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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - SHORT COMMUNICATION

5-HT_{3A} serotonin receptor in the gastrointestinal tract: the link between immune system and enteric nervous system in the digestive form of Chagas disease

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Abstract
Chagas disease is caused by *Trypanosoma cruzi* and remains one of the most neglected diseases in Latin America. One of its clinical forms is Chagas megacolon. Despite being known for more than half a century, detailed causes are still obscure. Recent evidence indicates a close relationship between the immune system and the enteric nervous system in the etiology of chagasic megacolon pathology. It is believed that low expression of the 5-HT_{3A} serotonin receptor on lymphocytes could be linked to megacolon development. To test this hypothesis, this work investigated the distribution of CD4, CD8, and CD20 lymphocytes and their 5-HT_{3A} receptor expression. The results demonstrated that Chagas patients without megacolon present a higher expression of the 5-HT_{3A} receptor in all analyzed lymphocytes compared with Chagas patients with megacolon. These data suggest that the high expression of this receptor may lead to immunomodulation and prevent the development of Chagas megacolon.

Keywords Chagas disease · Megacolon · Enteric nervous system · 5-HT_{3A} · Lymphocytes

Introduction

Chagas disease is caused by the intracellular protozoan *Trypanosoma cruzi*. The infection is characterized by an acute phase with high parasitemia and a chronic phase where an effective immune response is established. Chronic form of the disease affects the heart and gastrointestinal tract in approximately 30% of infected patients. In the gastrointestinal tract, disorders of enteric innervation lead to the development of intestinal constipation and colon dilatation, known as megacolon (Koberle 1968). When analyzing histological samples of Chagas patients, tissues with megacolon present a high degree of inflammation, destruction of neurons and neuronal fibers, fibrosis, and enlargement of the lumen, whereas non-carrier patients of megacolon present practically the same histological architecture as uninfected individuals (Tafuri 1971). Thus, it is curious to consider why are the clinical spectra in the same disease are so divergent? The mechanisms that lead from a functional intestine to a dilated and constipated intestine are still far from understood. Recently, the role of serotonin and its receptors in intestinal pathologies was studied, revealing that serotonin plays a critical role in enteric neurotransmission, in the initiation and propagation of intrinsic enteric reflexes, and in communication between the intestine and the brain (Johnston et al. 2014). It is believed to be one of the major chemical mediators between the enteric nervous system and the immune system (Walstab et al. 2010). In addition, serotonin's 5-HT_{3A} receptor might have direct action on the immune system with anti-inflammatory effect of several pathologies. Thus, the objective of this study was to evaluate the expression of the primary receptor of serotonin in the

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intestine, $5HT_{3A}$ receptor, and to relate it to the presence of CD_4-IR (immunoreactive) T lymphocytes, CD_8-IR T lymphocytes, and CD_{20}-IR B lymphocytes in the colon of Chagas patients. Furthermore, we also evaluated the presence of fibrosis in tissue samples with Gomori trichrome staining and neuron expression through the pan-neuronal marker, Peripherin.

Materials and methods

Samples of the colon wall obtained from carriers and non-carriers of chagasic megacolon (15 samples in each group) and non-chagasic individuals (15 samples) were submitted to necropsy or surgical procedures at Universidade Federal de Goiás (Goiânia, Minas Gerais, Brazil). Chagas patients with megacolon presented similar clinical conditions. Non-infected patients presented no inflammation or gastrointestinal symptoms, and no patients received any parasite- specific treatment. Informed consent was obtained from the patient or family members prior to tissue procurement, and this work was approved by Universidade Federal de Uberlândia Research Ethics Committee (ETIC nº 110/11) according to Guidelines and Norms for Research Involving Human Beings - Resolution CNS 466/2012 - Brazil. Tissue samples were collected from the rectosigmoid region and fixed in 4% neutral buffered formaldehyde solution. In order to visualize the tissue fibrosis process, the samples were embedded in paraffin, sectioned at 7 μm, and submitted to Gomori trichrome staining.

