Treatment with Benznidazole during the Chronic Phase of Experimental Chagas’ Disease Decreases Cardiac Alterations

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Chagas’ disease, caused by Trypanosoma cruzi infection, is one of the main causes of death due to heart failure in Latin American countries. Benznidazole, the chemotherapeutic agent most often used for the treatment of chagasic patients, is highly toxic and has limited efficacy, especially in the chronic phase of the disease. In the present study we used a mouse model of chronic Chagas’ disease to investigate the effects of benznidazole treatment during the chronic phase on disease progression. The hearts of benznidazole-treated mice had decreased parasitism and myocarditis compared to the hearts of untreated chagasic mice. Both groups of Trypanosoma cruzi-infected mice had significant alterations in their electrocardiograms compared to those of the healthy mice. However, untreated mice had significantly higher cardiac conduction disturbances than benznidazole-treated mice, including intraventricular conduction disturbances, atrioventricular blocks, and extrasystoles. The levels of antibodies against T. cruzi antigens (epimastigote extract, Pβ, and trans-sialidase) as well as antibodies against peptides of the second extracellular loops of β1-adrenergic and M2-muscarinic cardiac receptors were also lower in the sera from benznidazole-treated mice than in the sera from untreated mice. These results demonstrate that treatment with benznidazole in the chronic phase of infection prevents the development of severe chronic cardiomyopathy, despite the lack of complete parasite eradication. In addition, our data highlight the role of parasite persistence in the development of chronic Chagas’ disease and reinforce the importance of T. cruzi elimination in order to decrease or prevent the development of severe chagasic cardiomyopathy.

Chagas’ disease, caused by the protozoan Trypanosoma cruzi, is a serious health problem in Latin America, where it affects 16 million to 18 million people (43). In the acute phase of the disease in humans, clinical signs are usually mild, despite high levels of parasitemia in blood, which is characteristic of this phase of infection and which declines with the onset of immunity (18). After this phase, individuals recover to an apparently healthy status, but a mild focal myocarditis may persist and may last for years without signs of cumulative damage in the indeterminate phase of the disease (3). Many years after the primary infection, about 30% of these individuals develop the symptomatic chronic phase of Chagas’ disease. This phase is characterized by the presence of myocarditis and/or pathological disturbances in the peripheral nervous and gastrointestinal systems (15, 37).

The most common chronic form of the disease is chagasic cardiomyopathy, a fatal form for which no effective treatments are available. It is characterized by focal or disseminated inflammatory infiltrates, myocyteolysis, and myonecrosis and progressive deposition of fibrotic tissue (11, 29). The intensity of the myocarditis varies considerably from light cardiac symptoms to intense chronic cardiomyopathy, leading to heart failure and death (31). Patients with chronic chagasic cardiomyopathy may have diverse arrhythmias that cause heart malfunction. The most frequent alterations recorded are ventricular premature beats, complete right bundle branch block, left anterior fascicular block, and atrioventricular block (27).

The mechanisms responsible for the cardiomyopathy are not clearly understood, but the presence of chronic myocardial injury in the absence of parasitemia suggests the participation of autoimmune processes (21, 23, 35). Many hypotheses have been proposed to explain the cardiomyopathy of Chagas’ disease, and these proposed causes of cardiomyopathy may act alone or in combination. They include the breakdown of the tolerance to self-antigens induced by polyclonal activation of the immune system by the parasite (33) or by T. cruzi-specific immune responses cross-reactive to self-antigens through a mechanism of molecular mimicry (13, 24). T. cruzi-specific immune responses acting in the residual parasites during the chronic phase of infection (34), and microvascular abnormalities (30). Several T. cruzi antigens have epitopes similar to those of mammalian cardiac antigens, such as cardiac myosin and the second loop of the human β1-adrenergic receptor (16, 38). Antibodies from chronic chagasic patients induce β1-ad-
renergic and M<sub>2</sub>-muscarinic effects on the myocardium, resulting in alterations of electrocardiographic (ECG) findings correlated to electrical abnormalities, as described in the literature (12, 14). However, there is also evidence that parasite persistence or reinfection influences the severity of the disease (8, 41). This may be due to the direct participation of anti-T. cruzi immune responses and/or by its influence on autoimmune responses (35).

