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Article history:
Received 2 March 2008
Received in revised form 15 April 2008
Accepted 17 April 2008
Available online 22 May 2008

Keywords:
Canine visceral leishmaniasis
Inflammation and cell migration
Innate-immune response
Vaccine
Saponin

ARTICLE INFO

Vaccine 26 (2008) 3922–3931
Contents lists available at ScienceDirect
Vaccine
ejournal homepage: www.elsevier.com/locate/vaccine

ABSTRACT

The search for new immunobiologics against canine visceral leishmaniasis (CVL) has intensified in the last decade. However, it still remains to be elucidated that mechanisms of the innate immune response in situ after immunization (a.i.). The aim of this study was to investigate the kinetics of cell migration in the skin dogs with distinct antigenic compounds of the LBSap vaccine. Our major findings indicated that saponin adjuvant alone or combined with Leishmania braziliensis antigen induced strong local acute inflammatory reaction. However, these reactions not progressed to ulcerated lesions. Overall, the cell profile found in Sap and LBSap was composed of neutrophils, lymphocytes and eosinophils. There was also increased production of iNOS in Sap and LBSap groups. Thus, we can conclude that dogs immunized with LBSap and the saponin adjuvant elicited a potential innate-immune activations status compatible with effective control of the resistance to infection by Leishmania and contributing to a better understanding of the innate-immunity events induced by the LBSap vaccine.

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1. Introduction

Visceral leishmaniasis (VL), a disease caused by Leishmania (Leishmania) chagasi (syn. Leishmania (Leishmania) infantum), constitutes a serious health problem in various regions of the Mediterranean and the Americas [1]. Since dogs are the most important domestic reservoirs of L. (L.) chagasi [2] within Latin America and Europe, the natural history of canine visceral leishmaniasis (CVL) has been studied extensively with respect to parasite load in different tissues and immunopathological changes relating to the progression of clinical forms [3–8]. Whilst such data have proven to be valuable in the development of tools employed in the evaluation of chemotherapies and vaccines against CVL, current treatment strategies have failed to achieve a consistent parasitological cure for the disease owing to the presence of latently infected cells [9,10]. In this respect, a canine vaccine may represent the most practical and effective method by which to reduce the incidence of human VL, and it could also provide a basis for the development of a similar vaccine for humans [11–13].

In the search for a potential vaccine against CVL, various approaches involving the dog model have been employed, and the use of purified fractions from parasite extracts (e.g. fucose mannose ligand [FML] antigen) [14,15] and from parasite cultures (excreted/secreted antigens) [16,17] have shown particular promise. Additionally, some remarkable results have been obtained following vaccination of dogs with killed parasite vaccines [18–23], thus demonstrating that vaccines prepared from antigenic extracts still remain a reliable proposition in consideration of their cost, safety and broad spectrum of antigenicity. In this context, we have recently demonstrated that killed Leishmania braziliensis, together with saponin (LBSap vaccine; Instituto Nacional da Propriedade Intelectual—patent PI 0601225-6, 17 February 2006), or with saponin and saliva of Lutzomyia longipalpis (LBSapSal vaccine), generates very high levels of immunogenicity in dogs. Vaccinated animals exhibited increased numbers of circulating T-cells (CD5+, CD4+ and CD8+), B-cells (CD21+) and L. chagasi-specific CD8+ T-cells [21,22], representing the key resistance profile against CVL [6]. Moreover, animals that had received saponin as adjuvant presented only minor local swelling as the major adverse reaction, indicating that in dogs, overall tolerance to the candidate vaccine appears to be adequate [21,22]. However, further studies are still required to...
overcome potential problems in this area by searching for additional safety biomarkers associated with the use of saponin as vaccine adjuvant.

On the basis of the above, we have investigated the kinetics of the inflammatory reaction and the expression of inducible nitric oxide synthase (iNOS) occurring in the skin of dogs following inoculation with different antigenic compounds considering initial time after each inoculum (1, 12, 24, 48 and 96 h). The approach presented herein represents an important tool through which it is possible to explore the cell profile and to identify additional safety biomarker parameters during the early events in the dermis of dogs that have been immunized with LB Sap vaccine and its separate components.

