Biotherapies of rabbit serum modulate the immune response and decrease parasite load in mice infected with Trypanosoma cruzi

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The use of biotherapies as intervention in murine infection with Trypanosoma cruzi is a possible means to understand the effects of these highly diluted medications. This study evaluated the effects of biotherapies that were prepared from rabbit serum uninfected (BSNI13c group) and chronically infected with Y strain of T. cruzi (BSI13c group), dynamization 13c, in mice experimentally infected. Parasitological, histopathological, and immunological parameters were evaluated. BSNI13c group exhibited the best outcome, including decreases in parasitemia and parasite load/inflammation in the heart, with pronounced Th1 response on days 8 and 12 after infection (a.i.) that was attributable to decrease in IL-4 concentrations, with no increases in TNF-α and IFN-γ, associated to decrease in IL-17A compared to control.

In contrast, BSI13c group did not exhibit alterations in parasitemia but a significant decrease in parasite load/inflammation in the heart, with pronounced Th2 response on day 12 a.i. that was attributable to increase in IL-4 concentrations, with no changes in TNF-α and IFN-γ.
Introduction

Chagas disease is caused by the protozoan Trypanosoma cruzi. This infection has been considered a neglected disease that affects 6–7 million people worldwide, mostly in Latin America where it is endemic (WHO, 2015). Moreover, no totally effective drugs for the etiological treatment of this disease have been developed to date (Coura and Dias, 2009; Guedes et al., 2011).

Homeopathy is a complementary and alternative medicine that is commonly utilized worldwide. The in vitro and in vivo effects of homeopathy have been reported in several published review articles (Bellavite et al., 2006b; Witt et al., 2007; Bonamin and Endler, 2010; Clausen et al., 2011). One of the earliest and most notable innovations of homeopathy was isopathy (Bellavite et al., 2005). Isopathy is a specific therapeutic method that seeks to combat diseases using highly diluted medications, referred to as biotherapies, that are prepared according to homeopathic techniques from biological products that are produced by the disease itself or the healthy organism (i.e., from secretions, excretions, tissues, organs, and products of microbial or allergen origin) (Fontes et al., 2009; Brasil, 2011).

The use of biotherapies as an intervention in murine infection with T. cruzi is a possible means to understand the effects of these highly diluted medications and discover new candidates for the treatment of Chagas disease (Aleixo et al., 2012). Previous studies have investigated the prophylactic and therapeutic effects of biotherapies prepared from trypomastigotes of T. cruzi in murine acute infection (Queiroz et al., 2006; Almeida et al., 2008; Ferraz et al., 2011, 2014; Aleixo et al., 2012; Sandri et al., 2015). These studies demonstrate that highly diluted antigens can modulate the immune response when the organism is challenged with the related antigen, leading to an ensemble of actions that culminate in balance or disorder of this system (Queiroz et al., 2006; Almeida et al., 2008; Sandri et al., 2015).

These outcomes have stimulated the study of biotherapies that are prepared from different active ingredients. Thus, considering the immunomodulatory actions of these highly diluted medications in experimental infections (Bonamin et al., 2013; Rodrigues de Santana et al., 2014a; Ferraz et al., 2015; Sandri et al., 2015) we developed new biotherapies that were prepared from rabbit serum uninfected and chronically infected with Y strain of T. cruzi (Brener, 1965; Melo and Brener, 1978; Zingales et al., 2009).

Araújo Jorge and Castro, 2000).

The present study evaluated the parasitological, histopathological, and immunological effects of biotherapies that were prepared from rabbit serum uninfected and chronically infected with the Y strain of T. cruzi, dynamization 13c, in mice experimentally infected with this protozoan.

Material and methods

Ethics

The study was approved by the Ethics Committee for Experiments in Animals of Universidade Estadual de Maringá/UEM (protocol no. 080/2012 for mice and 051/2013 for rabbits). All of the recommendations of National Law no. 11794 (October 8, 2008) for the scientific management of animals were followed (Brasil, 2015a).

Animals and infection

Sixty-eight male Swiss mice, 28 days of age, were supplied by the Central Vivarium, UEM. The animals were from a conventional lineage with periodically controlled intestinal parasites and ectoparasites. The mice were maintained in the Parasitology Vivarium, UEM, under controlled temperature (22 ± 2 °C) and a 12 h/12 h light/dark cycle. The animals received food and water ad libitum.

The mice were intraperitoneally inoculated with 1400 blood trypomastigotes of the Y strain of T. cruzi (Silva and Nussenzweig, 1953). The Y strain (Discrete Typing Units – T. cruzi II) is one of the strains most used in experimental studies and has widely known biological and molecular properties (Brener, 1965; Melo and Brener, 1978; Zingales et al., 2009).

