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Acute *Strongyloides venezuelensis* infection did not prevent EAE development: implications for hygiene hypothesis

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Abstract. Prevalence of allergic and autoimmune pathologies is clearly increasing in developed countries. This has been attributed to a decreased exposure to certain microorganisms and been referred as hygiene hypothesis. In this study we evaluated if a previous infection with *Strongyloides venezuelensis* would alter the progression of experimental autoimmune encephalomyelitis (EAE) in Lewis rats. Animals were initially infected with 4000 L3 infective larvae of *S. venezuelensis* by subcutaneous route. Encephalomyelitis was then induced during the acute phase of the infection by immunization with myelin basic protein emulsified with Complete Freund's Adjuvant plus *Mycobacterium butyricum*. Previous infection downmodulated cytokine production but did not change clinical and histopathological EAE manifestations. Cytometric analysis with antibodies specific for CD4+CD25+Foxp3+ regulatory T cells indicated that infection also did not alter the frequency of these cells in spleen and regional lymph nodes. This finding could partly explain the failure of this worm to avoid EAE progression. Altogether these results demonstrated that infection with *S. venezuelensis* was not able to modify EAE progression in Lewis rats. In the context of the hygiene hypothesis, these results reinforce the necessity of a comparative study among different helminth species to identify the ones with immunoregulatory competence.

INTRODUCTION

A steady increase in the incidence of autoimmune, allergic and inflammatory diseases has been reported in the most developed countries (Sawczenko *et al.*, 2001; Back, 2002). This higher incidence has been attributed, in some degree, to a lack of contact between human beings and certain environmental agents and has been denominated hygiene hypothesis. According to this hypothesis, organisms as lactobacillus, mycobacteria and helminths are endowed with immunoregulatory properties that are able to regulate immune-mediated diseases (Manuel *et al.*, 2012; Versini *et al.*, 2015). At least three immunoregulatory mechanisms have been described to be triggered by these agents as polarization of immune response towards Th2 (Zheng *et al.*, 2008), induction of regulatory T cells (Zaccone *et al.*, 2009) and more recently, induction of alternatively activated macrophages (Espinoza-Jiménez *et al.*, 2010).

This theory has been supported by both, epidemiological and experimental data (Okada *et al.*, 2010) being multiple sclerosis

(MS) one of the autoimmune diseases that seem to be restrained by contact with these environmental agents. MS affects more than 2 million people worldwide (Browne *et al.*, 2014) by seriously compromising motor and sensory function through demyelination and axonal loss. Even though Th1 cells have been classically described as the main responsible for the central nervous system (CNS) destruction, emerging data suggest that Th17 cells also contribute to CNS autoimmunity in both, MS and the corresponding experimental autoimmune encephalomyelitis (EAE) model (El-behi *et al.*, 2010).

Epidemiological data is suggestive of a protective effect of helminth infections on MS (Sewell *et al.*, 2003). This preventive effect of helminths in MS was reinforced by experimental studies. For example, *Trichinella spirallis* infection and immunization with *Schistosoma* antigens were able to reduce EAE severity in Dark-Agouti rats and C57BL/6 mice, respectively (Sewell *et al.*, 2003; Gruden-Movsesijan *et al.*, 2008; Zheng *et al.*, 2008). In addition, an observational study indicated that parasite-infected MS patients showed a significantly lower number of relapses, more discrete changes in disability scores and also significantly lower magnetic resonance imaging activity. Interestingly, these improved clinical manifestations were related to increased frequency of CD4+CD25+Foxp3+ T cells (Correale & Farez, 2007).

One of the most common helminths found in the human population is *Strongyloides stercoralis*. The estimated number of people colonized with this worm is around 100 million (Genta, 1989; Siddiqui & Berk, 2003). This helminth may also lead to hyperinfection syndrome mainly in immunocompromised patients (Azira & Zeehaida, 2010). Much of the knowledge related to this parasite was obtained from rodent experimental infections with *S. venezuelensis* (Marra *et al.*, 2010; Marra *et al.*, 2011). The larvae of this parasite penetrate into the skin and reach the lungs where they develop to the fourth stage larvae. Then larvae migrate to the small intestine were they become adult parasites (Tindall & Wilson, 1988; Marra *et al.*, 2011).