2. Materials and methods

2.1. Animals

The details of the proposed study were presented to, and approved by, the Ethical Committee for the Use of Experimental Animals of the Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil. The animal population consisted of 12 mongrel dogs (males and females), 8–12 months old, which were born and raised in the kennels of the Institute of Biological Sciences, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil. The dogs were treated with an anthelmintic and vaccinated against rabies (Tecpar, Curitiba, PR, Brazil), canine distemper, type 2 adenovirus, coronavirus, parainfluenza, parvovirus and leptospira (Veterinary Health HTLP 5/CV-L; Pfizer Animal Health, New York, NY, USA). Two days prior to the experiments, the dorsal area of all animals was shaved and no local reactions were observed.

2.2. Immunization protocol

The study population was divided into four groups of three dogs per group. The Sap group was inoculated with 1 mg of saponin (Sigma Chemical Co., St. Louis, MO, USA), the LB group was inoculated with 600 μg of L. braziliensis promastigote protein, the LB Sap group was inoculated with a 600 μg of L. braziliensis promastigote protein and 1 mg of saponin, and the control (S) group was inoculated with an equivalent volume of sterile 0.9% saline solution. Adjuvants and antigens were diluted with sterile 0.9% saline solution to a total volume of 250 μL. Following application of a general anaesthetic (Thiopentax®; Cristália, Itapira, SP, Brazil; 7 mg/kg of body weight), the antigenic components of the LB Sap vaccine and the placebo were inoculated into the shaved dorsal area of the animals via intradermic injection. Skin biopsies in the inoculation areas were performed at 1, 12, 24, 48 and 96 h in order to evaluate the kinetics of cell migration, and alterations (i.e. nodules or ulcerous lesions) produced by the different substances in these areas were recorded. Skin fragments were fixed with 10% buffered formalin (pH 7.2), embedded in paraffin and cut into 5 μm sections for histochemical and histopathological analyses.

2.3. Immunohistochemical analysis and assessment of iNOS expression

Endogenous peroxide was blocked by incubating skin sections with 3% hydrogen peroxide (H₂O₂) in methanol for 30 min. Sections were then de-waxed by heating in a microwave oven (700 W) for 10 min to retrieve the antigens and cooled to room temperature. After washing with phosphate buffered saline (PBS), the sections were further blocked with normal horse serum (Vector Laboratories Burlingame, CA, USA) to reduce non-specific antibody binding, and incubated with the primary antibody against iNOS diluted 1:200 (Cat. No. sc-651; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4 °C overnight. After washing with PBS for 3 × 5 min, the sections were incubated with the secondary antibody conjugated with biotin (Elite ABC Kit, Vector Laboratories) for 30 min at 37 °C, washed again with PBS, and incubated with streptavidin–peroxidase complex for 30 min at 37 °C. The reaction products of peroxidase were visualized by incubation with PBS containing 50 mg 3,3′-diaminobenzidine (DAB) and 500 μL of H₂O₂. Finally the sections were stained for nuclei with Harris haematoxylin solution. Negative control slides were prepared in the absence of the primary antibody.

2.4. Histopathological analysis

For standard histological examination (morphometric analysis and leukocyte differential counting) sections were stained with haematoxylin and eosin (HE). The kinetics of cell migration was evaluated within three skin layers (outer dermis, inner dermis and hypodermis) by submitting the stained histological preparations to immunohistochemical analysis for iNOS and subsequent examination under an Olympus Optical Co. (Tokyo, Japan—model CH3RF100 optical microscope). The intensity and predominance of cells in the inflammatory infiltrate and their distribution within the skin layers were assessed, together with the distribution of iNOS-positive cells and the intensity of expression as registered semi-quantitatively (i.e. absent, discreet, moderate or intense).

A quantitative (morphometric) analysis of the inflammatory cells present in the skin layers was performed by acquiring digital images of pre-marked areas that had been found to be associated with iNOS expression. Images were captured at 40 and 100× magnification using a Leica DM500B micro-camera (Leica Microsystems-Switzerland Ltd., Heerbrugg, Switzerland) and Leica Application Suite software (version 2.4.0 R1), and analysed with the aid of Leica QWin (V3) software. Twenty random images (total area = 1.5 × 10⁴ μm²) were determined to be sufficient for the representative analysis of a slide. In order to identify which types of cells were recruited to the sites of different inoculations sites, the inflammatory cells (neutrophils, eosinophils, macrophages and lymphocytes) were counted and the results were expressed in percentages.

