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Short communication

Histological study of cell migration in the dermis of hamsters after immunisation with two different vaccines against visceral leishmaniasis

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ABSTRACT

Vaccine candidates, including live and/or killed parasites, *Leishmania*-purified fractions, defined recombinant antigens and antigen-encoding DNA-plasmids have been proposed to use as vaccine anti-*Leishmania*. More recently, the hamsters have been used to pre-selection of antigens candidate to apply in further experiments using canine model. In this report we evaluated the kinetics of cell migration in dermal inflammatory infiltrate, circulating leukocytes and the presence of nitric oxide (NO)/induced nitric oxide synthase during the early (1–24 h) and late (48–168 h) periods following inoculation of hamsters with antigenic components of anti-canine visceral leishmaniasis vaccines Leishmune® and *Leishmania braziliensis* antigen (LB) with and without saponin (Sap) adjuvant. Our results show that LB caused an early reduction of lymphocytes in the dermis while Sap and LBSap triggered a late recruitment, suggesting the role of the adjuvant in the traffic of antigen-presenting cells and the induction of lymphocyte migration. In that manner our results suggest that the kinetics of cell migration on hamster model may be of value in the selection of vaccine antigens prior the tests in dogs particularly in respect of the toxicity of the preparations.

1. Introduction

Visceral leishmaniasis (VL) caused by *Leishmania* (*Leishmania*) *chagasi* [syn *Leishmania* (*Leishmania*) *infantum*] is one of the most important of emergent diseases and is wide-spread in both tropical and subtropical areas. The disease can be particularly virulent, attaining a level of 98% mortality in certain non-treated human cases (Desjeux, 2004; Tesh, 1995).

Considering the central role of dogs in the control of zoonotic VL, a vaccine against canine visceral leishmaniasis (CVL) would represent an important tool in the immunoprophylaxis of the canine and human disease (Hommel et al., 1995). In this context, the natural history of CVL has been well described, particularly in respect of the parasite load in different tissues and of the immunopathological changes relating to the progression of clinical forms (Chamizo et al., 2005; Giunchetti et al., 2008a; Lage et al., 2007; Reis et al., 2006a,b). Recently, the first commercial vaccine of this type against CVL (Leishmune®; Fort Dodge Animal Health, Campinas, Brazil) has been

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Additionally, vaccines prepared from antigenic extracts of whole parasites still offer a reliable perspective by virtue of their broad spectrum of antigenicity, and their cost and safety (Giunchetti et al., 2007, 2008a,b; Mayrink et al., 1996; Ravindran and Ali, 2004). In this context, a study in which dogs were immunised with killed *Leishmania braziliensis* together with saponin (LBSap: patent number PI 0601225-6, 17/02/2006; Instituto Nacional da Propriedade Industrial, Rio de Janeiro, RJ, Brazil), or with LBSap together with the saliva of *Lutzomyia longipalpis* (LBSap-Sal), revealed an increase in the number of circulating T-cells ($CD5^+$, $CD4^+$ and $CD8^+$), B-cells ($CD21^+$) and *L. chagasi*-specific $CD8^+$ T-cells (Giunchetti et al., 2007, 2008b). Studies in our laboratories have revealed that the major adverse reaction to these candidate vaccines was local swelling in animals that had received saponin as adjuvant, thus indicating that the overall tolerance to the preparations appeared to be adequate (Giunchetti et al., 2007, 2008b). We previously shown in dogs that saponin adjuvant alone or combined with *L. 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 (Vitoriano-Souza et al., 2008).

In this context, the Hamster *Mesocricetus auratus* provide an excellent model for an overtly this parameters considering their capability to test a great number of vaccines antigens (Garg and Dube, 2006). So, our paper describes the detailed analysis of the histological dynamics following inoculation of two vaccines (Leishmune® and LBSap) and their separated components into the dermis and peripheral blood. Moreover, the application of this important system for vaccine studies against VL in the selection of vaccine antigens particularly with respect to their toxicities prior to tests in dogs has been investigated.

2. Materials and methods

2.1. Design of vaccine and study animals and treatments

In this study, two vaccines were used: (i) LBSap described previously by Giunchetti et al. (2007) and registered at the Instituto Nacional da Propriedade Industrial (patent: PI 0601225-6, Rio de Janeiro, RJ, Brazil) composed by *L. braziliensis* crude antigen and saponin adjuvant; (ii) Leishmune® composed by lyophilized FML (Fucose mannose ligand) antigen and saponin adjuvant (Parra et al., 2007).

