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Toxoplasma gondii infection causes structural changes in the jejunum of rats infected with different inoculum doses

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ABSTRACT

Aim: To evaluate the mucosal tunic and submucosal plexus of the jejunum of rats infected with different inoculum doses of *Toxoplasma gondii*.

Main methods: Rats were infected with different inoculum doses (50, 500, 1000 and 5000 oocysts) of the *T. gondii* for 30 days, while a control group (CG) received saline solution. Blood and feces were collected before euthanasia for analysis of blood and fecal leukocytes (LEs). Histological analysis of the mucosa, submucosa, villi, crypts and enterocytes were performed. Goblet cells, intraepithelial lymphocytes (IELs) and Paneth cells were quantified. Immunohistochemistry was used to assess enteroendocrine serotonergic (5HT-IR) cells, proliferative cells ($PCNA^+$) and mast cells. Whole mounts were obtained to determine the total submucosal neurons by Giemsa staining and metabolically active neurons ($NADH\text{-}d^+$), nitrergic neurons ($NADPH\text{-}d^+$) and glial cells (S100).

Key findings: An increase in blood LEs was observed 30 days post-infection (dpi). Fecal LEs were more abundant in the feces in all infected groups at 21 dpi when compared to the CG. The number of IELs, sulfomucin-producing goblet cells, Paneth cells, $PCNA^+$ cells and mast cells increased, whereas the number of 5HT-IR cells decreased. The jejunal architecture was altered, with atrophy of the mucosa, submucosa, villi and crypts. The number of total submucosal neurons decreased, but the $NADPH\text{-}d^+$ subpopulation increased.

Significance: The results show how chronic toxoplasmic infection affects the tissue and cellular composition of the rat jejunum. These structural changes tend to intensify with the inoculum dose, demonstrating the importance of the parasitic load on intestinal alterations.

1. Introduction

The complex dynamics of the structure and immunology of the intestinal mucosa in response to oral protozoan infections are not well understood. On this basis, our research group has questioned whether the increasing number of cases of inflammatory bowel disease in immunocompetent humans may be related to infections caused by protozoa.

Toxoplama gondii (*T. gondii*) infection is very common worldwide and affects 1/3 of the population [1]. However, the infection usually has few or no symptoms in immunocompetent individuals and may go unnoticed [1,2]. Thus, we questioned whether *T. gondii* could induce chronic inflammatory bowel disease in immunocompetent individuals. The parasite generally enters the body via the oral route, which occurs through the ingestion of oocysts or cysts present in contaminated water and food [2–5]. In the gastrointestinal tract (GIT), the parasite must cross the mucosal lining to reach the bloodstream and then migrate to other regions of the organism [6]. Understanding the mucosal tissue response to infection may help elucidate its possible relationship with inflammation, and studies with murine models will enable the testing of treatments for this condition in the future. The intestinal mucosa is the first mechanical and immune barrier and is responsible for maintaining a dynamic balance with the constituent elements of the microbiome [7]. In addition to neural and humoral organization and coordination, the different cell types of the mucosa make studies of this locus particularly challenging. Therefore, it is important to understand what

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structural changes can be triggered with different inoculum doses of *T. gondii* that can lead to possible functional alterations in the intestinal mucosa.

We explored the tissue and cellular structures of the jejunal mucosa and submucosa in rats orally infected with different inoculum doses of the ME-49 strain of *T. gondii* (genotype II) for 30 days. First, we showed that there was a direct relation between the inoculum doses and the most lesions provoked by the toxoplasmic infection. In addition, we found that progressive inoculum doses of *T. gondii* caused gradual loss of submucosal neurons and that the most of the remaining neurons were nitrergic.

2. Materials and methods

The experimental protocol was approved by the Ethics Committee on the Use of Animals in Experimentation of the State University of Maringá (Universidade Estadual de Maringá – UEM, protocol no. 081/2012).

2.1. Experimental design

Thirty-five male Wistar rats (*Rattus norvegicus*) 60 days of age weighing 269.63 ± 7.90 g were used. The animals were randomly assigned to five groups ($n = 7$). The control group (CG) received sterile saline solution (0.9% NaCl), and the infected groups received gavage suspensions containing 50 (G50), 500 (G500), 1000 (G1000) and 5000 (G5000) sporulated ME-49 strain of *T. gondii* (genotype II) oocysts. The oocysts were obtained from the veterinary parasitology laboratory of the State University of Londrina (Universidade Estadual de Londrina-UEL), Paraná, Brazil, and were sporulated in sulfuric acid solution prior to inoculation to render them viable [8]. The rats were kept in individual cages under a 12-h light and dark cycle (6 am–6 pm) and controlled temperature (24 °C ± 2 °C) and were fed rodent chow (Nuvilab, Colombo, PR, Brazil) and water ad libitum. Blood was collected from the animals before and after infection through the retro-orbital plexus puncture for the detection of IgG anti-*T. gondii* antibodies by direct agglutination; titers > 1:25 were considered positive [9]. In addition, chronic infection was confirmed by the qualitative presence of brain cysts. In this sense, the brains of the rats were macerated with a mortar and pestle in 1000 µL phosphate-buffered saline (PBS, pH 7.2). A 25-µL aliquot was placed between the slide and the coverslip, and the preparation was analyzed for the presence of cysts under a microscope at 200 × magnification [10].

