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LEISHMANIA AMAZONENSIS INFECTION DOES NOT INHIBIT SYSTEMIC NITRIC OXIDE LEVELS ELICITED BY LIPOPOLYSACCHARIDE IN VIVO

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ABSTRACT: Leishmaniasis is a parasitic disease that leads to chronic inflammation. Macrophages, depending on their activation state, are either hosts or killers of the parasites. Downregulation of nitric oxide (NO) synthesis by the parasite infecting the macrophages has been proposed to be an important evading mechanism based on in vitro studies. We confirmed inhibition of NO release by macrophages infected with *Leishmania amazonensis* in vitro. To examine the role of the parasite in regulating NO production in vivo, we monitored systemic NO levels elicited by challenging naive and *L. amazonensis*-infected BALB/c mice with lipopolysaccharide (LPS). Animals were challenged after 1, 2, 6, and 9 wk of infection. NO production was monitored by electron paramagnetic resonance spectroscopy as the levels of hemoglobin nitrosyl complexes (HbNO) present in the animal's blood. No significant differences in HbNO levels were observed between LPS-treated naive and inoculated mice at any time during infection. To control for increased macrophage numbers in infected mice, naive mice were injected with a macrophage cell line before LPS challenge; this treatment did not increase produced NO levels. The results argue against a major role for the parasite in downregulating NO production in vivo.

Nitric oxide (NO) is an endogenously synthesized free radical that has been implicated in a variety of physiological and pathological processes (Moncada and Higgs, 1993; Gross and Wolin, 1995; Mayer and Hemmens, 1997). Lymphokine-treated macrophages produce NO through oxidation of the amino acid L-arginine by an inducible NO synthase (iNOS) (reviewed by MacMicking et al., 1997; Bagdan, 1998). Because inhibitors of NO synthases reduce the cytotoxicity of macrophages against several pathogens, including *Leishmania, Trypanosoma cruzi,* and *Mycobacteria,* NO is considered to be a key weapon of these activated phagocytes against microorganisms (reviewed by Fang, 1997; Liew et al., 1997).

Leishmaniasis is an endemic parasitosis caused by protozoa of the genus *Leishmania.* All *Leishmania* species are obligate intramacrophage parasites that live within secondary phagolysosomes. In this manner, the parasite is able to multiply, lyse the host cell, and infect surrounding macrophages (Dubremetz and McKerrow, 1995; Mauel, 1996). The survival and development of *Leishmania* within the hostile environment represented by the macrophage is supposed to be mediated by several protective mechanisms including the inhibition of the macrophage respiratory burst (Mauel, 1996; Bogdan and Rollinghoff, 1998). Recently, Proudfoot et al. (1995, 1996) demonstrated that *Leishmania major* promastigotes and glycolipids extracted from parasites were able to regulate the synthesis of NO at the transcriptional level of iNOS expression in macrophages stimulated with interferon-γ (IFN-γ) and *Escherichia coli* lipopolysaccharides (LPS). These authors suggested that downregulation of NO release by infected cells may contribute to the survival of the parasite (Liew et al., 1997). The relevance of these observations for the in vivo infection with *Leishmania,* however, is not known. Indeed, the high concentrations (10–100 μM) of *L. major* glycosylphosphatidylinositol and lipophosphoglycan (LPG) required to affect NO production in macrophage cultures (Proudfoot et al., 1995, 1996; Tachado et al., 1996) may not be achieved in vivo (Reiner, 1994). Although LPG might regulate expression of iNOS in macrophages, such a molecule is unlikely to play a similar role in established infections because mammalian amastigotes do not produce LPG in quantities comparable to those produced by promastigotes (Turco and Descoteaux, 1992; Moody, 1993). In addition, recent studies have demonstrated that during the late stages of murine infection with *Leishmania amazonensis, L. major,* and *Leishmania donovani,* susceptible mice produce increased NO levels in spite of the high parasite loads (Augusto et al., 1996; Evans et al., 1996; Giorgio et al., 1996; Bories et al., 1997; Giorgio, Linares et al., 1998). Thus, in contrast with the in vitro observations, *Leishmania* appears not to be a downregulator of NO production in vivo.

