Nitric oxide production by *Peromyscus yucatanicus* (Rodentia) infected with *Leishmania (Leishmania) mexicana*

Elsy Nalleli Loría-Cervera/+, Erika Ivett Sosa-Bibiano, Liliana Estefanía Villanueva-Lizama, Nicole Raymonde Van Wynsberghe, Silvia Beatriz Canto-Lara, José Luis Batún-Cutz, Fernando José Andrade-Narváez

Laboratorio de Inmunología, Centro de Investigaciones Regionales Dr Hideyo Noguchi, Universidad Autónoma de Yucatán, Yucatán, México

Peromyscus yucatanicus *(Rodentia: Cricetidae) is a primary reservoir of* Leishmania (Leishmania) mexicana *(Kinetoplastida: Trypanosomatidae). Nitric oxide (NO) generally plays a crucial role in the containment and elimination of* Leishmania. *The aim of this study was to determine the amount of NO produced by* P. yucatanicus *infected with* L. (L.) mexicana. *Subclinical and clinical infections were established in* P. yucatanicus *through inoculation with 1 x 10^2 and 2.5 x 10^6 promastigotes, respectively. Peritoneal macrophages were cultured alone or co-cultured with lymphocytes with or without soluble* Leishmania *antigen. The level of NO production was determined using the Griess reaction. The amount of NO produced was significantly higher ($p \leq 0.0001$) in co-cultured macrophages and lymphocytes than in macrophages cultured alone. No differences in NO production were found between* P. yucatanicus *with subclinical* L. (L.) mexicana *infections and animals with clinical infections. These results support the hypothesis that the immunological mechanisms of NO production in* P. yucatanicus *are similar to those described in mouse models of leishmaniasis and, despite NO production,* P. yucatanicus *is unable to clear the parasite infection.*

Key words: nitric oxide - *Peromyscus yucatanicus* - *Leishmania (L.) mexicana*

Cutaneous leishmaniasis (CL) caused by *Leishmania (Leishmania) mexicana* Biagi, 1953, *emend*. Garham, 1962, is a seasonal wild zoonosis that is endemic in the Yucatan Peninsula, Mexico (Andrade-Narváez et al. 2003). In this area, CL, known as "chiclero's ulcer", produces a single ulcer in 84.5% of patients and is commonly (39.9%) located on the ear lobe (Seidelin 1912, Canto-Lara et al. 1998, Andrade-Narváez et al. 2001). The annual incidence rate of symptomatic infection, i.e., CL, in the state of Campeche is 0.5%, whereas the subclinical infection prevalence rate is 19% (Andrade-Narváez et al. 1990, Arjona-Villicaña 2002).

In Campeche, three rodent species have been identified as primary reservoirs of *L. (L.) mexicana: Ototylomys phyllotis* Merriam, 1901; *Peromyscus yucatanicus* JA Allen & Chapman, 1897, and *Heteromys gaumeri* JA Allen & Chapman, 1897. The two latter species are endemic to the Yucatan Peninsula (Chable-Santos et al. 1995, Canto-Lara et al. 1999, Van Wynsberghe et al. 2000, 2009).

P. yucatanicus has been adapted to captivity and a colony has been established for experimental studies in our institution. Previous studies have demonstrated that *P. yucatanicus* experimentally infected with 1 x 10^6 *L. (L.) mexicana* promastigotes showed clinical and histological features similar to those of humans with CL caused by the same parasite species (Sosa-Bibiano et al. 2012). These results support the utility of *P. yucatanicus* as a novel experimental model to study CL caused by *L. (L.) mexicana*.

In the laboratory mouse (*Mus musculus* Linnaeus, 1758) model of CL, the resistance of C57BL/6 mice to *Leishmania (Leishmania) major* infection has been associated with the classical activation of macrophages. Activated macrophages express inducible nitric oxide (NO) synthase and the up-regulation of this gene induces the synthesis of NO from L-arginine in a two-step process, with hydroxyl-arginine and citrulline as intermediates. NO is a potent cytotoxin involved in the killing of *Leishmania* parasites and therefore it is a key molecule in the control of the disease (Stenger et al. 1994, Fang 1997, Bogdan et al. 2000).

In 1976, Preston and Dumonde, based on a study about asymptomatic or subclinical infections in humans caused by *L. (L.) mexicana,* demonstrated that low doses (10^2, 10^3) of *Leishmania (Leishmania) tropica* induced subclinical infection in *M. musculus* (González & Biagi 1968, Preston & Dumonde 1976). Subsequently, it was demonstrated that the parasite dose determines the Th_1/Th_2 nature of the response to *L. (L.) major* independent of the infection route or the strain of the host or parasite (Bretscher et al. 1992, Doherty & Coffman 1996, Menon & Brestcher 1998).