In this context, this study was designed to evaluate the effect of a previous infection with *S. venezuelensis* on the development of experimental autoimmune encephalomyelitis.

MATERIAL & METHODS

Animals

Female Lewis rats weighing 110-130 g and with 4-6 weeks old were purchased from the CEMIB (UNICAMP, Campinas, SP, Brazil). The animals received sterilized food and water *ad libitum* and were manipulated in compliance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation, being the experimental protocol approved by the local Ethics Committee (protocol 607).

Experimental design

Rats were allocated into two groups (5-6 animals per group): EAE (non-infected rats with EAE) and EAE + *S. venezuelensis* (infected rats with EAE). At the 8th day after *S. venezuelensis* third-stage larvae (L3) infection (acute phase), the animals were submitted to EAE induction by immunization with myelin basic protein (MBP). Animals were daily evaluated for weight loss and clinical score and euthanized during EAE recovery phase (20th day) to assess the immune response and CNS inflammatory infiltration. Cytokine production and specific antibody levels (IgG1 and IgG2b anti-*S. venezuelensis*) were determined by ELISA. The frequency and absolute number of CD4+CD25+Foxp3+ regulatory T cells in regional lymph nodes and spleen were analyzed during the acute phase of the infection, that is, at the 8th day.

Parasite and infection

S. venezuelensis strain employed in this study was isolated from wild rats in 1980. It has been maintained in Wistar rats, routinely infected at the Parasitology Department of the Univ. Estadual Paulista (UNESP). For experimental infections, infective third-stage larvae (L3) of *S. venezuelensis* were obtained from faecal cultures using

sterilized horse manure as substrate. The cultures were incubated at 25°C for 72 h and L3 were collected and concentrated by using a Baermann apparatus. Recovered larvae were washed in phosphate-buffered saline (PBS), the number of viable infective larvae was estimated under optical microscopy and 4000 L3 were subcutaneously inoculated in each animal.

EAE induction and evaluation

Rats were immunized with 25 µg of MBP (Sigma Aldrich, St. Louis, MO, USA) emulsified with Complete Freund's Adjuvant (CFA) (Sigma Aldrich) containing 5 mg/mL of *Mycobacterium butyricum* (Difco, Detroit, MI, USA). Animals were injected in the hind left footpad with 50 µg of the emulsion and daily evaluated for body weight loss and clinical score. Signs of disease were graded as 0 (zero): no disease; 1: loss of tonicity in the distal portion of the tail; 2: total loss of tail tonicity; 3: hind limb weakness (partial paralysis); 4: complete hind limb paralysis and urinary incontinence and 5: moribund.

Quantification of inflammatory infiltrates

The histological analysis was performed during EAE recovery phase, i.e., 20 days after immunization with MBP. After euthanasia, brain and lumbar spinal cord samples were removed and fixed in a 10% solution of buffered formalin. Paraffin slides with 4-5 µg, were routinely stained with hematoxylin and eosin and analyzed with a Nikon microscope. Quantitative evaluation of perivascular inflammatory infiltrates was performed in a computerized system for image analysis (Qwin Lite 3.1, Leica Microsystems, Wetzlar, Germany). The total section area of each brain and lumbar spinal cord was measured to avoid any inter-animal variance. Further, perivascular mononuclear infiltrated areas of whole sections were assessed by point-counting morphometry, as described elsewhere (Bock *et al.*, 2003). The values were expressed as μm^2 of mononuclear infiltrate per mm^2 of organ section (μm^2/mm^2).