The main drug available for the treatment for Chagas’ disease is benznidazole, whose action eliminates T. cruzi parasites. This compound, however, has limited efficacy and a degree of high toxicity. In humans, benznidazole treatment is effective at parasite eradication mainly in the acute phase of infection but not in the prevalent chronic stage of the disease (10). Several studies have investigated the efficacy of benznidazole treatment during chronic infection in mice. In those studies (1, 2, 9, 32, 39), the investigators focused on the analysis of parasite eradication and confirmed in the mouse model that benznidazole treatment during the chronic phase does not completely eliminate the parasite. The effects of benznidazole treatment for experimental chronic chagasic myocardiitis is controversial. Segura et al. (32) did not observe a significant decrease in myocarditis in mice treated with benznidazole during the chronic phase of infection, whereas in the work by Andrade et al. (1), when parasitological cure was achieved, benznidazole-treated mice had a decreased incidence of myocarditis.

Given the lack of therapeutic options for Chagas’ disease, the potential benefits of benznidazole treatment in the chronic phase of the disease should be carefully examined. In the present study we used a murine model of chronic chagasic cardiomyopathy caused by a myotropic T. cruzi strain to re-evaluate the beneficial effects of benznidazole treatment during the chronic phase of Chagas’ disease. The goal of the benznidazole treatment was to cause a decrease in the residual parasite load present during the chronic phase of infection and analyze its influence on the development of heart alterations characteristic of this disease. The decrease in parasitism resulting from the benznidazole treatment were correlated to histopathological and ECG parameters, as well as to the levels of antibodies specific for T. cruzi antigens and β<sub>1</sub>-adrennergic and M<sub>2</sub>-muscarinic receptors, in order to determine if the treatment results in decreased heart pathology even in absence of a parasitological cure.

MATERIALS AND METHODS

Animals, infection, and chemotherapy. Two-month-old male BALB/c mice, raised and maintained in the animal facilities at the Gonçalo Moniz Research Center, Fundação Oswaldo Cruz, were used in the experiments and were provided with rodent diet and water ad libitum. Mice were infected by intraperitoneal injection of 100 trypomastigote forms of the Colombian strain of T. cruzi (17). Trypomastigotes were obtained by in vitro infection of LCC-MK2 cells. Parasitemia was evaluated at different times after infection by counting the number of parasites in peripheral blood aliquots placed between a glass slide and a coverslip (6). Forty-five days after infection, a group of 10 mice was treated by the oral route with 100 mg of benznidazole (Rochagan; Roche) per kg of body weight daily for 1 week, followed by weekly administrations for an additional 8 months. All animals were killed while they were under anesthesia and were handled according to the guidelines of the National Institutes of Health, for the ethical use of laboratory animals.

ECG records. ECGs were performed with a Bio Amp device PowerLab System (PowerLab 2/20; ADInstruments, Castle Hill, Australia), which recorded bipolar lead I. All animals were anesthetized by intraperitoneal injection of xylazine at 10 mg/kg of body weight and ketamine at 100 mg/kg of body weight. ECG recordings were obtained after the induction of general anesthesia. All data were acquired on a computer for further analysis by using Chart 5 for Windows software (Power Lab; ADInstruments). Wave durations (in milliseconds) were calculated automatically by the software after placement of the cursors. Measurements are average values determined from 14 consecutive ECG records. Records were filtered (1 to 100 Hz) through a band-pass filter to minimize environmental signal disturbances. The sampling rate was 1 kHz. The ECG analysis included the following measurements: heart rate, PR interval, P-wave duration, QT interval, QTc, atrioventricular block, intraventricular block, and other arrhythmias. The software used a derivative-based QRS detection algorithm to calculate the heart rate by detecting the peaks of the R waves automatically. As the T waves are normally not separated from the QRS complex in rodent ECGs (5), we measured the QT interval instead of the QRS complex duration. The QT interval was measured from the beginning of the QRS to the end of the T wave. The definition of the end of the T wave was the point where the signal returned to the isoelectric line (42). The QTc was calculated as the ratio of the QT interval to the square root of the RR interval.

