

## Vaccination of C57BL/10 mice against cutaneous leishmaniasis using killed promastigotes of different strains and species of *Leishmania*

Vacinação de camundongos C57BL/10 contra leishmaniose com promastigotas mortas de diferentes cepas e espécies de *Leishmania*

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**Abstract** Antigenic extracts from five *Leishmania* stocks were used to vaccinate C57BL/10 mice. The Leishvacin® and PH8 monovalent vaccine yielded the highest IFN- $\gamma$  levels in the supernatants of spleen cell culture from vaccinated animals. Each single strain immunized group showed evidence of protective immunity six months after the challenge with promastigotes of *Leishmania* (*Leishmania*) amazonensis. No differences were detected between the vaccinated groups. It can be concluded that vaccines composed of single *Leishmania* stocks can provide protection to C57BL/10 mice against *L. (L.) amazonensis* infection.

**Key-words:** *Leishmania*. Vaccine. Interferon- $\gamma$ . Interleukin-4.

**Resumo** Estudos anteriores revelaram que uma vacina preparada com promastigotas mortas de cinco cepas de *Leishmania* pode induzir uma imunidade protetora para a leishmaniose tegumentar americana no homem e em modelos experimentais. Um dos problemas do uso desta vacina é a complexidade de sua composição e a necessidade de se incorporar diferentes cepas de *Leishmania*. Por esta razão, extratos antigênicos de cada uma das cinco cepas constituintes da vacina foram preparados e usados individualmente em estudos imunológicos com camundongos C57BL/10. A Leishvacin® e a vacina monovalente PH8 induziram os maiores níveis de Interferon-g (IFN- $\gamma$ ) detectado no sobrenadante de células esplênicas dos animais vacinados. Cada grupo imunizado com vacinas monovalentes desenvolveram uma imunidade protetora seis meses após a infecção desafio com promastigotas de *Leishmania* (*Leishmania*) amazonensis e nenhuma diferença estatística foi observada entre os grupos vacinados. Pode-se concluir que vacinas compostas por cepas isoladas de *Leishmania* protegem camundongos C57BL/10 contra, pelo menos, da infecção por *L. (L.) amazonensis*.

**Palavras-chaves:** *Leishmania*. Vacina. Interferon-g. Interleucina-4.

Leishmaniasis are parasitic infections of animals and humans caused by several species of protozoans of the genus *Leishmania*. Cutaneous, mucocutaneous and visceral manifestations are clinically observed depending on the species involved and the host's response.

Visceral leishmaniasis is a serious form with a high mortality rate<sup>47</sup>, however, with treatment of patients, use of insecticides, elimination of dogs and epidemiological surveillance it is possible to control the transmission<sup>15 26</sup>. On the other hand, prevention of the American tegumentary leishmaniasis (ATL) is difficult because it is a zoonosis whose vectors and reservoirs are mainly sylvestric<sup>19 34</sup>. Thus, the measures for identifying and

combating the infected animals, as well as the use of insecticides, are difficult.

Considering this problem, World Health Organization experts admit that vaccination would be the only desirable, practical and safe measure for the control of tegumentary leishmaniasis<sup>34</sup>.

Attempts at human vaccination against cutaneous leishmaniasis have been tried in different parts of the world starting in the Old World<sup>5 27 38</sup>. In Brazil the first trials were carried out by Pessoa & Pestana<sup>40</sup>, with promising results, however 30 years elapsed until Mayrink *et al*<sup>9</sup> continued these studies using a vaccine of dead promastigotes constituted of five *Leishmania*

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strains, which were able to induce protection against ATL and did not show any side-effects<sup>3 13 28 30 37</sup>. Other recent researches support the Mayrink's findings. Thus, Mendonça *et al*<sup>62</sup> showed that humans, immunized against American tegumentary leishmaniasis using a vaccine made of whole antigens from killed promastigotes of five dermatotropic *Leishmania* strains, had a positive proliferative response of peripheral blood mononuclear cells (PBMC) and Interferon-g was also detected in the supernatants of stimulated PBMC. Nascimento *et al*<sup>67</sup> vaccinated Brazilian army conscripts with polyvalent vaccine made of five *Leishmania* strains and found a correlation of 0% between positive skin test results and positive lymphocyte stimulation indices. This same vaccine has been shown to protect susceptible mice<sup>12 13</sup>. Other animal models have been satisfactorily used. Kenney *et al*<sup>18</sup> used rhesus monkeys to assess the safety, immunogenicity and efficacy of a vaccine combining heat-killed *Leishmania (L.) amazonensis* with rIL-12 and aluminum hydroxide gel as adjuvants. This study demonstrated the development of a protective immunity in primate model.