For the immunohistochemical reactions, the anti-Peripherin antibody technique was utilized (Santa Cruz Biotechnology, SC-7604, Germany, 1:100), anti-$5HT_{3A}$ receptor (ACRIS, TA302602, Germany, 1:100), anti-CD_4 (ACRIS, UM800010, Germany, 1: 100), anti-CD_{20} (ACRIS, UM800001, Germany, 1:100), and anti-CD_8 (ACRIS, AM05901PU-S, Germany, 1:100).

For immunohistochemistry, tissue sections were first incubated in 10% normal donkey serum (NDS) plus 1% Triton X-100 for 1 h. Incubation with primary antibodies was carried out for 24 h at 4 °C with diluted antiserum containing 10% NDS. Following incubation in primary antiserum, preparations were rinsed in PBS (3 × 10 min) and then incubated for 1 h at room temperature with secondary antibodies (Table 1). Further 3 × 10-min washes in PBS were done before tissue was mounted in fluorescence medium. Analysis was performed considering the specifically labeled area of at least 10 neuronal ganglia per patient in both the submucosal and the myenteric plexuses. Prior to incubation, tissues were viewed with a laser scanning microscope (Nikon Eclipse E1000-M, Tokyo, Japan) equipped with a confocal system (Nikon Digital Eclipse C1) with three channels (laser configuration: 488 nm argon laser, 543 nm helium-neon laser [both from Melles Griot, Carlsbad, CA, USA], 638 nm diode laser [Coherent, Santa Clara, CA, USA]). A × 20 dry objective lens (numerical aperture, 0.75) was used with the zoom factor set to 2.0 in all scanning sessions. Pinhole and gain were adjusted equally in all negative control sessions and values were noted. The images were created using three different excitation wave lengths and the figure plates were prepared using the EZ-C1 FreeViewer program (Gold Version 3.30 build 647) from the Nikon Corporation.

Table 1 Secondary antibodies

Antibody	Company	Dilution
donkey anti-goat **ALEXA 488**	Molecular Probes	1:1000 μl
donkey anti-mouse **ALEXA 555**	Molecular Probes	1:1000 μl
donkey anti-rabbit **ALEXA 647**	Molecular Probes	1:1000 μl

Sections through ganglia were selected randomly in a meandering fashion until a total of 3 mm^2 were analyzed in each ganglionated plexus. Single optical section images on the same focus plane were created in the ganglia by applying three different excitation wavelengths (488 nm argon laser, 543 nm helium-neon laser, and 638 nm diode laser) Pictures were prepared using Confocal Assistant 4.02 and CorelDraw 13. For area analysis of reactive co-stimulation molecules, the pictures were analyzed individually and merged, and the positive area was counted in square micrometers. Statistical analysis was performed by the Anova one-way analysis of variance test. Differences were considered statistically significant at $p < 0.05$.

Results

Evaluation of colon innervation was performed by measurement of Peripherin immune-reactive area in tissue samples. Qualitative and quantitative analyses were executed in non-infected individuals and patients with Chagas disease. Non-infected individuals presented preserved neuronal ganglia, with typical shaped neuronal bodies and no sign of inflammatory process, while Chagas patients showed deformed ganglia and an increased number of neuronal bodies. The statistical analyses exhibited a significantly reduced quantity of neurons and neuronal filaments in the Peripherin immunoreactive area when compared to the non-infected group.

Analyses of fibrosis areas in the muscle layers demonstrated that Chagas patients with megacolon presented a diffuse increase in endomysial and perimysial connective tissue and frequent foci of fibrosis. Fibrous connective tissue was rare in the non-infected individuals group and was represented only by endomysial or perimysial connective tissue. In Chagas patients without megacolon, the appearance of the connective tissue in the muscle layers was similar to that observed in non-infected individuals. Statistical analyses demonstrated that the fibrous connective tissue area relative to the total

examined tissue area was greater in the Chagas patients with megacolon compared to Chagas patients without megacolon and the non-infected individuals. There was no statistically significant difference between non-infected individuals and the Chagas patients without megacolon. The pattern of all antibodies and coloration used are shown in Fig. 1.