Here, to examine further the role of the parasite in regulating NO production in vivo, we monitored systemic NO levels elicited by challenging naive and *L. amazonensis*-infected BALB/c mice with LPS. This exogenous stimulus is known to increase iNOS mRNA levels and NO production in various tissues of challenged mice mainly through macrophage-dependent mechanisms (Park et al., 1996; Salkowski et al., 1997). LPS-triggered NO production was monitored by the levels of hemoglobin nitrosyl (HbNO) complexes present in the blood of mice because these complexes reflect overall NO production in vivo (Chamulitrat et al., 1995; Inoue et al., 1996). There were no significant differences in the HbNO levels produced by the 2 animal groups. In contrast with the findings that *Leishmania* are able to anergize macrophages in vitro (Proudfoot et al., 1995, 1996; Liew et al., 1997), our results did not show an impairment of NO production by infected mice.

MATERIALS AND METHODS

Parasite

Leishmania amazonensis (MHOM/BR/73/M2269) amastigotes were obtained from footpad lesions of BALB/c mice as previously described (Barbiéri et al., 1993).

In vitro experiments

Preparation of peritoneal exudate macrophages from female C57Bl/6 or BALB/c mice was performed as previously described (Giorgio, Barão et al., 1998). The cells were cultured with Iscove's medium supplemented with 2 mM glutamine, 10 mM HEPES, 20 mM $NaHCO_3$, 100 U/ml penicillin, 100 g/ml streptomycin (all from Sigma Chemical Co., St. Louis, Missouri), and 5% fetal calf serum (Cultilab, SP, Brazil)

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in 24-well plates (5×10^5 macrophages/well) at 37 C in 5% CO_2/95% humidified air. Cells were stimulated with recombinant mouse IFN-γ (100 U/ml) and LPS (10 ng/ml) from *E. coli* (Sigma) before or 24 hr after infection with *L. amazonensis* (Ho et al., 1992). Amastigotes were added to the cultures at a 3:1 parasite-to-cell ratio for 2 hr. The macrophage cultures were then washed to remove free parasites, and the supernatants were analyzed for nitrite accumulation at different stimulation times. In the case of infection before stimulation, the cells were infected for 2 hr, free parasites were removed, and 24 hr later the cultures were stimulated. From then on, at 24, 48, and 72 hr the supernatants were removed for nitrite determination. In the case of stimulation before infection, macrophages were treated with LPS and IFN-γ. After 24 hr, the cells were washed and the parasites added. After 2 hr, free parasites were removed and macrophages were stimulated again. Supernatants were removed immediately and 24 and 48 hr after the second stimulation, that is, 24, 48, and 72 hr after the first stimulation, respectively.

Nitrite assay

Nitrite content in the supernatant of macrophage cultures was measured by the Greiss method (Green et al., 1984) using a colorimetric assay kit (Alexis Corporation, San Diego, California).

In vivo experiments

Female BALB/c mice (4–6 wk old) were injected subcutaneously (s.c.) into the right footpad with 2×10^6 *L. amazonensis* amastigotes. The course of infection was monitored by measuring the increase in footpad thickness, compared with the contralateral uninfected footpad, with a dial caliper.

Animals at different weeks after infection and normal animals (of the same age) were divided into 2 groups that received an intraperitoneal (i.p.) injection of 0.2 ml saline or LPS (10 mg/kg body weight) from *E. coli* serotype 0111:B4 (Sigma) (Wang et al., 1991). Four hours later, mice were anesthetized and blood was collected from the orbital plexus for low-temperature electron paramagnetic resonance (EPR) measurements of HbNO complexes. In some experiments, the animals were killed 6 hr after LPS challenge.

In the case of J774 cell transfer experiments, naive BALB/c mice were divided into 2 treatment groups. Animals received an intravenous (i.v.) injection of 0.5 ml DME medium 2 hr prior to LPS (10 mg/kg body weight, via i.p.) and the other group received an i.v. injection of 10×10^6 J774 cell line in 0.5 ml of DME medium 2 hr prior to LPS (10 mg/kg body weight, via i.p.). Four hours after endotoxin injection, mice were anesthetized and blood was collected from the orbital plexus for low-temperature EPR measurements of HbNO complexes. The macrophage cell line J774 obtained from BALB/c mouse was cultivated as described previously (Ralph and Nakoinz, 1975).