Histopathological analysis. The hearts of the benznidazole-treated mice and untreated controls were removed and fixed in buffered 10% formalin. Sections of paraffin-embedded tissue were stained by the standard hematoxylin-eosin and Sirius red staining methods for evaluation of inflammation and fibrosis, respectively. Paraffin sections were digitized with a color digital video camera (CoolSnap, Montreal, Quebec, Canada) adapted to a BX41 microscope (Olympus, Tokyo, Japan). The images were analyzed with the Image Pro program (version 5.0, Media Cybernetics, San Diego, Calif.) to integrate the number of inflammatory cells counted by the area. The cells in 10 fields per heart from each mouse in each group were counted.

Immunofluorescence for parasite detection. Five-micrometer-thick frozen heart sections were prepared in a cryostat on poly-l-lysine-coated slides and were fixed with cold acetone. Sections were incubated with phosphate-buffered saline (PBS)–5% bovine serum albumin for 30 min, followed by overnight incubation with anti-T. cruzi rat serum (1:400). After the sections were washed with PBS, they were incubated for 1 h with fluorescein isothiocyanate-conjugated rabbit anti-rat immunoglobulin G (1:100; Sigma) adsorbed with normal mouse serum. The sections were washed three times, counterstained with Evans blue, and mounted with Vectashield (Vector). Images were digitalized with a color digital video camera (DP-70) adapted to an Olympus AX-70 microscope without motorized stage xev (Media Cybernetics) and in a FV-500 confocal system with an IX 81 inverted microscope (Olympus). The images were analyzed by use of the Image Pro program (version 5.0, Media Cybernetics) so that the parasite foci could be counted and integrated by the area.

Antigen preparations. Epimastigotes of the Colombian strain of T. cruzi were obtained from axenic cultures grown in liver infusion tryptose medium. The parasites were washed three times in PBS (pH 7.4) and resuspended in distilled water, and then incubated to five cycles of freezing and thawing. The extract was made isotonic by addition of PBS concentrated 10-fold and was centrifuged at 30,000 rpm for 30 min. The supernatant was aliquoted and stored at −70°C. Recombinant P<sub>75</sub> was kindly donated by Mariano Levin. Recombinant trans-sialidase (TS) was produced and purified from Escherichia coli cells transformed with plasmid pTS-cat<sub>S</sub>, as described previously (28). Peptides of the second extracellular loops of β<sub>1</sub>-adrenergic (residues 197 to 222) and M<sub>2</sub>-muscarinic (residues 164 to 185) cardiac receptors were synthesized in an automatic peptide synthesizer by the 9-fluorenlymethoxy carbonyl technique by using solid-phase automatic synthesis. Their purities were checked by high-performance liquid chromatography after cleavage and desalting on a P2 column.

Antibody detection. Sera from untreated, benznidazole-treated, and untreated mice were tested for the presence of antibodies against T. cruzi antigen, TS, P<sub>75</sub>, and the M<sub>2</sub>-muscarinic and β<sub>1</sub>-adrennergic cardiac receptors by enzyme-linked immunosorbent assay. Microtiter plates (Maxisorb; Nunc) were coated with α-T. cruzi antigen (1 μg/ml), 1:1,200 (for TS), and 1:25 (for the M<sub>2</sub>-muscarinic and β<sub>1</sub>-adrenergic cardiac receptor peptides) in PBS–5% nonfat milk and incubated for 2 h at room temperature. After the plates were washed, 50 μl of peroxidase-conjugated anti-mouse polyclonal immunoglobulins (Sigma) diluted 1:1,000 was dispensed into each well and the plate was incubated for 30 min at room temperature. The plates were washed eight times in washing buffer, and the reaction was developed with the 3,3',5,5'-tetramethylbenzidine substrate (Sigma).
and read at 450 nm in a Spectramax 190 microplate reader (Molecular Devices, Sunnyvale, Calif.).

