-converted or non-vaccinated subjects[2 27]; (d) old vaccine preparations (four years old) proved to be equivalent to newly prepared ones in converting the results of skin tests from negative to positive as well as providing protection[21]; (e) protection of 50% of vaccinated individuals whose skin test converted from negative to positive after vaccination[2]; (f) strong correlation has been found between positive skin test results and lymphocyte stimulation indices (LSI) in vaccinated subjects[24]; (g) LSIs of vaccinated groups were significantly higher ($P<0.001$) than those of placebo group[26]; (h) eight major antigens with estimated molecular weights of 13.5 to 160kDa may play an important role in the immunity against tegumentary leishmaniasis[24]; (i) gp63 was the major protein present in our vaccine[24]; and (j) T lymphocyte-mediated immune response against *Leishmania* antigens, by Th1 profile response and by protection against the disease, was observed in 50% of subjects vaccinated[2 8 19 21 22 24].

This vaccine has become an important tool in the identification of the key parasite antigens that induce immunity against tegumentary leishmaniasis, and has also been successfully used as an immunotherapeutic agent[20 18].

In this study, we isolated proteins of estimated molecular weight of 42, 46, 63, 66, 73, 87, 97 and 160kD from Leishvacin®, by preparative polyacrylamide gel electrophoresis, and tested their ability to induce IFN-γ synthesis by T lymphocytes from vaccines. We also analyzed the ability of these isolated proteins to induce protective immunity in susceptible C57BL/10 mice against *L. (L.) amazonensis.*

MATERIAL AND METHODS

Leishvacin®. Leishvacin® was produced and supplied by BioBrás (Biochemistry of Brazil), according to Mayrink's group methodology of production[21] in GMP (good manufacturing practice) conditions. It is constituted by five Leishmania stocks: (*Leishmania (Leishmania) amazonensis* MHOM/BR/60/BH6, *Leishmania* major-like, MHOM/BR/73/BH121, *Leishmania* major-like MHOM/BR/71/BH49, *Leishmania (Leishmania) amazonensis* IFLA/BR/67/PH8, *Leishmania (Viannia) braziliensis* MHOM/BR/70/M1176[19 24]). Proteins from Leishvacin® were isolated on 10% SDS-PAGE[14]. The proteins were electroeluted from gel slices, dialyzed against PBS pH 7.2, concentrated and dialyzed again in the same buffer. Protein concentrations were determined according to Lowry's method[16].

C57BL/10 mice. Isogenic female mice aged 8-10 weeks were used in the vaccination experiments. These animals were maintained in the animal house of the Federal University of Minas Gerais, State of Minas Gerais, Brazil, under US National Institutes of Health guidelines for animal care.

Mice vaccination. Eight groups of seven 8-10 week mice, obtained from animal facilities at Federal University of Minas Gerais, Minas Gerais State, Brazil, were vaccinated subcutaneously into the left footpad with three doses of each identified and purified protein (30μg) at 15-day intervals combined with 250μg of live CP (*Corynebacterium parvum* – Fundação Athaulpho de Paiva, Rio de Janeiro, Brazil), as adjuvant. Three control groups with seven animals each received only 250μg of live CP, 100μl of PBS (phosphate buffer saline) or 100μg of Leishvacin® in 100μl of PBS, respectively, following the same immunization scheme.

Parasite for challenge infection. The *L. (L.) amazonensis* IFLA/BR/67/PH8 strain was used for experimental infections. This strain was maintained by continuous passages in hamsters for the preparation of infective promastigotes. Promastigotes were washed in 0.8% NaCl solution and 10^5 stationary phase promastigotes of *L. amazonensis* were used in the challenge infection for each C57BL/10 mice. The injection was done into the base of the tail, seven days after the last vaccination. The development of lesions was monitored at 15-day intervals for 180 days.

Lymphocyte stimulation index (LSI). Six months after challenging the animals, they were sacrificed and the spleens were transferred to Petri dishes containing complete RPMI 1640 medium (Gibco, USA), as previously described[23].