Blood collection and EPR measurements

Blood was collected from the orbital plexus with heparinized pipettes and immediately pipetted into EPR quartz tubes and the samples were frozen in liquid nitrogen. The EPR spectra were obtained with a Bruker ER 200 D-SRC spectrometer using fingertip liquid nitrogen Dewar, as previously described (Giorgio, Linares et al., 1998). The concentrations of HbNO complexes were determined by double integration of their EPR signal and comparison with the doubly integrated signal from samples of known concentrations prepared by adding saturated solutions of gaseous NO in phosphate buffer to deoxyhemoglobin solutions (Kruszyna et al., 1987).

Data analysis

Two to 3 independent experiments involving 3–6 mice per group were performed to quantify the levels of HbNO complexes. Data obtained with different treatments were analyzed statistically by 1-way ANOVA and Student's *t*-test ($P < 0.05$).

RESULTS

Production of nitrite by macrophages infected with *L. amazonensis*

In agreement with previous studies (Proudfoot et al., 1995; Balestieri et al., 1996), we observed that *L. amazonensis* infection decreased the amount of NO produced by IFN-γ plus LPS-stimulated murine macrophages (Fig. 1). NO production was measured by the levels of nitrite, one of its end products, present in cell supernatants (Proudfoot et al., 1995; Balestieri et al., 1996). Treatment of C57Bl/6 peritoneal macrophages with IFN-γ and LPS led to an increased nitrite production over a period of 72 hr, with most of the compound being synthesized within the first 48 hr. Nitrite production was inhibited when the macrophages were incubated with *L. amazonensis* amastigotes (the parasite : macrophage ratio of 3:1 resulted in cultures with 50–60% of infected macrophages). The inhibitory effect was more pronounced when the cells were stimulated with IFN-γ plus LPS after than before infection. In the first case, the values were 50, 51, and 37% inhibition at 24, 48, and 72 hr, respectively (Fig. 1). Similar results were obtained with macrophages from BALB/c mice (data not shown). To examine whether *L. amazonensis* is able to impair NO production in vivo, we selected LPS as the trigger of NO production and susceptible BALB/c mice as the hosts.

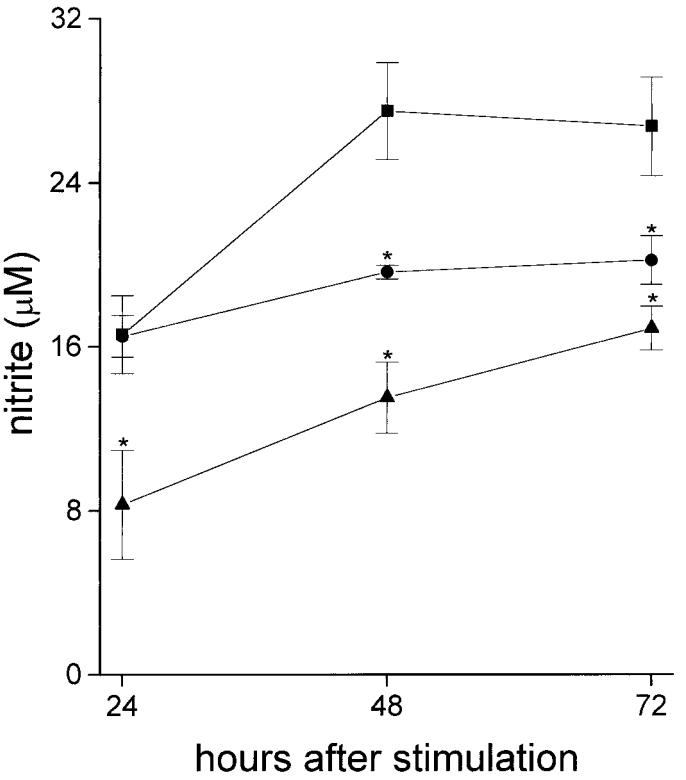

FIGURE 1. Effect of *Leishmania amazonensis* infection on nitrite production by murine peritoneal macrophages. Peritoneal macrophages noninfected (■) or infected with *L. amazonensis* amastigotes were stimulated by addition of interferon-γ (100 U/ml) and lipopolysaccharide (10 ng/ml) 24 hr before (●) or after (▲) infection. Nitrite concentrations were determined in the supernatants from cultures after specified stimulation times by the Griess reaction. The values represent the mean ± SE of 3 independent experiments. Significance of difference between control and infected macrophages is indicated in the figure. *$P < 0.05$.