2.5. Statistical analyses

Statistical analyses were performed with the aid of the software package Prism 4.0 (Prism Software, Irvine, CA, USA). Normality of the data was established using the Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA) and Tukey post hoc tests were used to investigate differences between groups with respect to cellular infiltrates in the dermal and hypodermal layers. Associations between leukocytes (%) or between iNOS and cell infiltrates in the dermis and hypodermis were investigated using Pearson's rank correlation. In all cases, differences were considered significant when P values were <0.05.

3. Results

3.1. Effects of the saponin adjuvant

Visual inspection of the experimental dogs revealed that none had developed adverse reactions against the inoculums except for local oedemas in individuals within the Sap group. Such alterations were observed onwards from 12 h following inoculation, and remained during the whole study period but did not evolve into ulcerated lesions. Histopathological examination showed that within the Sap and LB Sap groups, animals presented oedema, congestion, haemorrhage and degeneration of the muscle fibres in the
inoculated areas, together with signs of significant focal inflammation. Such alterations were observed particularly in the early period (1 h) following inoculation (Fig. 1D and G) and continued until the late period (up to 96 h) (Fig. 1F and I). Sap and LBSap inoculums induced a pronounced inflammatory reaction in the dermis and hypodermis of the study animals, and this was positively correlated with iNOS expression (Fig. 2, upper and lower right panels).

Significant increases in inflammatory infiltrate were observed in the dermis of animals of the Sap group after 1, 12, 24 and 96 h compared with control animals (S group). At 24 h, LB group animals also exhibited a significant increase in inflammatory infiltrate compared with the S group (Fig. 2, upper left panel). Longitudinal evaluation of the results showed that there were no differences within the groups concerning inflammatory infiltrate during the period of investigation. A positive correlation ($P = 0.0265/r = 0.6924$) between the total number of cell nuclei and the marked areas of iNOS expression could be established only in the Sap group (Fig. 2, upper right panel).

In the hypodermis, the inflammatory infiltrate increased significantly within the Sap and LBSap groups at 12, 24 and 48 h in comparison with the S and LB groups (Fig. 2, lower left panel and Fig. 3). It is important to emphasise that the increases observed within the Sap and LBSap groups occurred at similar times. At 1 and 96 h, the inflammatory infiltrate within the Sap group was significantly enhanced compared with the S group. Longitudinal analysis revealed that in the Sap and LBSap groups, inflammatory infiltrate was significantly increased within the period 12–48 h in comparison with the level at 1 h after inoculation. There was a positive correlation ($P = 0.0427/r = 0.5683$) between the total number of cell nuclei and the marked areas of iNOS expression in the hypodermis of animals within the LBSap group (Fig. 2, lower right panel).

![Fig. 1. Photomicrographs of the dermis of dogs immunized with saline (upper panels; control), saponin (middle panels) or LBSap (antigen of *L. braziliensis* plus saponin; lower panels) and recorded at 1, 24 and 96 h after inoculation (slides shown at 40× magnification; bar = 50 μm).](image-url)
3.2. Assessment of iNOS expression in the different skin layers

Anti-iNOS immunohistochemical reactions were observed in all three skin layers of Sap group as well as in the epidermis (keratinocytes) and annexes (sebaceous and sudoriparous glands). Such reactions varied on a semi-quantitative basis from moderate to intense in the keratinocytes, from discreet to intense in the dermis, and from absent to intense in the hypodermis. Similar levels of iNOS expression were also observed in the LB group. Within the LBSap group, iNOS expression was intense in the keratinocytes and dermis during the whole study period, whilst in the hypodermis, expression varied from moderate to intense. iNOS expression was also observed in the glands, fibroblasts and endothelial cells of all studied groups (Fig. 4B).

