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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original Research http://dx.doi.org/10.1016/j.apjtm.2016.07.007

Canova medication changes TNF-α and IL-10 serum levels in mice infected with *Trypanosoma cruzi* Y strain

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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form 16 Jun 2016
Accepted 1 Jul 2016
Available online 26 Jul 2016

Keywords:
Trypanosoma cruzi
Canova medication
Cytokines

ABSTRACT

Objective: To identify whether Canova medication changes TNF-α and IL-10 serum levels in mice infected with *Trypanosoma cruzi* Y strain.

Methods: Animals were divided into five groups: non-treated infected animals (I); benznidazole-treated infected animals (Bz; 100 mg/kg body weight, single daily dose by gavage); Canova medication (CM) treated infected animals (CM; 0.2 mL/animal, single daily dose by gavage); benznidazole- and Canova medication-treated infected animals with the above-mentioned dose (Bz+CM); and non-infected animals (C). TNF-α and IL-10 levels were determined in serum aliquots after 4, 7, 10, 13, and 29 days of infection. An ELISA technique was employed with R&D System Inc. antibody pairs.

Results: A high increase in TNF-α and IL-10 levels occurred in the infected and CM-treated groups within the treatment employed on the 10th day after infection, coupled with a IL-10 decrease on the 13th day after infection when compared with the other experimental groups.

Conclusions: CM may change the balance between plasma cytokine levels (TNF-α and IL-10) in mice infected with Y strain *T. cruzi*, with important consequences leading towards a more severe infection.

1. Introduction

The digenetic protozoon *Trypanosoma cruzi (T. cruzi)* and the etiological agent of Chagas disease cause systemic infection in humans and mammals. Its main defense mechanism is cell-mediated immunity, especially macrophages activated by T helper cells (Th1) derived cytokines [1].

Similar to other infections by intracellular pathogenic microorganisms, the host's infection by *T. cruzi* triggers multiple humoral and cellular mechanisms of the innate and acquired immunological response. Although the parasite is continually attacked and its multiplication is highly reduced, it remains indefinitely in the host's tissues, as does the immunological response. Tissue lesions caused by prolonged immunological activity may eventually lead towards muscular and nervous functional alterations that are proper to Chagas disease [2,3].

Cytokines have an important role in controlling the immunological response. They are involved in the resistance and in the immuno-pathological mechanisms of Chagas disease. Interferon gamma (IFN-γ), mainly produced by lymphocytes Th1 and natural killer cells (NK), has been extensively studied. It is a protector cytokine because it activates macrophages to produce other protector cytokines, such as tumor necrosis factor-alpha (TNF-α), and toxic metabolites for the parasite, such as nitric oxide and oxygen reactive species [4]. On the other hand, interleukin-10 (IL-10) may suppress the activation of macrophages induced by IFN-γ while inhibiting the release of toxic metabolites and the differentiation of Th1. Whereas TNF-α is involved in the resistance and in the genesis of tissue lesions, IL-1 and IL-6, which are associated with great alterations

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Peer review under responsibility of Hainan Medical College.

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in the endothelial cell functions, may be involved in the microvascular changes reported in the myocardiopathy of Chagas disease [3,5]. Furthermore, cytokines such as TNF-α, IL-6, and IL-1 modulate adhesion molecules that participate in the recruitment of lymphocytes for the inflammation sites [6]. Thus, quantitative or equilibrium changes between different cytokines may be related to resistance and to the development of different lesions reported in Chagas disease, mainly in its chronic phase [7,8].

Several studies show that the control of the acute phase of *T. cruzi*-induced infection depends on the activation of macrophages mediated by cytokines that trigger a number of events causing the parasite's death within the cell [9,10]. However, some studies report the occurrence of immunosuppression in Chagas disease especially during this phase. Deficiencies in the cell's immune response have been reported in animal experiments [11–13] and in patients suffering from Chagas disease [14]. Other researchers also suggest immuno-suppression against antigens not related to *T. cruzi* [15], which would have implications on the development of self-immunity of Chagas disease [16,17].

