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Canova medication modifies parasitological parameters in mice infected with *Trypanosoma cruzi*

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ABSTRACT

The goals of this study were to evaluate the effect of the Canova® medication, a homeopathic immune-system modulator, on the evolution of infection induced by the *Trypanosoma cruzi* Y strain in mice. The animals were divided into five groups: (i) untreated infected controls (I), (ii) infected animals treated with benznidazole (Bz), (iii) infected animals treated with the Canova medication (CM), (iv) infected animals treated with benznidazole and the Canova medication (Bz + CM), and (v) uninfected controls that received only the vehicle (grain alcohol) (C). The parameters evaluated were: parasitemia, mortality, control of cure, and tissue parasitism analysis. Our results showed that the evolution of the experimental infection was modified by treatment with CM, and that daily and consecutive doses were harmful to the animals, causing death in 100% of the infected animals in a brief period. The analysis of parasitism performed on the organs on the 12th day postinfection showed that in infected animals treated with CM, the number of amastigote/nests in the spleen was significantly reduced, while in cardiac tissue, intestine, and liver the number was significantly increased compared with infected control animals. These results indicate that CM has a negative influence on the host–parasite relationship, modifying the tropism of the parasite for tissues, and increasing the parasitemia peak in this experimental model.

1. Introduction

Chagas' disease is one of the most important tropical diseases in Brazil and Latin America, with a very high disease burden. Eight million people are estimated to be infected, and another 50,000 new cases are reported annually in the Americas (World Health Organization, 2002; Senior, 2007).

The current treatment for Chagas' disease relies on two nitroheterocyclic drugs, the nitrofuran Nifurtimox (Lampit®, whose production has been discontinued) and 2-nitroimidazole benznidazole (Rochagan®; Croft et al., 1997; Paulino et al., 2005). Both drugs, when administered during the acute phase of the disease, can cure 50–70% of patients. However, both drugs have limited efficacy in the treatment of the chronic phase of the disease, and are quite toxic to patients (Docampo and Moreno, 2001).

The existence of *Trypanosoma cruzi* strains that are naturally resistant to both drugs may be an important factor in explaining the low cure rates in chagasic patients (Filardi and Brener, 1987; Murta et al., 1998). However, little is known about the influences of host parameters on therapeutic failure. One of the major factors that may interfere with the efficacy of Chagas' disease treatment is the host immunological response through cooperative action with the drug (Berger and Fairlamb, 1992; Romanha et al., 2002; Targett, 1985). Studies have shown that the persistence of the parasite in tissues is important in infection maintenance and the clinical evolution of the disease (Anez et al., 1999; Consenso Brasileiro em Doença de Chagas, 2006).

Some studies have reported that certain products of natural origin are effective in the treatment of diseases, by maintaining an organism's resistance to infection (Bin-Hafeez Rizwanul et al., 2003). The mechanism by which these agents exert their effects appears to be the activation of the immune system (Mungantiwar et al., 1999; Bin-Hafeez Rizwanul et al., 2003). The newest forms of immunomodulatory therapy can be directed at specific cells or to their products that contribute to the immune response. These forms of immunotherapy are known as "biological response modifiers" (Ballow and Nelson, 1997).

The Canova® medication is indicated for patients whose immune systems appear to be depressed. Clinical observations of these patients have confirmed successful treatment with this medication (Buchi and Vecchio, 2002), which appears to enhance the individual's own immunity by triggering a particular immune response against several pathological conditions (Smit et al., 2009). Studies have investigated the immunomodulatory properties of this medication, and have demonstrated reductions in viral load

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and opportunistic illnesses in patients infected with the human immunodeficiency virus (HIV) (Di Bernardi, 2005; Stroparo, 2005).

Similarly, an improved immune response in mice treated with the Canova® medication was demonstrated in studies with Sarcoma 180. In this study, a reduction in sarcoma size was shown, and infiltration of lymphoid cells, granulation tissue, and fibrosis occurred around the tumor. All of the animals of the treated group survived, and 30% had total tumor regression (Sato et al., 2005).