On a quantitative basis, iNOS expression increased significantly within the Sap and LBSap groups at 12 h compared with the level determined at 1 h after inoculation (Fig. 4). Moreover, the area of iNOS expression was significantly increased within the LB group compared with the S group at 1 h after inoculation, whilst at 12 h both the Sap and LBSap groups presented significantly larger areas of iNOS expression than the S and LB groups. Increased intensities of iNOS were also observed between 24 and 96 h within the LBSap group and at 96 h within the Sap group, and these differences were statistically significant in comparison with the S and LB groups.

3.3. Mobilisation of inflammatory cells to the outer dermis

Longitudinal evaluation of cell migration revealed that the percentage of neutrophils increased significantly within the Sap group at 12 h compared with 1 h, the former value being significantly lower than that of the S group (Fig. 5, upper middle panel). The LBSap group presented a significantly reduced number of macrophages at 48 h compared with the S and LB groups. There was a positive correlation \( (P = 0.0445/r = 0.5876) \) between iNOS expression and the percentage of macrophages in the outer dermis of LB groups (Fig. 5, right panel).

In contrast to the above, the number of lymphocytes increased significantly in the outer dermis of members of the LBSap group at 48 h compared with the value determined at 1 h following inoculation, and this increase was statistically different from those determined in the S and LB groups at 48 h (Fig. 5, middle left panel). Additionally, the number of lymphocytes within the Sap and LBSap groups increased significantly at 24 h compared with the S and LB groups.

3.4. Mobilisation of inflammatory cells to the inner dermis

Longitudinal analysis showed that the number of neutrophils, macrophages and lymphocytes present in the inner dermis of animals within each group did not vary significantly during the study period. In contrast, significant variations in inflammatory cell profiles were recorded between the groups of animals. Thus, the number of neutrophils increased significantly at 48 h after inoculation within the Sap group compared with the LB group (Fig. 6, upper left panel). Furthermore, the numbers of macrophages were reduced significantly at 24 and 48 h within the LBSap group compared with the S group, and at 48 h compared with the LB group (Fig. 6, upper middle panel). Additionally, the percentage of macrophages was significantly reduced at 48 h within the Sap group compared with the S group. Regarding the eosinophils, there was a significant increase within the Sap group at 96 h after inoculation compared with the levels at 1, 12, 24 and 48 h (Fig. 6, lower left panel), and also within the LBSap group at 96 h after inoculation compared with the values at 12 and 24 h.

In the inner dermis of Sap animals, a positive correlation \( (P = 0.0402/r = 0.5742) \) was observed between the percentage of macrophages, a reduction was observed within the Sap group at 12 h compared with 1 h, the former value being significantly lower than that of the S group (Fig. 5, upper middle panel). The LBSap group presented a significantly reduced number of macrophages at 48 h compared with the S and LB groups. There was a positive correlation \( (P = 0.0445/r = 0.5876) \) between iNOS expression and the percentage of macrophages in the outer dermis of LB groups (Fig. 5, right panel).
eosinophils and the total number of cell nuclei (Fig. 6, lower right panel), whilst in members of the LBSap group there was a negative correlation ($P=0.0346/ r = -0.5880$) between the percentage of macrophages and iNOS expression (Fig. 6, upper right panel).