Experimental design and treatment regimen

Blind, controlled, and randomized trials were performed twice. To blind the experiments, each experimental group received a code so that the researchers were unaware of the treatment that was received by each group. The mice were divided into four experimental groups such that the mean initial weights of the animals in each group were statistically equal (n = 12–19 animals/group; 6–7 animals/group for the analysis of parasitological
and succussion (rhythmic agitations) was performed according to the Brazilian Homeopathic Pharmacopoeia, 3rd edition (Brasil, 2011), on a centesimal scale (c) until dynamization 13c. Succussions among dilutions were performed using (dynamization 13c) and unsuccussed, did not alter the course of the biotherapies when the host’s immunological system was already in contact with the parasite, on days that coincided with the prepatent period (i.e., from day 4 a.i.), parasitemia peak (i.e., day 7 or 8 a.i.), and period of high morbidity of the infected animals (i.e., from day 10 a.i.) (Silva and Nussenzeig, 1953; Magalhães et al., 1985; Araújo Jorge and Castro, 2000).

The medication vehicle (7% alcohol solution) was used as the treatment in the IC group because a previous study demonstrated that 7% alcohol solution and water, succussed (dynamization 13c) and unsuccessed, did not alter the course of T. cruzi infection (Ferraz et al., 2013).

Preparation of the biotherapies

Two male New Zealand white rabbits (Oryctolagus cuniculus), 3 months of age, were supplied by the Farm Vivarium, UEM. The animals were maintained in the Parasitology Vivarium, UEM, under controlled conditions. One of the rabbits was intraperitoneally inoculated with 4 × 10^6 trypomastigotes of the Y strain of T. cruzi. On day 14 a.i., the presence of the parasite in the bloodstream was detected by a fresh blood test. On day 60 a.i. (i.e., the chronic phase of infection), the animal was intramuscularly anesthetized with 35 mg/kg ketamine chloride + 5 mg/kg xylazine (Faria Neto and dos Santos, 2008), and blood was aseptically collected by cardiac puncture. Uninfected rabbit blood was collected on the same day using the same procedures. After blood clotting, the samples were centrifuged at 3000 rotations per minute (rpm) for 10 min, and sera were collected under sterile conditions (i.e., under a laminar flow hood), stored at –20 °C, and subsequently used for the preparation of the medications.

The biotherapies were prepared under sterile conditions (laminar flow hood). The dynamization process (i.e., dilution and succussion [rhythmic agitations]) was performed according to the Brazilian Homeopathic Pharmacopoeia, 3rd edition (Brasil, 2011), on a centesimal scale (c) until dynamization 13c. Succussions among dilutions were performed using a mechanical stirrer (AUTIC Mod. Denise 10-50, Campinas, Brazil). The first 1:100 dilution (v/v) of the pure rabbit serum (uninfected or chronically infected with T. cruzi) was performed in sterile water, with 100 succussions, resulting in dynamization 1c; thus, the 1:100 dilution (v/v) plus 100 succussions of 1c was used to produce dynamization 2c. In the first three dynamizations (1c to 3c), the vehicle that was used for the dilutions was sterile water (i.e. component present in higher quantities in the serum, in which the substances present in serum are soluble). Dynamizations 4c to 12c were similarly prepared, and the vehicle that was used for the dilutions was 70% alcohol solution. The final dynamization, 13c, was prepared in a 7% alcohol solution, considering that it was used to treat the animals. The biotherapies were prepared and stored in sterile amber commercial glass flasks at room temperature.

Dynamization 13c was selected based on the clinical experience of a veterinary homeopath and based on the beneficial results that were reported by Temporini et al. (2012) after screening that evaluated three other dynamizations (6c, 13c, and 30c) using the same experimental protocol as the one used in the present study (unpublished data).

Microbiological tests (Microbiology Laboratory, UEM) of the inert ingredients were negative, according to regulations of the Brazilian Ministry of Health – RDC no. 67 (Brasil, 2015b).

Parasitological parameters

Parasitemia was assessed using Brener’s technique (Brener, 1962). The parasite count was performed daily from day 4 a.i. until death of the animals. The parasitemia curve was plotted using the mean of parasitemia in each group. The results are presented as the day when parasitemia was detected in fresh blood, parasitemia peak (i.e., the mean of the sum of parasitemia of each mouse over the entire experiment), mortality rate (i.e., the total number of dead animals relative to the number of infected animals), and survival time (i.e., the mean time that the animals survived, in days).