Anti-S. venezuelensis antibody levels

Serum samples were collected at the recovery EAE phase and tested by ELISA for the presence of antibodies against *S. venezuelensis* antigen, which was prepared according to the procedure described by Negrão-Corrêa *et al.* (2004). Briefly, infective larvae (L3) were extensively washed with PBS and resuspended in RPMI containing a mixture of protease inhibitors (Protease Inhibitor Cocktail Tablets, Roche, Boehringer Mannheim, Indianapolis, IN). The larvae were mixed with glass beads, submitted to vortexing (5 cycles of 1 min each) and then subjected to sonication (10 cycles of 1 min each immersed in an ice bath). The insoluble material was removed by centrifugation and protein concentration was determined by BCA method – bicinchoninic acid, using a commercial kit (Bicinchoninic Acid Kit for Protein determination, Sigma). Plates were coated with 100 µg/mL of L3 antigen in coating solution (Na_2CO_3/ $NaHCO_3$; pH 9.6) overnight at 4ºC. Non-specific antibody binding was blocked by incubation with 0.05% Tween 20, 10% fetal calf serum in PBS (200 µL per well) for 1 h at 37°C. Subsequently the plates were incubated overnight at 4°C with dilutions (1:10) of rat serum samples. For detection of specific serum IgG1 and IgG2b, the plates were incubated with class specific biotinylated mouse anti-rat antibodies (Oxford Biotechnology, Oxford, UK). Plates were then incubated for 30 min at room temperature with StreptAB (kit from Dako, Carpinteria, CA, USA) and revealed by adding H_2O_2 + OPD (Sigma). Color development was stopped with H_2SO_4 and optical density was measured at 492 nm.

IFN-γ and IL-10 production

Lymph node (popliteal + inguinal) cells were collected and adjusted to $2.5x10^6$ cells/mL. Cells were cultured in complete RPMI medium (RPMI supplemented with 10% of fetal calf serum, 2 mM of L-glutamine and 40 mg/L of gentamicin), in the presence of 10 µg/mL of MBP or 5 µg/mL of concanavalin

A (ConA, Sigma). Cytokine levels were evaluated 72 h later in culture supernatants by ELISA according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Sensitivity of ELISA for IFN-γ and IL-10 was 19 and 31 pg/mL, respectively.

Frequency and absolute number of CD4+CD25+Foxp3+ regulatory T cells

Spleen and lymph node (popliteal + inguinal) cells were collected and the red blood cells were lysed with Hank's buffer containing NH_4Cl. The cell suspension was washed once in RPMI 1640 and adjusted to $2.5x10^6$ cells. Cells were then incubated with 0.5 µg of fluorescein isothiocianate anti-rat CD4 (clone OX35) and 0.25 µg of allophycocyanin anti-rat CD25 (clone OX39) for 20 min at room temperature. The staining for Foxp3 was then performed by using the phycoerythrin anti-mouse/rat Foxp3 Staining Set (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. After incubation, the cells were fixed in paraformaldehyde 1%. The cells were analyzed by flow cytometry using the FACSCanto II (Becton Dickinson, San Jose, CA) and FlowJo software (TreeStar, Ashland, OR, EUA).

Statistical analysis

Data were expressed as mean ± SE. Comparisons were made by Student's t test or one way ANOVA with post-hoc Holm-Sidak test. Significance level was $p<0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc., Bedford, UK).

RESULTS

S. venezuelensis infection triggers a Th2 polarized response in Lewis rats

Infection with *S. venezuelensis* determined an acute infection that was identified by the highest number of eggs in faecal samples at the 7th day of infection (not shown). Recovery occurred around the 21st day of infection and was associated with a significant production of IgG1, but not IgG2b, specific antibodies (Figure 1a). The amount of regulatory CD4+CD25+Foxp3+ T cells that was assessed in spleen and lymph nodes at the acute phase of the infection was not modified by the previous infection with *S. venezuelensis* (Figures 1b and 1c).