Analysis of the results demonstrated significant differences between the distribution of the classes of lymphocytes and the distribution of the 5-HT_{3A} receptors in analyzed groups. The distribution of CD4 lymphocytes presented a similar pattern and numbers of analogous cells in non-infected individuals, Chagas patients without megacolon and Chagas patients with

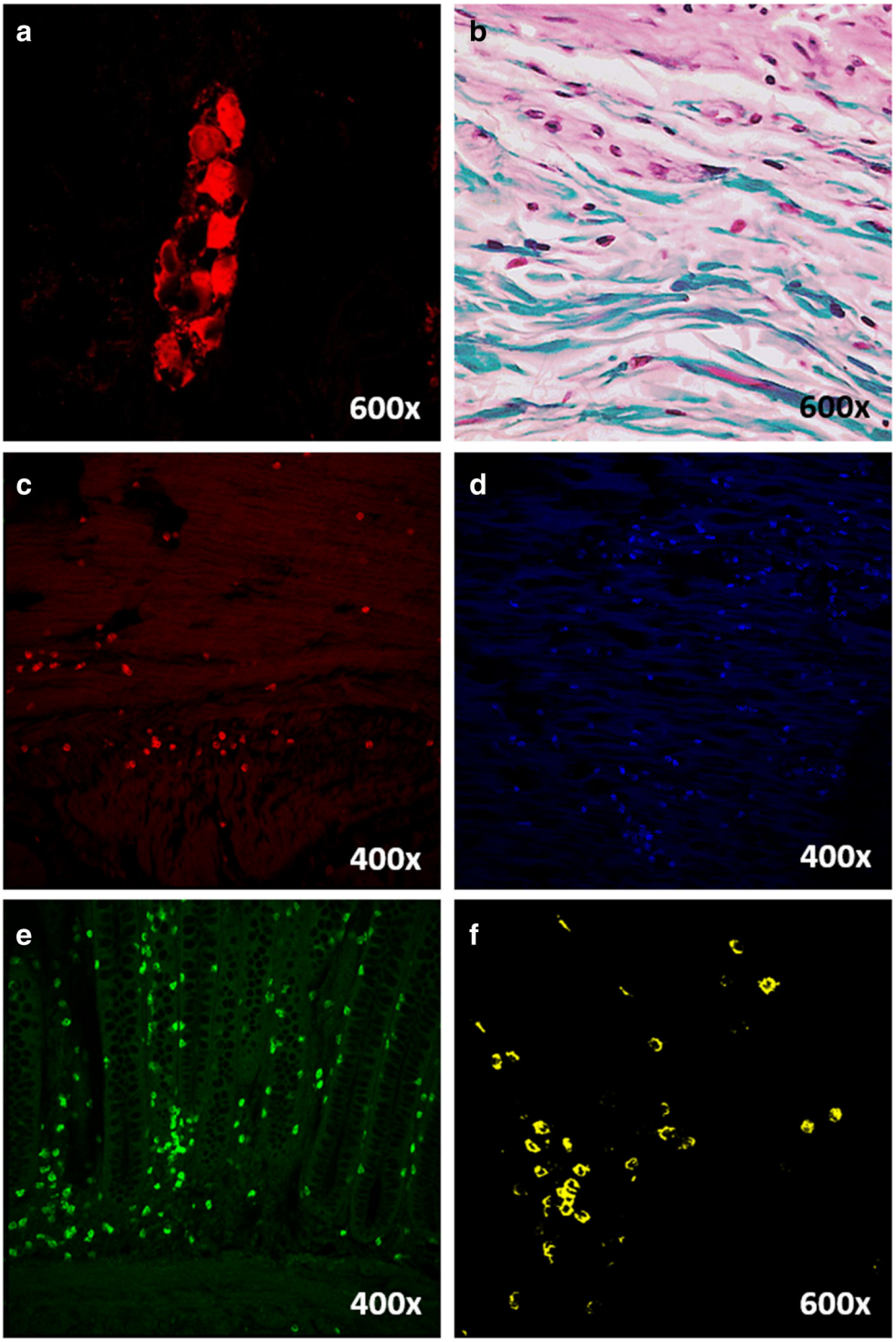

Fig. 1 Pattern of all antibodies and coloration used in this study. **a** Peripherin label showing neuronal ganglia in myenteric plexus. **b** Gomori trichrome demonstrating fibrosis (blue) in inner muscular of colon. **c** Anti-CD4 antibody presenting lymphocytes CD4 distribution (red). **d** Anti-CD8 antibody (blue) and **e** Anti-CD20 antibody (green). **f** Anti-5-HT_{3A} receptor distribution in colon sample

Table 2 Distribution of CD4, CDS, and CD2O lymphocytes and the 5-HT_{3A} receptor in the colon samples from chagasic patients and non-infected individuals

Patients	CD4	$5Ht_{3A}$	CD8	$5Ht_{3A}$	CD2O	$5H^{-}1_{3A}$
Non-infected individuals	26 ± 4	102 ± 9	28 ± 3	65 ± 5	33 ± 2	69 ± 5
Chagasic patients without megacolon	30 ± 5	163 ± 12[b]	32 ± 5	112 ± 9[b]	35 ± 4	144 ± 10[b]
Chagasic patients with megacolon	29 ± 5	98 ± 8	64 + 7[a]	73 ± 5	70 + 7[a]	64 + 5

[a] Statistically significant difference between Chagas patients with megacolon and the other groups

[b] Statistically significant difference between Chagas patients without megacolon and the other groups

Data presented positive area in square micrometer. All statistical analyzes were performed with using one-way analysis of variance ($p < 0.05$)

megacolon groups. The presence of CD8 and CD20 lymphocytes was significantly higher in Chagas patients in the megacolon group compared to Chagas patients without megacolon and non-infected individuals groups. The distribution of serotonin 5-HT_{3A} receptor in all analyzed lymphocytes was more pronounced in Chagas patients without megacolon than in non-infected individuals and Chagas patients with megacolon (Table 2).

Discussion