We previously demonstrated that in experimental infection with *L.* (*L.*) *amazonensis* and *Paracoccidioides brasiliensis*, the Canova® medication had an immunomodulatory effect, reducing the progression of the infection and limiting its dissemination (Pereira et al., 2005; Takahachi et al., 2006a,b).

Canova® has been shown to activate macrophages *in vitro* and *in vivo*, with resulting enhanced spreading of the cells and formation of microvillus extensions (Fuentes et al., 2001; Piemonte and Buchi, 2002; Abud et al., 2006; Lopes et al., 2006). Other studies demonstrated that Canova® causes activation of monocytes with differentiation into large macrophage-like cells (Smit et al., 2008), and induces proliferation of lymphocytes when stimulated by activated human macrophages treated with Canova® (Burbano et al., 2009).

Aleixo et al. (2008) evaluated the random amplification of the polymorphic DNA (RAPD) profile of *T. cruzi* II under the influence of Canova® and benznidazole. The authors found that the RAPD profiles of parasites treated with Bz and Canova® + Bz were different from those in the control group and in the group treated with Canova®. The results showed no direct effect of Canova®, but suggested that Canova® interacts with benznidazole. The differences in the RAPD profile of the Y strain of *T. cruzi* produced by Bz, Canova® + Bz, and the natural course of the infection suggest selection/suppression of populations.

The present study evaluated the effect of Canova® on the evolution of infection induced by the Y strain of *T. cruzi* in mice.

2. Materials and methods

2.1. Canova medication

The Canova® preparation is a homeopathic immunomodulator described in the Brazilian Homeopathic Pharmacopeia (CRFB, 1997; Lacerda, 1998). Experiments were performed with commercial Canova® purchased from *Canova do Brasil*, a Brazilian company that holds the international patent. The compound is presently registered as a magistral formula (registration No. 5991/73). Mother tinctures are purchased from authorized agencies approved by the Brazilian Health Ministry. The final product, an aqueous, odorless, and colorless solution, contains extracts from the bark of *Thuja occidentalis* (Cupressaceae), from fresh roots of *Bryonia alba* (Curcubitaceae), from fresh preparations of the intact plant (including the roots at the beginning of flowering) of *Aconitum napellus* (Ranunculaceae), as well as *Arsenicum album* (arsenic trioxide) and venom of *Lachesis muta* (Viperidae). These matrices have a warranty certificate that ensures the quality (endotoxin-free) and physicochemical composition. No toxicity or mutagenic effects have been reported (Seligman et al., 2003).

2.2. Animals

Male Swiss mice, 4 weeks old, were supplied by the Biotério Central of the State University of Maringá (UEM), Paraná. The animals had free access to food and water. All recommendations of the National Law (No. 6638, November 5, 1979) for scientific management of animals were observed, and the UEM Committee for Animal Studies approved all procedures.

2.3. Infection of mice

Mice were inoculated intraperitoneally with 10,000 blood trypomastigotes/animal of the Y strain of *T. cruzi* (Pereira da Silva and Nussenzweig, 1953). The number of parasites in the inocula was determined according to Brener (1962).

2.4. Treatment regimens

The animals were divided into five groups with 20 animals each: (i) untreated infected controls (I), (ii) infected animals treated with benznidazole (Bz, 100 mg/kg of body weight, p.o., daily), (iii) infected animals treated with Canova medication (CM; 0.2 ml/animal, p.o., daily), (iv) infected animals treated with benznidazole and Canova medication at the same doses as above (Bz + CM), and (v) uninfected controls that received the same volume of the vehicle (grain alcohol) (C). The treatment began on the 5th day after infection, and continued for 20 consecutive days.

2.5. Parameter evaluation

Parasitemia: Parasitemia was evaluated among infected animals before and during treatment and until the 20th day after treatment. The blood was obtained from the mouse tails and the number of parasites was estimated according to Brener (1962) The patent period (PP), maximum peak of parasitemia (MP), day of maximum parasitemia (DMP), total parasitemia (TP), and parasitemia curve (PC) were determined.