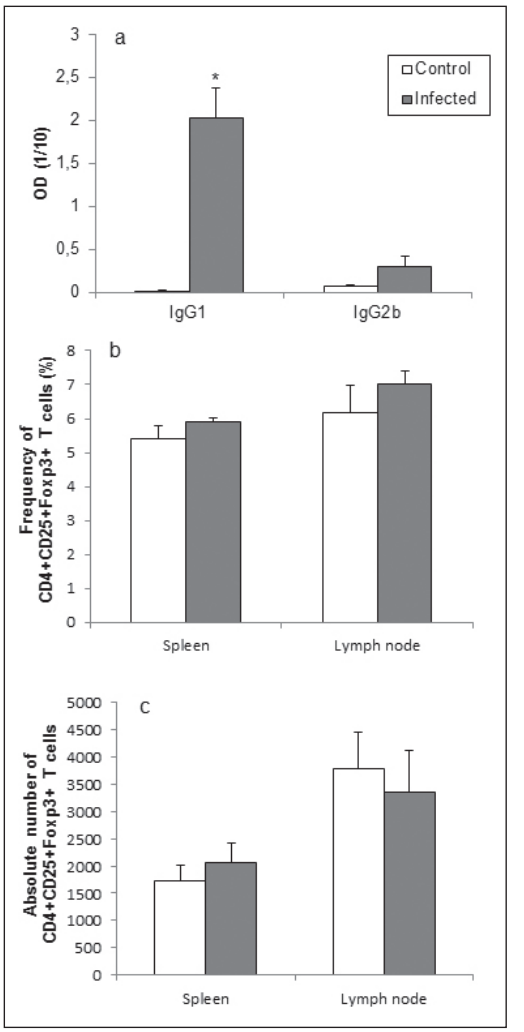

Figure 1. Infection of Lewis rats with *S. venezuelensis*: antibody production and effect on regulatory T cells.

Serum levels of anti-*S. venezuelensis* IgG1 and IgG2b antibodies (a), percentage (b) and absolute number (c) of CD4+CD25+Foxp3+ regulatory T cells in lymph nodes (popliteal and inguinal) and spleen cells. Data were presented by mean±SE of 5 rats. *$p<0.05$.

Clinical and histopathological EAE parameters are not modified by previous infection with S. venezuelensis

As expected, Lewis rats immunized with MBP associated with CFA developed encephalomyelitis characterized by significant weight loss and elevated clinical scores, as illustrated in Figures 2a and 2b, respectively. These animals also presented accentuated inflammatory infiltrates in both, brain and lumbar spinal cord (Figure 2c). Prior infection of the rats with L3 did not affect these clinical and histopathological parameters as can be observed in Figures 2a and 2c. The morphometric analysis showed in Figure 2d indicates that the intensity of inflammation was similar in EAE and infected+EAE groups.

Production of IFN-γ and IL-10 induced by MBP is downregulated by previous infection with S. venezuelensis

The production of IFN-γ and IL-10 by lymph node cell cultures stimulated with MBP was significantly downmodulated by previous helminthic infection as illustrated in Figures 3a and 3c, respectively. On the other hand, the levels of these cytokines were not altered in cell cultures stimulated with ConA (Figures 3b and 3d).

DISCUSSION

In this investigation we evaluated if a previous infection with *S. venezuelensis* would interfere with development of EAE in Lewis rats. This approach is relevant because *S. stercoralis* is one of the most prevalent parasites in human population and also because helminths are considered masterful immunoregulators (Vadlamudi *et al.*, 2006; Hewitson *et al.*, 2009; Montes *et al.*, 2010). In this context, female Lewis rats were infected with *S. venezuelensis* and then, at the acute phase of the infection, they were subjected to EAE induction. The number of eggs in the feces indicated the establishment of an acute infection one week after infection, as we already described before (Chiuso-Minicucci *et al.*, 2010). Rats immunized with MBP associated with CFA developed the expected characteristics of encephalomyelitis as weight loss, paralysis and the presence of