Statistical analyses. Histological and serological data were analyzed by Student’s $t$ test, one-way analysis of variance, and the Newman-Keuls multiple-comparison test, as indicated in the text, with Prism Software (version 3.0; GraphPad Software, San Diego, Calif.). Statistical analyses of the cardiac parameters were performed with SPSS software (version 9.0; SPSS Inc., Chicago, Ill.). The values for all continuous variables are presented as means ± standard errors. To test if the variables were normally distributed, we performed the Kolmogorov-Smirnov test. Nonparametric tests (the Kruskall-Wallis and/or the Mann-Whitney test) were used because the variables were not normally distributed by two-tailed hypothesis testing. Differences were considered significant if $P$ was equal to or less than 0.05.

RESULTS

Decreased parasitism and myocarditis in benznidazole-treated mice. In order to investigate the effects of benznidazole treatment in the chronic phase of $T. cruzi$ infection, BALB/c mice were treated with benznidazole for 8 months after the acute phase of infection (Fig. 1). The hearts of mice in both the untreated and the benznidazole-treated groups had detectable parasite foci 10 months after infection (Fig. 2A and B). How-

FIG. 1. Parasitemia during the acute phase of infection with the Colombian strain of $T. cruzi$ in BALB/c mice. BALB/c mice were infected with 100 trypomastigotes of the Colombian strain of $T. cruzi$. Parasitemia was determined at different times after infection. Values represent the medians for five mice. The arrow indicates the beginning of benznidazole administration.

FIG. 2. Parasitism and histopathological analyses of heart sections of $T. cruzi$-infected mice. Heart sections of untreated (A and C) or benznidazole-treated (B and D) $T. cruzi$-infected mice were analyzed for the presence of parasite foci by confocal microscopy (A and B; magnification, ×60) or inflammation by staining with hematoxylin-eosin (C and D; magnification, ×40).
ever, the number of parasite foci was threefold higher ($P < 0.01$) in the hearts of untreated mice than in those of benznidazole-treated mice (Fig. 3A). Histopathological analysis of heart sections also demonstrated a twofold reduction ($P < 0.02$) in the numbers of inflammatory cells in benznidazole-treated mice (Fig. 3B). No significant differences were observed ($P > 0.05$) when the fibrotic areas of the hearts from untreated and benznidazole-treated mice were compared (Fig. 3C).

**Benznidazole treatment prevents the development of ECG alterations in chronic Chagas’ disease.** To investigate whether benznidazole treatment in the chronic phase of infection ameliorates the cardiac function, ECG analysis was performed 10 months after infection. Both groups of *T. cruzi*-infected mice had significant alterations ($P < 0.05$) in various ECG parameters compared to those for the healthy mice, such as an increased intrinsicoid deflection and an increased QT interval (Table 1 and Fig. 4). Alterations in these parameters indicate a delay in the conduction of the electric impulse and an increase in the duration of the ventricular action potential. More importantly, the ECGs of all untreated *T. cruzi*-infected animals showed severe cardiac conduction disturbances, such as intraventricular conduction disturbances, atrioventricular blocks, and extrasystoles (Table 1 and Fig. 4A). In contrast, only 20% of the benznidazole-treated mice had cardiac conduction disturbances (intermittent intraventricular conduction disturbances), while the remaining animals did not have any conduction disturbances (Fig. 4B). Intermittent conduction disturbances are clinically relevant but are much less severe than the permanent intraventricular conduction disturbances observed in the untreated infected animals. The heart rates of the healthy mice were lower than those of the untreated and benznidazole-treated mice, but the differences between the healthy and chagasic mice were not statistically significant (Table 1).