Mortality: The mortality rate was expressed as the cumulative percentage of death within the period of 120 days after inoculation.

Treatment evaluation: To evaluate the treatment, two independent tests that consisted of blood culture and polymerase chain reaction (PCR) were performed.

2.6. Polymerase chain reaction

Mice were bled from the orbital venous sinus, and 200 μl of blood was collected on the 30th and 60th day post-treatment. Four-hundred microliters of 6.0 M/0.2 M guanidine/EDTA was added to this volume (Ávila et al., 1991). The samples were maintained at ambient temperature for 1 week. After this period, the material was boiled for 7 min and kept at ambient temperature until use. The PCR assay was performed according to Gomes et al. (1998).

2.7. Hemoculture

Thirty days after the end of the treatments, hemoculture was performed according to Filardi and Brener (1987). The mice were bled from the orbital venous sinus, and 500 μl of blood was collected and placed in two tubes containing 3 ml of liver infusion tryptose medium (Camargo, 1964). The tubes were incubated at 28 °C for 90 days and examined after different periods (30, 45, 60, and 90 days).

2.8. Tissue parasitism analysis

For each experimental group, five mice were killed on the 7th and 12th days after infection. Tissue from the heart, skeletal muscle, gastrointestinal tract (small and large intestine), liver, spleen, and diaphragm was collected. This material was processed routinely and embedded in paraffin. After hematoxylin–eosin staining, the presence of tissue parasitism was evaluated in loci by examining 50 random microscopic fields (400× magnification) in each

section. Spleen sections were examined under oil immersion (1000× magnification) for the detection of parasites.

2.9. Statistical analysis

The data were subjected to Student's *t*-test or analysis of variance (ANOVA) followed by the Tukey test. All analyses were done using GraphPad Prism (GraphPad, San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

3. Results

In uninfected animals treated with CM, no alterations were observed in the animals' physical activity, behavior, or survival, and no significant changes were found in growth rate (data not shown).

3.1. Blood parasitism analysis

The patent period (PP, period with positive fresh blood examination) in the untreated infected group (I) was 15.0 ± 0.5 days. In the infected group treated with CM, the PP was 12.1 ± 0.8 days). In the infected group treated with Bz, the PP was significantly less (PP = 2.0 ± 0.4 days) compared to the untreated infected group (I). Similar results were found in the infected group treated with the Bz + CM combination (PP = 2.0 ± 0.4 days) (Table 1).

The day of maximum parasitemia (DMP) of the untreated infected animals (I) occurred on the 12th day postinfection (89.9×10^3 trypomastigotes/0.1 mL of blood). The infected group treated with CM showed a DMP on the 8th day postinfection (257.8×10^3 trypomastigotes/0.1 mL of blood), and the infected groups treated with Bz or Bz + CM, DMP on the 5th day postinfection, showed a greatly reduced number of parasites (1.6×10^3 and 3.3×10^3 trypomastigotes/0.1 mL of blood, respectively) (Table 1).

The total parasitemia (TP) in animals infected with *T. cruzi* and treated with CM was similar when compared with untreated infected animals (I). In contrast, infected animals treated with Bz or Bz + CM showed a significant reduction in TP compared with the control group. The reduction was even more evident in the Bz-only group compared with the Bz + CM group (Table 1).

The group of untreated infected animals (I) displayed curves of parasitemia (CP) that peaked on the 12th day postinfection. Infected animals treated with CM had increased CP compared with group I. In groups of infected animals treated with Bz and BZ + CM, we observed a significant decrease in CP compared with group I (Fig. 1).

3.2. Mortality rate

The mortality rate of the untreated infected animals (I) was 100%. The same occurred in the infected group treated with CM. All animals infected and treated with Bz or Bz + CM survived the acute infection for 120 days. Importantly, infected animals treated with CM died more quickly (between the 12th and 16th day postinfection) than untreated infected animals (between the 14th and 20th day postinfection) (Table 1, Fig. 2).

