CHRONIC CHAGAS’ DISEASE IN Rhesus Monkeys (Macaca mulatta): Evaluation of Parasitemia, Serology, Electrocardiography, Echocardiography, and Radiology

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Abstract. Severe chronic damage to the heart and gastrointestinal tract in patients with Chagas’ disease are often observed 10–20 years after the acute phase. The course of long-lasting infection with the Colombian strain of Trypanosoma cruzi was studied in seven rhesus monkeys infected for 15–19 years. Subpatent parasitemia was detected in all studied animals, using hemoculture (two of seven), artificial xenodiagnosis (three of seven), and a polymerase chain reaction PCR (six of six). High titers of specific IgG antibody to T. cruzi persisted throughout the chronic phase of infection. Abnormal electrocardiographic (three of six) and echocardiographic (one of six) patterns detected in the T. cruzi-infected monkeys were possibly related to parasite-triggered myocardial damage. The results suggest that rhesus monkeys experimentally infected with T. cruzi, besides reproducing the acute phase of Chagas’ disease, also develop chronic chagasic cardiomyopathy.

INTRODUCTION

Chagas’ disease, which is caused by a flagellate protozoan parasite, Trypanosoma cruzi, and transmitted to humans by blood-sucking triatomine insects and by blood transfusions, is endemic in many regions of Latin America. The disease has a high social impact, enhancing morbidity and mortality, especially in patients with the chronic form of infection.¹ The pathogenesis of chronic chagasic cardiomyopathy is poorly understood, due to the lack of a suitable animal model that fully reproduces the disease processes.

The rhesus monkey (Macaca mulatta) is closely related to human phylogenetically and physiologically. These monkeys have been used as experimental models for numerous human pathologies, including cardiovascular and infectious diseases.²–¹⁰

The acute phase of T. cruzi infection in rhesus monkeys is similar to that which occurs in humans.¹¹–¹³ Our previous studies described the acute and early chronic phases of infection in these monkeys over a three-year period of experimental infection with the Colombian strain of T. cruzi. Chagoma, patent parasitemia, circulating IgM and IgG antibodies specific for T. cruzi, and hematologic alterations (leukocytosis and lymphocytosis) were observed in the acute phase. Electrocardiographic alterations were minor and transient, similar to those observed in non-lethal human acute chagasic myocarditis up to the fifth month of infection. The heart muscle cells present various degrees of degenerative alterations and a striking increase in the number of lysosomal profiles that exhibit acid hydrolytic reaction products. A strong inflammatory reaction with lymphocytic infiltrate and eosinophils associated with ruptured cells was present.¹³,¹⁴

Considering that approximately one-third of T. cruzi-infected humans develop severe chronic disease with irreversible damage to the heart and/or gastrointestinal tract with dilation and disorders of nerve conduction, it is crucial to understand the mechanisms leading to these organ-specific pathologies.¹⁵ Because these alterations are observed 10–20 years after the acute phase, we have examined long-lasting (15–19 years) T. cruzi experimental infection in seven male rhesus monkeys that were initially evaluating for the clinical, parasitologic, serologic, and hematologic aspects of the chronic infection.

MATERIALS AND METHODS

Animals. Seven male Macaca mulatta, 22.7 ± 3.2 (mean ± SD) years old and experimentally infected for 16.69 ± 1.48 (mean ± SD) years, were maintained in non-human primate modular isolation units (Double L Group, Ltd., Monoma, IA) of the Department of Primatology at the Center for Laboratory Animal Breeding of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). Monkeys were caged individually, provided water ad libitum, and fed a standard commercial chow (Nuvilib Primates 6030; Nuvital, Comabo, Brazil) supplemented with fresh fruits, eggs, and vegetables. Temperature, humidity, and light/dark cycles were controlled to provide standard conditions. Two age-matched male monkeys were maintained uninfected as controls and were simultaneously analyzed.

