Toxoplastic Infection-induced Injury in the Ileal Myenteric Plexus in Rats Depends on the Dose of Toxoplasma gondii Oocysts

L. M. T. Tironi1 E. J. Beraldi1 S. C. Borges1 C. L. Massocato1 S. L. V. Vieira1 D. M. G. Sant’ana1 E. J. A. Araújo2 N. C. Buttow1

1 Departamento de Ciências Morfológicas, Universidade Estadual de Maringá – UEM, Jardim Universitário, Maringá, Paraná, Brazil
2 Departamento de Histologia, Universidade Estadual de Londrina – UEL, Londrina, Paraná, Brazil

Address for correspondence L. M. T. Tironi, Departamento de Ciências Morfológicas, Universidade Estadual de Maringá – UEM, Avenida Colombo, 5790, Jardim Universitário, CEP 87020-900, Maringá, PR, Brazil (e-mail: liamaratironi@gmail.com).


Abstract

Introduction The present study evaluated the effects of different inocula of Toxoplasma gondii oocysts on the ileal myenteric plexus in rats.

Materials and Methods Male Wistar rats, 60 days old, were distributed into four groups: control group (CG; which received saline solution) and groups that were infected with 100 (TG100), 1000 (TG1000), and 5000 (TG5000) T. gondii oocysts. Thirty days after infection, the rats were sacrificed, and the ileum was collected to make whole-mount preparations that were subjected to immunofluorescence staining to observe the general neuronal population (HuC/D), nitrergic neurons (nNOS), and enteric glial cells (S100). Morphometric and quantitative analyses of myenteric neurons were performed.

Results The infections with different T. gondii inocula did not cause neuronal or glial loss, but cause neuronal hypertrophy in general population and nitrergic subpopulation in infected groups. Conclusion: Changes in neuronal morphology were observed in the TG5000 group, including the presence of vacuoles, translocation of Hu protein to the nucleus, and dendritic distortions, suggesting functional alterations in these cells.

Introduction

Toxoplasma gondii is an intracellular parasite that causes toxoplasmosis and is considered one of the most evolutionarily successful protozoa on Earth.1 One-third of the world’s population has had contact with this parasite.2 In Brazil, the prevalence of anti-T. gondii immunoglobulin G (IgG) antibodies in the human population varies from 59% to 91% in different regions.3,4 Based on SAG2 gene analysis, T. gondii has three main distinct clonal lineages: genotypes I, II, and III.5,6 The majority of human infections is associated with type II genotype strains,5 and its transmission can occur when the host ingests tissue cysts that are present in under-cooked meat or oocysts in contaminated water and food and via the vertical transmission of tachyzoites.1,7 When sporulated oocysts are ingested by any warm-blooded animal, sporozoites are released in the small intestine, and the parasite penetrates enterocytes within 30 minutes.8 Tachyzoites can be found in the blood 4 hour after the ingestion of oocysts, and most tachyzoites remain in the lamina propria where they multiply inside several cell types, with the exception of erythrocytes.9 The presence of these parasites in the intestine can cause sloughing of the intestinal mucosa and enteritis, with edema and necrosis in the lamina propria.9 Unknown is how often T. gondii infection causes gastrointestinal symptoms in humans.
Materials and Methods

All of the procedures that involved the use of animals were approved by the Ethics Committee on the Use of Animals in Experimentation of the State University of Maringá (CEAE, Universidade Estadual de Maringá, no. 081/2012) and were in accordance with the ethical principles adopted by the Brazilian Society of Science in Laboratory Animals (SBCAL).

Experimental Design

Twenty-five male Wistar rats (Rattus norvegicus), 60 days old, were randomly distributed into four groups: control group (CG; n = 5; which received saline solution) and groups infected with 100 (TG100, n = 6), 1000 (TG1000, n = 7), and 5000 (TG5000, n = 7) Toxoplasma gondii oocysts of the ME49 strain (genotype II). The oocysts were obtained from the Veterinary Parasitology Laboratory, Universidade Estadual de Londrina (Londrina, PR, Brazil) and previously sporulated and resuspended in 1 mL of sterile saline solution. Control rats received only the sterile saline solution. The rats were then maintained for 30 days in an animal house with controlled temperature (22 ± 2°C) and a 12/12 hour light/dark cycle and fed standard rodent chow (NuVilab, Quintia SA, Colombo, PR, Brazil) and water ad libitum.