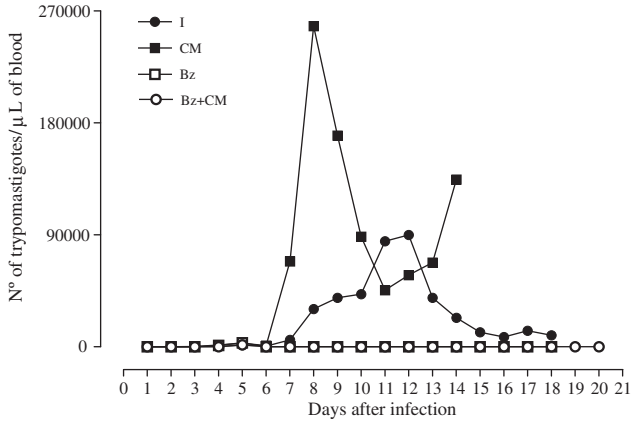

Fig. 1. Curve of mean parasitaemia in group of untreated infected animals (I) and infected animals treated with Canova medication (CM – 0.2 mL/animal/day), with benznidazole (Bz – 100.0 mg/kg/day), or association Bz + CM (100.0 mg/kg/day + 0.2 mL/animal/day). The treatment (p.o.) began on the 5th day of confirmed infection and was continued for consecutive days.

3.3. Treatment evaluation

Considering the results from the hemoculture and PCR, Bz treatment induced a significant reduction in parasitemia levels in animals infected with the Y strain of *T. cruzi*, but a parasitological cure was not completely achieved. Treatment with Bz + CM was less effective, with minimal parasitological cure (Table 2). The infected group treated with CM lost more weight than the other experimental groups (data not shown).

3.4. Tissue parasitism (TP)

On the 7th day postinfection, the tissue parasitism analysis did not show significant differences between experimental groups (I, CM, Bz, Bz + CM) (data not shown). Moreover, on the 12th day postinfection, the number of organ amastigote/nests in the untreated infected animals was 14.2/1.2 in the intestine, 24.2/4.0 in the liver, 46.7/5.2 in the heart, 49.5/5.0 in the skeletal muscle, and 1488.5/115.7 in the spleen. In the infected group treated with CM, a significant increase in the number of amastigote/nests was detected in the intestine, liver, and heart. A significant reduction in the number of amastigote/nests was observed in the spleens of these animals (Fig. 3). The number of amastigote/nests was 208.5/23.2 in the intestine, 126.0/10.0 in the liver, 522.2/27.5 in the heart, 53.2/3.0 in the skeletal muscle, and 935.0/70.0 in the spleen (Table 3). When we determined the total number of amastigote/nests in this set of organs, no significant differences were

Table 1
Parasitological parameters evaluated in infected mice.

Group	Patent period-days (PP)	Day of maximum parasitemia (DMP)	Maximum peak of parasitemia (MP – trypomastígotas/0.1 mL)	Total parasitemia (TP)	Mortality
I	15.1 ± 0.5	12th	$89.9 \pm 31 \times 10^3$	$989 \pm 108 \times 10^3$	15/15
Bz	2.0 ± 0.4**	5th**	$1.6 \pm 0.5 \times 10^{3**}$	$0.6 \pm 0.3 \times 10^{3**}$	0/15
CM	12.1 ± 0.8	8th*	$257.8 \pm 65 \times 10^{3**}$	$1036 \pm 183 \times 10^3$	15/15
Bz + CM	2.0 ± 0.4**	5th**	$3.3 \pm 0.8 \times 10^{3**}$	$2.8 \pm 0.4 \times 10^{3**}$	0/15

Mice were infected with trypomastigotes/0.1 mL of the Y strain of *T. cruzi*. Each value represents the means ± SEM of infected mice and treated with benznidazole (Bz – 100.0 mg/kg/day), Canova medication (CM – 0.2 mL/animal/day) and association Bz + CM (100.0 mg/kg/day + 0.2 mL/animal/day). The treatment (p.o.) began on the 5th day of confirmed infection and was continued for consecutive days.

