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Previous contact with *Strongyloides venezuelensis* contributed to prevent insulitis in MLD-STZ diabetes

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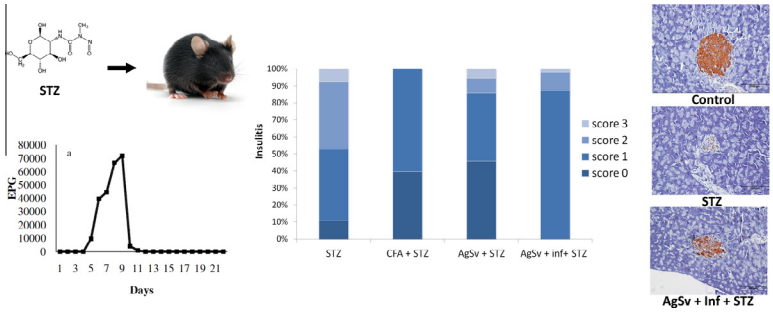
HIGHLIGHTS

- Recovery from *S. venezuelensis* infection is associated with Th2 polarized response.
- Contact with *S. venezuelensis* contributed to prevent insulitis in MLD-STZ diabetes.
- Protection associated with *S. venezuelensis* was linked to IL-5 and IL-10 production.

GRAPHICAL ABSTRACT



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ABSTRACT

Epidemiological and experimental studies support the idea that helminth infections can induce a protective effect against the development of autoimmune and allergic diseases. In this study we characterized the immune response induced by *Strongyloides venezuelensis* infection in C57BL/6 mice and then evaluated the effect of a previous contact with this helminth in the outcome of type 1 diabetes. Animals were initially infected with 2000 L3 larvae from *S. venezuelensis* and euthanized 22 days later. An acute phase, identified by a high amount of eggs per gram of feces, was established between days 7 and 9 post-infection. Recovery from infection was associated with a Th2 polarized response characterized by a significant level of serum IgG1 specific antibodies and also a significant production of IL-5 and IL-10 by spleen cells stimulated with *S. venezuelensis* soluble antigen. Immunization with soluble *S. venezuelensis* antigen associated with complete Freund's adjuvant followed by infection with *S. venezuelensis* protected mice from diabetes development induced by streptozotocin. Protection was characterized by a higher body weight gain, lower glycemic levels, much less severe insulitis and preserved insulin production. Together, these results indicate that *S. venezuelensis* contributed to protect C57BL/6 mice against experimental diabetes induced by streptozotocin.

1. Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disorder associated, in genetically susceptible individuals, with generation and activation of autoreactive T cells that recognize pancreatic β-cell autoantigens. Self-reactive CD_4^+ and CD_8^+ T lymphocytes infiltrate

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the pancreas and selectively destroy the insulin producing β-cells in the islets (Chentoufi et al., 2008). T1D has clearly increased in prevalence over the last several years in developed countries (Shapira et al., 2010). Mice and rats have been widely used as experimental models to investigate the contribution of helminths and other environmental agents to regulate the immune system during allergy and autoimmune diseases. Epidemiological evidences strongly suggest that improved standards of living are associated with an increased incidence of immune system-mediated diseases as allergies and autoimmune pathologies. A theory known as the "hygiene hypothesis" suggests that improved health standards through sanitation and vaccination may in part be responsible for the apparent increase in immune-mediated diseases due to decreased microbiological and parasitic infections in humans, particularly in children (Whary and Fox, 2004). Thus, parasitic infections might somehow shape the immune system to avoid exaggerated inflammatory responses (Cooke et al., 2004; Romani, 2008; Vercelli, 2006). Epidemiological and experimental studies have supported the idea that helminth infections can induce a protective effect against the development of both autoimmune and allergic diseases (Zaccone et al., 2008).

Species of *Strongyloides* are important intestinal parasites of humans and domestic animals (Grove, 1996; Júnior et al., 2006). *S. venezuelensis* is a rodent parasite, usually found in rats and is very useful as a model to study nematode infections (Marra et al., 2010; Maruyama et al., 2006). Infective larvae of *S. venezuelensis* penetrate into the skin and migrate to the lungs where they achieve the fourth stage. Then, these larvae reach the small intestine where they finally become adult parasites (Tindall and Wilson, 1988). Our previous experience with this parasite indicated that it was able, as many other nematodes, to induce a strong Th2 kind of response in Lewis rats (Chiuso-Minicucci et al., 2010). Streptozotocin (STZ) is a nitrosourea antineoplasic agent that exhibits direct pancreatic β-cell cytotoxicity (Rerup, 1970). This substance has been widely used to induce two distinct experimental forms of diabetes. An inflammatory form of diabetes with clinical and immunohistological features similar to those found in human type 1 diabetes, can be induced by injection of multiple low doses of STZ in susceptible strains of mice (Kolb, 1987; Like and Rossini, 1976).