Histopathological parameters

On days 0 a.i. (i.e., without infection), 8 a.i. (i.e., parasitemia peak), and 12 a.i. (i.e., a period of high morbidity, when the animals exhibit attempts to initiate a reaction to the infection), four animals per group were euthanized by deep anesthesia with 300 mg/kg ketamine chloride + 30 mg/kg xylazine, i.p. (Faria Neto and dos Santos, 2008). On each day, the heart, large intestine (distal colon), skeletal muscle from the left hind leg, and liver were collected. The organs were fixed in 10% formalin and preserved in 70% ethanol. After dehydration and diaphanization, the organs were embedded in paraffin. Histological semi-serial sections (5 μm thickness at 20 μm intervals) of the collected organs were performed and stained with hematoxylin-eosin (HE).

Four histological semi-serial sections were obtained for each organ. Ten microscopic fields (at two-field intervals) were analyzed for each section under an Olympus CX31 optical microscope (400× magnification; Olympus, Tokyo, Japan), for a total of 40 fields/organ. Tissue parasitism, i.e., the number of parameters; 12 animals/group for the analysis of immunological and histopathological parameters). The experimental groups were the following: UI (uninfected; n = 12), IC (infection control; the animals were treated on days 4, 7, and 10 after infection [a.i.] with a 7% alcohol solution [medication vehicle], n = 19), BSN13c (the animals were treated on days 4, 7, and 10 a.i. with a biotherapy that was prepared from rabbit serum uninfected with T. cruzi, dynamization 13c, n = 18 animals), and BSI13c (animals were treated on days 4, 7, and 10 a.i. with a biotherapy that was prepared from rabbit serum chronically infected with T. cruzi, dynamization 13c, n = 19).

The medications were diluted in water (10 μl/ml) and offered ad libitum in a sterile amber bottle according to Aleixo et al. (2012) for 16 consecutive hours (medications were available to the animals from 5:00 PM to 9:00 AM). The treatment regimen was based on the action of the medication that is linked to its immunological effects and specific evolution of the Y strain of T. cruzi in Swiss mice (Aleixo et al., 2012). Thus, this treatment regimen assessed the action of the biotherapies when the host’s immunological system was already in contact with the parasite, on days that coincided with the prepatent period (i.e., from day 4 a.i.), parasitemia peak (i.e., day 7 or 8 a.i.), and period of high morbidity of the infected animals (i.e., from day 10 a.i.) (Silva and Nussenzeig, 1953; Magalhães et al., 1985; Araújo Jorge and Castro, 2000).

The medication vehicle (7% alcohol solution) was used as the treatment in the IC group because a previous study demonstrated that 7% alcohol solution and water, succussed (dynamization 13c) and unsuccessed, did not alter the course of T. cruzi infection (Ferraz et al., 2013).
amastigote nests and number of amastigotes/nest, were counted in each field analyzed and the inflammatory process was classified according to intensity and distribution as the following: (1) intensity according to the number of inflammatory cells per field (absent, 0–9 cells; discreet, 10–25 cells; moderate, 26–50 cells; intense, >50 cells) and (2) distribution of inflammatory infiltrate per field (focal, one focus per field; multifocal, more than one focus per field; diffuse, inflammatory cells that were diffusely distributed throughout the field) (Michailowsky et al., 2001).

**Immunological parameters**

On days 0, 8, and 12 a.i. (as detailed in the Histopathological parameters section above), before the organs were removed, peripheral blood was collected from four animals per group. The uninfected group (UI group) provided the basal levels of cytokines. After blood clotting, the samples were centrifuged at 3000 rpm for 10 min, and sera were separated and stored at −20°C (Gholamnezhad et al., 2014) for subsequent cytokine determination.

Cytokines were analyzed to understand the proinflammatory (interferon-γ [IFN-γ], tumor necrosis factor α [TNF-α], and interleukin-17A [IL-17A]) and antiinflammatory (interleukin-4 [IL-4]) balance (Munoz-Fernandez et al., 1992; Santos-Lima et al., 1997; Miyazaki et al., 2010) in Swiss mice that were infected with the Y strain of *T. cruzi* and treated with the biotherapies. The levels of IFN-γ, TNF-α, IL-4, and IL-17A were determined by an enzyme-linked immunosorbent assay according to the manufacturer’s instructions (eBiosciences®, San Diego, USA). Plates were read at 450 nm using a Multiskan GO reader (Thermo Scientific, Waltham, USA).