* $P < 0.05$.

** $P < 0.005$ (ANOVA, Tukey's test) compared to untreated infected controls.

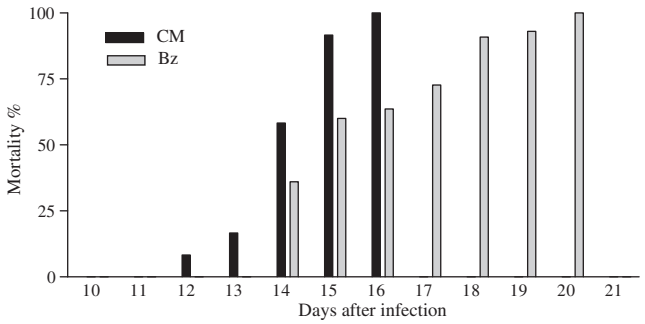

Fig. 2. Mortality rates of untreated infected animals (I) and infected animals treated with Canova medication (CM – 0.2 mL/animal/day). The treatment (p.o.) began on the 5th day of confirmed infection and was continued for consecutive days.

Table 2
Control of parasitological cure in mice infected and treated with Bz and CM + Bz.

Control of cure	Bz (% of cure)	CM + Bz (% of cure)
Haemoculture	100.0	66.7
PCR	68.6	22.0

Mice were infected with trypomastigotes/0.1 mL of the Y strain of *T. cruzi*. Bz: infected mice treated with benznidazole (100.0 mg/kg/day) and CM + Bz: infected mice treated with association benznidazole (100.0 mg/kg/day) + Canova medication (0.2 mL/animal/day). The treatment (p.o.) began on the 5th day of confirmed infection and was continued for consecutive days.

found between the two groups (Table 3). In the infected groups treated with Bz and Bz + CM, parasites were not detected during the evaluation period (12th day postinfection).

4. Discussion

This study showed that the evolution of experimental infection induced by the Y strain of *T. cruzi* in mice was modified by treatment with the Canova® medication (CM). We also demonstrated that treatment with CM in daily and consecutive doses was harmful to the animals, causing death in 100% of the infected animals in a shorter time than the untreated infected animals.

In the present study, Swiss mice infected with the Y strain of *T. cruzi* exhibited the classical pattern of the disease. Intense parasitemia was observed in the acute phase, with no survivors on the 20th day postinfection, consistent with other studies (Pereira da Silva and Nussenszweig, 1953; De Souza et al., 2000; Olivieri et al., 2002, 2005). We also confirmed previous reports (Andrade and Figueira, 1977; De Souza et al., 2000; Olivieri et al., 2002, 2005) showing that benznidazole treatment is effective in reducing the level of parasitemia, with a survival rate of 100%.

In contrast, when infected animals were treated with CM, the parasitemia peak increased substantially compared with untreated animals. The data showed that CM did not suppress parasitemia induced by *T. cruzi* in the acute phase of the infection. Comparing the parasitemia curves of infected mice treated with CM and the untreated group, we found altered parasitemia kinetics. The number of circulating parasites was threefold higher in treated mice (CM).

In the present study, infected animals treated with CM lost more weight during the progression of the infection than animals in the other groups (Bz and Bz + CM). Moreover, the 100% mortality rate of infected animals treated with CM occurred more quickly than the untreated infected group.

In acute *T. cruzi* infection, the parasite/host relationship is characterized by intracellular multiplication of the parasite and by an immune response of the host, involving an important inflammatory component. After they penetrate the host, the parasites are actively internalized by macrophages. As the infection progresses, the macrophages are activated by various cytokines, which depends on the CD4 response (Minoprio et al., 1991) and γ-interferon (Wirth et al., 1985; Plata et al., 1987). Accentuated production of H_2O_2, O_2, and OH^- and nitric-oxide metabolites occurs, together with intracellular destruction of the parasites (Gazzinelli et al., 1992; Vespa et al., 1994).