The study population consisted of 120 hamsters (*M. auratus*), 60 males and 60 females, with 8 weeks of age and average weight 120 g. The animals were divided into five groups of 24 individuals each. The S group was inoculated with sterile 0.85% saline solution; the Sap group was inoculated with 100 μg of saponin (Sigma Chemical Co., St. Louis, MO, USA) diluted with sterile 0.85% saline solution; the LB group was inoculated with 60 μg of *L. braziliensis* antigen diluted with sterile 0.85% saline solution; the LBSap group was inoculated with a mixture of 60 μg *L. braziliensis* antigen and 100 μg of saponin diluted with sterile 0.85% saline solution; and the Leishmune® group was inoculated with Leishmune® vaccine (150 μg of lyophilised FML antigen of *L. donovani* and 50 μg saponin reconstituted in 2 mL of sterile 0.85% saline solution). Vaccines were inoculated into the abdomen of study animals via intradermal injections (200 μL). This study was approved by the Ethical Committee for the Use of Experimental Animals of the Universidade Federal de Ouro Preto, Ouro Preto-MG, Brazil.

2.2. Collection of blood and skin samples

After 1, 12 and 24 h (early period), and 48, 96 and 168 h (late period) following inoculation, blood and skin samples were collected from four animals from each of the five experimental groups per time period. Aliquots (1 mL) of blood were collected by intracardiac puncture using a 1 mL syringe and needle. Skin fragments were collected from the inoculated areas in the abdomen, fixed with 10% buffered formalin (pH 7.2), and embedded in paraffin.

2.3. Differential leukocyte counting in blood samples

Blood-coated glass slides were freshly prepared and stained with *Giemsa*. A total of 200 cells were counted on each glass slide using an optical microscope CH30 (Olympus Corporation, Tokyo, Japan) at 100× magnification, and the percentage of polymorphonucleated (neutrophils and eosinophils) and mononucleated (lymphocytes and monocytes) cells were determined.

2.4. Measurement of serum NO

Blood was centrifuged at 1500 rpm and the serum separated and stored under appropriate conditions until the determination of the NO concentration, according to Griess method (Green et al., 1982).

2.5. Histopathological analysis of skin sections

Histological sections were prepared, stained with haematoxylin eosin and observed under the light microscope at 40× magnification in order to determine the number of neutrophils, macrophages and lymphocytes present in the inflammatory infiltrate.

2.6. Assessment of expression of induced nitric oxide synthase (iNOS) in skin sections

Endogenous peroxide was blocked by incubating skin sections with 3% hydrogen peroxide (H_2O_2) in methanol. Sections were then heated in a microwave oven (700 W) and cooled to room temperature. After this, the sections were further blocked with normal horse serum (Vector Laboratories Burlingame, CA, USA), incubated overnight at 4 °C with the primary polyclonal antibody against iNOS (Cat. No. sc-651; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), washed in PBS and incubated with the secondary polyclonal antibody conjugated with biotin followed by