HbNO levels in the blood of infected BALB/c mice challenged with LPS

Murine cutaneous leishmaniasis can have distinct outcomes depending on the parasite species and mouse strain (Milon et

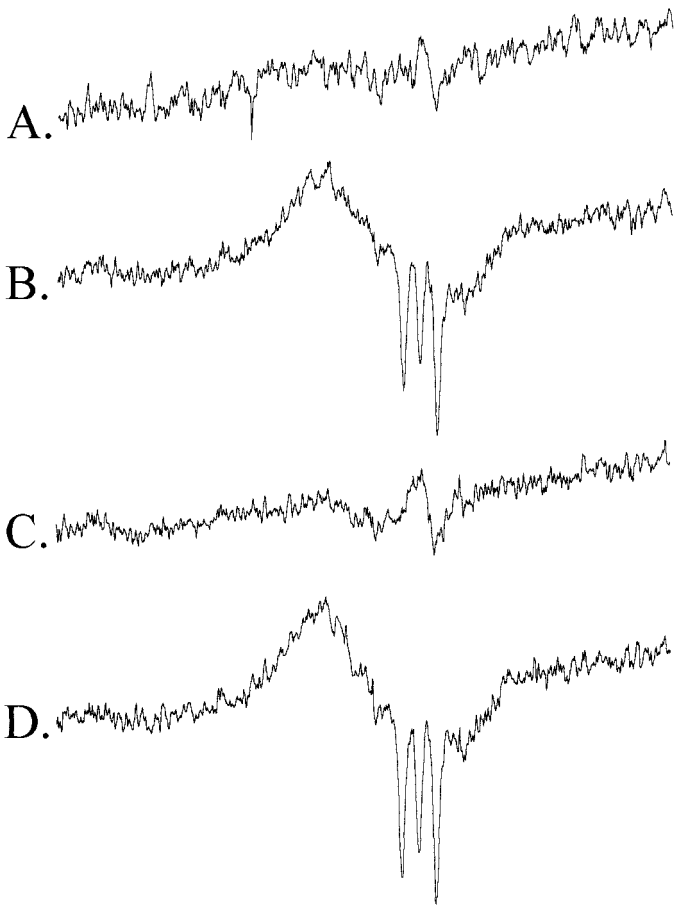

FIGURE 2. Representative electron paramagnetic resonance spectra of blood drawn from naive and *L. amazonensis* BALB/c mice 4 hr after different intraperitoneal injections. Blood from a naive mouse injected with saline (**A**) and lipopolysaccharide (LPS) (**B**); blood drawn from an infected mouse injected with saline (**C**) and LPS (**D**). The spectra were run at 77 K. Instrument conditions: microwave power, 10 mW; modulation amplitude, 0.5 mT; time constant, 1 sec; scan rate 0.12 mT/sec gain, 10×10^5.

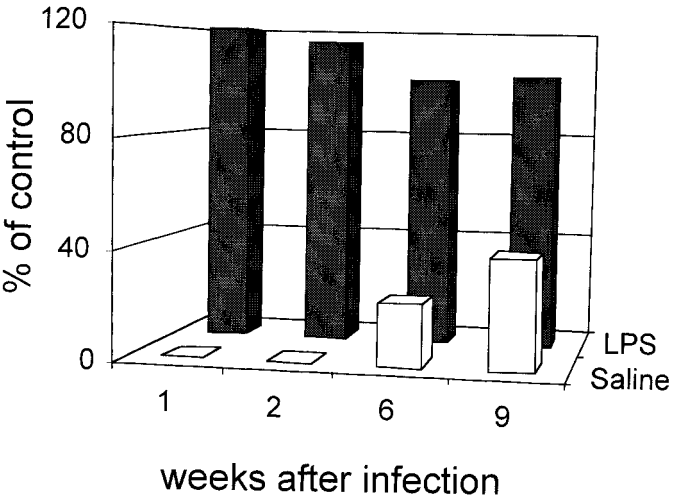

FIGURE 3. Effect of *Leishmania amazonensis* infection on hemoglobin nitrosyl (HbNO) complex levels by BALB/c mice stimulated with lipopolysaccharide (LPS). At the specified week of *L. amazonensis* infection, mice were injected with saline or LPS (intraperitoneally) and sacrificed 4 hr later; HbNO complex levels were quantified in the drawn blood as described in the Materials and Methods. In parallel, naive mice with the same age for each infected group were similarly treated with LPS (controls). Blood HbNO complex levels of infected animals treated either with saline (open bars) or LPS (hatched bars) were expressed as percentage of corresponding levels found in LPS-stimulated naive mice (% of control). The values represent the average of the values obtained for different animals ($n \geq 3$). Data are the percentages of blood HbNO levels compared with those from LPS-stimulated naive mice (with the same age for each infected group) set as 100%. At least 3 mice were used for each time point and the values presented are the average values whose SE were consistently below 23%.