**Statistical analysis**

The data were compared using BioEstat 5.0 software at the significance level 2α = 0.05. The results were expressed as mean ± standard deviation. The D’Agostino Pearson or Shapiro-Wilk test was performed to determine normality of the data for the parasitological, histopathological, and immunological parameters. Comparisons between data with a normal distribution were performed using analysis of variance (ANOVA), followed by the Least Significant Difference test (LSD). Nonparametric data were analyzed using the Kruskal-Wallis test, followed by the Student-Newman-Keuls test. Statistical comparisons of inflammatory processes were performed using Fisher’s exact test. The Pearson correlation test was performed to correlate tissue parasitism in specific organs (i.e., the number of amastigote nests and number of amastigotes/nest) that showed significant differences among groups and cytokine concentrations on day 8 and/or 12 a.i. Correlations with ‘r’ value up to 0.299 were considered weak, r = 0.300–0.499 were considered intermediate and, r = 0.500–0.999 were considered strong. The sign (+ or −) indicates the direction, i.e., whether the correlation is positive (direct) or negative (inverse). The log-rank test was used to compare survival curves. The analysis was performed using R 3.1.1 software.

**Results**

**Parasitological parameters**

The parasitemia curves showed a characteristic profile of infection with the Y strain of *T. cruzi* in all of the groups. The animals in the BSNI13c group exhibited lower total parasitemia and a decrease in the parasitemia peak (day 8 a.i.) compared with the IC group. The BSNI13c group had a longer survival time and higher expected survival (11% and 34%, respectively), although these were not significantly different from the IC group (Table 1). No significant differences were observed in the parasitological parameters between the BSI13c and IC groups (Table 1).

**Table 1 – Parasitological parameters assessed in groups of mice infected with the Y strain of *T. cruzi* and submitted to the treatment with biotherapies of rabbit serum.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Prepatent period (days)</th>
<th>Patent period (days)</th>
<th>Parasitemia peak (trypomastigotes/ml)</th>
<th>Total parasitemia (trypomastigotes/ml)</th>
<th>Survival (days)</th>
<th>Expected survival (days)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>4.0 ± 0.0*</td>
<td>10.8 ± 0.8*</td>
<td>(10.7 ± 3.4) × 10^5a</td>
<td>(22.4 ± 4.6) × 10^5a</td>
<td>14.0 ± 0.6*</td>
<td>4.8*</td>
<td>100</td>
</tr>
<tr>
<td>BSNI13c</td>
<td>4.7 ± 1.2*</td>
<td>11.3 ± 1.5*</td>
<td>(5.3 ± 3.0) × 10^5b</td>
<td>(14.2 ± 2.0) × 10^5b</td>
<td>15.7 ± 1.9*</td>
<td>7.2*</td>
<td>100</td>
</tr>
<tr>
<td>BSI13c</td>
<td>4.0 ± 0.0*</td>
<td>10.3 ± 1.7*</td>
<td>(8.2 ± 3.6) × 10^5ab</td>
<td>(20.4 ± 4.6) × 10^5ab</td>
<td>13.7 ± 2.0*</td>
<td>5.5*</td>
<td>100</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± standard deviation. IC – treated with 7% alcohol solution, BSNI13c – treated with biotherapy of rabbit serum uninfected with *T. cruzi*, dynamization 13c and BSI13c – treated with biotherapy of rabbit serum infected with *T. cruzi*, dynamization 13c. Different lowercase letters in a column = statistically significant.
significant decreases in the number of amastigote nests and amastigotes/nest in the heart compared with the IC group (Table 3). Moreover, heart inflammation was significantly lower in the BSNI\textsubscript{13c} group and BSI\textsubscript{13c} group compared with the IC group. The BSNI\textsubscript{13c} and BSI\textsubscript{13c} groups presented intensity and distribution of the majority of inflammatory processes classified as discreet and diffuse (30% and 33%, respectively), while the IC group presented intensity and distribution of the majority of inflammatory processes classified as moderate and diffuse (53%; Fig. 1). In the large intestine, skeletal muscle, and liver, the number of amastigote nests, amastigotes/nest (Table 3), and inflammatory processes were not significantly different among groups on day 12 a.i. The intensity and distribution of the inflammatory processes in the intestine and skeletal muscle were predominantly discreet and focal in the BSNI\textsubscript{13c} group (11\% and 17\% for the intestine and skeletal muscle, respectively), BSI\textsubscript{13c} group (6\% and 14\%, respectively), and IC group (8\% and 18\%, respectively). In the liver, all of the groups presented intensity and distribution of the inflammatory processes classified as moderate and diffuse (100\%). On days 0, 8, and 12 a.i., no signs of inflammatory reactions were observed in the organs in the UI group.

**Immunological parameters**

Serum TNF-α levels in the IC, BSNI\textsubscript{13c}, and BSI\textsubscript{13c} groups during the course of infection were significantly higher on days 8 and 12 a.i. compared with day 0. The BSNI\textsubscript{13c} group exhibited TNF-α concentrations that were significantly lower than in the IC and BSI\textsubscript{13c} groups. On day 12 a.i., no significant differences were observed among the IC, BSNI\textsubscript{13c}, and BSI\textsubscript{13c} groups. The UI group (basal value) exhibited lower TNF-α concentrations on days 8 and 12 a.i. compared with the infected groups (Fig. 2).