Interferon gamma assay (IFN-γ). Spleen cells were obtained from C57BL/10 mice of each vaccinated group, as well as control mice at six months after challenge infection. Cells were prepared as previously described[11]. Cells were isolated by Ficoll-hypaque gradient centrifugation and suspended at 1.5×10^6cells per ml in complete RPMI 1640 medium (200mM L-glutamine,

100UI of penicillin per ml, 50μg of streptomycin per ml, 10mM HEPES [N-2-hydroxyethylpiperasine-N-2-ethanesulfonic acid]) containing 10% inactivated fetal calf sera (FBS). Cell culture was carried out in flat-bottomed 24-well plates and maintained at 37°C in a 5% CO_2 incubator for two days. The IFN-γ measurement was performed by ELISA on supernatant pools of triplicate cultures stimulated with each protein (20μg/ml), Leishvacin® (50μg/ml) and *Corynebacterium parvum* (50μg/ml) at 48 hours after stimuli. Results are presented as means of cytokine concentrations (UI/ml) determined for seven vaccinated individuals in each group.

Vaccine efficacy. Clinical observations of the animals and lesion development were carried out during 180 days after the challenge infections at seven-day intervals. Lesion measurements were done at 15-day intervals using a micrometer (Mitutoyo Sul Americana, São Paulo, Brazil). The results were expressed as percentages of protection, which represent the animals without lesions per group. The animals were sacrificed and smears from footpad skin were Giemsa stained for the presence of parasites. For histopathological examinations a biopsy was taken at the site of infection fixed in 10% formalin in PBS, washed in water for 4 hours, dehydrated and embedded in paraffin, cut (3-4μm thick) and stained with hematoxylin and eosin for optical microscopic examination[35].

Statistical analysis. Statistical significance was determined by Pearson chi-square test, Student t test[10], and Statistical Epinfo analysis version 6.0.

RESULTS

Eight proteins with estimated molecular weights of 42, 46, 63, 66, 73, 87, 97 and 160kD were purified from Leishvacin® (Figure 1A,B).

All proteins were tested *in vitro* to stimulate peripheral blood monocyte cells (PBMC) from vaccinated mice in order to determine their ability to induce synthesis of IFN-γ. The results showed that the levels of IFN-γ, produced by protein stimulation in culture supernatants, were not statistically significant (P>0.05) ranging from 19.8UI/ml to 15.8UI/ml (Table 1). The levels of IFN-γ induced by each protein were comparable to those induced by Leishvacin®. These proteins were also able to induce cell proliferation and no statistically significant differences were observed between them with regard to the amplitude of proliferation induced (Table 1).

Vaccination of C57BL/10 mice with each of these purified proteins resulted in different levels of protection, as shown in Table 2. Lesion development was followed