The medication Canova (CM) is a homeopathic, non-mutagenic, non-toxic drug that is highly diluted and dynamic [18]. Its active components are *Aconitum napellus*, *Arsenicum álbum*, *Bryonia alba*, *Lachesis trigonocephalus*, and *Thuya occidentalis*. CM is an immunomodulator that is clinically employed, associated with other medical agents, in the treatment of diseases in which the patient's immunological system is impaired, as in the case of Acquired Immunodeficiency Syndrome and in cancers [19,20]. Although there is experimental evidence showing that CM controls the progression of infection by intracellular parasites, such as *Leishmania amazonensis* and *Paracoccidioides brasiliensis* [21–23], we have recently demonstrated a negative effect of CM on Y strain *T. cruzi* infection. CM modified the evolution of acute infection induced by the Y strain of this parasite, causing increased parasitism and the early death of all animals [24]. The mechanisms involved in this negative effect were not yet investigated. However, there is substantial evidence to suppose that CM may affect cytokine production by defense cells like macrophages, which are the main cells infected by this protozoan and which play a central role in infection control [25]. As observed by Piemonte and Buchi [26], the macrophages treated with this drug become larger, with more cellular projections and a substantial increase of cytoplasmic volume. NADPH oxidase and inducible nitric oxide synthase activities are also increased as a result of CM treatment [27]. Regarding cytokine production, some authors have reported *in vitro* inhibition of TNF-α release by macrophages infected with *T. cruzi* and treated with CM [28]. The influence of these phenomena observed *in vitro* on the progression of the *in vivo* infection is not well established.

Therefore, the aim of the present work was to investigate the influence of CM medication alone or in association with benznidazole treatment on plasma cytokine levels (TNF-α and IL-10) in mice infected with Y strain *T. cruzi*.

2. Materials and methods

2.1. Canova medication

Canova is a homeopathic pharmaceutical product, developed by Homeopathic Pharmacies of Brazil. The method for the preparation of Canova followed Hahnemannian homeopathy described in the Brazilian Homeopathic Pharmacopeia [29,30]. Decimal dilutions, represented by DH units, were used. The number in front of DH indicates the number of decimal dilutions, *i.e.*, 10 DH represents a dilution of 1×10^{-10}. Canova's formula is composed of *Thuya occidentalis* (Cupressaceae) 19 DH, obtained from the bark of the tree; *Bryonia alba* (Curcurbitaceae) 18 DH, obtained from fresh roots; *Aconitum napellus* (Ranunculaceae) 11 DH, obtained from fresh preparations of the entire plant, including roots, at blooming; *Arsenicum album* (arsenic trioxide) 19 DH, and *Lachesis muta* (Viperidae) poison 18 DH. Active compounds were extracted and diluted with equal parts in 70% ethanol.

2.2. Parasites

Strain Y of *T. cruzi* was employed [31]. Parasites were obtained from the mice's blood on the seventh day of infection. Blood was collected, and an inoculum consisting of 10 000 trypomastigotes per animal was produced. Inoculation of parasites was carried out intraperitoneally.

2.3. Animals

Four-week-old male Swiss mice, weighing approximately between (28–30) g, were used. The experiment protocol was approved by the Ethics Committee in Animal Experimentation (CEAE/UEM 008-2005).

2.4. Treatment

The animals were divided into five groups with twelve animals each: (1) non-treated infected animals (I); (2) benznidazole-treated infected animals (Bz; Roche; 100 mg/kg body weight, single daily dose by gavage); (3) Canova medication-treated infected animals (CM; Canova of Brazil; 0.2 mL/animal, single daily dose, by gavage); (4) benznidazole- and Canova medication-treated infected animals with the above-mentioned dose (Bz+MC); and (5) non-infected animals that received the same volume of alcohol (C). Treatment started on the fifth day after infection and lasted for 20 d. The treatment started on the 5th day from the initiation of the infection, considering that this procedure is used by most researchers to evaluate medicines in experimental infection with *T. cruzi* [32–35].