3.5. Mobilisation of inflammatory cells to the hypodermis

Longitudinal analysis showed that the percentage of neutrophils in the hypodermis was significantly reduced within the LBSap group at 96 h compared with the levels at 1 and 24 h after inoculation (Fig. 7, upper left panel). Moreover, compared with the $S$ group, the numbers of neutrophils within the Sap and LBSap groups were significantly increased at 1 and 24 h after inoculation, whilst in the LB group the neutrophil number was significantly increased at 24 h compared with the $S$ group. Additionally, the LB group exhibited a significant reduction in the number of neutrophils at 48 and 96 h compared with the 1, 12 and 24 h levels. It is important to mention that $S$ (saline group) presented a tendency in increasing the counts of neutrophils at 12 h, suggesting a possible interference of this inoculum in the neutrophils migration during 12 h may be caused by tissue injury. Regarding the macrophages, the percentages were significantly reduced within the LB, Sap and LBSap groups at 1 h after inoculation compared with the $S$ group (Fig. 7, upper right panel). Diminished numbers of macrophages were still observed within the Sap and LBSap at 24 h compared with the $S$ groups. At 48 h, the percentage of macrophages within the Sap and LBSap decreased when compared with the $S$ and LB groups. The numbers of eosinophils increased significantly within the Sap group at 48 and 96 h compared with the values determined at 1, 12 and 24 h. At 96 h, the percentage of eosinophils was particularly high compared with those recorded at other times (Fig. 7, lower left panel). Although there were no differences between the groups concerning the number of lymphocytes, a significant increase was observed.
Fig. 4. Upper panel: kinetics of expression of iNOS in the skin of dogs at various times after inoculation with saline (control group S; □), saponin (Sap group; △), antigen of *L. braziliensis* (LB group; ▲) or antigen of *L. braziliensis* plus saponin (LBSap group; ●). Significant differences (*P* < 0.05) between the values associated with the S and LB groups are indicated by the letters “a” and “c”, respectively. Lower panel: photomicrographs showing the immunohistochemical detection of iNOS expression in the dermis (plate 1) and the hypodermis (plate 2) of dogs and recorded 12 h after immunization with LBSap: plate 3 shows the negative control of the reaction (slides shown at 40× magnification; bar = 50 μm). Anti-iNOS immunohistochemical reactions were observed in all three skin layers as well as in the epidermis (keratinocytes), fibroblasts and inflammation cells.

Fig. 5. Left and middle panels: comparative analyses of the selective migration of cells to the outer dermis of dogs at various times after inoculation with saline (control group S; □), saponin (Sap group; △), antigen of *L. braziliensis* (LB group; ▲) or antigen of *L. braziliensis* plus saponin (LBSap group; ●). Significant differences (*P* < 0.05) between the values associated with the S, LB and LBSap groups are indicated by the letters “a”, “c” and “d”, respectively. Right panel: correlation between the percentage of macrophages and iNOS activity in the outer dermis of dogs in the LB group (*r* = Pearson correlation coefficient).
4. Discussion

The emergent and re-emergent character of VL results from the failure of authorities fully to control reservoirs and vectors, and also from opportunistic infection by the parasite of vulnerable individuals, particularly those affected by AIDS [24–26]. Moreover, the escalation of the disease has been aggravated by the development of drug-resistant strains of Leishmania [27]. Numerous anti-CVL vaccines containing diverse antigens and adjuvants have been tested in Brazil, and some have shown promising results [14,18,21–23,28–32]. In our continuing effort to develop a vaccine against CVL that is both efficient and safe, we have conducted studies on two new preparations, namely, LBSap and LBSapSal. The
results obtained so far have revealed that these vaccines possess strong immunogenic capacities and can induce high levels of anti-
Leishmania IgG (IgG1 and IgG2) as well as lymphocytes, particularly
T CD8+ (circulating and in vitro L. chagasi-specific) cells. Furthermore,
we have demonstrated that these vaccines induce an immune
response that is compatible with the control of the etiological agent
of CVL, i.e. intense proliferative activity of L. chagasi-specific lymphocytes and increased production of NO during in vitro stimulation
by soluble L. chagasi antigens [21,22].

In the present study, the inflammatory processes induced in the
inoculation area by the antigen and adjuvant present in LBSap vac-
cine were evaluated during the initial 96 h period. On the basis that
immunization against infectious agents requires the participation
of innate and adaptive immune responses, the determination of
the kinetics of cell migration to the inoculation area is extremely
relevant since the number and types of cells recruited immedi-
ately after inoculation will stimulate the innate-immune system and
will influence the development of acquired immunity [33]. Although most studies tend to focus on the cytokine profile of the
inflammatory reaction [34–36], knowledge of the cell profile of the
local inflammatory infiltrate can provide information concerning
the immune response in the microenvironment of the inoculation
[37,38].

The local oedema observed following inoculation with Sap or
LBSap [21] suggests an acute inflammatory response to the saponin
adjuvant, and this is important for inducing a specific immune
reaction [39]. However, the oedemas did not evolve into ulcerated
lesions, thus demonstrating that saponin (in isolation or in combi-
nation with the L. braziliensis antigen) is safe and can be employed
as an adjuvant. Giunchetti et al. [21] observed a similar reaction in
dogs vaccinated with LBSap and demonstrated that the nodules that
emerged after vaccination disappeared during the later stages, thus
indicating that LBSap vaccine was well tolerated despite the pres-
ence of the saponin adjuvant. Following inoculation with an FML
antigen vaccine (Leishmune®), dogs presented moderate adverse
reactions, including pain, anorexia and local puffiness, which sponta-
neously disappeared before the second immunization [40].