**Decreased anti-*T. cruzi* antibody levels after benznidazole treatment.** The levels of antibodies against different *T. cruzi* antigens were investigated. Sera from benznidazole-treated mice had decreased levels of antibodies against *T. cruzi* extract ($P < 0.001$) compared to those in the sera of untreated *T. cruzi*-infected mice (Fig. 5A). The levels of antibodies against TS, an immunodominant *T. cruzi* antigen, were also significantly lower ($P < 0.01$) in benznidazole-treated mice (Fig. 5B). Benznidazole-treated mice also had lower levels of antibodies against P$_{2}$B ($P < 0.01$), a member of the acidic ribosomal protein family from the protozoan parasite *T. cruzi* with structural similarities with cardiac receptors (Fig. 5C). The levels of antibodies against peptides from the second extracellular loops of the β$_1$-adrenergic and the M$_{2}$-muscarinic receptors were also decreased in benznidazole-treated mice compared to the levels in untreated *T. cruzi*-infected mice (Fig. 5D and E).

**DISCUSSION**

Parasite persistence occurs in most individuals infected with *T. cruzi*. This can be demonstrated by diagnostic techniques and can be illustrated by the recrudescence of the infection in many patients with heart failure due to Chagas’ disease who
FIG. 4. ECG recordings from BALB/c mice. ECGs of untreated (A) and benznidazole-treated (B) T. cruzi-infected mice were recorded 10 months after infection. (C) ECG for a healthy BALB/c mouse. △, P wave; Δ, QRS complex; *, second-degree atrioventricular block.

TABLE 1. Electrocardiographic parameters measured in the experimental groups

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Heart rate (no. of beats/min)</th>
<th>PR interval (ms)</th>
<th>P-wave duration (ms)</th>
<th>PR segment (ms)</th>
<th>Intrinsicoid deflection (ms)</th>
<th>QT interval (ms)</th>
<th>QT</th>
<th>Cardiac conduction disturbances (no. of mice)</th>
</tr>
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<tbody>
<tr>
<td>Infected (8)</td>
<td>270.3 ± 30.3</td>
<td>39 ± 2.6</td>
<td>18.5 ± 2.4</td>
<td>21.9 ± 1.4</td>
<td>16.1 ± 2.9</td>
<td>41 ± 2.5</td>
<td>2.9 ± 0.2</td>
<td>IVCD (5), AVB (2), ES (1)</td>
</tr>
<tr>
<td>Infected and treated with benznidazole (10)</td>
<td>256.7 ± 15.7</td>
<td>35.5 ± 2.1</td>
<td>21.6 ± 0.9</td>
<td>13.2 ± 1.4</td>
<td>15.8 ± 1.7</td>
<td>43.5 ± 2.8</td>
<td>2.6 ± 0.2</td>
<td>IT-IVCD (1)</td>
</tr>
<tr>
<td>Healthy (18)</td>
<td>215.3 ± 18.5</td>
<td>36.5 ± 1.2</td>
<td>19.7 ± 0.7</td>
<td>16.9 ± 0.8</td>
<td>9 ± 0.7</td>
<td>32.2 ± 1.4</td>
<td>1.9 ± 0.9</td>
<td>IT-IVCD (1), ASI (1)</td>
</tr>
</tbody>
</table>

Values represent the means ± standard errors of the means for individual mice. IVCD, intraventricular conduction disturbance; AVB, atrioventricular block; ES, extrasystole; IT-IVCD, intermittent intraventricular conduction disturbance; ASI, acute subendocardial ischemia.

P < 0.05 compared to the results for untreated infected mice.