2.5. Determination of cytokines serum level

Concentrations of cytokines (TNF-α and IL-10) were determined in serum aliquots from animals of the different groups after 4, 7, 10, 13, and 29 days of infection. An enzyme-linked immunosorbent assay (ELISA) technique was employed with antibody pairs from R&D System Inc. (Minneapolis, USA). The technique was developed according to protocols by the manufacturer, with slight modifications. Microplates of 96 wells (Nunc-MaxiSorp) were sensitized with anti-cytokine monoclonal antibody and incubated overnight at 4 °C. Non-specific sites were blocked with non-fatty milk powder (Molico) dissolved in PBS-Tween (0.5 mL Tween-20/L) for 2 h at 37 °C. Samples were then added and incubated for 2 h at 37 °C. Polyclonal anti-cytokine biotinylated antibodies were

employed as detecting antibodies. The wells were then incubated with a solution containing Streptavidin-HRP (horseradish peroxidase) in a dilution of 1:10 000 in PBS-Tween for 1 h at 37 °C. Final reaction was determined by adding 50 μL TMB-tetrametylbenzidin (TMB single solution chromogen for ELISA). Enzyme reaction was blocked with 50 μL $H_2SO_4 \cdot 2N$. Reading was undertaken in 492 nm. Cytokine concentrations were determined according to a standard curve from recombinant mice cytokine (R&D System), and results were given in pg/mL.

2.6. Evaluation of animal weight

Animals from the different groups were weighed before infection onset and on the 10th day after onset to calculate weight gain.

2.7. Statistical analysis

Results were given as mean ± SEM and analyzed by Student's *t* test to compare two means or by ANOVA, followed by Tukey's test for multiple comparisons.

3. Results

3.1. Effect of Canova medication on the serum level of cytokines

Table 1 show the serum levels of TNF-α and IL-10, respectively.

Animals infected with the Y strain of *T. cruzi* showed an increase in serum levels of cytokines, beginning on the 10th day after the protozoan inoculation. TNF-α levels in the infected group (I) were high on the 10th day (139.3 ± 35.0) and on the 13th day (217.3 ± 38.0) after infection. Similarly, the IL-10 levels in this group of animals (I) increased on the 10th day (252.5 ± 53.5) and on the 13th day (368.9 ± 50.4) after infection.

CM treatment caused an augmentation of TNF-α and IL-10 levels on the 10th day after infection. The amount of TNF-α was approximately three-fold higher and IL-10 levels were almost two-fold higher in the CM-treated group (CM) when compared with the infected group (I) at this period. The levels of both cytokines diminished on the 13th day of infection. However, TNF-α levels were still high, as they were not statistically different from the infected group (I) on the same day. Otherwise, IL-10 levels were greatly decreased on day 13, reaching almost basal levels (day 0). On the other hand, TNF-α and IL-10 serum levels were almost totally suppressed in the group of infected animals treated with benznidazole (Bz) or benznidazole associated with CM (Bz+CM) during the entire experimental period.

It is also worth noting that the animals treated with benznidazole alone (Bz) or in association with CM (Bz+CM) survived till the 29th day of infection. Importantly, infected animals treated with CM (CM) and non-treated infected animals (I) died more quickly (from the 12th day after infection – data not shown).

3.2. Animals' weight gain

The animals' weight gain was calculated by the difference between the weight on the 10th day of infection and the weight on the day prior to infection. Figure 1 shows the animals' weight gain in the different experimental groups. When compared with control group animals without any infection (C), the animals of all other experimental groups had a significant difference, or rather, a lower weight gain. There was a significant difference in groups treated with Bz+CM when compared with the non-treated and CM-treated groups. Group CM had the lowest weight gain.

