ROLE OF NITRIC OXIDE IN THE IMMUNOSUPPRESSIVE EFFECT OF \textit{TRYPANOSOMA LEWISI} ON MULTIPLICATION OF \textit{TOXOPLASMA GONDII} IN WHITE RATS

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ABSTRACT
The role of nitric oxide in the immunosuppressive effect of \textit{Trypanosoma lewisi} on the response to \textit{Toxoplasma gondii} in WISTAR rats was evaluated. Two groups of rats were infected with \textit{T. gondii} tachyzoites. One of these groups had been infected with \textit{T. lewisi} four days earlier. A third group was infected with \textit{T. lewisi} only. The concentration of nitrates, as a reflection of nitric oxide production, was measured in serum during 10 days after infection with \textit{T. gondii}. The results show that rats infected with \textit{T. lewisi} only, do not at any time display altered levels of serum nitrate. \textit{T. lewisi} infection does, however, partially inhibit the increase of serum nitrate levels caused by \textit{T. gondii} 2 days after infection, a time point at which \textit{T. gondii} multiplication in \textit{T. lewisi} infected rats is exacerbated.

KEYWORDS
Nitric oxide, interferon-\(\gamma\), immunosuppression, \textit{Toxoplasma gondii}, \textit{Trypanosoma lewisi}. 
RESUMEN
El papel del óxido nítrico sobre la inmunosupresión de Trypanosoma lewisi en la respuesta contra Toxoplasma gondii fue evaluada. Se infectaron dos grupos de ratas con taquizoitos de T. gondii. Uno de estos fue infectado con T. lewisi cuatro días antes de la infección con T. gondii. Un tercer grupo fue infectado con T. lewisi solamente. La concentración de nitratos fue medida como reflejo de la producción de óxido nítrico durante 10 días después de la infección con T. gondii. Los resultados muestran que ratas infectadas solo con T. lewisi a través de los días de muestreo no presentan alteración en los niveles de nitratos en suero. Sin embargo, los sueros de ratas previamente infectadas con T. lewisi y posteriormente infectadas con T. gondii, muestran una inhibición parcial en el incremento de los niveles de nitratos en suero 2 días después de la infección por T. gondii. Esta inhibición, coincide con una exacerbación en la multiplicación del parásito.

PALABRAS CLAVES
Óxido nítrico, interferon-gamma, inmunosupresión, Toxoplasma gondii, Trypanosoma lewisi.

INTRODUCTION

Different mechanisms underlying the phenomenon have been suggested such as parasite-induced alterations of lymphocyte function, polarization towards a T helper (Th) 2 type response, macrophage suppression, and down-regulation of interleukin-2 (IL-2) (Albright & Albright, 1991, Darji et al., 1992) and interferon-γ (IFN-γ) production (Chinchilla et al., 2005), all of which are reflected in a decrease in the activity of inflammatory cytokines involved in the control of intracellular parasite infections.

Trypanosoma lewisi is a blood borne protozoan parasite that infects rats. The animals generally remain free of disease due to appearance of 3 antibodies in the infected animals. One is a reproduction-inhibiting antibody known as ablastin and the other antibodies are trypanocidal and gradually kill the adult trypanosomes between 1 and 4 months.
after infection. The immunity resulting from the infection continues throughout the life of the rat (Ndarathi, 1991, 1992). Previous studies from this laboratory have shown that between day 4 and 5 after infection with *T. lewisi*, rats otherwise resistant, become susceptible to infection with *Toxoplasma gondii* (Chinchilla *et al*., 2005, Guerrero *et al*., 1997). Further investigation of this model demonstrated increased multiplication of *T. gondii* in peritoneal macrophages isolated from rats infected with *T. lewisi* as compared to macrophages from control animals (Catarinella *et al*., 1999, Chinchilla *et al*., 2004). This was later associated with a concomitant decrease of serum concentrations of IFN-γ, a key mediator of resistance to *T. gondii* (Chinchilla *et al*., 2005). An important effector mechanism in the defense against the parasite is the generation of reactive nitrogen intermediates (RNI), including nitric oxide (NO) (James, 1995, Scharton-Kersten *et al*., 1997, Filisetti & Candolfi, 2004). During infection with *T. gondii*, NO is produced as a result of activation of inducible NO synthase (iNOS), which is induced by IFN-γ (Luder *et al*., 2003, Pepper & Hunter, 2007, Silva *et al*., 2009). The major cell populations involved in the production of early IFN-γ are natural killer (NK) cells (Une *et al*., 2000, Korbel *et al*., 2004), NK T cells (Nakano *et al*., 2002) and, in some cases, macrophages (Stafford *et al*., 2002).