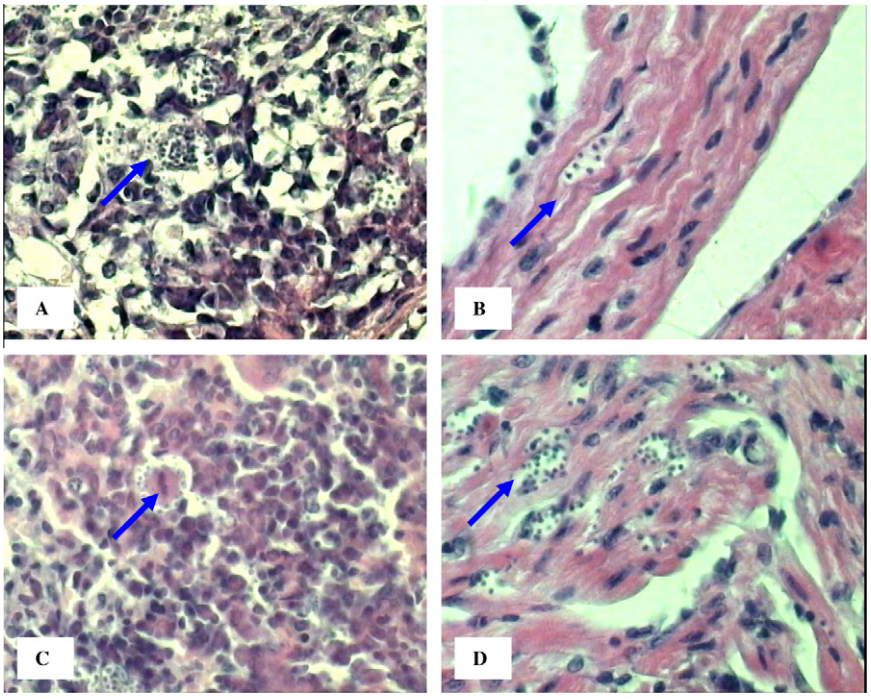

Fig. 3. Spleen (A) and heart (B) of animals inoculated with *Trypanosoma cruzi* and untreated. Spleen (C) and heart (D) of animals inoculated with *Trypanosoma cruzi* and Canova medication treated. Observed in optical microscopy (H&E staining; increase Original: 400×).

Table 3
Number of amastigotas/nests in tissues of the infected mice.

Organ	Untreated infected mice Number of amastigotes	Infected mice treated with CM	Untreated infected mice Number of nest	Infected mice treated with CM
Heart	46.7 ± 1.5	522.2 ± 18.9*	5.2 ± 0.1	27.5 ± 0.7*
Liver	24.2 ± 2.6	126.0 ± 4.7**	4.0 ± 0.3	10.0 ± 0.6*
Spleen	1488.5 ± 82.3	935.0 ± 44.2*	115.7 ± 0.6	70.0 ± 2.1*
Intestine	14.2 ± 1.2	208.5 ± 17.9*	1.2 ± 0.1	23.2 ± 0.8*
Skeletal muscle	49.5 ± 0.9	53.2 ± 0.7	5.0 ± 0.2	3.0 ± 0.2
Total	1623.2 ± 86.5	1844.5 ± 49.2	131.25 ± 0.3	133.7 ± 2.8

Mice were infected with trypomastigotes/0.1 mL of the Y strain of *T. cruzi*. Treatment, p.o., with Canova medication (CM – 0.2 mL/animal/day) began on the 5th day of confirmed infection and was continued for consecutive days. Each value represents the means ± SEM of five animals/group, sacrificed on the 12th day of infection.
* $P < 0.05$.
** $P < 0.005$ (Student's *t*-test) compared to untreated infected controls.

In addition to these factors, $CD8^+$ lymphocytes are also able to produce cytokines that activate macrophages (Tarleton, 1990, 1991). The activated macrophages are the elements that are most important for vertebrate animals for controlling the multiplication of parasites. However, macrophages cannot always control the infection, and the parasites are able to reach the microcirculation to become phagocytes or actively penetrate, not into phagocytic cells, but rather into cardiac and skeletal tissue.