al., 1995). *Leishmania amazonensis* amastigotes produced rapidly developing skin lesion in BALB/c mice as attested by the continuous increase in footpad thickness (data not shown). These animals experienced ulceration of cutaneous lesions, dissemination of the parasite to the lymph nodes, spleen, and liver, and increased levels of HbNO complexes in the blood that became detectable (≥ 2 μM) after 6 wk of infection and increased thereafter (data not shown) (Giorgio, Linares et al., 1998).

The levels of HbNO complexes present in the blood of BALB/c mice infected with *L. amazonensis* were compared with those present in the blood of naive mice submitted to the same LPS dose (10 mg/kg) (Figs. 2, 3). The experiments were performed at different times of infection by using naive animals of the same age as the control for each infected group because age-related differences in LPS sensitivity have been previously reported (Chorinchath et al., 1996). Blood drawn from both group of mice challenged with LPS presented the characteristic EPR spectra of HbNO complexes (Fig. 2B, D). The unchallenged mice did not present detectable levels of HbNO complexes in the blood (Fig. 2A, C) except in the case of inoculated mice with infection times of 6 and 9 wk (Fig. 3) (Giorgio, Linares et al., 1998). No significant differences in blood HbNO levels were observed between LPS-treated naive and inoculated mice at any infection time (Fig. 3). The HbNO levels in the blood of infected mice at 1, 2, 6, and 9 wk after infection were 122, 115, 101, and 102% of the corresponding controls (Fig. 3), respectively. The slightly higher values at weeks 1 and 2 compared to the controls, however, were not significant ($P > 0.05$). The levels of HbNO present in the blood of naive and infected animals were higher at 6 hr than at 4 hr after LPS challenge (data not shown), but again, the values did not significantly vary between groups. These results suggest that systemic NO production elicited by endotoxin was not inhibited by *L. amazonensis* infection.

HbNO levels in the blood of macrophage-injected mice

The levels of LPS-induced NO production were similar in normal and infected mice (Fig. 3). This does not exclude that the parasite inhibited NO production by macrophages because there is a continuous enhancement in the number of macrophages in the lesion and affected organs during *L. amazonensis* infection in BALB/c mice (McElrath et al., 1987). An increased number of macrophages not accompanied by an overall enhancement of NO levels after LPS stimulation (Figs. 2, 3) could be a consequence of more macrophages synthesizing less NO due to parasite downregulation. To address this problem, we tried to increase macrophage numbers in naive mice by inject-

TABLE I. Blood hemoglobin nitrosyl (HbNO) complex levels in response to lipopolysaccharide (LPS) in normal and macrophage-injected mice.

Treatment*	HbNO (μM)†
DME medium/LPS	5.1 ± 0.2
10×10^6 J774/saline	ND‡
10×10^6 J774/LPS	5.0 ± 0.7

* BALB/c mice were injected with DME medium or 10×10^6 J774 macrophages (intravenously), 2 hr before challenge with saline or LPS (10 mg/kg; intraperitoneally). After 4 hr, blood HbNO complex levels were quantified by low-temperature electron paramagnetic resonance as described in the Materials and Methods.
† Data are the arithmetic mean ± SE of the values obtained from 3 mice.
‡ Nondetectable.

ing them with a macrophage cell line (J774) and measuring their ability to synthesize NO in response to LPS. The results show that animals transferred with J774 cells and challenged with LPS were not capable of producing higher levels of HbNO complexes than mice injected with medium alone and challenged with the endotoxin (Table I). Similar results were obtained when both groups of animals were treated with a higher dose of LPS (30 mg/kg) (data not shown).