Despite these promising results, the complex composition of the vaccine (5 strains) has made it difficult to standardize as well as to identify those components that are responsible for inducing the specific immune response in vaccinated individuals. Thus, a "Vaccine

Advisory Group of Discussion" was organized and supported by World Health Organization in September 1991 in Belo Horizonte, MG, Brazil with the participation of representatives of national and international organizations interested in developing an anti-ATL vaccine. It was suggested that studies with the vaccine should continue, however using only one strain, the *L. (L.) amazonensis* (IFLA/BR/67/PH8). This strain was chosen because its antigens induced high stimulation indexes for lymphocytes from vaccinated volunteers it is easy to grow in noncellular media, it is internationally known and it is taxonomically well defined.

In addition, other studies have shown that the resolution of the lesions in mice, requires the induction of effective cell-mediated immunity against common antigens found in different strains of *Leishmania*<sup>7 12 39 42</sup>. Because of this, it becomes relevant to produce individual antigenic extracts with each one of the 5 strains which are components of the vaccine described by Mayrink *et al* 1979<sup>29</sup> and to carry out a comparative study of its immunogenicity using an animal model with the purpose of evaluating the humoral and cellular immunity as well as the immunoprotection induced by different vaccines facing the *L. (L.) amazonensis* challenge. Thus, we may define which strain(s) is (are) immunogenic by evaluating with immunological parameters.

## MATERIAL AND METHODS

**Parasites.** The following strains were used to prepare the vaccines: *L. (L.) amazonensis* (IFLA/BR/67/PH8); *L. (L.) mexicana* (MHOM/BR/60/BH06); *L. (Viannia) guyanensis* (MHOM/BR/70/M1176); *L. sp* [(MHOM/BR/71/BH49) – *L. major*-like]; *L. sp* [(MHOM/BR/73/BH121) – *major*-like]. Promastigote cultures were initiated from biopsies of the border of leishmanial lesions in experimentally infected golden hamsters. They were grown in an NNN medium overlaid with LIT (NNN/LIT) culture medium<sup>7</sup> at 23°C. Infective promastigotes were obtained by the culture of hamster lesion fragments in an NNN/LIT medium at 23°C for 14 days. The challenge consisted of 1 X 10<sup>5</sup> *L. (L.) amazonensis* promastigotes.

**Mice.** Female C57B1/10 mice (8-12 week old) were obtained from the breeding facilities at the Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais state, Brazil, were used.

**Preparation of vaccines.** Vaccines were prepared according to previously described methods<sup>3 29</sup>. Briefly, promastigotes of five different stocks were grown separately in LIT medium and harvested at stationary phase (7 days of culture) by centrifugation. After washing with sterile saline, the pellet was divided in two: one part was disrupted by sonication and the other was then mixed and diluted with the appropriate amount of merthiolate phosphate buffered solution to give a final concentration of 240mg/ml of total nitrogen<sup>20</sup> and 1:10,000 merthiolate. In this way, five monovalent vaccines (PH8, M1176, BH06, BH121 and BH49) were

prepared and the original Mayrink's polyvalent vaccine (Leishvacin®) which is composed of all five strains.

**Immunization of mice.** Groups of 30 isogenic C57BL/10 mice per group were vaccinated according to Costa<sup>13</sup>. Each animal received two subcutaneous inoculations, at an interval of seven days, each dose containing 100mg of protein vaccine plus 250mg of *Corynebacterium parvum*. Twenty-eight days after the second dose, the animals received a further 10mg of vaccine, without adjuvant. Seven days after the booster, 10 animals were challenged with 1 x 10<sup>5</sup> *L. (L.) amazonensis* promastigotes inoculated subcutaneously into the base of their tails. The animals were examined at two-week intervals for a total of 180 days after the challenge to observe the appearance of lesions and their subsequent development. The 20 remaining animals were used to assess the humoral and cellular immune responses, seven days after booster. Ten mice were exclusively used for the delayed type hypersensitivity assay and ten other animals reserved to lymphocyte proliferation assay, evaluation of specific antibodies and cytokine quantification.