The adverse events following immunizations to be defined
are fever, local reactions, intussusceptions, intractable crying,
seizure, hypotonic hyporesponsive episode, allergic reaction, rash,
arthritis, paresthesia, myalgia and idiopathic thrombocytopenia in
humans [60]. Biomarkers in local reactions associated with safety
role in the immunogenic response induced by saponin-containing
vaccine. Indeed, the macrophages are known to play an important
role in the activation of the innate response has recently been
stressed by Berg and Forman [48]. According to those authors,
the migration of CD8+ T lymphocytes represents an additional
inoculation with Sap. This finding reinforces the assumption that
saponin modulates the immune response by stimulating antibody
production and non-specific reactions, such as inflammation, and
cell traffic [41,43]. The numbers of cells observed in the hypodermis
of Sap and LBSap animals were larger than in the dermis, probably
by virtue of the more extensive vascularisation in the former layer.
As demonstrated in the present study, the inflammatory infiltrate
increased within the Sap and LBSap groups at 12, 24 and 48 h com-
pared with the S and LB groups, and also in the Sap group at 1
and 96 h compared with the S group. Since, throughout the whole
experimental period, there was greater cell recruitment within the
Sap group than in the LBSap group, the initial assumptions regard-
ing the role of saponin in the transfer of inflammatory cells to the
inoculation area are strengthened.

Within the Sap and LBSap groups, increases in the numbers of
neutrophils and lymphocytes and decreases in the number
of macrophages were observed at various times during the study
period. In the late period following inoculation, there were
increases in the numbers of eosinophils in the inner dermis and
hypodermis of Sap animals, and in the inner and outer dermis of
LBSap animals. Based on these results it is possible to hypothesise
that neutrophils function at the front line of the immune system,
responding immediately upon request, and direct the migration
of other cells to the inoculation area some hours afterwards. It
appears, therefore, that neutrophils participate effectively in the
adaptive immune response from the very beginning of the process.
This assumption is supported by several studies in which the role
of neutrophils has been considered [44,45]. According to Appel-
berg [44], neutrophils not only act as phagocytes but also determine
the inflammatory immune response and cooperate with other cells
in the amplification of such response. Moreover, neutrophils play
a role in acquired (or adaptive) immunity since they are antigen-
recognising cells (APCs) that can activate naïve T lymphocytes [45].
In this context, neutrophils have been shown to migrate to the infec-
tion site before macrophages and dendritic cells, and are the first
cells to interact with CD8+ T lymphocytes [44]. Moreover, together
with saponin [41], the neutrophils stimulate the activation of T
CD8+ lymphocytes, hence contributing to the entire immunogenic
process. Some reports have suggested that neutrophils operate
not only in an indirect manner as APCs, but are also instrumen-
tal in the recruitment of other cells (i.e. T lymphocytes, monocytes,
macrophages and immature dendritic cells) through the production
of chemokines [46].

The increase in the number of eosinophils within the Sap and
LBSap groups at the end of the experimental period (48 h and 96 h)
suggests that immunization with the LB antigen and saponin gener-
at ed a mixed cell mediated immune response. Based on this finding,
it is possible to hypothesise that eosinophils, together with neu-
rophils, participate effectively in the innate-immune response at the
inoculation area.

With respect to macrophages, the reduction in the numbers
of these cells was possibly due to the intense migration of neutrophils
to the inoculation area, which occurs in any acute process [47], and
not because they were inhibited by the antigenic components of the
vaccine. Indeed, the macrophages are known to play an important
role in the immunogenic response induced by saponin-containing
vaccines [41].