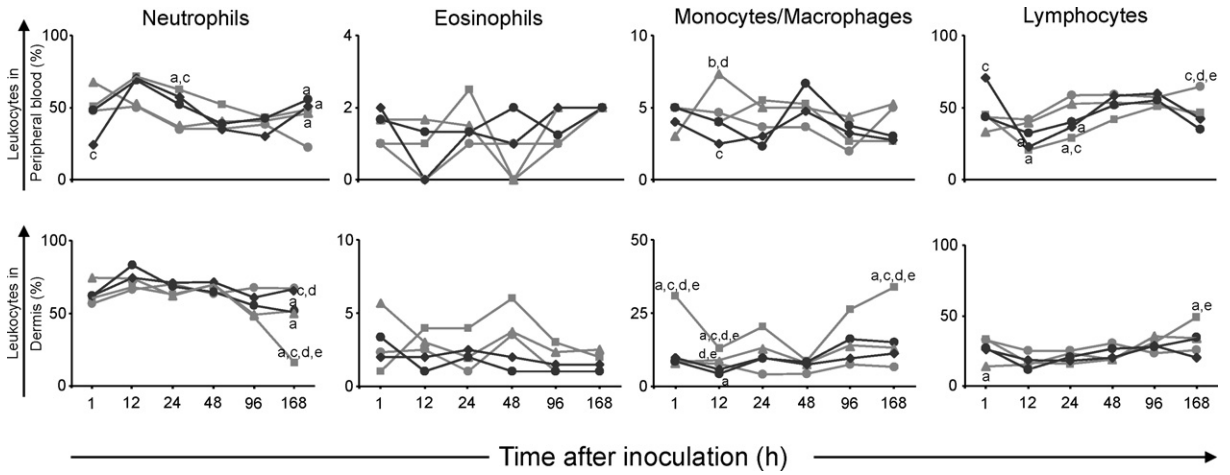

Fig. 1. Profiles of leukocytes (neutrophils, eosinophils, monocytes/macrophages and lymphocytes) in the peripheral blood and dermis of hamsters ($n = 4$/time) inoculated with saponin (Sap; ■), *Leishmania braziliensis* antigen (LB; ▲), LB plus Sap (●), Leishmune® (◆) or sterile saline as control (S; ●). Significant differences ($P < 0.05$) between the groups are indicated by the letters a, b, c, d, e, related to the groups S, Sap, LB, LBSap and Leishmune®, respectively.

incubation with streptavidin–peroxidase complex (Elite ABC Kit, Vector Laboratories). The reaction products of peroxidase were visualised by incubation with 3.3′-diaminobenzidine (DAB). Finally the sections were lightly counterstained with Harris's haematoxylin solution.

Morphometric analysis was performed by quantifying all of the cell nuclei and marked areas of iNOS expression in skin sections over 20 random fields (total area = 1.5×10^6 μm^2). The images were visualised under 40× magnification and digitised using a Leica DM5000B micro-camera (Cambridge, UK) and Leica Application Suite software (version 2.4.0 R1). Images were analysed with the aid of Leica QWin (V3) software.

2.7. Statistical analyses

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

3. Results and discussion

To better understanding how antigens and vaccine adjuvants interact with the innate immune response, we tested two vaccines in the hamster model. Since there are few reports concerning the kinetics of cell migration after inoculation of different vaccines and adjuvants, the objective of the investigation was to assess such migration in hamsters and to determine the levels of NO after inoculation with the distinct antigenic components of Leishmune® and LBSap.

One hour following inoculation, the number of neutrophils in the peripheral blood of hamsters of Leishmune® group was lower when compared to LB group. Sap group presented higher number of neutrophils 24 h after inoculation in comparison to LB and S groups. However, in the late period (168 h), this cell type increased in the blood of Leishmune®, LBSap and LB-treated animals compared with the S group (Fig. 1, upper panel). The increase in the percentage of neutrophils within the Sap group during the early period (24 h) indicates the participation of this cell type in the innate immunity against inoculation with the adjuvant. Giunchetti et al. (2007) evaluated the cell migration response of dogs inoculated with Sap, LB and LBSap, and reported an increase in circulating neutrophils within the LB group in comparison with the control group 15 days after the first immunisation.

In the present study, no differences were observed with respect to the numbers of eosinophils in the peripheral blood of any of the experimental groups of animals. However, inoculation with LB antigen stimulated the mobilisation of circulating monocytes in the early period (12 h) after inoculation in relation to LB and LBSap groups, whilst Leishmune® induced the opposite effect when compared to LB group (Fig. 1, upper panel). The recruitment of monocytes plays an important role in the immune reaction against *L. major* infection since the presence in the infected area of dendritic cells differentiated from monocytes are responsible for the induction of an immune protective response (Leon et al., 2005, 2007).

The Leishmune® group presented higher percentage of lymphocytes 1 h after inoculation when compared to LB group. Sap and Leishmune® vaccine promoted an early reduction (12 and 24 h) in circulating lymphocytes in peripheral blood suggesting a systemic immune response following immunisation. A similar reduction in the number of lymphocytes was induced by LB antigen, LBSap

and Leishmune® in the late period (168 h) following inoculation in relation to S group (Fig. 1, upper panel). The lowering of blood lymphocyte levels induced by the vaccines at two different periods may be explained by the concomitant mobilisation and selective migration of lymphocytes and neutrophils to the inoculated area. Giunchetti et al. (2007) observed that dogs inoculated with LB and Sap presented reduced numbers of lymphocytes 15 days after the first immunisation compared with the control group, whilst animals inoculated with LBSap showed an increase in lymphocyte numbers during the same period. Such alterations appear to be fundamental and may directly influence the local response in the inoculation area.