**Controls.** The control group consisted of mice that received only *C. parvum* with the same immunization schedule. Ten of them were challenged with *L. (L.) amazonensis* as described above and the ten remaining mice were used as control for immunological assays.

**Humoral immune response.** This was assessed by examining sera obtained 42 days after the first dose of

vaccine, by ELISA, performed as described by Nascimento *et al*<sup>37</sup> using 2mg/well of the respective vaccine as an antigen, and goat anti-mouse IgG and IgM peroxidase conjugate (SIGMA, USA).

**Cellular Immune Response.** These evaluations were done 42 days after the first dose of vaccine.

**Lymphocyte proliferation assay.** Lymphocyte proliferation assays were performed as described by Costa *et al*<sup>3</sup>. Basically, spleen cells were harvested in an RPMI 1640 (SIGMA, USA) medium containing 10% fetal calf serum (SIGMA, USA), 2mM L-Glutamine, 50mM 2-mercaptoethanol, 10,000 units of penicillin, 10mg streptomycin and 250mg fungizone (SIGMA, USA) per ml. Red blood cells were removed by lysis in a 0.144M NH<sub>4</sub>Cl buffer. The cells (4 x 10<sup>5</sup>/well) were then incubated in triplicate in 96-well plates for 5 days either with 20mg/ml of each vaccine antigens without merthiolate or 0.5µg/ml of Concanavalin A (SIGMA, USA) in an atmosphere of 5% CO<sub>2</sub> at 37°C. Proliferation was assayed by a [3H] thymidine incorporation (0.2mCi/well, specific activity 5.0 mCi/ml – Dupont, NEN Research Products, USA). The results were expressed as stimulation index. A positive response showed a stimulation index equal or higher than 2.5<sup>32</sup>. This assay was done with a cell pool from each group.

**Cytokine quantification.** A commercially available kit (Genzyme Corporation, USA, with 2.5 pg/ml of sensitivity ) for Interleukin-4 (IL-4) quantification was used to assay the levels of this cytokine on mononuclear cell supernatant harvested from splenic cell culture after a two-day stimulus with homologous *Leishmania* antigens. For IFN-γ quantification in 72-hour mononuclear cell culture supernatant, we employed the same methodology previously described by Scott<sup>44</sup>. Dr. Phillip Scott (Pennsylvania University, USA) kindly provided the reagents. This assay has 2 pg/ml of sensitivity.

**Delayed type hypersensitivity test.** This test, which measures the footpad swelling test, was performed by the subcutaneous inoculation of 50µl containing 20mg of protein of the PH8 strain antigen in the sole cushion of the right paw of vaccinated mice (42<sup>nd</sup> day), and the same volume of merthiolated saline was inoculated in the left paw<sup>39</sup>. Measurements were performed at 24/48 hours after the inoculation with a micrometer (MITUTOYO, number 2046-08, Japan). The analyses of the right paw thickness (test) and of the left paw (control) were then compared, considering the mean difference between the footpads.

**Statistical analysis.** Analysis of antibody production in each group were made by paired two tail Student's t test. The remaining data were analyzed by univariate analysis and Scheffe's test. P values ≤ 0.05 were considered significant.

## RESULTS

**Evaluation of humoral immune response.** According to Figure 1, we could verify that the immunization induced the production of anti-*Leishmania* antibodies with higher

levels of IgM than of IgG, in all immunized groups (p<0.05), except in the group of mice which was vaccinated with Leishvacin<sup>®</sup> and in the control group.

