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Antigen, antibody and immune complex detection in serum samples from rats experimentally infected with *Strongyloides venezuelensis*

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ABSTRACT

In order to establish an antigen, antibody and immune complex detection by enzyme-linked immunosorbent assay (ELISA) in serum samples, normal or immunocompromised Wistar rats experimentally infected with *Strongyloides venezuelensis* were used. The microtitre plates were coated with IgG anti-*S. venezuelensis* for antigen and immune complex detection and with alkaline parasite extract for antibody detection. Analysis revealed at least 12.5 μg/mL of *S. venezuelensis* specific antigens in serum samples. Assay for antigen detection was not a good approach for evaluating infection in normal or immunocompromised rats. In normal rats IgG specific for *S. venezuelensis* was preferentially detected during the first 13 days post-infection (p.i.) and immune complex detection was significantly reduced in 21 p.i. day. On the other hand, in immunocompromised rats, IgG and immune complex were detected during the entire kinetic (5, 8, 13 and 21 p.i). These results suggest that immune complex screening seems to be an alternative for early strongyloidiasis diagnosis in immunocompromised individuals.

1. Introduction

Strongyloides stercoralis is an intestinal nematode with worldwide distribution, particularly in tropical and subtropical regions, that infects dogs, cats, monkeys and humans (Grove, 1996). Infection usually results in intestine asymptomatic chronic disease, which can remain undetected for decades (Concha et al., 2005). Due to larvae shedding fluctuations in *S. stercoralis* infected individuals parasitological methods have shown low sensitivity. Thus, repeated stool examinations are necessary (Dreyer et al., 1996; Uparanukraw et al., 1999).

In strongyloidiasis immunological assays, such as enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and Western blotting are useful tools for evaluating host immune response, to diagnose asymptomatic cases and to epidemiological surveys (Lindo et al., 1994; Boscolo et al., 2007; Mota-Ferreira et al., 2009).

Strongyloides venezuelensis, a rodent parasite, has been used as an experimental model to study human infection (Machado et al., 2008; Feliciano et al., 2010; Gonzaga et al., 2011) and to develop innovative procedures for strongyloidiasis diagnosis (Gonçalves et al., 2008, 2010). In experimental infections, infective larvae from *S. venezuelensis* migrate to the lungs before relocating in duodenal mucosa. Thus, this specie migration in the rodent host is similar to that observed during *S. stercoralis* migration in humans (Negrão-Corrêa et al., 2003; Ferreira et al., 2007).

In order to establish an antigen, antibody and immune complex detection system in serum samples, we used normal or immunocompromised Wistar rats experimentally infected with *S. venezuelensis*.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 100–120 g were used in the experiments, fed with laboratory chow, given tap water to drink *ad libitum* and maintained in the animal facilities of the *Centro de Experimentação e Utilização de Animais, UFU*. All experiments were conducted in accordance with animal ethics guidelines and were approved by the *Comitê de Ética na Utilização de Animais* of the *Universidade Federal de Uberlândia* (CEUA 096/10).

2.2. Parasites

S. venezuelensis third-stage infective larvae (L3) were obtained from charcoal cultures of infected rat feces. The cultures were stored at 28 °C for 48 h, and the infective larvae were collected and concentrated using the Rugai method (Rugai et al., 1954).

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The pellet (3 mL) from the conical cup were diluted 10 times in distilled water and larvae were counted using stereomicroscopy. Larvae were quantified according to the following formula: $N \times 10 \times 60$ (N = the number of larvae counted, 10 = dilution factor and 60 = correction factor when using pipettes of 50 μL). For each infection, 1500 *S. venezuelensis* L3 larvae were inoculated subcutaneously in rats abdominal cavity.

2.3. Experimental groups

Animals were divided into four groups: normal and infected rats (n = 24); immunocompromised and infected rats (n = 24); normal control rats (n = 24) and, immunocompromised control rats (n = 24). Six animals were used for each time point (5, 8, 13 and 21 post-infection day [p.i.]). Before infection, immunocompromised groups received 5 μg/mL of dexamethasone disodium phosphate, in water, for 5 days as described previously (Romand et al., 1998), then, animals from infected groups were inoculated subcutaneously in the abdominal cavity with 1500 larvae from *S. venezuelensis* L3.

During each time point, rats were anesthetized with 60 mg/kg ketamine and 7 mg/kg xilazine s.c, and blood samples were collected by cardiac puncture. Blood was then centrifuged, and the serum obtained was stored at −20 °C.

2.4. Control of infection

Eggs/g of feces were counted on days 5, 8, 13 and 21 p.i. in order to certify the animal infection. The parasitological examination was performed three times, and the average of the three results was recorded.