Inbred laboratory strains of mice have been helpful in elucidating the cell types, cytokines, signal transduction cascades and antileishmanial effector mechanisms involved in the clinical resolution and progression of disease. Although these experimental models have had the major advantage of allowing control over the genetics of both the parasite and the host, they do not recapitulate the pathology observed in human disease.

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+ Corresponding author: nalleli.cervera@uady.mx
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A new approach to study host-parasite relationships has been the use of wild animals, particularly primary reservoirs, as experimental animal models. Wild animals are similar to humans in that they are genetically polymorphic and thus represent an emerging system for the genetic analysis of the physiological and behavioural bases of habitat adaptation (Guénet & Bonhomme 2003).

Laboratory studies using natural hosts as experimental models of *Leishmania* could increase our understanding of the mechanisms involved in immune activation during nonpathogenic and pathogenic infections. The study of dogs, the main reservoir of visceral leishmaniasis, has led to increased interest in the immune responses and the *Leishmania* antigens implicated in protective cellular immunity in canine visceral leishmaniasis (Alvar et al. 2004, Baneth et al. 2008).

The possible role of NO in *P. yucatanicus* infected by *L. (L.) mexicana* remains unknown. Therefore, as part of our continuing effort to develop *P. yucatanicus* as a novel experimental animal model to study CL caused by *L. (L.) mexicana*, in the present study, we determined the amount of NO produced by peritoneal macrophages and macrophages and lymphocytes co-cultured in the presence or absence of soluble *Leishmania* antigen (SLA). The macrophages and lymphocytes were obtained from *P. yucatanicus* animals that were clinically or subclinically infected with *L.* (*L.*) *mexicana*.

MATERIALS AND METHODS

Animals - Wild *P. yucatanicus* animals adapted well to captivity and a colony derived from wild progenitors captured in the state of Campeche has been maintained for experimental studies in our institution since 1998. Six-18-month-old *P. yucatanicus* animals of both sexes were selected. The animals were maintained in the animal care facility of the Regional Research Centre of the University of Yucatan. The mice were individually housed in small cages (19 x 29 x 12 cm) lined with wood shavings and were fed rodent chow *ad libitum* (2018S Harlan, Wisconsin). For enrichment and food balance, the mice were given fresh fruit or vegetables once per week. The animals were kept at 22ºC ± 1ºC with a 12/12 h light cycle. Physical enrichment was provided weekly in the form of cardboard tubes for use as hiding places and soft paper for use as nesting material. The animals were euthanised with an overdose of sodium pentobarbital (100 mg/kg IV).

Parasites and antigen - The *L. (L.) mexicana* MHET/MX/97/Hd18 strain was selected and its infectivity was restored by passage in Syrian golden hamsters (Chable-Santos et al. 1995, Canto-Lara et al. 1999, Van Wynsberghe et al. 2000, Dumonteil et al. 2003). Promastigotes were grown in Senekjie medium for seven days at 23ºC. Stationary phase promastigotes were washed three times in RPMI-1640 (RPMI medium, Gibco) before being counted and adjusted to the concentrations needed for inoculation. SLA was obtained from stationary promastigotes that were washed twice in phosphate buffered saline (PBS), resuspended in PBS and phenylmethanesulfonyl fluoride and subjected to five freeze-thaw cycles at -70ºC and 37ºC. The protein content was determined by the Bradford method. The final concentration of SLA used for the in vitro stimulation of the co-cultures was 2.5 µg/mL.

Experimental infection - To study NO production, three groups of 14 *P. yucatanicus* mice were inoculated in the base of the tail as follows: Group 1, RPMI medium (control), Group 2, 1 x 10^2 promastigotes of *L. (L.) mexicana* (subclinical group), and Group 3, 2.5 x 10^6 promastigotes of *L. (L.) mexicana* (clinical group). The evolution of the infection was followed up weekly for 12 weeks. All animals were examined for the following clinical signs that are suggestive of *Leishmania* infection: oedema, erythema, ulcers and scars.