Serum IFN-γ concentrations in the IC, BSNI\textsubscript{13c}, and BSI\textsubscript{13c} groups during the course of infection were significantly higher on days 8 and 12 a.i. compared with day 0. On days 8 and 12 a.i., no significant differences were observed among the IC, BSNI\textsubscript{13c}, and BSI\textsubscript{13c} groups. The UI group (basal value) exhibited lower IFN-γ concentrations compared with the infected groups (Fig. 2).

The groups presented different evolutions of serum IL-4 levels during the course of infection. The IC group did not present significant changes in IL-4 concentrations during the days of assessment. The BSNI\textsubscript{13c} group presented a significant decrease in IL-4 concentrations on day 12 a.i. compared with day 0. The BSI\textsubscript{13c} group presented a significant increase on days 8 and 12 a.i. compared with day 0 and on day 12 a.i. compared with day 8 a.i. On day 8 a.i., the BSNI\textsubscript{13c} group exhibited IL-4 concentrations that were significantly lower compared with the IC and BSI\textsubscript{13c} groups, but these values were not different from the basal value. The IC and BSI\textsubscript{13c} groups presented higher serum IL-4 concentrations compared with the basal value (Fig. 2). On day 12 a.i., the BSI\textsubscript{13c} group exhibited lower IL-4 concentrations compared with the IC and BSI\textsubscript{13c} groups and basal value. In contrast, IL-4 concentrations were higher in the BSI\textsubscript{13c} group than in the IC and BSNI\textsubscript{13c} groups and the basal value (Fig. 2).

The BSNI\textsubscript{13c} group exhibited a different evolution of serum IL-17A levels during the course of infection compared with the IC and BSI\textsubscript{13c} groups. The BSNI\textsubscript{13c} group exhibited a significant decrease in IL-17A concentrations on day 12 a.i. compared with day 0. In contrast, the IC and BSI\textsubscript{13c} groups did not exhibit significant changes in IL-17A concentrations among the days of assessment. On day 8 a.i., no significant difference was

**Table 2 – Numbers of amastigote nests and amastigotes/nest assessed in different organs, on day 8 after infection (a.i.), in groups of mice infected with the Y strain of T. cruzi and submitted to the treatment with biotherapies of rabbit serum.**

<table>
<thead>
<tr>
<th>Organs</th>
<th>IC</th>
<th>BSNI\textsubscript{13c}</th>
<th>BSI\textsubscript{13c}</th>
<th>IC</th>
<th>BSNI\textsubscript{13c}</th>
<th>BSI\textsubscript{13c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>0.02 ± 0.05*</td>
<td>0.02 ± 0.05*</td>
<td>0.08 ± 0.11*</td>
<td>1.90 ± 3.26*</td>
<td>0.30 ± 0.71*</td>
<td>2.54 ± 2.96*</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.01 ± 0.05*</td>
<td>0.01 ± 0.03*</td>
<td>0.00 ± 0.00*</td>
<td>0.06 ± 0.23*</td>
<td>0.07 ± 0.21*</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.00 ± 0.00*</td>
<td>0.01 ± 0.03*</td>
<td>0.00 ± 0.00*</td>
<td>0.00 ± 0.00*</td>
<td>0.03 ± 0.10*</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>Liver</td>
<td>0.01 ± 0.03*</td>
<td>0.00 ± 0.00*</td>
<td>0.00 ± 0.00*</td>
<td>0.09 ± 0.32*</td>
<td>0.00 ± 0.00*</td>
<td>0.00 ± 0.00*</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± standard deviation. IC – treated with 7% alcohol solution, BSNI\textsubscript{13c} – treated with biotherapy of rabbit serum uninfected with T. cruzi, dynamization 13c and BSI\textsubscript{13c} – treated with biotherapy of rabbit serum infected with T. cruzi, dynamization 13c. Different lowercase letters in a line = statistically significant.

**Table 3 – Numbers of amastigote nests and amastigotes/nest assessed in different organs, on day 12 after infection (a.i.), in groups of mice infected with the Y strain of T. cruzi and submitted to the treatment with biotherapies of rabbit serum.**