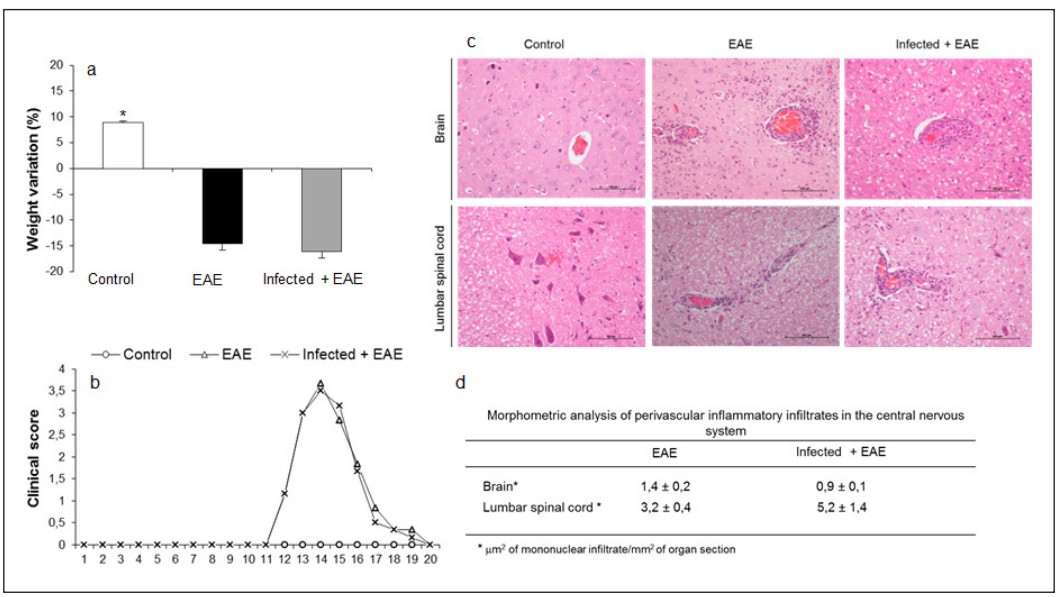

Figure 2. Effect of previous infection with *S. venezuelensis* on EAE development.
Female Lewis rats were infected with *S. venezuelensis* and EAE was induced during the acute phase (8th day) of the infection. Weight variation (a), clinical score (b), histopathological analysis (c) and morphometric analysis (d) of brain and lumbar spinal cord were evaluated 20 days after EAE induction. Data were presented by mean±SE of 5-6 rats. *p<0.05.