L. (L.) mexicana kDNA detection in subclinically infected animals by polymerase chain reaction (PCR) - To verify the subclinical infection, another group of 22 *P. yucatanicus* mice inoculated with 1 x 10^2 *L. (L.) mexicana* promastigotes were sacrificed at 18 weeks post-infection and biopsy samples were obtained from the skin for parasite DNA detection by PCR. Briefly, DNA was extracted from tissue biopsy samples after homogenisation with 100 mM Tris-HCl, pH 8.0, 100 mM ethylenediamine tetraacetic acid (EDTA), 100 mM NaCl, 1% sodium dodecyl sulfate and 4 µL of 10 mg/mL proteinase K (Sigma). The DNA precipitate was dissolved in 200 µL of Tris-EDTA. Total DNA (300 ng) was amplified in 100 mM KCl, 20 mM Tris-HCl, 0.1 mM EDTA, 1 mM dithiothreitol and 1.75 mM $MgCl_2$ in the presence of 0.2 mM of each deoxyribonucleotide, 10 pmol of each primer and 2.5 units of *Taq* DNA polymerase in a final volume of 25 µL. Primers 13A (5'GTGGGGGAGGGGCGTTCT3') and 13B (5'ATTTTACACCAACCCCCAGTT3') were used to amplify a conserved region of the kinetoplast DNA (kDNA) minicircle (120 bp) of *L.(L.) mexicana* (Kerr et al. 2006). The positive PCR control was DNA extracted from a logarithmically growing culture of *L. (L.) mexicana* strain MHET/MX/97/Hd18 and the negative control was the PCR mixture without DNA. PCR products were separated by electrophoresis in 1% agarose gels in tris-borate-EDTA (45 mM tris-borate, 1 mM EDTA).

Macrophages and lymphocytes - All *P. yucatanicus* mice included in the NO experiments were inoculated intraperitoneally with incomplete Freund´s adjuvant 10 days before being euthanised. Macrophages were obtained by peritoneal cavity lavage with RPMI. The cells were washed three times in RPMI and their viability was determined by the trypan blue exclusion test. Macrophages were adjusted to 1 x 10^5/mL in complete culture medium containing RPMI supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 0.1% β-mercaptoethanol and 100 U/mL penicillin-streptomycin. Splenocytes were obtained by maceration, washed three times with RPMI and depleted of erythrocytes by treatment with lysis solution (Promega Z3141). Macrophages were separated from lymphocytes by adherence to glass dishes at 37ºC at 5% CO_2 for 2 h. Lymphocytes were washed in RPMI and adjusted to 1 x 10^6 in complete culture medium. Macrophages alone or in co-culture with autologous lymphocytes were plated in

24-well flat-bottom culture plates with RPMI. Another co-culture was stimulated with 2.5 µg/mL SLA. All groups were incubated at 37ºC at 5% CO_2 for 96 h. The in vitro experiments were repeated once.

NO quantification - The culture supernatants were collected and the amount of NO produced was indirectly determined by the quantification of the nitrite (NO_2^-) concentration using Griess reagent. Briefly, after 96 h of incubation, cell culture supernatants from macrophages were mixed with an equal volume of Griess reagent and the absorbance was spectrophotometrically measured at 540 nm. The NO_2^- concentration was determined using a standard $NaNO_2$ curve and was expressed as µM/mL.

Statistical analysis - Analysis of variance (one-way ANOVA) with a p-value < 0.05 was used to compare the levels of NO production among the three culture types (peritoneal macrophages alone and co-cultures of macrophages and autologous lymphocytes with or without SLA stimulation) and among the doses of infection: no parasite (control), 1 x 10^2 promastigotes of *L. (L.) mexicana* (subclinical infection) and 2.5 x 10^6 (clinical infection).

Ethics - All animals were handled according to the Mexican Law for the use of laboratory animals (fmvz.unam.mx/fmvz/principal/archivos/062ZOO.PDF) and the Guide for the Care and Use of Laboratory Animals (NRC 1996) and the proposal was approved by our institutional bioethics committee.

RESULTS

Experimental infection - Subclinical infection was induced in 100% of *P. yucatanicus* mice inoculated with 1 x 10^2 *L. (L.) mexicana* parasites and 92.3% (12/13) of rodents inoculated with 2.5 x 10^6 parasites developed one or more suggestive signs of CL beginning at two weeks post-infection. The most frequent symptoms were oedema, induration, ulcers and, later, scars at the inoculation site. No lesions were found in the control group inoculated with RPMI.

L. (L.) mexicana kDNA detection in subclinically infected animals by PCR - The PCR results were positive for 12 of the 22 *P. yucatanicus* (54.5%) mice inoculated with 1 x 10^2 *L. (L.) mexicana* promastigotes.