DISCUSSION

Experimental in vitro models are commonly used to assess the strategies developed by microorganisms to adapt to intracellular life (Reiner, 1994; Mauel, 1996; Kotwal, 1997; Bogdan and Rollinghof, 1998). Here, we confirmed that nitrite production by macrophage cultures stimulated with IFN-γ and LPS was inhibited by *L. amazonensis* amastigotes (Fig. 1) (Proudfoot et al., 1995, 1996; Balestieri et al., 1996; Liew et al., 1997). However, the relevance of these in vitro observations for the in vivo infection with *Leishmania* remained unexplored. To examine whether an impairment of NO production was detectable in *L. amazonensis*-infected BALB/c mice, the animals were challenged with endotoxin. LPS-treated naive and infected mice showed the characteristic EPR spectra of HbNO complexes in the blood (Fig. 2) as could be anticipated from previous studies on rodent response to endotoxin (Wang et al., 1991; Chamulitrat et al., 1995). However, no significant differences were observed in blood HbNO levels between LPS-treated naive and inoculated mice at any infection time (Fig. 3) (see also, Results). In contrast with the in vitro observations (Fig. 1) (Proudfoot et al., 1995, 1996; Balestieri et al., 1996; Liew et al., 1997), *Leishmania* appears not to be a downregulator of NO production in vivo (Figs. 2, 3).

A possible reason for the discrepancy between in vitro and in vivo results may be the continuous enhancement of the number of macrophages in the lesion and affected organs during *L. amazonensis* infection in BALB/c mice (McElrath et al., 1987). An increased number of macrophages in infected mice not accompanied by an overall enhancement of HbNO levels after LPS stimulation (Figs. 2, 3) could indicate parasite inhibition of NO production. The difference in the number of macrophages in naive and infected mice is difficult to approach experimentally. An alternative would be to increase the number of macrophages in naive mice by injecting them with J774 macrophages. Such an approach is likely to increase macrophage numbers in transferred animals because J774 is a tumor cell line able to metastasize to liver, lungs, and abdominal organs (Ralph and Nakoinz, 1975). In addition, previous studies have demonstrated that transfered macrophages and lymphocytes repopulate tissues and maintain their effector activities (Shand and Bell, 1972; Titus et al., 1984; VanFurth and Sluiter, 1986). However, mice transferred with J774 cells produced the same levels of HbNO complexes as those produced by control mice upon LPS challenge (Table I). This result suggests that increased macrophage numbers in the host are not enough to upregulate NO synthesis after LPS challenge. Such a conclusion is in line with studies indicating that high levels of circulating cytokines and proinflammatory cytokines such as tumor necrosis factor, IFN-γ, interleukin (IL)-1, IL-2, and IL-6 are required to upregulate NO production in vivo (Estrada et al., 1998).

Explanations other than the high number of macrophages as compared with controls in in vivo infection models are likely to be responsible for the discrepancy between the results obtained in vitro (Fig. 1) (Proudfoot et al., 1995, 1996; Balestieri et al., 1996; Liew et al., 1997) and in vivo (Figs. 2, 3; Table I). One possibility is that, in vivo, the *Leishmania*-induced defective NO production by macrophages is compensated for NO synthesis by other cell types responsive to LPS. This appears unlikely because the LPS-induced NO response in in vivo models is ~55–85% dependent on macrophages (Salkowski et al., 1997). Another possibility is to consider that systemic HbNO levels measured in in vivo models do not reflect localized NO production at the lesion sites that could be inhibited by the parasites. This possibility cannot be excluded but previous studies have demonstrated that HbNO levels found in the blood are correlated with nitrosyl complexes found in the lesions, reflecting localized NO production (Giorgio, Linares et al., 1998). Finally, it is possible that measurements of systemic NO levels lack the sensitivity required to measure in vivo effects that are likely to be less pronounced than those that can be obtained in in vitro systems.

In summary, the results reported here did not show inhibition of NO production by *Leishmania*-infected mice challenged with LPS. Although in contrast with the findings that *Leishmania* are able to anergize macrophages in vitro (Proudfoot et al., 1995, 1996; Balestieri et al., 1996; Liew et al., 1997), our results are in line with other in vivo studies demonstrating an increase in systemic NO levels during leishmaniasis progression (Augusto et al., 1996; Evans et al., 1996; Giorgio et al., 1996; Bories et al., 1997; Giorgio, Linares et al., 1998). The difficulty of reconciling in vitro and in vivo models only emphasizes that much ingenuity will be required to elucidate the strategies used by *Leishmania* to evade killing by the host.

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