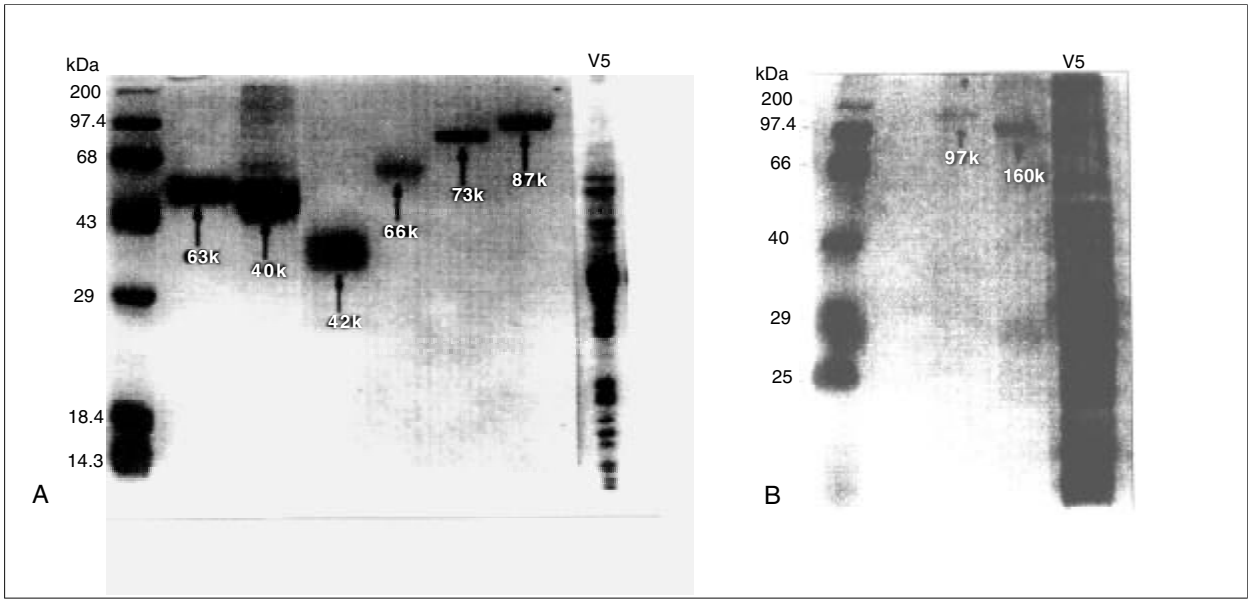

Figure 1 - Proteins with estimated molecular weights of 63, 46, 42, 66, 73 and 87kD (A) and 97, 160kD (B) in 10% polyacrylamide gel electrophoresis (SDS-PAGE) purified from Leishvacin® (V5). MW - molecular weight markers.

over a period of 180 days after challenge with 10^5 promastigotes of *L. amazonensis*. Non-vaccinated mice that received only *C. parvum* developed progressive infections on the footpads. The proteins of 63, 87 and 160kD induced protection of 4/7 (57.14%) mice, similar to the protection obtained with Leishvacin®. Protective rates of 3/7 (42.86%) mice were obtained by those animals vaccinated with proteins of 46, 66, 73 and 97kD. The protein of 42kD induced protection of only 2/7 (28.57%) mice. In the other two control groups, all animals were infected.

Table 1 – Gamma interferon (IFN-γ) production by human peripheral blood monocytes from vaccinated subjects and lymphocyte stimulation index (LSI) from vaccinated C%&BL/10 mice.

Group (7 mice)	Protein (kD)	IFN-γ (UI/ml)	LSI (Ratio)
1	42 + CP	17.5	3.7
2	46 + CP	16.4	8.0
3	63 + CP	17.6	7.5
4	66 + CP	17.1	9.6
5	73 + CP	16.6	5.8
6	87 + CP	17.3	6.5
7	97 + CP	19.8	8.3
8	160 + CP	15.8	12.2
9	Leish + CP	14.4	11.2
10	CP	4.6	1.5
11	PBS	5.5	1.8

CP: *Corynebacterium parvum*; Leish: Leishvacin®; PBS: phosphate buffer saline

Table 2 – Protection of C57BL/10 mice induced by proteins purified from Leishvacin® after challenge infection with 10^5 promastigotes of Leishmania (Leishmania) amazonensis

Group (7 mice)	Protein (kD)	Infection (Protection - %)	Pearson (Chi square)
1	42 + CP	5/7 (28.57)	$P > 0.05$
2	46 + CP	4/7 (42.86)	$P < 0.05$
3	63 + CP	3/7 (57.14)	$P < 0.02$
4	66 + CP	4/7 (42.86)	$P < 0.05$
5	73 + CP	4/7 (42.86)	$P < 0.05$
6	87 + CP	3/7 (57.14)	$P < 0.02$
7	97 + CP	4/7 (42.86)	$P < 0.05$
8	160 + CP	3/7 (57.14)	$P < 0.02$
9	Leish + CP	3/7 (57.14)	$P < 0.02$
10	CP	7/7 (00.00)	-
11	PBS	7/7 (00.00)	-

CP: *Corynebacterium parvum*; Leish: Leishvacin®; PBS: phosphate buffer saline

Good correlation was found between protection and LSI in the groups immunized with the purified proteins and in the group immunized with Leishvacin®, in comparison with the other control groups (only CP and PBS). Such data could be observed for the proteins of 46, 63, 66, 87, 97 and 160kD, whereas the 42kD proteins produced only a slight protective effect (Table 1).