The kinetics of cell migration to the dermis involved a reduction in the neutrophils numbers during the late period (168 h) following inoculation with LBSap and LB compared with the S group. Moreover, in the Sap group the lower percentage of neutrophils were observed in relation to other groups. Additionally, animals inoculated with Leishmune® present higher percentage of neutrophils when compared with the LB and LBSap groups (Fig. 1, lower panel) and presented a positive correlation between the number of neutrophils in peripheral blood and dermis ($P = 0.0222/r = 0.5079$). Tafuri et al. (1993) investigated histopathological aspects of the Montenegro skin intradermic reaction in dogs following inoculation with Leishvacin® and P10.000G antigens, and demonstrated that neutrophils were the predominant inflammatory cells in the dermis within 24 h. Such studies substantiate the role of neutrophils as the first line of defence against infections (Ribeiro-Gomes et al., 2004), and verify their participation in the protection induced by vaccines. Indeed, neutrophils are the predominant cells in most acute inflammatory processes during the first 24 h (Collins, 2000), forming the initial response to diverse stimuli and acting as microbiocides. In the present study, the participation of neutrophils in the innate immune response following sensitisation with antigens and vaccinal adjuvants has been demonstrated.

There were no observable alterations in the percentage of eosinophils in the dermis of inoculated animals. In contrast, inoculation with Sap induced an early and persistent recruitment of monocytes, whilst treatment with LBSap and Leishmune® induced no alterations in the numbers of monocytes in the dermis (Fig. 1, lower panel). This finding is of interest because it shows that cell migration is immunomodulated by the addition of the Sap adjuvant to *L. donovani* (Leishmune®) and/or *L. braziliensis* (LBSap) antigens. Following activation, the macrophages produce various cytokines that perform diverse functions during the evolution or resolution of the inflammatory process and play significant roles in the control and modulation of the response. The macrophages also form an important part of the immune response since they act as antigen-presenting cells and produce inflammatory and chemostatic mediators (Johnston, 1988; Laskin et al., 1994). Moreover, IFN-γ-activated macrophages produce IL-2, TNF-α, IL-12 and NO, which improve the ability of the cells to control the multiplication and growth of intracellular pathogens (Brennan et al., 2004).

Regarding the presence of lymphocytes in the dermis, their numbers initially (1 h after inoculation) were lower within the LB group in relation to S group. Sap group presented higher percentage of lymphocytes 168 h after inoculation when compared to S and Leishmune® groups (Fig. 1, lower panel). A positive correlation between circulating and dermal lymphocytes could be established within the Leishmune® group ($P = 0.0150/r = 0.5352$).

The present study demonstrates that saponin adjuvant contributes strongly to the recruitment of cells containing antigen receptors during all periods following inoculation, since the migration of lymphocytes occurred even in the late period. The increase in lymphocytes within the Sap group and the reduction within the Leishmune® group suggest that the composition of the inflammatory infiltrate was modulated by the FML antigen present in the Leishmune® vaccine. In contrast, LBSap vaccine contains a profusion of soluble and fractionated *L. braziliensis* antigens that may lead to a diminished mobilisation of circulating leukocytes at some point in the kinetics.

Morphometric analysis of the dermis of the animals inoculated with Sap and Leishmune® revealed that inflammatory infiltrate increased in the late period after inoculation strengthening the hypothesis that saponin plays a major role in the composition of such infiltrate and supporting its application as a vaccinal adjuvant (Fig. 2, upper panel). Indeed, various studies have demonstrated the importance of saponin as adjuvant either as a