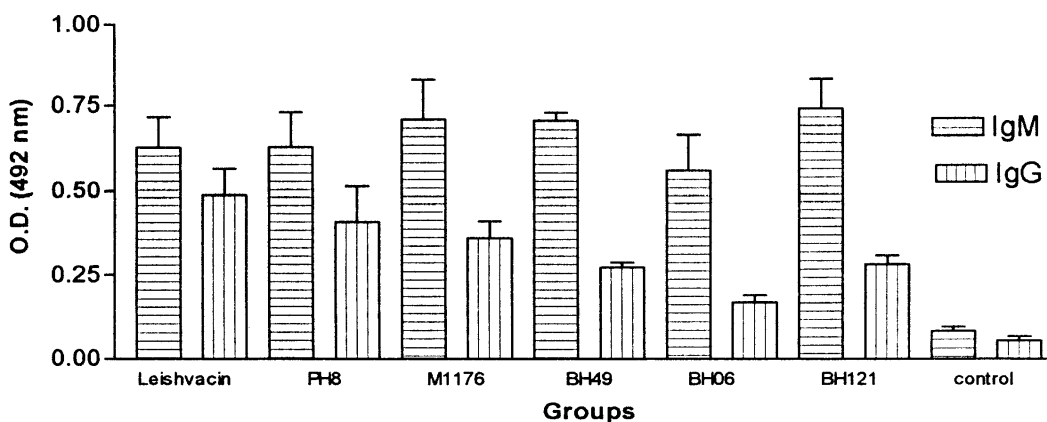


Figure 1 - Levels (mean ± SD) of IgG and IgM detected in sera obtained from mice immunized with different vaccine formulations. Antibody levels were determined by ELISA using extracts of the promastigotes (2µg/well) of each strain. The data represent the absorbance of serum collected from 10 animals/group, at a dilution of 1/50. Control = serum of mice inoculated with *C. parvum* only. O.D. = Optical density. In all immunized groups, levels of IgM were always higher than IgG (p < 0.05). The error bars were represented in each column.

**Test of Footpad Swelling Test.** Ten animals in each group were inoculated with antigens of the parasite in the right paw and with merthiolated saline in the left paw, performing the analyses after 24 and 48 hours of inoculation and effectuating the difference among the

swelling analyses of each paw. The mean of these differences between paws was determined. In Table 1 we observed that in the exam after 24 hours no statistically significant differences were noted between the mean size of swelling in the paws (p>0.05) in all of

the groups studied. Forty-eight hours after the antigen inoculation, we observed a reduction in relation to the analysis obtained after 24 hours, however without presenting significance ( $p > 0.05$ ). The analysis 2 days after inoculation was not different between any of the vaccinal groups ( $p > 0.05$ ).

Table 1 - Footpad swelling test.

| Groups     | Reading after |               |
|------------|---------------|---------------|
|            | 24h<br>(n=10) | 48h<br>(n=10) |
| Leishvacin | 0.410 ± 0.016 | 0.220 ± 0.019 |
| PH8        | 0.470 ± 0.033 | 0.360 ± 0.036 |
| M1176      | 0.410 ± 0.020 | 0.300 ± 0.034 |
| BH49       | 0.430 ± 0.037 | 0.210 ± 0.023 |
| BH06       | 0.370 ± 0.034 | 0.290 ± 0.030 |
| BH121      | 0.420 ± 0.034 | 0.270 ± 0.030 |
| Control    | 0.030 ± 0.003 | 0             |

The data represent the average of the differences (mm) between the thickness of right foot (inoculated with antigen) and left foot (inoculated with saline solution). N = 10 mice per group. Values represent mean ± 1 SD

**Lymphoproliferative response.** In order to evaluate the level of cellular immunity induced by vaccination, groups of 10 mice C57BL/10 were immunized with different *Leishmania* antigenic preparations plus *C. parvum*. Spleen cells from each immunized and control groups were incubated with the homologous antigen preparation. The proliferative response was measured by [3H]-Thymidine incorporation. Figure 2 shows that the specific antigen proliferation of spleen cells from immunized mice was at least 3.1 times higher than the one in control animals. All the ConA stimulated spleen cells always showed a stimulation index above 4.4, suggesting that the culture cell system was carried out under good viability.

**Evaluation of immunoprotection.** C57BL/10 mice were immunized with different vaccinal preparations associated with *C. parvum*. The ability of each vaccine to induce a persistent protection in C57BL/10 mice against a challenge with  $1 \times 10^5$  infective *L. (L.) amazonensis* promastigotes is presented in Figure 3.