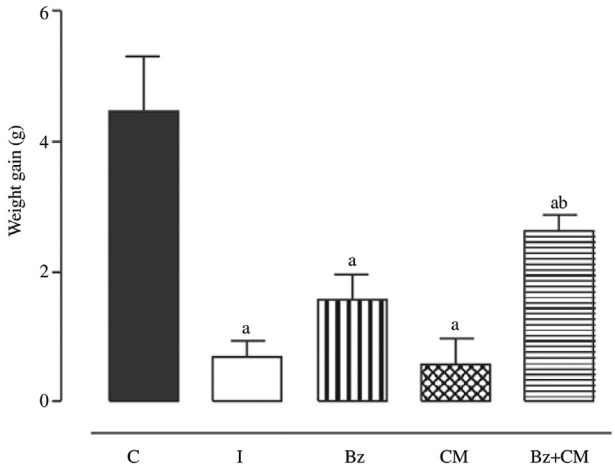

Figure 1. Weight gain of infected untreated 4-week-old male Swiss mice. Each data point represents the mean SEM of 6–12 animals. [a]$P < 0.01$, compared with control animals; [b]$P < 0.05$, compared with untreated infected animals and infected animals treated with CM.

Table 1
Serum TNF-α and IL-10 levels in 4-week-old male infected untreated Swiss mice (pg/mL).

Group	TNF-α				IL-10			
	7	10	13	29	7	10	13	29
I	0.0 ± 0.0	139.3 ± 35.0	217.3 ± 38.0	–	6.7 ± 2.6	252.5 ± 53.5	368.9 ± 50.4	–
CM	0.0 ± 0.0	326.7 ± 81.2	135.1 ± 44.7	–	4.2 ± 4.2	437.5 ± 82.0[c]	80.2 ± 29.6[d]	–
Bz	0.0 ± 0.0	40.0 ± 31.7[a]	19.3 ± 12.4	2.0 ± 1.9	0.0 ± 0.0	58.1 ± 14.2[a]	21.5 ± 5.6[b]	41.0 ± 6.0
Bz+CM	0.0 ± 0.0	30.6 ± 3.5[a]	13.4 ± 3.3	3.4 ± 3.3	0.0 ± 0.0	13.4 ± 2.1[a]	41.0 ± 6.0	9.7 ± 6.8

Each data point represents the mean ± SEM of 5 animals. [a]$P < 0.05$, compared with infected animals (I) and infected animals treated with CM on the 10th day; [b]$P < 0.05$, compared with infected animals (I) on the 13th day of infection; [c]$P < 0.05$, compared with infected animals treated with Bz or Bz+CM on the 10th day of infection; [d]$P < 0.05$, compared with infected animals (I) on the 13th day of infection (ANOVA followed by Tukey's test).

4. Discussion

The findings of the present work give evidence to support the hypothesis that cytokines serum levels are associated with the negative influence of CM on Y strain *T. cruzi* infection in mice, as we had previously reported [24]. In our previous study it was observed that the peak of parasitemia in the group of CM-treated infected mice was on the 8th day post-infection. In the present study the results clearly show a massive release of TNF-α and IL-10 on the 10th day when infected mice were treated with CM. This result suggests that probably the release of cytokines by inflammatory cells is stimulated as a consequence of the higher amount of parasites in these animals. It is currently well established that cells and mechanisms of the immune system are responsible for the control of the parasite's multiplication in tissues. These data, however, are not sufficient to explain why CM-treated mice present a higher parasitemia. However, it can clarify the weight loss in CM-treated mice as well as the high mortality rate.

TNF-α is a pro-inflammatory cytokine whose main source are activated macrophages and monocytes, which operates in inflammatory and immune processes, in addition to regulating the growth and differentiation of some cell types [36]. The production of TNF-α can be induced directly by the parasite or by their surface antigens, being dependent on the activation of the transcription factor NF-κB, as shown by Ropert *et al.* [37]. Although it is crucial to the regulation of the cytokine cascade that provides a rapid form of host defense against infection, including intracellular parasites such as *T. cruzi*, it is fatal in excess and is intimately associated with cachexia [38–40].