The increased numbers of lymphocytes observed in the outer
dermis of Sap and LBSap animals at 24 h after inoculation (and also
at 48 h in the LBSap group) further demonstrates the role of saponin
in the rapid and effective recruitment of cells to the inoculation
area [41]. The co-participation of cells associated with the adaptive
response in the activation of the innate response has recently been
discussed by Berg and Forman [48]. According to those authors,
the migration of CD8+ T lymphocytes represents an additional
selective recruitment of lymphocytes, particularly IFN-γ cytokines that take part in the adaptive response and control of Leishmania infection [49]. Based on such evidence [48,49], it is possible to infer that the selective recruitment of lymphocytes, particularly IFN-γ-producing CD8+ T-cells, contribute to the protective immune response against CVL. Another feature that demonstrates the importance of the saponin adjuvant in prompting the immunogenicity of the LB antigen is the fact that within the LB group the recruitment of inflammatory cells to the inoculation area was rather weak. The contribution of inflammatory cells to the creation of a microenvironment suitable for immune reaction against Leishmania infection has also been investigated by examining the activity of iNOS in the inoculation area. It is known that iNOS expression is a key factor in the immune adaptive response to external stimuli and virulent pathogens [50,51], and that NO is particularly important in the control of Leishmania parasites [52]. Semi-quantitative analysis demonstrated that iNOS was expressed in the skin layers of all animal groups including those of the control group S. This finding leads us to believe that all stimuli have the capacity to increase iNOS expression, contributing to the production of NO and participating in a broad range of processes ranging from non-specific reactions to the modulation of the immune system [53]. Quantitative analysis of iNOS expression showed that at 1 h after inoculation the area of expression of the enzyme was enlarged within the LB group. This observation can be explained by the presence of a higher number of macrophages, cells that are considered to be important NO producers, recruited to the outer dermis during this period in response to the LB inoculum. In contrast, the Sap and LBSap groups showed larger areas of iNOS expression at 12 h onwards in comparison with the other groups. The increased iNOS expression in these groups may be explained by the great number of NO-producing cells recruited to the inoculation area, activation of which would contribute to the eradication of the parasite. The correlation between the number of nuclei and iNOS expression in the dermis and hypodermis of animals of the Sap and LBSap groups confirms these assumptions. It is important to emphasise that iNOS is expressed in many types of cells (macrophages, neutrophils and fibroblasts) in response to diverse stimuli including cytokines and lipopolysaccharides [54]. Indeed, numerous reports have demonstrated that type 1 cytokines (IFN-γ, TNF-α and IL-18) induce iNOS expression [55,56], whilst type 2 cytokines (IL-4, IL-13 and IL-10) diminish leishmanicidal activity in murine models and in humans by downregulating iNOS expression [57]. In the present study it was shown that in the outer dermis of LB animals, the percentage of macrophages was positively correlated with the area of iNOS expression suggesting that the LB inoculum activated NO-producing macrophages. In addition, within the Sap group there was a positive correlation between the number of cell nuclei and eosinophils present in the inner dermis, suggesting that these cells contribute to the innate response induced by the strong adjuvant power of saponin. The role of NO in inflammatory reactions of the skin is very complex since at low levels it induces the dilatation of blood vessels and the migration of neutrophils, whilst at high levels it down regulates the production of cell adhesion molecules, suppresses the activation of inflammatory cells and induces their apoptosis [58]. Hence, NO influences the balance between type 1 and type 2 responses. The high levels of NO observed in the groups inoculated with saponin may be related to the high levels of type 1 cytokines (IFN-γ and TNF-α) [59] that are essential for the activation of effector mechanisms such as the production of NO for the control of Leishmania parasites.

The results presented in this paper demonstrate that the LBSap vaccine and the isolated saponin adjuvant were able to induce intense cell migration in the skin of inoculated dogs, thus triggering the initial immunogenic events. Moreover, the components of the vaccine were shown to be safe since no ulcerated lesions were observed at the inoculation sites during the study period. The selective recruitment of neutrophils to the skin, and the intense iNOS expression exhibited by animals in the Sap and LBSap groups, were similar to the reactions observed in animals resistant to infection by Leishmania.

Acknowledgements

The study was supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil (PRONEX 2007). RCO and P-SCB thank CNPq for fellowships. The authors wish to extend their gratitude to the staff of the kennels at the Federal University of Ouro Preto for their help and dedication throughout the execution of this project. The authors are also grateful for the use of facilities at CEBIO, Universidade Federal de Minas Gerais and Rede Mineira de Bioterismo (FAPEMIG), and for support with the provision of experimental animals.

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