The presence of parasites in the footpad lesions of all mice was evaluated by histopathological studies involving serial sections of the footpad tissue. No parasites were found in vaccine-protected mice, while in the non-vaccinated ones parasites were observed inside macrophages or among the cells. Amastigote forms were found through histopathological examination in the control groups (Figure 2).

DISCUSSION

In this study, we demonstrated that eight proteins, isolated from Leishvacin®, induced synthesis of IFN-γ by lymphocyte from vaccinated mice and induced lymphocyte proliferation in vaccinated animals. Previous studies have shown that human individuals vaccinated with Leishvacin® developed *Leishmania*-specific T cell responses and protective immunity against this parasite. Characterization of cytokine production and lymphocyte phenotype involved in this immune response indicated that PBMC from vaccinated subjects induced IFN-γ but not IL-4[22]. Moreover, in subjects vaccinated one year before, CD8+ T cells proliferated preferentially in response to *L. (V.) braziliensis* antigen[22].

Different levels of protection were observed in C57BL/10 mice vaccinated with each protein. C57BL/10 mice are resistant to *L. major,* but susceptible to *L. amazonensis* infection, developing a chronic infection[31]. A polarized Th1 response is detected after *L. major* infection, but this response is absent following *L. amazonensis* infection, indicating that parasite-associated factors are able to modify the T helper response profile induced in C57BL/10 mice[30].

Characterization of the immune response associated with experimental murine leishmaniasis has led to the identification of two immunoregulatory subsets of T helper lymphocytes (Th1 and Th2) that influence the outcome