<table>
<thead>
<tr>
<th>Organs</th>
<th>IC</th>
<th>BSNI\textsubscript{13c}</th>
<th>BSI\textsubscript{13c}</th>
<th>IC</th>
<th>BSNI\textsubscript{13c}</th>
<th>BSI\textsubscript{13c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1.30 ± 0.50*</td>
<td>0.40 ± 0.20*</td>
<td>0.70 ± 0.10*</td>
<td>27.40 ± 10.60*</td>
<td>8.70 ± 4.60*</td>
<td>15.6 ± 4.50*</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.50 ± 0.70*</td>
<td>0.40 ± 0.20*</td>
<td>0.40 ± 0.20*</td>
<td>3.60 ± 3.40*</td>
<td>2.60 ± 1.90*</td>
<td>2.80 ± 1.40*</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.02 ± 0.08*</td>
<td>0.02 ± 0.06*</td>
<td>0.01 ± 0.04*</td>
<td>0.16 ± 0.61*</td>
<td>0.98 ± 3.25*</td>
<td>0.70 ± 1.85*</td>
</tr>
<tr>
<td>Liver</td>
<td>0.04 ± 0.07*</td>
<td>0.02 ± 0.04*</td>
<td>0.02 ± 0.04*</td>
<td>0.94 ± 2.17*</td>
<td>0.38 ± 0.94*</td>
<td>0.38 ± 0.89*</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± standard deviation. IC – treated with 7% alcohol solution, BSNI\textsubscript{13c} – treated with biotherapy of rabbit serum uninfected with T. cruzi, dynamization 13c and BSI\textsubscript{13c} – treated with biotherapy of rabbit serum infected with T. cruzi, dynamization 13c. Different lowercase letters in a line = statistically significant.
observed among groups (Fig. 2). On day 12 a.i., the BSNI13c group exhibited lower IL-17A concentrations compared with the IC and BSI13c groups and basal value. The BSI13c group also exhibited significantly lower IL-17A concentrations than the IC group, but these values were not different from the basal value (Fig. 2).

The TNF-α/IL-4 and IFN-γ/IL-4 ratios were also calculated to evaluate the Th1/Th2 cytokine balance (Fig. 3). On days 8 and 12 a.i., the BSNI13c group exhibited significant increases in the TNF-α/IL-4 and IFN-γ/IL-4 ratios, with a more pronounced Th1 response that was attributable to a reduction of IL-4 concentrations, with no significant increase in TNF-α or IFN-γ concentrations compared with the IC and BSI13c groups. In contrast, on day 12 a.i., the BSI13c group exhibited a significant decrease in the IFN-γ/IL-4 ratio (Fig. 3), with a more pronounced Th2 response that was attributable to an increase in IL-4 concentrations, with no significant changes in TNF-α or IFN-γ concentrations compared with the IC and BSNI13c groups.

**Correlation between tissue parasitism in the heart and cytokine concentrations on day 12 a.i.**

In the BSNI13c group, the decrease in tissue parasitism in the heart was strongly correlated with a decrease in IL-17A ($r = 0.8783$), and the increase in tissue parasitism in the heart was strongly correlated with an increase in serum IL-4 concentrations ($r = 0.8430$) and decrease in serum TNF-α concentrations ($r = -0.8622$; Fig. 4). In the IC group, the increase in tissue parasitism in the heart was strongly correlated with a decrease in serum TNF-α concentrations ($r = -0.6866$; Fig. 4).

**Discussion**

The use of biotherapy that was prepared from rabbit serum uninfected with the Y strain of *T. cruzi*, dynamization 13c (BSNI13c group), in mice experimentally infected with this protozoan produced immunomodulatory action that was clearly related to a beneficial effect in the host. This immunomodulatory action involved a decrease in serum IL-4 concentrations on days 8 and 12 a.i. and decrease in IL-17A concentrations on day 12 a.i. compared with the IC and BSI13c groups. Interestingly, on day 12 a.i., the concentrations of both cytokines were lower than the basal values measured in the UI group. Serum TNF-α and IFN-γ concentrations significantly increased during the course of infection but were not different among the infected groups. Moreover, the analysis of the Th1/Th2 balance revealed increases in the TNF-α/IL-4 and IFN-γ/IL-4 ratios on days 8 and 12 a.i., with a more pronounced Th1 response.
response that was attributable to a reduction in IL-4 concentrations compared with the IC and BSI13c groups. In Chagas disease, the proinflammatory Th1 response is required for parasite control and characterized by the production of cytokines (e.g., TNF-α and IFN-γ) that act in concert to activate macrophages to kill intracellular amastigotes through the production of nitric oxide and nitrogen-free radicals (Munoz-Fernandez et al., 1992; Santos-Lima et al., 1997). However, an excessive inflammatory response also significantly impacts the pathogenesis of experimental Chagas disease (Hunter et al., 1997; Hölscher et al., 2000).