Sample Collection and Tissue Preparation

T. gondii infection was confirmed by the presence of anti-T. gondii IgG in serum using the direct agglutination method. Thirty days after infection, the rats were deeply anesthetized by the presence of halothane vapor. Laparotomy was performed to collect the distal ileum, which was tied at both ends and filled with 4% buffered paraformaldehyde (pH 7.4) and distended with 0.1 M sodium cacodylate buffer (pH 7.4) with 0.08% sodium azide. The whole-mount preparations were incubated with mouse anti-HuC/D (a marker of the general population of myenteric neurons) or rabbit anti-nNOS (a marker of the nitrergic subpopulation) or rabbit anti-S100 (a marker of glial cells) antibodies (Table 1). Double-labeling was performed for HuC/D/nNOS and HuC/D/S100 in separate preparations. The whole-mount preparations were washed (2 x 10 minute) in PBS plus 0.5% Triton X-100 (PBS-T) and placed in a blocking solution (PBS-T, 2% bovine serum albumin (BSA) and 10% donkey serum) for 1 hour. They were then incubated for 48 hour with the primary antibodies diluted in PBS-T, 2% BSA, and 2% donkey serum at room temperature under shaking. The whole-mount preparations were then washed in PBS-T (3 x 10 minute) and incubated with secondary antibodies (Table 1) for 2 hour at room temperature under shaking while protected from light. Lastly, they were washed in PBS-T (3 x 10 minute) and mounted on glass slides using Prolong Gold Antifade Reagent (Molecular Probes, Life Technologies, Eugene, OR, USA).

Morphoquantitative Analysis

The neuronal density and morphometry analyses were performed on images that were captured under a fluorescence microscope with a high-resolution camera coupled to a computer. The analyses were performed using ImagePro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). The images were captured by randomly sampling across all of the whole-mount preparations, with no specific visual fields chosen, and the same field was not captured more than once. Immunoreactive neurons (HuC/D+ and nNOS+) and glial cells (S100+) were counted in 30 images per animal. The area of each image was 0.36 mm², and the total quantified area was 10.82 mm² per animal. The results are expressed as the number of enteric neurons or glial cells per cm². For morphometry, the area of 100 HuC/D+ cell bodies and 100 nNOS+ cell bodies per animal were measured, and 100 HuC/D+/nNOS- neurons per animal were also measured. Only neurons for which we could clearly delimit the cell body were chosen, and the area is expressed in μm².

Statistical Analysis

The data were analyzed using the Statistica 7.1 and GraphPad Prism 3.1 software. The quantitative data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s test. Morphometric data were set in delineation blocks (ANOVA) followed by Tukey’s test. All of the statistical tests considered a 5% level of significance. The results are expressed as mean ± standard error.

Table 1 Primary and secondary antibodies used for immunofluorescence

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Secondary Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>Host</td>
</tr>
<tr>
<td>HuC/D</td>
<td>Mouse</td>
</tr>
<tr>
<td>nNOS</td>
<td>Rabbit</td>
</tr>
<tr>
<td>S100</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>

Abbreviations: HuC/D, general neuronal population; nNOS, nitrergic subpopulation; S100, enteric glial cells.
Results

Clinical Aspects
None of the characteristic clinical signs of toxoplasmosis were observed in the infected rats. The TG100, TG500, and TG5000 groups presented serum anti-\( T.\) gondii IgG 30 days after inoculation, whereas the CG remained negative.

Neuronal and Glial Density
None of the myenteric cellular populations (neurons and glial cells) presented significant population density changes in the infected groups (TG100, TG1000, and TG5000) compared with the CG (\( \text{Table 2} \)).

Morphometric Analysis
The general HuC/D\(+\) neuronal population and HuC/D\(+\)/nNOS\(-\) subpopulation presented hypertrophy in all of the infected groups compared with the CG \( (p < 0.01) \), most prominently in the TG100 group (\( \text{Table 2} \)). The nNOS\(+\) subpopulation showed hypertrophy of the cell bodies in the TG100 and TG5000 groups \( (p < 0.01) \). Some of the rats in the TG1000 group exhibited translocation of Hu protein to the nucleus, causing a reduction of cytoplasmic immunoreactivity and compromising visualization of the cell body limits. Special attention was necessary to ensure that the entire area of the neuronal body, including the nucleus and cytoplasm, was being measured.

Qualitative Analysis
HuC/D\(+\)/nNOS\(+\) neuronal immunoreactivity was generally weak in the infected rats (\( \text{Fig. 1} \)). Higher HuC/D\(+\) nuclear immunofluorescence was observed in neurons in the TG1000 group, indicating the translocation of Hu protein from the cytoplasm to the nucleus (\( \text{Fig. 2A} \)). Some neurons in the TG5000 group presented an irregular shape and no clear definition of the cell body limits (\( \text{Fig. 2B} \)). A substantial presence of intra-cytoplasmic vacuoles was observed in neurons in the infected groups (\( \text{Fig. 2C} \)). Some nNOS\(+\) neurons in the infected groups were swollen and presented distortions in the dendritic projections (\( \text{Fig. 2D} \)). Although the HuC/D antibody that was used is considered a pan-neuronal marker, we were able to observe some nNOS\(+\) neurons that did not show immunoreactivity to Hu protein (\( \text{Fig. 3} \)).