NO production - The level of NO production was significantly higher ($p \leq 0.0001$) in co-cultured macrophages and lymphocytes than in the monocultured macrophages for all three groups of *P. yucatanicus* (Table). The level of NO production was significantly higher ($p \leq 0.05$) in subclinically infected *P. yucatanicus* mice than control mice (Fig. 1). No differences in NO production were found between *P. yucatanicus* mice inoculated with 1 x 10^2 parasites and those inoculated with 2.5 x 10^6 parasites at 12 weeks post-infection (Fig. 2).

DISCUSSION

In the present study, we determined that NO was produced by peritoneal macrophages and by co-cultured macrophages and lymphocytes from *P. yucatanicus* mice infected with *L.* (*L.*) *mexicana* and grown in the

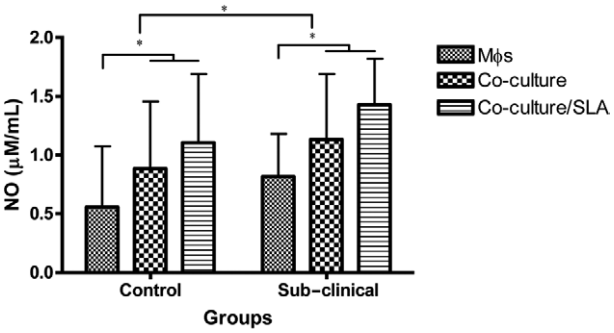

Fig. 1: nitric oxide (NO) productions by control and sub-clinical infected *Peromyscus yucatanicus*. Data in sub-clinical rodents were registered at 12 week post-infection. Two samples were analysed for each animal. Fourteen animals per group were used. Results are expressed as means ± standard deviation. Asterisks mean $p < 0.05$. SLA: *Leishmania* antigen.

TABLE

Nitric oxide productions in all groups

Groups	Stimulus	Mean	SD
Healthy	MΦs	0.5553	0.51833
	Co-culture	0.8835[a]	0.57388
	Co-culture/SLA	1.1064[a]	0.58228
Subclinical	MΦs	0.8159	0.36438
	Co-culture	1.1321[a]	0.55636
	Co-culture/SLA	1.4287[a]	0.39042
Clinical	MΦs	0.6521	0.43567
	Co-culture	1.0058[a]	0.31240
	Co-culture/SLA	1.2500[a]	0.48972

a: $p < 0.05$. Two samples were analyzed for each animal. Fourteen animals per group were used. Results are expressed as means ± standard deviation (SD). SLA: *Leishmania* antigen.

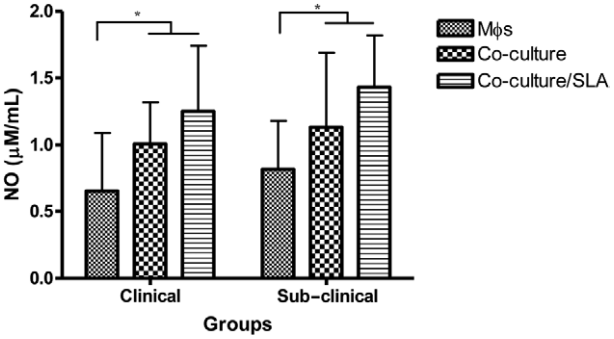

Fig. 2: nitric oxide (NO) productions by clinical and sub-clinical infected *Peromyscus yucatanicus* at 12 week post-infection. Two samples were analysed for each animal. Fourteen animals per group were used. Results are expressed as means ± standard deviation. Asterisks mean $p < 0.05$. SLA: *Leishmania* antigen.

presence or absence of SLA, but the mice were unable to clear the infection. This study is the first to document NO production by *P. yucatanicus* mice infected experimentally with a low or high dose of *L.* (*L.*) *mexicana* promastigotes.

As expected, none of the *P. yucatanicus* mice inoculated with 1 x 10^2 (low dose) *L.* (*L.*) *mexicana* promastigotes exhibited symptoms of the disease by the end of the study. However, the PCR was positive for 54.4% of *P. yucatanicus* mice inoculated with the low dose of *L. (L.) mexicana* promastigotes. The criteria for subclinical infection in humans include living in an area endemic for cutaneous leishmaniasis, the absence of CL symptoms or a scar and a positive Montenegro skin test (González & Biagi 1968). The detection of *Leishmania* genus and subgenus kDNA by PCR must be added as a criterion for wild rodent or mouse models of subclinical leishmaniasis infection. Because *P. yucatanicus* is a primary reservoir of *L. (L.) mexicana,* the identification of parasite kDNA in subclinically infected animals was expected.