This study was performed to determine the role of NO in the immunosuppressive effect caused by *T. lewisi* on the multiplication of *T. gondii* in the white rat.

**MATERIALS AND METHODS**

**Experimental animals:** Male WISTAR Hannover rats (HsdBrf1flan: WIST) weighing 250 - 300g and bred at facilities of the Biological Assay Laboratory (LEBi), University of Costa Rica were used for the experimental parasite infections.

**Parasites:** Tachyzoites of the *T. gondii* RH strain (5174 genotype 1, American Type Culture Collection) were obtained from peritoneal exudates of previously infected mice, washed with sterile saline solution at 0.85%, counted in a Neubauer chamber and adjusted to a concentration of 10^7/ml. Trypomastigotes of the *T. lewisi* TL2 strain (isolated in Costa Rica in 1977 from a grey rat *Rattus norvegicus* and
maintained by weekly passages in Sprague-Dawley rats were isolated from blood, counted in a Neubauer chamber and adjusted to a concentration of $10^6$/ml.

**Inoculation of the animals:** A total of ten experiments (nine rats for experiments) were performed in order to evaluate the three different experimental infection protocols (see Table 1): I. Inoculation of $10^6$ *T. lewisi* trypomastigotes at day 0. II. Inoculation of $10^7$ *T. gondii* tachyzoites at day 4. III. Inoculation of $10^6$ *T. lewisi* trypomastigotes at day 0 followed by inoculation of *T. gondii* tachyzoites at day 4. All inoculations were intraperitoneal.

**Obtention of sera:** Blood was drawn from the tail of each animal into Microtainer gold tubes with serum separator (Becton Dickinson) at day 0, 5, 6, 7 and 14 of the experiment. The blood samples were centrifuged at 14000 rpm for 10 minutes, the sera was separated and stored in -20°C until used.

**Determination of nitrate concentrations in serum:** Serum nitrate was reduced to nitrite by the enzyme Nitrate Reductase isolated from *Aspergillus niger* (Sigma, N7265) according to Gilliam *et al.* Briefly, after centrifugation at 14000 rpm for 10 min, 25 µl of the sera was added in duplicates to a flat bottom 96 well plate and mixed with 40 µl of phosphate buffer, 1 µl of 12.5 mM NADPH, 30 µl distilled water, and 4 µl Nitrate Reductase (3.5 U/ml) and incubated for 10 min at room temperature (Gilliam & Shernan, 1993). The nitrite was subsequently quantified by the Griess reaction. Equal volumes (50 µl) of sulfanilamide (Sigma, N9125) dissolved in phosphoric acid and N-1-naphtyl-ethylenediamine (Sigma, N9125) were added to the wells. The optical density was determined in a microplate reader (Lab Systems) at 540 nm. The concentration was calculated from a standard curve based on readings of known concentrations of NaNO$_3$ on the same plate (Green *et al.*, 1982, Sun *et al.*, 2003, Tsikas, 2005).

**Statistical analysis**
The results were analyzed by a Factorial ANOVA with repeat measures with one factor. The factor was the infection (I- *T. lewisi*, II- *T. gondii* and III- *T. lewisi-T gondii*) and different levels were the days.
of experiments (0, 5, 6, 7, and 14 days). The analysis was performed with a confidence coefficient of 0.05 ($\alpha = 0.05$).

RESULTS
Infection with *T. lewisi* (group I) alone did not affect serum nitrate levels at any time point after infection (Table 1). Baseline concentrations of 20-40 $\mu$M were maintained throughout the 14 day duration of the experiment.

Both the animals infected with *T. gondii* only (group II) and those infected with *T. gondii* after previous inoculation with *T. lewisi* (group III) showed a significant increase in nitrate levels at days 5, 6 and 7 post-infection with *T. lewisi* as compared to control values at day 0 (Table 1). The highest concentrations were registered at day 6 after which there were a decline and values returned to control levels at day 14 post-infection. Also, at days 5-7, all animals infected with *T. gondii* displayed nitrate concentrations which were significantly higher than those of rats infected only with *T. lewisi* (Table 1). However, at day 6, the animals that were infected with *T. lewisi* before being inoculated with *T. gondii* had significantly less nitrate in the blood than those only infected with *T. gondii* (Table 1), indicating a possible mechanism for the immunosuppressive effect.

DISCUSSION
The immune response mobilized against *T. lewisi* is characteristic of a response to extracellular pathogens and involves complement activation and Th2 type antibody formation (Ndarathi, 1991). As production of RNI is a hallmark of cellular immunity, it was to be expected that the concentrations of nitrate remain normal during the infection with *T. lewisi* (Chinchilla *et al.*, 2005).