This cytokine balance resulted in decreases in total parasitemia, parasitemia peak (day 8 a.i.), parasite load, and inflammatory process (discreet and diffuse) in the heart in the BSNI13c group on day 12 a.i. In this experimental model, in which animals are extremely susceptible to infection (i.e., 100% mortality in infected animals), parasitemia means morbidity (Araújo Jorge and Castro, 2000). A decrease in

Fig. 2 – Serum TNF-α, IFN-γ, IL-4 and IL-17A concentrations (pg/ml) assessed, on day 0, 8 and 12 after infection, in mice infected with the Y strain of T. cruzi and belonging to the groups: UI (animals uninfected), IC (treated with 7% alcohol solution), BSNI13c (treated with biotherapy of rabbit serum uninfected with T. cruzi, dynamization 13c) and BSI13c (treated with biotherapy of rabbit serum infected with T. cruzi, dynamization 13c). *Statistically significant (comparisons were made among the days during the course of infection for each group). Different lowercase letters = statistically significant (comparisons were made among groups for each day evaluated).

Fig. 3 – TNF-α/IL-4 and IFN-γ/IL-4 ratios (Th1/Th2 balance) obtained for the groups: IC (treated with 7% alcohol solution), BSNI13c (treated with biotherapy of rabbit serum uninfected with T. cruzi, dynamization 13c) and BSI13c (treated with biotherapy of rabbit serum infected with T. cruzi, dynamization 13c). Different lowercase letters = statistically significant (comparisons were made among groups for each day evaluated).
The use of biotherapy that was prepared from rabbit serum chronically infected with the Y strain of T. cruzi, dynamization 13c (BSNI13c group), in mice experimentally infected with this protozoan also produced an immunomodulatory action that was different from the one produced by the biotherapy that was prepared from rabbit serum uninfected and the infection control. The immunomodulatory action involved an increase in serum IL-4 concentrations and decrease in IL-17A concentrations on day 12 a.i. Serum TNF-α and IFN-γ concentrations significantly increased during the course of infection. However, on day 8 a.i., TNF-α concentrations were significantly lower compared with the IC and BSNI13c groups. The analysis of the Th1/Th2 balance revealed a significant decrease in the IFN-γ/IL-4 ratio on day 12 a.i., with a more pronounced Th2 response that was attributable to an increase in IL-4 concentrations compared with the IC and BSNI13c groups. In Chagas disease, the cytokine IL-4 is involved in the differentiation of T lymphocytes toward an anti-inflammatory Th2 response (Seder and Paul, 1994), with the subsequent production of several cytokines (e.g., IL-4, IL-5, IL-10, IL-13, among others) that are related to disease susceptibility in the acute phase (Gazzinelli et al., 1992) and control excessive inflammation (Hunter et al., 1997; Hölscher et al., 2000).

This cytokine balance did not alter parasitemia in the treated animals but significantly decreased the parasite load and inflammatory process (discreet and diffuse) in the heart on day 12 a.i. compared with the IC group. The correlation analysis between tissue parasitism in the heart and cytokine concentrations on day 12 a.i. showed that the decrease in serum IL-17A concentrations was important for the significant reduction of tissue parasitism in the heart. The increase in IL-4 concentrations and decrease in TNF-α concentrations were strongly correlated with the increase in tissue parasitism in the heart.

The balance between the Th17 response and Th1 or Th2 responses was not assessed because little (and contradictory) information about the interactions between these responses have been reported (Monteiro et al., 2007; da Matta Guedes et al., 2010; Miyazaki et al., 2010; Erdmann et al., 2013). Although the cytokine IL-17A has been linked to the pathogenesis of several inflammatory and autoimmune diseases (Stumhofer et al., 2006; Zelante et al., 2007; Tesmer et al., 2008), the pathogenic or protective role of IL-17A in mice infected with T. cruzi is still controversial. Previous studies in mice IL-17A−/− or treated with anti-IL-17 demonstrated the protective role of IL-17A against T. cruzi infection, in which these mice presented premature mortality compared with wildtype mice (da Matta Guedes et al., 2010; Miyazaki et al., 2010), with higher recruitment of inflammatory cells in heart tissue, despite a reduction of cardiac parasitism (da Matta Guedes et al., 2010). Other studies showed that the increase in IL-17A expression was associated with susceptibility to T. cruzi infection in a murine model that utilized bradykinin receptor 2−/− mice (Monteiro et al., 2007) and associated with an increase in liver pathology in mice experimentally infected with T. cruzi (Erdmann et al., 2013). In the present study, the decrease in IL-17A concentrations on day 12 a.i. was considered beneficial and strongly correlated with the decrease in tissue parasitism in the heart. Importantly, the cytokines that are produced in Chagas disease act in concert, the final balance of which is a determining factor in whether the development of pathological alterations is mild or aggressive.

In fact, the biotherapies of rabbit serum exerted parasitological, histopathological, and immunological effects in infected mice compared with the infection control (this group presented effects only of the infection itself). The inclusion of experimental groups that are uninfected and treated with the biotherapies would serve as a control that will allow us to determine whether the biotherapies can modulate the immune system without the presence of infection. This possibility will be pursued in future experiments, but we will have to optimize the number of animals because the ethics
committees for experiments in animals are increasingly stringent in approving experiments with very large numbers of experimental groups.