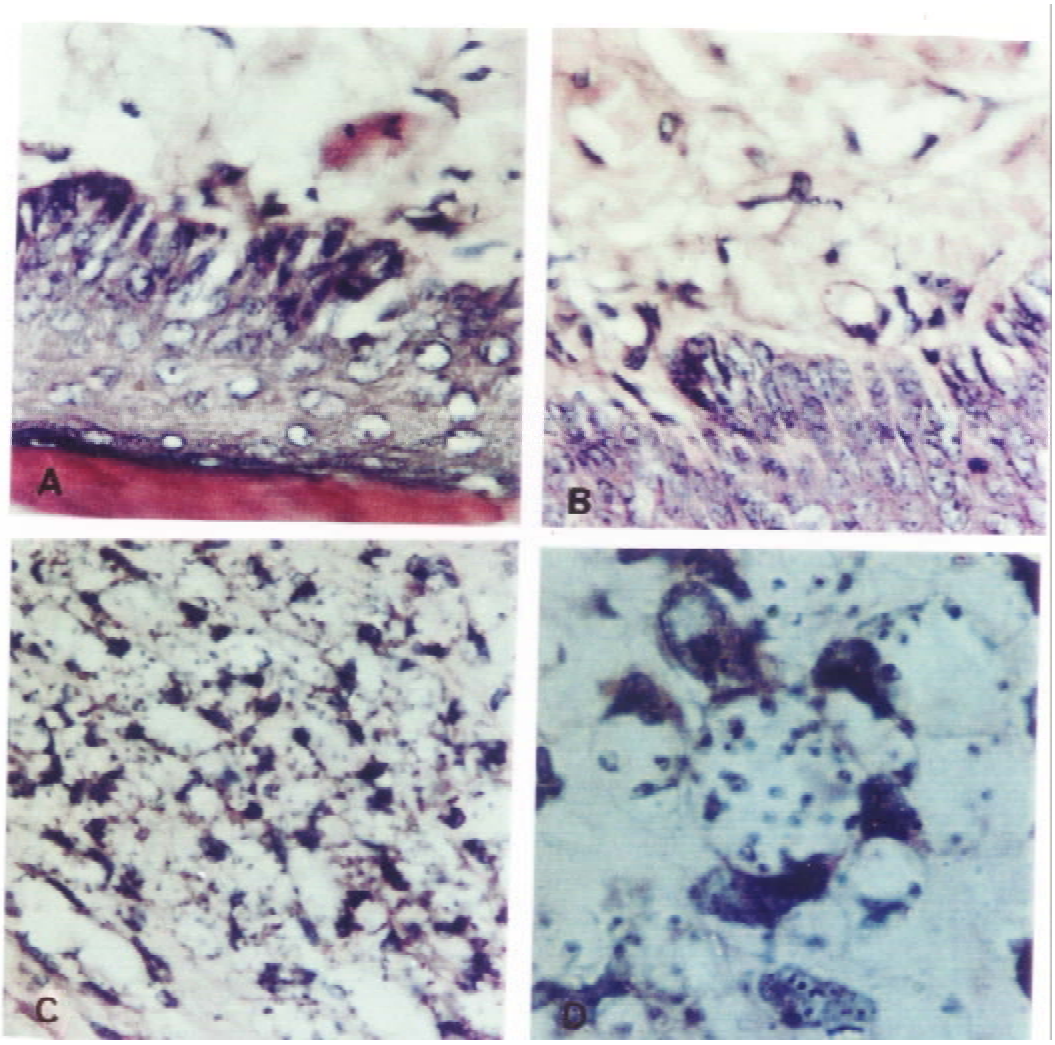

Figure 2 - Histological pattern of footpad skin from C57BL/10 mice. A: non-infected control mouse with normal hypodermis and dermis (x80). B: mouse from vaccinated protected group with absence of amastigotes and normal histological tissue. C: mouse from vaccinated non protected group with presence of several macrophages vacuolated with many amastigotes (x80). D: mouse from infected control group with intense parasitism of amastigotes (x1000). Stained with hematoxylin-eosin technique.

of infection. Th1 lymphocytes and synthesis of IFN-γ and IL-2 are associated with mild or self-healing disease, whereas the expansion of IL-4- and IL-10-producing Th2 lymphocytes is associated with disseminated infection[31]. A similar distinction has been observed in patients with different disease manifestations. T cells present in lesions of patients with localized and self-healing cutaneous leishmaniasis predominantly secrete IFN-γ and other Th1 cytokines, but not IL-4[5 27 31]. CD8+ T cells also synthesized IFN-γ, even though the protective role of these cells in leishmaniasis is not completely clear. The analysis of *in vitro* IFN-γ production in response to purified antigens therefore provides a reliable parameter for the ranking of immunodominant antigens.

Among the eight proteins evaluated, gp46 and gp63 have already received considerable attention and are known to induce protective immunity in susceptible mice, as well as synthesis of IFN-γ. Furthermore, in a previous report, C57BL/10 mice were immunized with purified proteins from *L. (L.) amazonensis* and showed an induction of a protective immunity and an elevated synthesis of IFN-γ by spleen cells, suggesting a protection against the parasite[23].

However, in our study, no positive correlation between levels of IFN-γ and protection was found. In addition, the amounts of IFN-γ production observed in susceptible mice were equivalent to the levels induced by Leishvacin®. This result also indicates a positive correlation between proliferation response and protection. These proteins are *Leishmania* immunodominant antigens and also important components of Leishvacin®.

We must consider, however, that each protein fraction evaluated in this study may consist of a mixture of different proteins. Leishvacin® is composed of five different stocks of *Leishmania*, made from different species. Although high levels of homology may exist among them, the presence of immunossupressive protein or epitopes cannot be ruled out[24]. Therefore, further studies are required to investigate the immunogenic potential of these proteins isolated from the *Leishmania* strains that comprise Leishvacin®.

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