P < 0.05 compared to the results for the T. cruzi-infected groups.
undergo cardiac transplantation. When these patients are immunosuppressed, myocarditis usually develops in the transplanted heart and *T. cruzi* parasites are detected in the blood and tissues (22, 36). Benznidazole is the main drug used for the treatment of *T. cruzi* infection. However, although it is effective when it is administered during the acute phase of the infection, treatment with benznidazole is not believed to be able to eliminate the parasite during the chronic phase of infection (10). It

![Graphs showing antibody levels](http://aac.asm.org/)

**FIG. 5.** Decreased levels of antibodies in sera from benznidazole-treated mice. Sera of untreated (I) or benznidazole-treated (I+B) *T. cruzi*-infected and health (N) BALB/c mice were tested by enzyme-linked immunosorbent assay in order to determine the levels of antibodies against *T. cruzi* antigen (A), TS (B), P₂β (C), the second extracellular loop of the human M₁-muscarinic cardiac receptor (D), and the second extracellular loop of the human β₁-adrenergic cardiac receptor (E). The data represent the means ± standard deviations for 5 to 10 mice for each group. *, P < 0.05 compared to the results for the untreated infected group by one-way analysis of variance followed by Newman-Keuls multiple-comparison test. O.D., optical density.
has been proposed that latent forms of the *T. cruzi* parasite present in tissues during the chronic phase of infection are not eliminated by the chemotherapy (7).

In this report we demonstrated with a mouse model of infection that although parasite eradication could not be achieved with benznidazole treatment in the chronic phase, a decrease in the parasite load could be observed. More importantly, we demonstrated for the first time that benznidazole treatment caused a decrease in cardiac dysfunction (ECG alterations) and myocarditis, indicating that parasite persistence plays an important role in the pathogenesis of chronic chagasic cardiomyopathy. The observations of Bustamante and coworkers (8) that reexposure to parasites through repeated infections aggravates heart dysfunction corroborates this idea.

The beneficial effects of benznidazole treatment were achieved by using a prolonged administration of a low dose in mice infected with the Colombian strain of *T. cruzi*, a chemotherapy-resistant strain (1, 40). Thus, it is likely that benznidazole treatment is even more effective against chronic infections caused by benznidazole-sensitive *T. cruzi* strains. In addition, due to the high degree of toxicity of benznidazole, a low-dose treatment similar to the one used in this work may be more tolerable for humans.

Our results demonstrate that the sera of benznidazole-treated mice had lower levels of antibodies specific to *T. cruzi* due to the high degree of toxicity of benznidazole, a low-dose treatment similar to the one used in this work may be more tolerable for humans.

The lower antibody levels in benznidazole-treated mice may account for the better performance in cardiac function in these animals. These data are in accordance with those from previous reports which demonstrated that mice immunized with recombinant *T. cruzi* ribosomal Pβ protein, which reproduced the typical anti-P profile characteristic of chronic infections, had ECG alterations without cardiac inflammatory lesions (4). Thus, in untreated mice, the higher levels of antibodies and heart inflammation could explain the finding of cardiac conduction disturbances in the animals in this group, whereas in benznidazole-treated mice these aggressive factors were decreased, resulting in better heart performance.

Chronic chagasic cardiomyopathy is a dilated cardiomyopathy accompanied by acute and chronic inflammation, fibrosis, and vasculitis. The mechanism by which this persistent inflammatory reaction occurs is not clearly known and occurs in only about 25% of *T. cruzi*-infected individuals. Several reports have recently demonstrated a role of autoimmune responses in experimental models of chronic chagasic myocarditis (19, 23, 26). However, although the intensities of tissue parasitism and inflammation do not have a direct correlation, parasite persistence is probably required for disease development and maintenance (35). In this study, we observed a correlation between decreased inflammation and parasitism in the myocardium of benznidazole-treated mice, which reinforces the importance of residual parasitism in the development of chronic chagasic myocarditis. As shown in a previous study (35), the profile of the host’s immune response during *T. cruzi* infection is also important in the development of the disease.

In conclusion, our results demonstrate the importance of benznidazole treatment in chronic chagasic patients in order to decrease or retard the development of chagasic cardiopathy, even though complete parasite eradication is not achieved. In addition, it reinforces the need for the discovery of new anti-*T. cruzi* drugs with higher degrees of efficacy against the chronic infection and fewer collateral effects. This will allow the more efficient elimination of the parasite from *T. cruzi*-infected individuals and proper treatment of those patients who cannot be treated with benznidazole due to its high degree of toxicity.

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