In contrast, 92.3% of *P. yucatanicus* mice inoculated with 2.5 x 10^6 promastigotes developed clinical infections, with lesions similar to those found in human CL patients infected with the same parasite species (Andrade-Narváez et al. 2001). This high disease rate was most likely due to the low temperature (22ºC). Parasite multiplication in *P. yucatanicus* was correlated with lower ambient temperature in the animal care facility in a two-year study of the persistence of *L.* (*L.*) *mexicana* in naturally infected rodents (Van Wynsberghe et al. 2000).

In laboratory studies, the control of *L. (L.) major* infection has been demonstrated to be mediated by high NO levels in a resistant *M. musculus* strain (C57BL/6). Moreover, the enhancer effect of autologous lymphocytes on macrophage NO production has already been demonstrated in dogs (*Canis familiaris*). The exposure of *Leishmania (Leishmania) infantum*-infected macrophages to autologous lymphocytes from dogs immunised with purified secreted antigens of *L.* (*L.*) *infantum* promastigotes resulted in a significant leishmanicidal effect due to the activation of macrophages by interferon (IFN)-γ (Lemesre et al. 2005). Furthermore, an enhanced NO-mediated antileishmanial activity of canine macrophages in response to higher IFN-γ production by T-cells was demonstrated in co-cultures (Holzmuller et al. 2005).

In contrast, in susceptible animals (BALB/c), the exacerbation of infection is associated with the production of NO inhibitory cytokines (Trinchieri 1995, Locksley et al. 1999, Aguilar-Torrentera & Carlier 2001, Rogers et al. 2002, Sacks & Noben-Trauth 2002, Lang et al. 2003). CL studies that consider only the traditional model in which *M. musculus* mice are infected with *L. major* cannot address the interesting immunobiological characteristics associated with the variation among *Leishmania* spp. In fact, *Leishmania (Leishmania) amazonensis* and *L.* (*L.*) *mexicana* are associated with disease patterns that differ greatly from that of *L. (L.) major* in the mouse model. For example, the lesions of C57BL/6 or C3H mice infected with *L. (L.) major* heal, whereas these same mouse strains develop chronic disease when infected with either *L. (L.) amazonensis* or *L. (L.) mexicana* (Aguilar-Torrentera 2002, Pinheiro & Rossi-Bergmann 2007). Nevertheless, NO has been considered a potent cytotoxin involved in the killing of *Leishmania* parasites and is therefore a key molecule in CL control (Fang 1997, Stenger et al. 1994, Bogdan et al. 2000).

Therefore, we focused on NO production by the endemic CL reservoir *P. yucatanicus* infected with *L.* (*L.*) *mexicana* from Yucatan Peninsula. In our in vitro experiments, autologous lymphocytes enhanced the NO production of macrophages collected from *P. yucatanicus* mice infected with either 1 x 10^2 or 2.5 x 10^6 promastigotes. This finding may be due to the release of specific cytokines, such as IFN-γ and tumour necrosis factor alpha from lymphocytes. These cytokines exert an activating effect on *Leishmania*-infected macrophages and regulate NO production, as already demonstrated in the traditional mouse model.

Laboratory studies using natural hosts as experimental models could provide a better understanding of the dynamics of infection, especially concerning the ability of the immune system to address the infection. However, experiments using wild rodents are limited, primarily due to the difficulty of managing wild mammals in captivity and the absence of reagents of defined specificity available to carry out these studies.

The experimental infection of *Thrichomys laurentius* with *Leishmania* species from different complexes, *L. mexicana* and *Leishmania donovani*, demonstrated the ability of both *Leishmania* species to invade and maintain themselves in the viscera and skin of *T. laurentius* and no rodent displayed any lesion, histological changes or clinical evidence of infection. Nevertheless, the immune response and the dynamics of infection in this wild *Leishmania* reservoir are unknown (Roque et al. 2010).

The lack of a difference in the amount of NO produced by *P. yucatanicus* mice with clinical or subclinical infections indicates that as a primary reservoir of *L. (L.) mexicana*, this deer mouse maintains circulating parasites, even when subclinically infected. *Leishmania* is an obligate intracellular parasite of macrophages and even in humans, parasite persistence is well known (Fagundes et al. 2007, Colomba et al. 2009). Further investigation is needed to elucidate the role of the lymphoproliferative response and cytokines in the establishment of subclinical and clinical *L.* (*L.*) *mexicana* infections in *P. yucatanicus* mice.

Finally, the results obtained support the use of *P. yucatanicus* as a novel experimental animal model to study CL caused by *L.* (*L.*) *mexicana* in subclinically and clinically infected mice. However, to reach this goal, it is necessary to study the expression kinetics of immune genes in *P. yucatanicus* mice infected with *L. (L.) mexicana*.

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