2.2. Euthanasia and collection of biological material

Thirty days post-infection (dpi), the rats were euthanized by deep anesthesia with halothane vapor (Tanohalo®, Cristália, Itapira, SP, Brazil) [11]. After vertical laparotomy the jejunum was removed, and its length and diameter were measured.

2.3. Quantification of blood and fecal leukocytes (LEs)

2.3.1. Quantification of blood LEs

Total LEs were counted by diluting 20 µL of blood from each animal in 380 µL of Turk's fluid. After erythrocytes hemolysis, a mirrored Neubauer chamber was filled, and the LEs present in the four lateral quadrants were counted. The number of cells found was multiplied by the correction factor to obtain the total LEs per mm^3 of blood. Blood smears were stained using the May-Grünwald Giemsa technique to count the relative (per 100 LEs) and absolute numbers of polymorphonuclear (PMN) and mononuclear (MN) LEs.

2.3.2. Quantification of fecal LEs

Feces were collected from all the rats on 0, 7, 14, 21 and 30 dpi. A pellet of feces was suspended in 1 mL of 0.9% NaCl. Smears were dried and then stained with May-Grünwald Giemsa. LEs were counted in 100 microscopic fields at 1000 × magnification.

2.4. Histochemistry and immunohistochemistry for intestinal wall analysis

Two 2-cm rings of the proximal jejunum of each rat were used. One ring was fixed in Bouin's fluid for 6 h, and the other ring was fixed in 4% buffered paraformaldehyde for 24 h. The segments were then subjected to routine histological processing to obtain semi-serial cross-sections with a 4-µm thickness.

The sections obtained from the material fixed in Bouin's fluid were stained with hematoxylin and eosin (HE) for quantification of intraepithelial lymphocytes (IELs) and measurements of the submucosa, mucosa, height and width of the villi, enterocytes and their respective nuclei, and depth and width of the crypts. The sections fixed in Bouin's fluid were also stained with periodic acid-Schiff (PAS) for the detection of neutral mucins and labile sialomucin, with Alcian blue (AB, pH 2.5) for sialomucins and sulfomucins and AB (pH 1.0) for sulfomucins analysis [12], and with AZAN trichrome stain for the determination of collagen fibers.

The sections obtained from material fixed in 4% buffered paraformaldehyde were stained with HE for the quantification of Paneth cells. The immunohistochemical technique was also applied on these sections to label mitotic cells (by proliferating cell nuclear antigen - PCNA), immunoreactive serotoninergic enteroendocrine cells (by 5-hydroxytryptamine immunoreactivity - 5-HT/IR) and mast cells [13] by using anti-serotonin antibody produced in rabbit (Sigma-Aldrich).

2.4.1. Morphometric analysis of the jejunal wall

2.4.1.1. Morphometry of the mucosa and submucosa. Images were captured from the HE-stained sections using an Olympus BX50 optical microscope coupled to a digital Pro series 3CCD camera and were processed using Image-Pro Plus software version 4.5.0.29 (Media Cybernetics, Silver Spring, MD, USA). Images captured using a 4 × objective were used to measure the mucosa, the height and width of the villi, and the depth and width of the crypts, images captured using a 20 × objective were used to measure the submucosa, and images captured using a 100 × objective were used to analyze the enterocytes and their respective nuclei. A total of 100 measurements were taken for each parameter.

2.4.2. Analysis of jejunal epithelial cells

2.4.2.1. Quantitative analysis of goblet cells and IELs. The goblet cells and IELs present among 2500 consecutive epithelial cells were counted. The proportions of goblet cells and IELs/100 epithelial cells were calculated.

2.4.2.2. Quantitative analysis of paneth cells. The segments were divided into four quadrants, and Paneth cells in the bottom of 64 crypts were counted.

2.4.2.3. Quantification of proliferation cells (PCNA), serotonergic Enteroendocrine (5HT/IR) and mast cells. Five-micrometer-thick sections were dewaxed, rehydrated and blocked against endogenous peroxidase activity with a solution of 3% hydrogen peroxide (H_2O_2) diluted in methanol. The slides were then washed in 0.01 M PBS (pH 7.4) and incubated with 10% non-immune goat serum blocking solution (Reagent A, Histostain-Plus Kit, Invitrogen®, Carlsbad, CA, USA) for 10 min. After blocking, individual sections for each technique were incubated with primary antibodies for PCNA and serotonin (1:500) for 12 h at room temperature. Subsequently, two washes of 5 min each in PBS were performed, followed by incubation for 2 h with the biotinylated secondary antibody (Reagent B, Histostain-Plus Kit, Invitrogen®, Carlsbad, CA, USA). The sections were washed twice for 5 min and incubated with streptavidin-peroxidase enzyme conjugate for 10 min (Reagent C, Histostain-Plus Kit, Invitrogen®, Carlsbad, CA,