IL-10, on the other hand, is one of the most important anti-inflammatory immune-regulating cytokines, which is produced by macrophages and T lymphocyte regulators. This cytokine inhibits the production of IFN-γ by T lymphocytes and the production of various cytokines and chemokines by macrophages through blockade of the accessory functions of these cells that activate T cells [41]. High levels of IL-10 have been detected in cultures of cells from whole blood infected with the trypomastigote form of the Y strain of *T. cruzi*, and some authors consider that the induction of IL-10 synthesis by the parasite is an escape mechanism of the immune response [42].

Different results were obtained when the serum levels of cytokines were analyzed in groups of animals infected with the Y strain of *T. cruzi* and treated with CM, Bz, or Bz+CM. Interestingly, in serum from animals infected with the Y strain of *T. cruzi* and treated with CM, the levels of TNF-α and IL-10 were markedly increased (approximately three times) on day 10 compared with infected animals that received no treatment. Furthermore, all of the infected animals that were treated with CM died prematurely, *i.e.*, day (12–16) post-infection. The weight loss observed in infected mice untreated and treated with CM is likely related to high levels of TNF-α, which appears to be involved in cachexia and mortality in animals infected with *T. cruzi* [38–40]. This hypothesis is supported by the fact that cytokine levels in infected animals treated with Bz or with Bz+CM were significantly reduced when compared with the levels in non-treated infected animals (I) or infected animals treated with CM (CM). Additionally, these animals (Bz or Bz+CM) survived until the end of the experimental period. Benznidazole is a nitro-heterocyclic drug clinically used for the treatment of Chagas disease [43,44]. Several studies show that the benefit of benznidazole in infections by *T. cruzi* does not merely depend on its tripanocide effects but also on its immunomodulating ones [45–48].

Cells and effector mechanisms of the immune system are well established to be responsible for controlling both the growth of the parasite in tissues and local lesions that result from antiparasitic activity. The importance of this regulatory mechanism can be clearly seen in a study in which the strain Tulahuen of *T. cruzi* was used to infect IL-10-deficient mice. These animals were able to control with great efficiency the parasitemia and the parasite load in the cardiac tissue. However, IL-10-deficient animals in this model die early due to the toxic effects caused by an excess in TNF-α production. The production of an excess of TNF-α in infections by *T. cruzi* is linked to immune hyper-reactivity, metabolic changes, and early death of animals [49]. Therefore, the production of IL-10 and other cytokines is required to neutralize the deleterious effects of the type 1 cytokines produced [50]. Additionally, the balance between these cytokines is crucial for the success of infection control and survival.

In the present study, CM administration in mice infected with the Y strain of *T. cruzi* increased the synthesis of both TNF-α and IL-10. These findings led us to speculate that CM can alter the balance between these cytokines, with important consequences related to worsening of the infection.

Some studies have shown that drugs and the immune system can interact during the etiological treatment of parasitic infections [51–54]. This interaction may occur through a synergistic action between the drug and immunological components in the host, leading to the induction of healing and protection. Immunotherapy may also enhance the effectiveness of treatment in some cases. However, the drug may induce immunosuppression by changing the response produced by the host and exacerbate the disease.

Given these observations, our results indicate a possible differential immunomodulatory effect of CM that depends on the treatment regimen. The results also indicate the need to further investigate the mechanism by which CM interferes with the production of different cytokines or mediators and the immunopathogenic role of cytokines or mediators in murine infection induced by the Y strain of *T. cruzi*.

Overall, the current study shows that CM treatment in mice infected with the Y strain of *T. cruzi* induced a concomitant increase in the synthesis of the two cytokines (TNF-α and IL-10) on the 10th day after infection and a significant IL-10 decrease on the 13th day after infection. These alterations are probably intimately related to the deleterious effect of CM on *T. cruzi* infection in mice. Further investigations should be carried out to better elucidate the mechanism by which CM interferes in the production of different cytokines or mediators and the immunopathogenic role of these cytokines or mediators in mouse infection induced by Y strain of *T. cruzi*.

Conflict of interest statement

We declare that we have no conflict of interest.

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