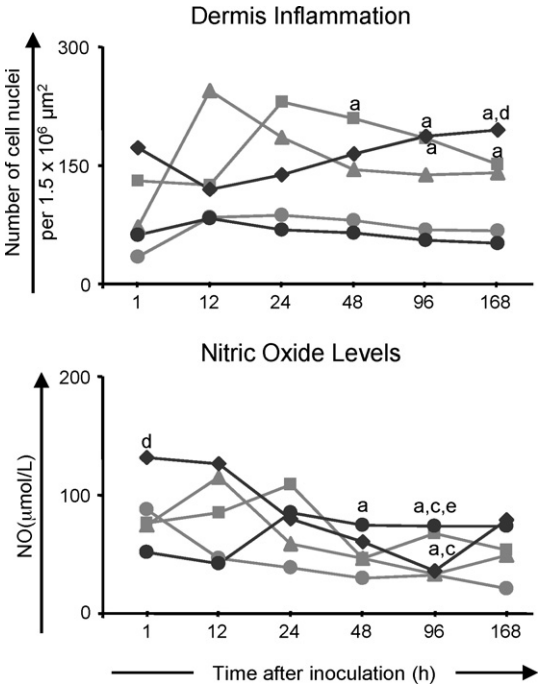

Fig. 2. Number of cell nuclei (dermis inflammation), and nitric oxide levels in the dermis of hamsters ($n = 4$/time) inoculated with saponin (Sap; ■), *L. braziliensis* antigen (LB; ▲), LB plus Sap (●), Leishmune® (◆) or sterile saline as control (S; ●). Significant differences ($P < 0.05$) between the groups are indicated by the letters a, c, d, e, related to the groups S, LB, LBSap and Leishmune®, respectively.

modulator of the immune response mediated by leukocytes or as a stimulator of antibody production and non-specific immune reactions such as in acute inflammatory processes (De Oliveira et al., 2001; Haridas et al., 2001; Oda et al., 2000) (Fig. 3).

The production of NO by iNOS allows the maintenance of antimicrobial activity of stimulated macrophages against intracellular pathogens (Rivera et al., 2001). According to Brandonisio et al. (2001) protection against leishmaniasis is associated with elevated expression of