2.5. Alkaline parasite extracts

Alkaline extracts were prepared using ~300,000 *S. venezuelensis* larvae, as previously described (Machado et al., 2003). The protein content of the supernatant was determined by the Lowry method (Lowry et al., 1951).

2.6. Production of immune serum and conjugate

Two rabbits were immunized for anti-*S. venezuelensis* immune serum production. Immunization and anti-*S. venezuelensis* specific IgG purification and horseradish peroxidase conjugation was carried out as previously described (Gonçalves et al., 2010).

2.7. Measurement of antigen in serum samples

Preliminary experiments were carried out in order to determine the optimal conditions for ELISA through serial dilution of the reagents (antibody, control serum, and conjugate). After, polystyrene microtitre plates were coated overnight at 4 °C with 40 μg/mL of IgG anti-*S. venezuelensis* alkaline extract in 0.06 M carbonate–bicarbonate buffer (pH 9.6). Plates were washed three times for 5 min with PBS containing 0.05% Tween 20 (PBS-T). After washing, plates were incubated with undiluted serum samples for 2 h at 37 °C and subsequently with the secondary antibody consisting of peroxidase-labeled rabbit anti-*S. venezuelensis* IgG at the optimal dilution of 1:5 for 1 h at 37 °C. The reaction was revealed by adding the enzyme substrate (0.03% H_2O_2 and *o*-phenylenediamine [OPD] in 0.1 M citrate–phosphate buffer, pH 5.0) and incubated for 15 min at room temperature. The reaction was stopped by adding 2 N H_2SO_4 and the optical density (OD) was determined at 492 nm in a plate reader (Titertek Multiskan; Flow Laboratories, McLean VA). Results were arbitrarily expressed as reactivity index (RI), previously reported in research on human strongyloidiasis according to the following formula: RI = OD sample/cut off, where the cut off was established as the mean OD of three negative control sera plus two standard deviations. Sera with RI > 1.0 were considered positive.

2.8. Measurement of antibodies in serum samples

Preliminary experiments were carried out in order to determine the optimal conditions for ELISA through serial dilution of the reagents (antigen, control serum, and conjugate). Polystyrene microtitre plates were coated overnight at 4 °C with 10 μg/mL of *S. venezuelensis* alkaline extract in 0.06 M carbonate–bicarbonate buffer (pH 9.6). Plates were washed three times with PBS-T. After washing, plates were incubated with serum samples (1:10) for 45 min at 37 °C and subsequently with the secondary antibody consisting of peroxidase-labeled goat anti-rat IgG (Sigma) at the ideal dilution of 1:8000 for 45 min at 37 °C. The reaction was revealed as described in Section 2.7. Sera with RI > 1.0 were considered positive.

2.9. Measurement of immune complex in serum samples

Preliminary experiments were carried out in order to determine the optimal conditions for ELISA through serial dilution of the reagents (antibody, control serum, and conjugate). Polystyrene microtitre plates were coated for 18 h at 4 °C with 50 μL/well of IgG obtained from immunized rabbits (40 μg/mL) in 0.06 M carbonate–bicarbonate buffer (pH 9.6). After three washes with PBS-T, 50 μL/well of serum samples (1:10) were added and incubated for 45 min at 37 °C. Positive and negative serum samples to immune complex were used as control. The positive serum samples consisted of a positive sample for IgG anti-*S. venezuelensis*. After incubation for 45 min at 37 °C and another three washes, the peroxidase-labeled goat anti-rat IgG (Sigma) was added (50 μL well) at the ideal dilution of 1:8000 for 45 min at 37 °C. The reaction was revealed as described in Section 2.7. Sera with RI > 1.0 were considered positive.

2.10. Statistical analysis

Each experiment was performed twice. The results are expressed as mean + SEM. Statistical variations were analyzed using ANOVA, followed by Tukey's test. The criterion for statistical significance was set at $P < 0.05$.

3. Results

3.1. Monitoring of infection

It was observed that the initial time point for egg shedding was 5 days p.i. for both groups. The number of eggs recovered in the feces at 8 days p.i was significantly higher for normal and immunocompromised rats. After 13 days p.i., the number of eggs decreased in both groups. However, it was observed that on the 21st day p.i. the infection was eliminated in normal animals, while in the immunocompromised, the infection persisted.

3.2. Antigen, antibody and immune complex detection

Results showed that at least 12.5 μg/mL of *S. venezuelensis* specific antigens was detected in serum samples.

Antigen detection rates were bellow the cut-off limits for normal and immunocompromised rats during the kinetics of infection (Fig. 1A).