The results demonstrate that infection with *T. lewisi* prior *T. gondii* results in a partial inhibition of the peak levels at day 6 of nitrate concentrations in serum induced by the latter parasite. Thus, rats infected with both parasites suffer from a dysfunction of the cellular immune response directed against *T. gondii*. This observation confirms previous work in this experimental model where an exacerbation of *T.
*T. gondii* multiplication was provoked in various tissues of rats that had been infected with *T. lewisi* and where the surviving animals present recuperation with lymphocyte infiltration in those tissues 10 days after infection with *T. gondii* (day 14) (Catarinella *et al.*, 2001). The data obtained is in agreement with the observations by Chinchilla *et al.* 2004, showing that in rats infected with *T. lewisi* only, IFN-γ can-not be detected in serum whereas the sera of animals infected with *T. gondii* contain significant amounts from 24 hours after infection (Chinchilla *et al.*, 2005).

It is known that *T. gondii* stimulates the production of IL-12 (Scharton-Kersten *et al.*, 1996), which in turn triggers the synthesis and release of early IFN-γ by various innate effectors cells such as NK cells, NKT cells and macrophages (Suzuki *et al.*, 1988, Scharton-Kersten *et al.*, 1997, Yap & Sher, 1999, Pepper & Hunter, 2007). IFN-γ is recognized as the principal mediator of innate resistance during the acute phase of the infection. Many studies confirm that RNIs, including NO, represent an important microbicidal mechanism generated by the activation of effectors cells by IFN-γ (Gazzinelli *et al.*, 1991, Hayasi *et al.*, 1996, Scharton-Kersten *et al.*, 1997, Sherstha *et al.*, 2006). There are several factors operating to counteract and balance the inflammatory response involving IFN-γ and NO (Adams *et al.*, 1990, Holän *et al.*, 2001, Seabra *et al.*, 2002, Silva *et al.*, 2009), among those the production of transforming growth factor (TGF)-β (Bermúdez *et al.*, 1993) and eicosanoids (Thardin *et al.*, 1993, Noverr *et al.*, 2003) which may be produced by macrophages infected with *T. gondii*. Moreover, both these immune mediators have been shown to inactivate macrophages that have been activated by IFN-γ, thereby indirectly inhibiting the generation of NO (Bermúdez *et al.*, 1993, Thardin *et al.*, 1993, Noverr *et al.*, 2003). Another possible mechanism for inactivation of a Th1 pathway implicating IFN-γ and NO is early IL-10, the production of which is activated by many pathogens as a way of evading cellular immune responses. IL-10 down-regulates IFN-γ production by CD4+ Th1 cells, CD8+ T cells, and NK cells during infection with several parasites, including *T. gondii* (Gazzinelli *et al.*, 1992, Lu *et al.*, 2003), and is therefore a potent inhibitor of the microbicidal activities of macrophages. It was reported that this effect occurs simultaneously to the suppressive of NO production by the effectors cells.
The *T. lewisi*-mediated immunosuppression model in white rats has the advantages of being inexpensive and rapid in comparison with administration of corticosteroids or other synthetic immunosuppressive substances. It has the potential to evaluate different infections in immunocompromized hosts. In addition to infection with *T. gondii* the model has been used to investigate interaction with *Cryptococcus neoformans* in our laboratory (Gross et al., 2006).

The model is now being further investigated in order to determine the role of reactive oxygen intermediates, IL-10, IL-12 and other cytokines in the impairment of cellular immunity caused by *T. lewisi*.

Table 1. Nitrate concentration (μM) in rat serum.

<table>
<thead>
<tr>
<th>Day of experiment</th>
<th>I (T. lewisi)</th>
<th>II (T. gondii)</th>
<th>III (T. lewisi-T.gondii)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean [NaNO₃]</td>
<td>S.E.M</td>
<td>Mean [NaNO₃]</td>
</tr>
<tr>
<td>0</td>
<td>32 ± 3.7</td>
<td></td>
<td>30 ± 3.6</td>
</tr>
<tr>
<td>5</td>
<td>25 ± 1.3</td>
<td></td>
<td>44 ± 4.7</td>
</tr>
<tr>
<td>6</td>
<td>31 ± 3.5</td>
<td></td>
<td>172 ± 33.1</td>
</tr>
<tr>
<td>7</td>
<td>35 ± 7.6</td>
<td></td>
<td>70 ± 11.2</td>
</tr>
<tr>
<td>14</td>
<td>24 ± 1.1</td>
<td></td>
<td>25 ± 1.9</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM of 10 experiments.

- a p<0.05 versus II and III
- b p <0.05 versus I and III
- c p<0.05 versus I and II
- d p<0.05 versus I
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