Discussion

Infection with different inocula of \( T.\) gondii (ME49 strain, genotype II) did not cause neuronal or glial cell loss but caused morphologic changes in myenteric neurons of the ileum in rats, such as hypertrophy, the translocation and degradation of Hu protein, the presence of vacuoles, and the distortion of dendrites in nitrergic neurons. The neuronal alterations that were observed in infected animals in this experimental model were not sufficient to provoke clinical signs of toxoplasmosis, such as diarrhea. Toxoplasmosis is often a subclinical infection in both rats and humans,\textsuperscript{12} which has also been observed in previous rat studies.\textsuperscript{13–16} Our data showed that the infection did not cause neuronal loss in the general population (HuC/D\(+\)) or nitrergic subpopulation (nNOS\(+\)). \( T.\) gondii infection in rats after 30 days does not compromise the survival of myenteric neurons, regardless of the infective stage of the parasite that is used for the inoculation, which was also demonstrated in previous studies that evaluated the ileum (acute and chronic phase) and colon (chronic phase) in rats that were infected with tachyzoites.\textsuperscript{16–18} The density of myenteric glial cells also did

Table 2  Quantification of the general population (HuC/D\(+\)) and subpopulations (nNOS\(+\) and nNOS\(-\)) of myenteric neurons and glial cells (S100 \(+\)) in the distal ileum in rats infected with different inocula of \( T.\) gondii for 30 days. The results are expressed as neurons per cm\(^2\) (mean \( \pm \) standard deviation)

<table>
<thead>
<tr>
<th>Group</th>
<th>HuC/D(+) general population</th>
<th>HuC/D(+)/nNOS(-) subpopulation</th>
<th>HuC/D(+)/nNOS(+) subpopulation</th>
<th>S100(+) Glial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16829 ( \pm ) 3966</td>
<td>12839 ( \pm ) 3322</td>
<td>3988 ( \pm ) 689</td>
<td>25752 ( \pm ) 3063</td>
</tr>
<tr>
<td>TG100</td>
<td>15749 ( \pm ) 2096</td>
<td>11333 ( \pm ) 1981</td>
<td>4295 ( \pm ) 742</td>
<td>25475 ( \pm ) 2350</td>
</tr>
<tr>
<td>TG1000</td>
<td>19443 ( \pm ) 2309</td>
<td>14760 ( \pm ) 1533</td>
<td>4691 ( \pm ) 790</td>
<td>26454 ( \pm ) 2508</td>
</tr>
<tr>
<td>TG5000</td>
<td>17116 ( \pm ) 1864</td>
<td>12848 ( \pm ) 1251</td>
<td>4299 ( \pm ) 685</td>
<td>24773 ( \pm ) 1812</td>
</tr>
</tbody>
</table>

No significant difference was found between groups \( (p < 0.05; \text{ANOVA}) \).

Table 3  Cell body area (\( \mu m^2 \)) of the HuC/D\(+\) general population, nNOS\(-\) subpopulation, and nNOS\(+\) subpopulation of myenteric neurons in the distal ileum in rats infected with different inocula of \( T.\) gondii for 30 days

<table>
<thead>
<tr>
<th>Group</th>
<th>HuC/D(+) general population</th>
<th>HuC/D(+)/nNOS(-) subpopulation</th>
<th>HuC/D(+)/nNOS(+) subpopulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>290.7 ( \pm ) 117.3</td>
<td>289.9 ( \pm ) 121.5</td>
<td>300.0 ( \pm ) 87.82</td>
</tr>
<tr>
<td>TG100</td>
<td>351.1 ( \pm ) 112.9a</td>
<td>364.6 ( \pm ) 131.4a</td>
<td>321.1 ( \pm ) 93.99a</td>
</tr>
<tr>
<td>TG1000</td>
<td>318.5 ( \pm ) 111.0ab</td>
<td>333.0 ( \pm ) 122.3ab</td>
<td>296.7 ( \pm ) 87.27b</td>
</tr>
<tr>
<td>TG5000</td>
<td>322.9 ( \pm ) 133.2ab</td>
<td>317.1 ( \pm ) 138.4ab</td>
<td>325.6 ( \pm ) 101.6c</td>
</tr>
</tbody>
</table>
The dose of parasites affected the myenteric neuronal population in the duodenum. Rats that were infected with *T. gondii* presented progressive, dose-dependent general neuronal loss in the duodenum in ascending order (10, 100, 500, and 5000 oocysts). The nitrergic myenteric neuronal subpopulation