Specific IgG for *S. venezuelensis* was preferentially detected in normal rats group during the first 13 days p.i. which was no longer

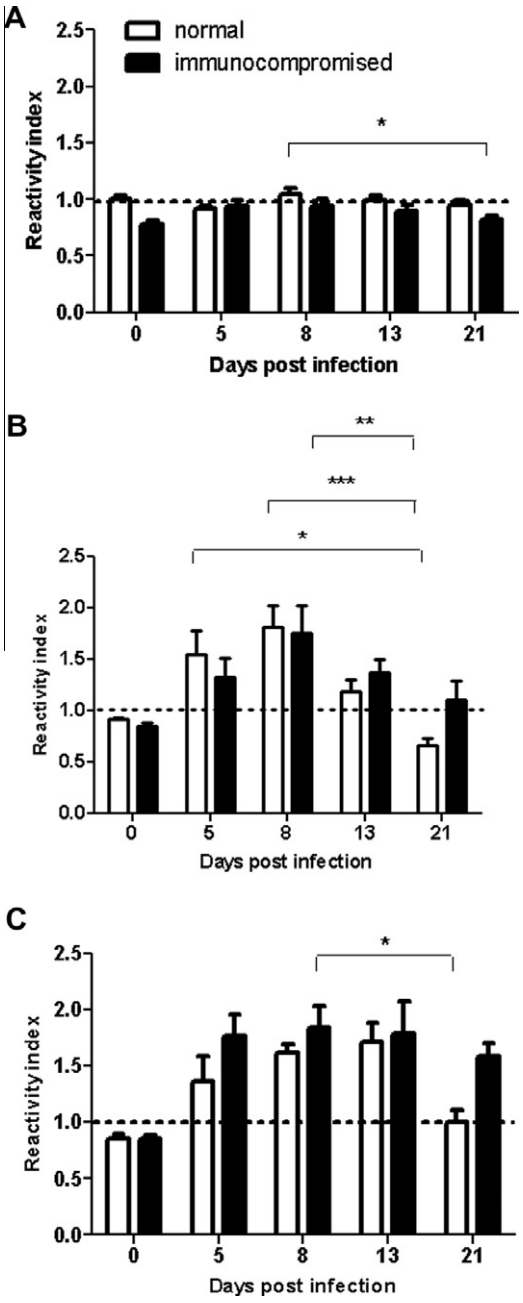

Fig. 1. Kinetics of *S. venezuelensis* antigen (A), antibody (B) and immune complex (C) detection in serum samples from normal or immunocompromised rats. Day zero (0) means the controls. Dashed lines indicate the detection limit for the antigen, antibody or immune complex. Data are expressed as mean ± standard error of the mean (SEM) ($n = 6$). $^{*}p < 0.05$; $^{**}p < 0.01$.

detected at 21 days p.i. In the immunocompromised rats IgG was detected during all the experimental kinetics without statistical difference (Fig. 1B).

Immune complex detection in normal group was significantly reduced in 21 p.i. days, but in the immunocompromised group it was detected during the entire kinetic (Fig. 1C).

3.3. Comparative analysis of antigen, antibody and immune complex detection

In normal group circulating antigens were not detected on 5 p.i. On the other hand, on 8 and 13 p.i. 33.3% of animals showed positivity and on 21 p.i. 16.7% were positive. Antibody detection was effective on 5, 8 and 13 days p.i., positivity detected on these time points were about 66.7%, 83.3% and 66.7%, respectively. However, on 21 day p.i. antibody detection was negative. Immune complex detection was positive during the entire kinetics and showed 83.3% positivity on 5 day p.i., 100% on days 8 and 13 p.i. and 33.3% on 21 day p.i. Statistically significant differences were observed for antigen and antibody detection on 5 day p.i. ($p < 0.05$) and also for antigen and immune complex detection ($p < 0.01$). Moreover, on days 8 and 13 p.i. statistically significant difference was observed for antigen and immune complex detection ($p < 0.05$) (Fig. 2A).

On immunocompromised group, antigen was detected from day 5 to 13 p.i. and showed 16.7% of positivity during these three first time points and negative on 21 day p.i. Antibody and immune complex detections were sensible during all time points. Immune complex detection showed 100% positivity during the entire kinetics. Statistically significant differences were observed on days 5 and 8 p.i. for antigen and antibody detection ($p < 0.05$) and for antigen and immune complex findings ($p < 0.01$). Additionally, on days 13 and 21 p.i. the differences found were between antigen and antibody detections ($p < 0.001$ and $p < 0.05$, respectively) and also between antigen and immune complex detections ($p < 0.001$) (Fig. 2B).