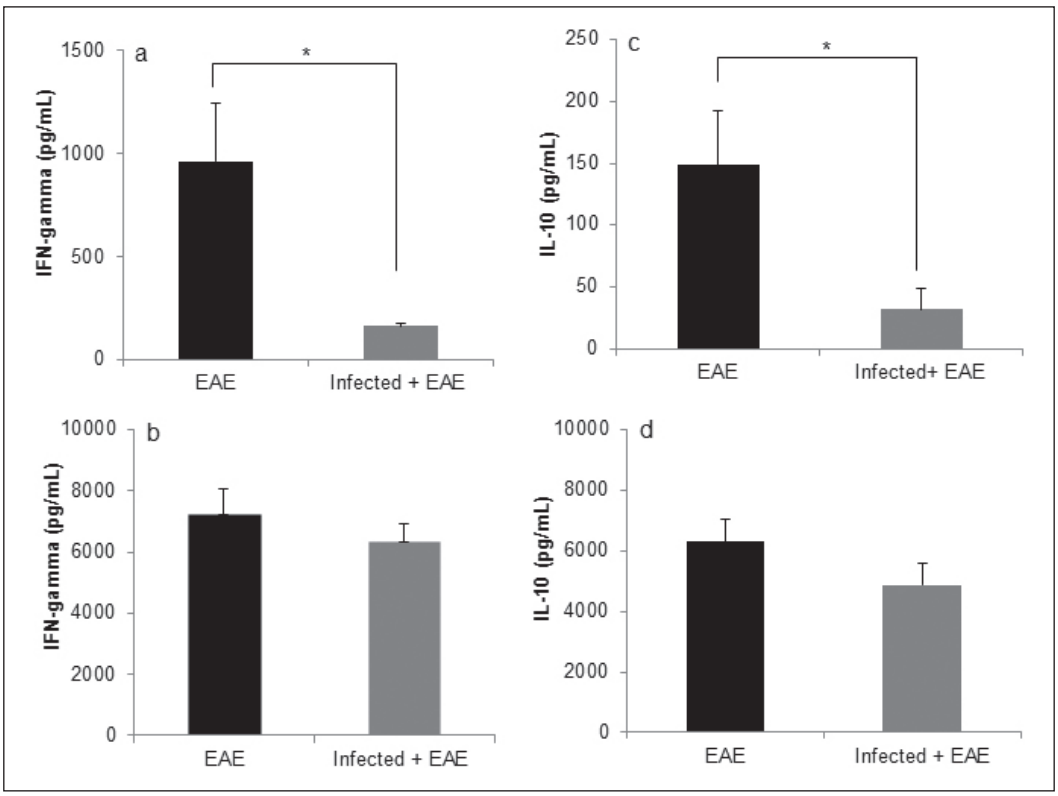

Figure 3. Effect of previous infection with *S. venezuelensison* on IFN-γ and IL-10 production. Female Lewis rats were infected with *S. venezuelensis* and EAE was induced during the acute phase (8th day) of the infection. Cytokine production was assayed 20 days after EAE induction. IFN-γ and IL-10 production by lymph node cells stimulated with MBP (a and c) or ConA (b and d). Data were presented by mean±SE of 5-6 rats. *p<0.05.

accentuated inflammatory infiltrates in both, brain and spinal cord as previously observed (Zorzella-Pezavento *et al.*, 2010). Contrarily to our initial hypothesis, previous infection with *S. venezuelensis* did not downmodulated EAE development. The prior contact with the worm was not able to alter the encephalomyelitis, neither clinically nor histopathologically. Similar weight losses and clinical scores were present in both experimental groups. In addition, a quantitative analysis of inflammatory infiltrates in brain and lumbar spinal cord sections indicated a comparable degree of inflammation in both groups.

In a previous article, by employing a model of multiple infections that would, allegedly, mimicry better the constant contact with the worm that happens in endemic areas, we already demonstrated this failure of *S. venezuelensis* to downmodulate EAE development (Chiuso-Minicucci *et al.*, 2011). With the acute infection model, used in this investigation, we expected to optimize any possible immunoregulatory potential of this helminth. Nonetheless, this approach was also not able to delay or reduce EAE severity. Even though the helminthic infection was clearly established and able to downmodulate cytokine production induced by MBP, it was incapable to trigger expansion of regulatory T cells. One possible explanation for this failure to reduce EAE development would be, therefore, the inability of this parasite to increase the number of this regulatory cell subset. At first view, this finding seems contradictory to the available literature. Several murine and human studies showed expansion of this T cell subset during ongoing

helminth infections (Babu *et al.*, 2006; Beiting *et al.*, 2007; Blankenhaus *et al.*, 2011). Association between higher parasite survival and regulatory T cell induction has been attributed to interference in the genesis of a protective immune response (Taylor *et al.*, 2007). In this scenario, we could think that *S. venezuelensis*, similarly to other parasites that do not remain in their hosts in a chronic fashion, does not need to stimulate an anti-inflammatory response. Consequently, its immunomodulatory properties, including the effect over regulatory T cells, would be weaker.

We recently confirmed this low immunomodulatory potential of *S. venezuelensis* in another autoimmune condition. Previous infection with this helminth triggered a very discrete protective effect against experimental diabetes induced by streptozotocin in C57BL/6 mice (Peres *et al.*, 2013). A similar outcome was recently described by Ortiz-Flores *et al.* (2013). These authors demonstrated that *Taenia crassiceps* infection does not modify the development of experimental rheumatoid arthritis. On the other hand, this worm reduced the incidence and severity of type 1 diabetes (Espinoza-Jiménez *et al.*, 2010) and EAE (Reyes *et al.*, 2011). Interestingly, we observed that immunization with soluble *S. venezuelensis* antigens emulsified with CFA, which contains mycobacteria, followed by infection with *S. venezuelensis* triggered a significant protective effect against diabetes development (Peres *et al.*, 2013). Altogether, these reports raise important aspects that need further investigation. In this sense we hypothesize that protection mediated by helminths or other environmental agents could be triggered by both, isolated agents or particular associations between them. These weaker or stronger potential for protection against inflammation would depend upon their antigenic composition, life cycles and also their intrinsic interplay with the host immune system. Another aspect to be considered would be the inflammatory disease itself, that is, some immunopathogenetic routes could be more resistant to the immunomodulatory mechanism displayed by certain parasites.

Overall, this investigation indicates that a previous infection with *S. venezuelensis* was not able to modify EAE progression in Lewis rats. We don't see these results as a drawback to the hygiene hypothesis. We rather believe that they reinforce the need for a careful and systematical comparison of the immunoregulatory potential presented by the numerous helminth species.

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