The mechanisms of action of homeopathic preparations on biological systems are still unknown. The present results and previous studies suggest that these medications can regulate inflammatory and immunopathological processes (Pedalino et al., 2004; Bellavite et al., 2006a; Bonamin and Endler, 2010; Rodrigues de Santana et al., 2014a,b; Sandri et al., 2015), likely through the participation of nanostructures in highly diluted medications that are beyond Avogadro’s number (Chikramane et al., 2010; Bell and Koithan, 2012). In the present study, the biotherapies were administered on days that coincided with the prepatent period (i.e., from day 4 a.i.), parasitemia peak (i.e., day 7 or 8 a.i.), and period of high morbidity in infected animals (i.e., from day 10 a.i.). Interestingly, the biotherapies were able to modulate the host’s immune system, which had already been activated by the presence of the parasite (Aleixo et al., 2012).

The biotherapy that was prepared from healthy rabbit serum appears to provide homeostasis information to the susceptible organism (Swiss mice that are infected with the Y strain of T. cruzi). Such homeostasis information from a resistant organism (i.e., the rabbit) includes an “equilibrium pattern” that comprises all biological molecules that are found in a state of non-infection, thus producing a balanced Th1 response, with evident benefits to the host. In contrast, the biotherapy that was prepared from the serum of rabbits that were chronically infected with T. cruzi provides to the susceptible organism (infected Swiss mice) information on the immune response against infection (i.e., a “disease pattern” that comprises all biological molecules that are found in the state of infection) from a resistant organism, thus resulting in a different immunomodulatory profile (a Th2 response), with less evident beneficial effects.

Moreover, previous studies that were performed by our group showed that a biotherapy that was prepared from healthy mouse serum (dynamization 13c) provided to the susceptible organism (Swiss mice that were infected with the Y strain of T. cruzi) homeostasis information from another susceptible organism (i.e., Swiss mice), producing a less balanced Th1 response and no change in the course of infection. However, the use of the biotherapy that was prepared from mouse serum that was infected with T. cruzi provided to the susceptible organism (infected Swiss mice) information on the immune response against infection (i.e., “disease pattern”) from another susceptible organism, thus producing a less balanced Th2 response, with an increase in parasitemia and early death in treated animals (Ferraz et al., 2015).

The outcomes of these biotherapies that were prepared from rabbit and mouse serum suggest that the ways in which the host’s immune system is modulated depends of the type of information that is provided by the species-specific serum that is used to prepare the biotherapies, including information of health/homeostasis, disease/imbalance, and resistance or susceptibility. The pattern of immunomodulation that was observed in this experimental model shows that biotherapies that are prepared from healthy serum primarily stimulate the Th1 response and this response, in turn, becomes more balanced and beneficial to the host when the serum is derived from a host that is more resistant to infection with T. cruzi (i.e., the rabbit). However, biotherapies that are prepared from serum that contains information on the immune reaction against T. cruzi stimulates primarily the Th2 response and this response, becomes more intensive and harmful to the host when the serum is derived from a host that is susceptible to T. cruzi infection (i.e., Swiss mice) (Ferraz et al., 2015). The challenge is to understand how this information translates to the infected organism because the outcomes of each of these highly diluted medications also are directly associated with the specific preconditions of the living system under study, i.e., susceptibility or resistance of the organism to infection.

Conclusions

In summary, this study suggest that the use of biotherapy that was prepared from rabbit serum uninfected with the Y strain of T. cruzi, dynamization 13c, in Swiss mice experimentally infected presented the best outcome, including decreases in parasitemia and parasite load/inflammation in the heart, with pronounced Th1 response on days 8 and 12 a.i. that was attributable to decrease in IL-4 concentrations, with no increases in TNF-α and IFN-γ, associated to decrease in IL-17A compared to control. In contrast, the use of biotherapy that was prepared from rabbit serum chronically infected with the Y strain of T. cruzi, dynamization 13c, in Swiss mice infected did not alter the parasitemia but significantly decreased the parasite load/inflammation in the heart, with pronounced Th2 response on day 12 a.i. that was attributable to increase in IL-4 concentrations, with no changes in TNF-α and IFN-γ, associated to decrease in IL-17A compared to control. The results represent a substantial contribution and suggest that these highly diluted medications differentially modulate the immune system in mice that were infected with the Y strain of T. cruzi, producing different evolutions of infection with evident beneficial effects and providing evidence of the actions of these medications.

Conflict of interests

The authors declare that they have no competing interests.

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