The purpose of this study was to determine if *S. venezuelensis* infection was able to induce a strong Th2 response in C57BL/6 mice as we observed in Lewis rats. In addition, as accentuated Th2 profiles have been associated with immunomodulatory ability, we also tested the effect of this infection on the outcome of experimental autoimmune diabetes.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice were purchased from CEMIB (UNICAMP, Campinas, SP, Brazil). Mice 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 84/08).

2.2. General experimental protocol

Mice were initially infected with *S. venezuelensis* to determine the kinetics of the infection. They were euthanized 22 days later to characterize the immune response. The effect of a previous contact with this helminth on the development of experimental diabetes was then evaluated. This contact was established by immunization with soluble *S. venezuelensis* antigens followed by infection with this worm. Diabetes was induced seven days later by giving STZ during 5 consecutive days by intraperitoneal route. Twenty-one days after last STZ injection, animals were euthanized and diabetes intensity was quantified by increase in body weight, glycemic levels, insulitis score and *in situ* insulin production.

2.3. Immunization and infection with S. venezuelensis

The *S. venezuelensis* strain was isolated from wild rats in 1980. The strain was then maintained in Wistar rats, routinely infected in the Parasitology Laboratory of the Univ. Estadual Paulista (UNESP). For the experimental infections, infective third-stage larvae (L3) of *S. venezuelensis* were obtained from fecal cultures using sterilized horse manure as substrate. The cultures were incubated at 25 °C for 72 h and the infective larvae were collected and concentrated by using a Baermann apparatus. Recovered larvae were washed in phosphate-buffered saline (PBS) and their number was estimated under stereo-microscopy. These L3 were used for both, soluble antigen preparation and infection. To obtain soluble antigen as previously described (Fernandes et al., 2008), larvae were resuspended in RPMI medium containing a protease inhibitor cocktail (Roche Applied Science, Mannnheim, Baden-Wurttemberg, Germany) and disrupted by vortexing with glass beads (five cycles of 1 min each) followed by sonication (10 cycles of 1 min) with a cell sonic disrupter (Vibra Cell Ultrasonic Processor VCX-400). After removing the insoluble particles by centrifuging the larvae homogenate, the recovered supernatant was filtered through a 0.22 μm membrane (Millipore) and aliquots were stored at −80 °C. The protein concentration was determined by bicinchoninic acid assay (Bicinchoninic Acid Kit for protein determination-Sigma, St. Louis, MO, USA). This antigen was used to estimate antibody levels, to stimulate spleen cell cultures *in vitro* and also to immunize the animals. Mice were immunized with 50 μg of L3 antigen in complete Freund's adjuvant (CFA) and one week later they were subcutaneously infected at the abdominal region with 2000 L3 larvae.

2.4. Fecal egg count

Infection intensity was determined by counting the number of eggs per gram of feces (EPG) by a modified Cornell McMaster method (Gordon and Whitlock, 1939). Fecal samples were daily collected until 22 days after infection. Infected animals were allocated in a box and their feces were collected three hours later to determine the number of eggs.

2.5. Parasite-specific antibodies

Serum parasite-specific IgG1 and IgG2a were estimated by ELISA. Briefly, plates (Nunc, Life Tech. Inc., USA) were coated with 100 μg/mL of L3 antigen in coating solution (Na_2CO_3/$NaHCO_3$, pH 9.6), at 4 °C, overnight. Non-specific protein binding was blocked by incubation with 200 μl of 0.05% tween 20, 10% fetal calf serum (FCS) in PBS for 1 h at 37 °C. Subsequently, plates were incubated with serum diluted 1:10 during 1 h at 37 °C. For the detection of specific IgG1 and IgG2a subclasses, the plates were incubated with biotinylated rat antimouse antibodies specific for each isotype (PharMingen, BD Biosciences, USA) for 1 h at 37 °C. Plates were then incubated for 30 min at room temperature with Strept AB (kit from Dako, Carpinteria), and revealed by adding H_2O_2 and o-phenylenediamine (Sigma, USA). Color development was stopped with H_2SO_4 and optical density was measured at 492 nm.

2.6. Cytokine production by spleen cells

Spleen cells were collected and adjusted to 5×10^6 cells/mL. They were then cultured in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine and 40 mg/L of gentamicin, in the