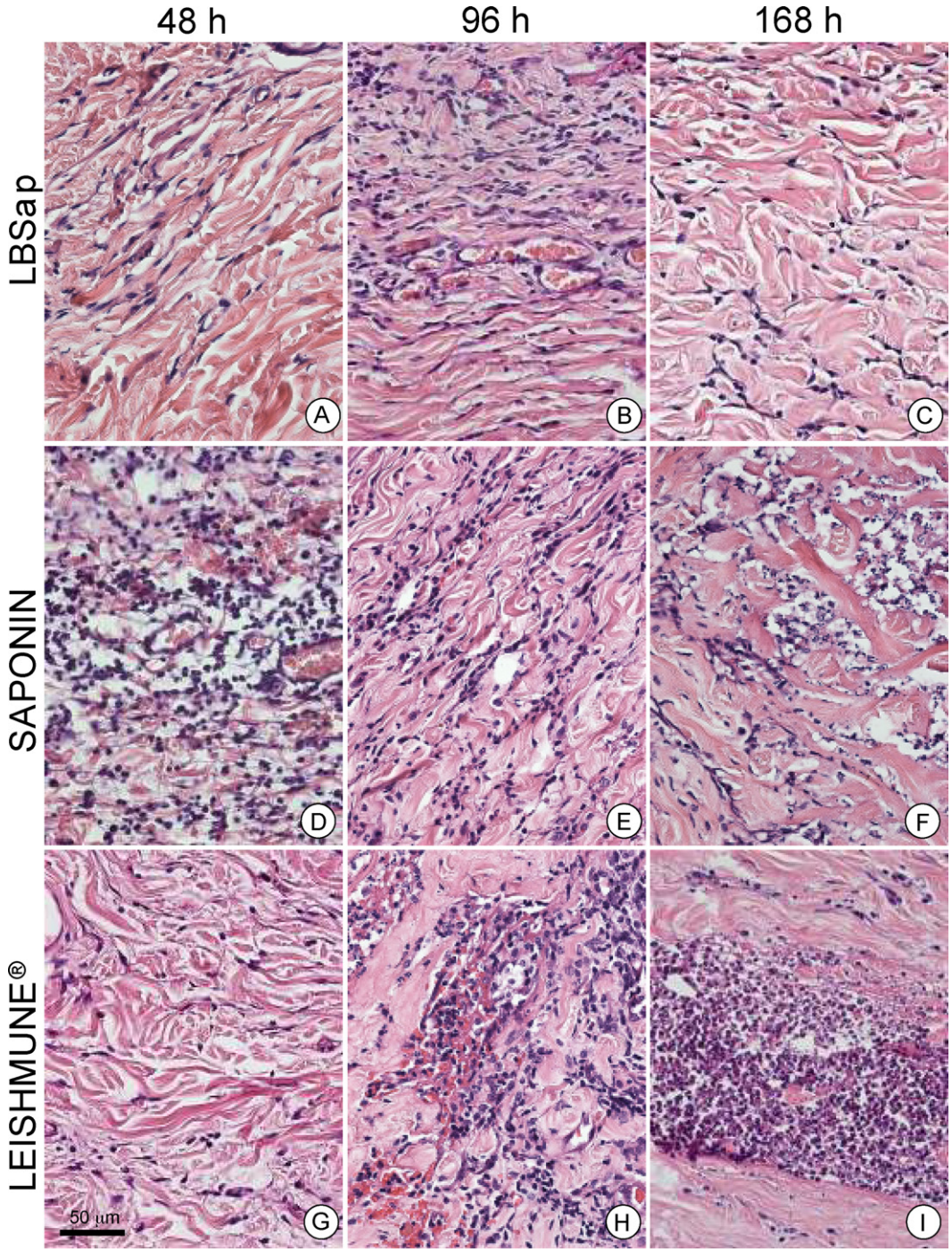

Fig. 3. Histopathology of the dermis of hamsters inoculated with Leishmune®, saponin (Sap) or *L. braziliensis* antigen plus saponin (LBSap). Following 48, 96 and 168 h, respectively, of inoculation the LBSap group presented profiles of inflammatory infiltration varying from discreet (A and C) to moderate with haemorrhagic areas (B), the Sap group presented intense inflammatory infiltration with haemorrhagic areas (D), moderate infiltration with haemorrhagic areas (E) and moderate infiltration (F), whilst the Leishmune® group presented discreet infiltration with rare inflammatory cells (G) to intense infiltration with haemorrhagic areas (H and I). Slides were stained with haematoxylin eosin; bar = 50 μm.

iNOS and higher levels of NO. In the present study, the expression of iNOS in the dermis was investigated in order to show possible compartmentalised responses to the different inoculums and to establish the time profile during the experimentation period.

Within the LB group, iNOS expression showed a positive correlation with the number of lymphocytes ($P = 0.0371$/ $r = 0.4941$) and macrophages ($P = 0.0236$/$r = 0.5303$) in the dermis, and a negative correlation with respect to the number of neutrophils ($P = 0.0382$/$r = -0.4918$). Although, animals inoculated with LBSap presented a smaller area of iNOS expression at 96 h, the concentration of serum NO at this time was increased when compared to S, LB and Leishmune® groups. In contrast, Leishmune® induced NO production immediately (1 h) after inoculation in relation to LBSap group following by a reduction in 96 h when compared to S and LB groups (Fig. 2, lower panel), whilst the correlation between the number of neutrophils and iNOS expression in the dermis was again negative ($P = 0.0483$/$r = -0.4852$). This finding supports the assumption that iNOS expression is induced by lymphocytes and macrophages as reported previously (Liew et al., 1990; Taylor-Robinson et al., 1994). Moreover, the results presented in this paper, together with additional evidence (Palatnik de Sousa et al., 1994), indicate that neutrophils do not contribute significantly to the production of NO *in situ*, and that the early production of serum NO is an indication of the development of a protective immune response against the VL agent.

The biological role of serum NO in the immunisation process is still unclear. However, Giunchetti et al. (2007) observed a significant increase in the levels of NO in dogs inoculated with LBSap using soluble *L. chagasi* antigen-stimulated in vitro cell proliferation cultures indicating that LBSap vaccine could induce a compatible response towards the etiological agent of CVL.

In the manner, the determination of the kinetics of cell migration in this model may be of value in the selection of vaccine antigens, particularly in respect of the toxicity of the preparations. Our results demonstrate that the hamster model represents an important tool in the discovery of potential antigens that could be used in the control of CVL. Further investigation will focus on the efficacy vaccination in protection against experimental challenge with *L. chagasi* using hamster and canine models.

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