Megacolon is the most common alteration in the digestive tract caused by Chagas disease. Currently, denervation is accepted as one of the main causes of megacolon development. Neuronal destruction in the acute phase occurs due to parasite concentration in the tissue, but in the chronic phase it is also related to the inflammatory process observed in this phase of Chagas disease. However, the destruction process and the enteric nervous system response to this inflammatory process are still obscure. Results presented in this study were in agreement with previous studies that characterize the denervation process in Chagas patients, as well as the presence of fibrosis in the muscular layers of the colon of these patients.

Furthermore, previous reports indicated the value to investigate the role of serotonin and its 5-HT_{3A} receptor in the immune system of the gastrointestinal tract (Cirillo et al. 2011; Simonin et al. 2012). In recent years, serotonin has been identified as one of the main articulators between the immune system and the enteric nervous system. Its absence has been related to pathological alterations such as intestinal constipation, inflammatory bowel diseases, and more severe forms of Chagas disease (de Freitas et al. 2015; Freitas et al. 2017; Sikander et al. 2009). However, the manner by which serotonin functions as a mediator of the inflammatory process is still unclear. In an attempt to elucidate some of these questions, this study aimed to evaluate the presence of the 5-HT_{3A} receptor in the main lymphocyte classes of colon samples from Chagas patients that did not have megacolon. Results presented here suggest the possibility that serotonin acts in the immune system through the 5-HT_{3A} receptor located in lymphocytes, which would lead to their inactivation and thus suppressing the exacerbation of the inflammatory process commonly observed in the Chagas megacolon. In addition, Chagas patients that do not develop megacolon appear to express a greater number of these serotonin receptors, which, in addition to preserving intestinal motility, could also be one of the causes of lower numbers of inflammatory cells in their tissues. From these results, a new direction of research on inflammatory bowel diseases is proposed with the intention of identifying targets that will promote homeostasis between the immune systems and the enteric nervous system**.** Among the possibilities, testing whether serotonin and its 5-HT_{3A} receptors could be major candidates as targets for new therapies.

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