Some studies have demonstrated that CM has a modulating effect on the immune system, activating macrophages both *in vivo* and *in vitro* (Lopes et al., 2006). CM stimulates the activity of the endosomal/lysosomal system, as well as the phagocytic activity of the macrophages when they interact with *Saccharomyces cerevisae* and *T. cruzi* epimastigotes (Lopes et al., 2006). Moreover, immunomodulatory effects of CM have been observed *in vivo* and *in vitro* in experiments with *L.* (*L.*) *amazonensis* and *P. brasiliensis*, controlling the progression of the infection and limiting its dissemination (Pereira et al., 2005; Takahachi et al., 2006a,b). Clinical studies that have been fundamental in elucidating the immunomodulatory properties of this medication have demonstrated a reduction in viral load and opportunist illnesses in HIV patients (Di Bernardi, 2005; Stroparo, 2005). Similarly, studies with Sarcoma 180 demonstrated that rats treated with CM showed a more efficient immune response compared with animals that had not received CM (Sato et al., 2005).

An important question is whether the immunomodulatory effect of the Canova medication can possibly contribute to a favorable response in hosts infected with *T. cruzi*. The CM medication exacerbated the infection in this experimental model, with a less efficient cure compared with conventional benznidazole treatment.

Benznidazole, a nitroheterocyclic drug, is known to reduce parasitism levels and eliminate acute-phase symptoms, thus shortening the course of the infection (for review, see Coura and De Castro, 2002). Moreover, some studies suggest that the beneficial effects of benznidazole in this infection may depend not only on its trypanocidal effect but also on immunomodulatory influences (Piaggio et al., 2001). Benznidazole exerts a number of effects on the host immune response to *T. cruzi* infection, including enhanced macrophage-associated phagocytosis and proinflammatory cytokine production (Murta et al., 1998), selective expansion of effector and memory $CD8^+$ T-lymphocytes (Olivieri et al., 2002), and decreased levels of both P-selectin and vascular cell adhesion molecule 1 (Laucella et al., 1999). Additionally, host immune factors, including interleukin-12 (Michailowsky et al., 1998) and γ-interferon (Romanha et al., 2002), are important for maximum efficacy of benznidazole therapy during infection.

We also found it important to investigate the number of parasites/nests in some organs of infected animals treated with CM, in order to more fully understand the increase in mortality rate during an early period of the acute infection process. Examination of the organs on the 12th day postinfection showed that in infected animals treated with CM, the number of amastigote/nests in the spleen was significantly reduced, whereas in skeletal muscle the medication had no effect compared with infected control animals that did not receive treatment. However, the number of amastigote/nests in the cardiac tissue, intestine, and liver of infected animals treated with CM was significantly increased compared with infected control animals, which traditionally show tropism, mainly for the spleen, during the acute phase of the infection (Andrade and Rocha da Silva, 1995).

When we evaluated the total number of parasites/nests in the organs, also on the 12th day postinfection, no differences were detected between untreated (1623.2/131.25) infected animals and infected animals treated with CM (1845.0/133.75). This finding reflected the decrease in number of nests in the spleen and skeletal muscle and the increase in number of nests in the heart, intestine, and liver of infected animals treated with CM.

Additionally, animals that had larger numbers of amastigotes in the heart died more quickly. These results indicate that CM has a negative influence on the host-parasite relationship in this experimental model, expressed by an alteration of tissue tropism.

In summary, this study provided strong evidence that CM can modify the response of mice to infection by *T. cruzi* Y strain. For the understanding of the mechanisms of action of this medication, other investigations will be necessary, using different genetic lines of *T. cruzi*, different lines of mice, and different doses and/or different regimens of administration of the medication. In addition, it will be important to investigate the effect of this medication on the chronic experimental infection induced by *T. cruzi*, evaluating parameters involved in regulation of the immune system.

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