4. Discussion

Diagnosis of *S. stercoralis* is often delayed owing to patients presenting nonspecific gastrointestinal complaints, a low parasite load and irregular larval output. Although several diagnostic methods exist to detect the presence of *S. stercoralis* there is no gold standard. Current parasitologic methods for the detection of parasites are at insufficient sensitivity for accurate infection detection in the immunocompromised host (Devi et al., 2011). In patients with malignancy, organ transplantation or concurrent human T-cell-lymphocytic virus 1 infection, those on corticosteroid therapy or HIV infection, autoinfection can go unchecked with large numbers

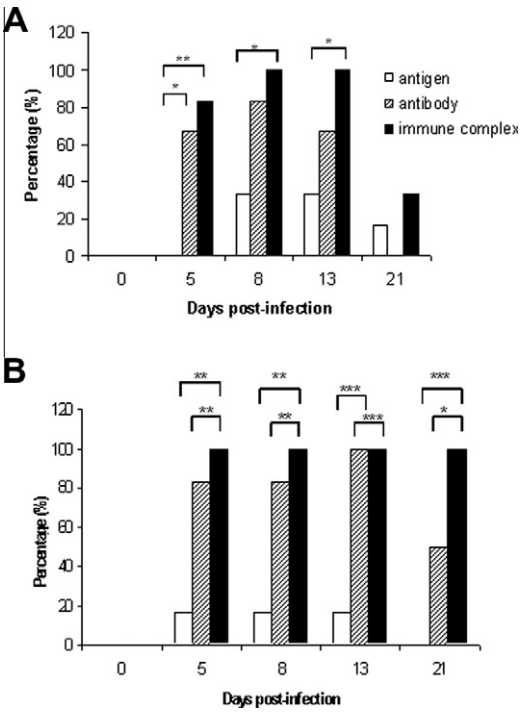

Fig. 2. Percentage of circulating antigen, antibody and immune complex detected on serum samples during *S. venezuelensis* infection kinetic. (A) Normal rats and (B) immunocompromised rats. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

of invasive *Strongyloides* larvae disseminating widely and causing hyperinfection with dissemination, which can be fatal (Montes et al., 2010).

Recent advances in the diagnosis of *S. stercoralis* include a luciferase immunoprecipitation system that shows increased sensitivity and specificity to detect *S. stercoralis*-specific antibodies and a real-time quantitative PCR method to detect *S. stercoralis* in fecal samples (Montes et al., 2010). However, these methods are out of the economic reality of the population under risk of infection. An innovative procedure to detect coproantigen in fecal samples by ELISA has been recently described. This study was conducted experimentally in normal or immunosuppressed rats and it was shown that the anti-*S. venezuelensis* is able to detect coproantigen in fecal samples from immunosuppressed rats earlier than normal rats (Gonçalves et al., 2010).

Attempts have been made over the last years to improve strongyloidiasis serum diagnosis. A method to detect levels of parasite-specific immunoglobulin M (IgM), IgA, IgE, total IgG and IgG1 in serum samples from MHC I and MHC II deficient mice infected by *S. venezuelensis* was described. It was observed that the levels of these immunoglobulins were also significantly reduced in the sera of MHC $II^{-/-}$ infected mice (Rodrigues et al., 2009).

In the present study, the spontaneous elimination of infection in normal rats on the 21st day p.i. is an interesting field for further investigations, considering the substantial difference with the constant autoinfection provoked by *S. stercoralis* in the human host.

Here, another alternative for strongyloidiasis diagnosis in immunocompromised rats is brought about. The approach was to evaluate antigen, antibody and immune complex detection in serum samples from immunocompromised and normal rats infected with *S. venezuelensis*. The results showed that antigen detection in serum samples may not be a good approach for strongyloidiasis diagnosis, because the detection rates were below the cut-off limits for normal and immunocompromised rats during the kinetics of infection. On the other hand, the results appeared to be promising concerning antibody and immune complex detection in serum samples from both groups. Moreover, the detection of antibody and immune complex in immunocompromised rats was possible until the end of the kinetics and for the normal rats the ability of detection was significantly decreased 21 days p.i. Immune complex detection is innovative and this is the first attempt to use this method for strongyloidiasis diagnosis.

Comparative analysis of antigen, antibody and immune complex detection in serum samples revealed that immune complex detection was more sensitive in all time points in immunocompromised animals, suggesting that more immune complex are formed than free antigen or antibody to be detected in circulation, thus, showing its significance for experimental strongyloidiasis diagnosis.

Novel diagnostic methods are expected to improve epidemiological studies and control efforts for prevention and treatment of strongyloidiasis. Based on this concept, we present here a novel approach which can be used for early strongyloidiasis diagnosis. The immune complex seems to be an alternative for early strongyloidiasis diagnosis in immunocompromised individuals.

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