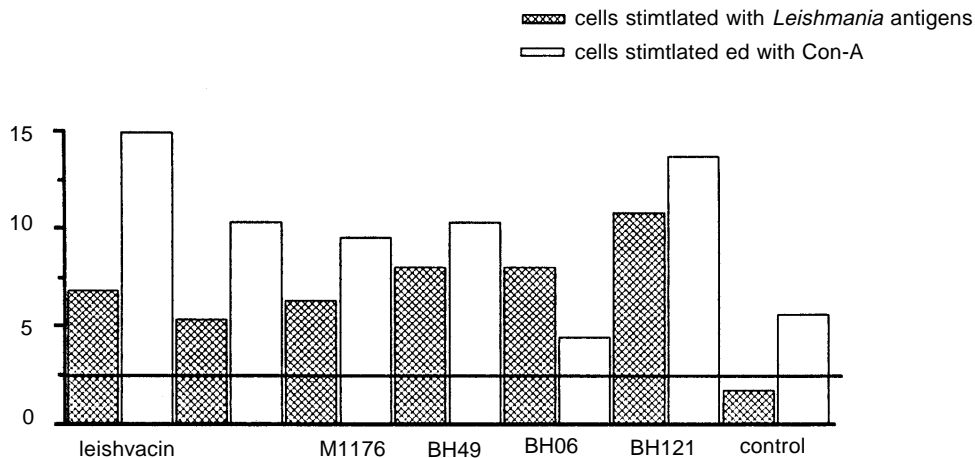


Figure 2 - Lymphoproliferative responses of *Leishmania* antigen (20 µg/ml) or ConA (0.5 µg/ml) stimulated splenic cells from mice submitted to immunization with different vaccines (Leishvacin®, PH8, M1176, BH49, BH06 and BH121). The bars represent the stimulation index (S.I.) of each group. The control was performed using splenic cells from mice inoculated with *C. parvum* and stimulated with ConA. N = 10 mice per group. Positive lymphoproliferative response showed a stimulation index equivalent or higher than 2.5 as represented by the horizontal line.

After the *Leishmania* inoculation, the animals were then followed-up for the appearance of lesions at the site of the inoculation (base of tail). The animals were considered protected when no lesion was observed. As shown in Figure 3, about 60 days after the challenge, almost 80% of control animals developed lesions (only at the site of the inoculation) compared with a maximum of 20% in the vaccinated group. After 150 days, about 40% of the animals in the vaccinated groups presented lesions. It should be mentioned that the lesions developed by vaccinated and non-protected mice were about ten-fold smaller than those in control animals (data not shown).

**Cytokine quantification.** In order to evaluate the cytokine production induced in C57BL/10 mice by the immunization with different isolated strains and the

multivalent vaccine, we determined the cytokines through the immunoenzymatic reaction (ELISA), in the supernatants collected, after a 24 and 72-hour culture of splenic cells stimulated with homologous antigens of *Leishmania* for dosing IL-4 and IFN-γ respectively. Figure 4 shows that all the vaccinated groups produced IFN-γ in average levels higher than  $570 \pm 141.72$  pg/ml, with the animals vaccinated with Leishvacin® and the monovalent vaccine PH8 presenting the highest levels of this cytokine, nevertheless there was no statistical difference between these two ( $p > 0.05$ ). On the other hand, these groups (Leishvacin® and PH8) produced a quantity of IFN-γ which was significantly higher than the other vaccinal groups: M1176, BH06, BH49 and BH121 ( $p < 0.05$ ). Figure 5 shows that all

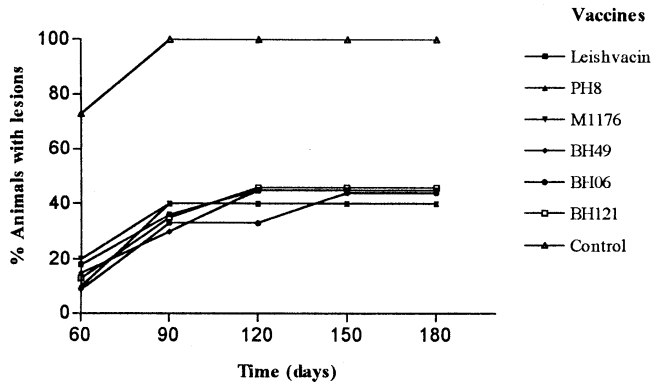


Figure 3 - Percentages of C57BL/10 mice immunized with different vaccines, developing leishmanial lesions after challenge by inoculation of promastigotes of *L. (L.) amazonensis*. Control represent animals inoculated with *C. parvum* only. N = 10 mice per group. All immunized groups developed a significantly lower number of lesion than the control group ( $p < 0.05$ ).