not change as a result of toxoplasmic infection. The survival of myenteric glial cells may have contributed to the maintenance of the number of neurons in the general population and nitrergic subpopulation because glial cell loss usually precedes neuronal loss.\textsuperscript{19}
was shown to increase at doses of 100, 500, and 5000 T. gondii oocysts. Although infection with different doses of T. gondii oocysts in mice is more harmful to the ileum than the duodenum, we did not know whether this holds true for rats. Studies by our group suggest that duodenal myenteric neurons in rats are more sensitive than ileal myenteric neurons to toxoplasmic infection. Within the general neuronal population (HuC/D+), we found that ~25% of the neurons were nitrergic (nNOS+) in all of the groups. The different doses of T. gondii oocysts did not cause any changes in the proportion. Although HuC/D is considered a pan-neuronal marker, we observed the absence of Hu protein immunoreactivity in some neurons that were immunoreactive to nNOS protein (~ Fig. 3). Similar results have also been reported in humans. Although we did not observe myenteric neuronal loss, toxoplasmic infection caused hypertrophy in HuC/D+, nNOS+, and HuC/D+/nNOS- neurons. This hypertrophy might have occurred in response to the inflammation that was caused by T. gondii in the intestine, a remarkable feature of this parasitic infection. In fact, the cell body area increased in all of the groups but in descending order of the dose of the parasites. This indicates that higher parasite doses might impair neuronal plasticity. The mechanisms that are involved in these effects should be explored in further studies. Other studies by our group found morphometric alterations in myenteric neurons during toxoplasmic infection. For example, we observed hypertrophy in myenteric neurons in the distal ileum in rats that were infected with tissue cysts of the ME-49 strain of T. gondii for 24 hour. We also observed hypertrophy in nitrergic subpopulation and total population of myenteric neurons of proximal jejunum in rats that were infected for 36 days with tissue cysts of the ME-49 strain of T. gondii.

Despite the fewer morphometric alterations in myenteric neurons in rats in the TG1000 and TG5000 groups, we suggest that toxoplasmic infection compromises neuronal plasticity in the ileum when it is exposed to higher doses of the parasites. Considering that neuronal plasticity is important for the survival and physiologic balance of neurons, myenteric neurons in rats that received higher doses of the parasites may be more impaired because of the infection. The qualitative analysis appears to support this hypothesis.

We observed the nuclear translocation of Hu protein especially in the TG1000 group. This could indicate an imbalance in the trafficking of molecules between the cytoplasm and nucleus, thus compromising cellular metabolism. Hu protein is an RNA-binding protein that is present in both the cytoplasm and nucleus, with many functions in neuronal biology, such as cellular development and plastic adaptations. Changes in the localization of Hu protein may occur in response to stimuli that change the mRNA regulation by Hu or modify or selectively degrade Hu protein, thus modifying its immunoreactivity. The consequences of translocation of this protein are not yet completely understood. This event may be indicative of neuronal injury (Rivera et al., 2011) and Hu protein loss in the cytoplasm may indicate changes in general protein synthesis. Some HuC/D+ neurons presented a reduction of cytoplasmic immunoreactivity, including almost no labeling in some cases in the TG1000 group. Some HuC/D+, nNOS-, and nNOS+ neurons in the infected groups also showed the presence of vacuoles in their cytoplasm. Although cytoplasmic vacuolation occurs in neurons that are exposed to injury, the presence of these vacuoles is not necessarily related to neuronal death. Additionally, HuC/D+, nNOS-, and nNOS+ neurons with an abnormal aspect were frequently observed in the TG5000 group. These neurons presented distortion and no distinct demarcation of the cytoplasm.

Conclusion
The different doses of T. gondii oocysts did not cause glial or neuronal death in the ileal myenteric plexus in rats, but they provoked hypertrophy of their cell bodies. The higher doses (1000 and 5000 oocysts) caused less hypertrophy. The appearance of cellular injury signals, such as cytoplasmic vacuoles, Hu nuclear translocation, and dendritic distortion, may indicate less neuronal plasticity that is caused by this infection.

Acknowledgments
This work was supported by the governmental agencies Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Programa de Apoio à Pós-graduação (PROAP). The authors thank Professor João Luiz Garcia (Universidade Estadual de Londrina – Londrina, PR, Brazil) for donating the oocysts used in this study.
References