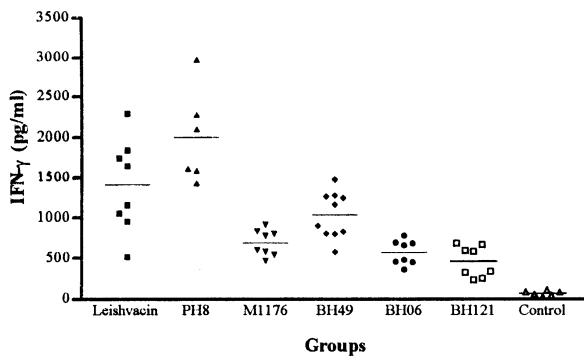


Figure 4 - IFN- $\gamma$  level (pg/ml) in the supernatant of homologous *Leishmania* antigen stimulated splenic cells from mice immunized with different vaccines (Leishvacin®, PH8, M1176, BH49, BH06 and BH121) for 72 hr to each animal. Horizontal bars represent the mean value for each group.

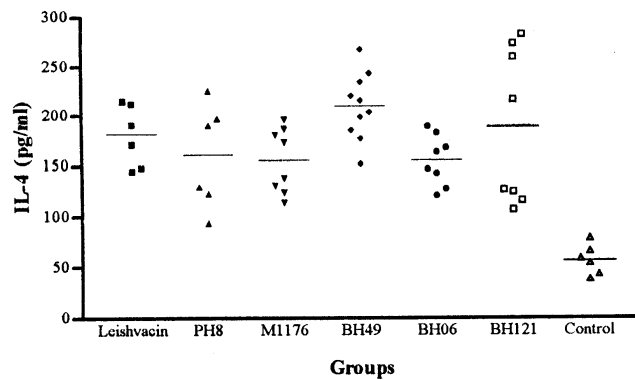


Figure 5 - IL-4 level (pg/ml) in the supernatant of homologous *Leishmania* antigen stimulated splenic cells from mice immunized with different vaccines (Leishvacin®, PH8, M1176, BH49, BH06 and BH121) after 24hr for each animal. Horizontal bars represent the mean value for each group.

groups produced consistently low and equivalent levels of IL-4 ( $< 210 \pm 32.56$  pg/ml) with no

significant differences when compared among themselves ( $p > 0.05$ ).

## DISCUSSION

Besides the promising results obtained in the studies of vaccination against American tegumentary leishmaniasis developed a long time ago<sup>3 29 31 37</sup> obtaining initially satisfactory results, the complexity of the vaccinal extract has made the process of industrialization difficult. This work, together with others related to the vaccination in humans<sup>32</sup> gives the support which is necessary to solve this problem, since it enabled us to evaluate the immunogenic potential of each strain which constitutes the original vaccine.

The isogenic C57BL/10 mouse was used as it is susceptible to the infection with *L.(L.) amazonensis*. Costa<sup>12</sup>, has shown that it is possible to protect 50% of the animals by using the vaccine described by Mayrink *et al*<sup>29</sup> adding *C. parvum* as adjuvant. Later, Costa *et al*<sup>13</sup> observed that the vaccine industrialized by BIOBRÁS S. A. was equally effective in inducing immunoprotection against *L.(L.) amazonensis*. Posterior studies showed that the vaccine could be simplified by using only one strain in its composition<sup>30</sup> as was suggested by the representatives of the organizations which participated in the "Vaccine Advisory Group of Discussion". Since then, studies have been realized using only one strain, the *L.(L.) amazonensis* (IFLA/BR/67/PH8). This work was performed with a view to finding the immunogenicity of each strain in isolation and through various immunological approaches.

Regarding the humoral immune response in the development of resistance to *Leishmania*, Mitchell<sup>33</sup> showed that there is no clear evidence of the participation of antibodies in the protection against *L. major* in mice. Costa<sup>12</sup> showed in C57BL/10 that besides having found high levels of antibodies no relation was observed between these levels, cutaneous test and protection. On the other hand, several authors considered necessary an association of the cellular and humoral responses to establish the cure or resistance to the parasite. Among them, Preston & Dumonde<sup>42</sup> verified the transference of immune cells, together with immune serum, rendered CBA mice more resistant to *L. major* than it would be if the transference were made only with cells. Our results reveal that there was humoral immune response in all groups tested, with higher levels of IgM than of IgG which may be related to the immunization scheme adopted, similar to that employed by Nascimento *et al*<sup>67</sup> who also found high levels of IgM in the serum of patients vaccinated with Leishvacin®.

The correlation between delayed hypersensitivity and protective immunity in *L.(L.) amazonensis* was reported by Andrade *et al*<sup>2</sup> who verified a persistent hypersensitivity in A/J mice, considered resistant to this species while BALB/c are known to be susceptible. They observed a failure to establish this kind of response. Our data also showed an association between these

two parameters. Grimaldi & Moriearty<sup>16</sup> observed that the analyses of the cutaneous tests after 24 hours presented a large infiltration of neutrophils instead of mononuclear cells, which present a peak in 48 hours. Because of this, we also effected the analysis in this period, in which the intensity of the response was shown to be maller in relation to that at 24 hours, however with no differences in intensity of this response in the two points of analysis and in the different groups.

Recently, several studies have demonstrated that cellular immunity plays an important role in the cure of tegumentary leishmaniasis<sup>8 9 11 14 32 41</sup>. Most of this knowledge was consolidated from the study of experimental infections in murine models<sup>1 14 17 23 24</sup>. Thus, in mice, the cure or resistance to the disease is directly related to the activation of macrophages with the production of nitric oxide to destroy intracellular parasites, with IFN- $\gamma$  and tumor necrosis factor - alpha (TNF- $\alpha$ ) as the main cytokines acting in this process<sup>1 22 46</sup>. On the other hand, IL-4 and Interleukin 10 (IL-10) block this activity inhibiting the activation of macrophages, allowing the proliferation of intracellular amastigotes and the aggravation of the infection<sup>1 4 21</sup>. In this model, the susceptibility of the BALB/c mouse is related to the induction of the immune response by CD4-TH2 cells while the resistance observed in CBA mice is associated to the induction of CD4-TH1 response<sup>23 45</sup>. This dichotomy between protective and non-protective immune response could be influenced by cytokine patterns produced by different lymphocyte subpopulations: Th1 producing mainly IFN- $\gamma$  and IL-2, while Th2 produces IL-4, 5 and 10<sup>36</sup>. Our results show that all vaccinal formulations induced lymphocytic proliferation. Or rather, splenic cells of vaccinated mice, after a specific *in vitro* antigenic stimulus, presented significant lymphoproliferation (stimulation index (SI)  $> 2.5$ )<sup>32</sup>. Our results also show that all vaccinal formulations stimulated T cells to synthesize IFN- $\gamma$  *in vitro*. It was demonstrated by variance analysis that PH8 and Leishvacin® induced equivalent levels of IFN- $\gamma$  ( $p = 0.446$ ). Besides, PH8 induced more IFN- $\gamma$  than M1176, BH06, BH49, and BH121 vaccines ( $p < 0.05$ ). Regarding IL-4, significant quantities were not found in any of the groups studied. This cytokine outline found suggests that the immunization of C57BL/10 mice with different strains of *Leishmania* leads to a significant stimulation of T cells with predominance of the Th1 type response. It is interesting to observe that, in this case, there was a cellular immune response against antigens of the extract of promastigotes of *L.(L.) amazonensis* developed by cells of animals immunized with the different strains of *Leishmania* in isolation, showing that all vaccinal formulations presented immunogenic potential similar to the immune response induced by the multivalent vaccine.

However, both Leishvacin® and the monovalent PH8 vaccine induced the highest levels of IFN- $\gamma$ . This cytokine together with TNF- $\alpha$  are the cytokines which are responsible for establishing resistance to the parasite. another interesting result was observed when the animals which were immunized with the different strains of *Leishmania* were submitted to challenge: about 60% of the animals of all immunized groups were not infected, without any significant difference between the groups. This is a very important data, as the protection rate obtained was similar to that observed when the animals were vaccinated with the complex vaccinal extract<sup>13</sup>. In similar studies, Preston & Dumonde<sup>42</sup>

obtained protection against *Leishmania tropica* in CBA mice using sonicated promastigotes. In their model and in our experiments, some of the mice showed no lesions at all, while others had smaller lesions than those of the control animals. The reason for this partial protection is still unknown by us.

Finally, it must be highlighted that our data support the "Vaccine Advisory Group of Discussion" suggestion of *Leishmania (L.) amazonensis* to be the component of monovalent vaccine actually being tested in a clinical trial in humans, since its antigens elicited an immune response better or similar to the other *Leishmania* single strain vaccines used in this study.

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