Animal infection. Details of the infection methodology have been previously reported.¹³ Briefly, metacyclic trypomastigotes forms of the Colombian strain of T. cruzi were used to infect monkeys subcutaneously in the antero-lateral face of the arm.¹⁶ All manipulations were performed under anesthesia using ketamine chloride (Vetaset; Fort Dodge, Campinas, Brazil) (10 mg/kg of body weight) given intramuscularly and according to standard guidelines.¹⁷ All animals were analyzed 16.69 ± 1.48 (mean ± SD) years after infection with T. cruzi and followed for 20 months. Body weight and temperature were measured regularly. Blood was obtained by puncture of the femoral vein and collection into appropriate tubes (Vacutainer⁰; Becton-Dickinson, Franklin Lakes, NJ). Standard techniques were used for hematologic evaluation (hemoglobin, hematocrit, red blood cell count, total and differential white cell count).
Direct parasitemia. Two blood smears from each monkey were stained with Giemsa and examined for patent parasitemia twice over an 11-month interval.

Hemoculture. Whole blood collected in EDTA (0.5 mL) was placed in 3 mL of NNN medium covered with 2 mL of liver infusion tryptose medium, mixed with 10% fetal calf serum and 140 mg/mL of gentamicin sulfate (Merck AS, Rio de Janeiro, Brazil), in quadruplicate.18–20 The cultures were incubated at 28°C and analyzed twice a month for seven months. In negative results were obtained, the animals were analyzed two more times at intervals of seven months.21

Artificial xenodiagnosis. Thirty fourth and fifth instar nymphs of Triatoma infestans and Panstrongylus megistus were fed with blood of rhesus monkeys that was collected in sodium heparin.22 The bugs were dissected and examined after 45 days for the presence of parasites. Those monkeys that yielded negative results were analyzed three more times at intervals of two months.

Extraction of DNA and polymerase chain reaction (PCR) conditions. Ten milliliters of blood were mixed with an equal volume of 6 M guanidine hydrochloride/200 mM EDTA buffer.23 The mixture was immersed for 15 minutes in boiling water and DNA was purified using two aliquots (200 μL) of each sample after extraction with phenol-chloroform and precipitation with ethanol.24 A 7.5-μL aliquot of DNA resuspended in water was PCR amplified using T. cruzi-specific minicircle primers (#121 5′-AAATAATGTACGG(T/G)GAGATGCA-TGA-3′ and #122 5′-GGTCTGAT-TGGGTTGGTGTAATATA-3′). The reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4.0 mM MgCl₂, 200 μM dNTPs, 0.4 μM of each oligonucleotide primer, and 2.5 units of Taq DNA polymerase in a final volume of 100 μL. The PCR was conducted in a Perkin-Elmer System 9600, using two cycles at 98°C for one minute and 64°C for two minutes, 33 cycles at 94°C for one minute and 64°C for one minute, followed by one extension step at 72°C for 10 minutes. Samples that were negative after T. cruzi-specific amplification were checked for possible inhibition with human β-globin specific primers (#PC03 5′-ACACAATGGTGCAGTAC-3′ and #PC04 5′-CAACCTCATCCACGTCACC-3′), using the same aforementioned protocol.25 Samples showing DNA inhibition were retested after a new DNA extraction.

Serology. Specific IgG antibodies to T. cruzi were measured by indirect immunofluorescence (IIF) and an enzyme-linked immunosorbent assay (ELISA), using commercial kits produced by Bio-Manguinhos (Oswaldo Cruz Foundation, Rio de Janeiro, Brazil). These were performed in accordance with manufacturers’ instructions. Two analyses were performed at an interval of 11 months.

Radiology. To evaluate cardiac and gastrointestinal alterations, we performed radiologic examinations (Polymat Plus 30/50; Siemens, Rio de Janeiro, Brazil) by means of contrast radiographs of the thorax (front view) and contrast radiographs of the esophagus-stomach transit area. The large intestine was also examined using barium sulfate perfusion. An approximate indication of heart size relative to chest size (the cardiothoracic index [CTI]) was obtained according to the procedure of Falasca and others.26 Two analyses were performed at an interval of 16 months.

Electrocardiograph (ECG) and echocardiograph. The classic 12-lead human ECG system was used; tracings were made at 25 mm/second and at a voltage of 1 mV standardized to 1 cm (ECG-6; Ecafix, Sao Paulo, Brazil). Two-dimensional and M-mode echocardiography were performed on a regular basis, recorded on a videotape, and printed on multi-image camera (Ultrasound Scanner EUB-555, Hitachi Medical Corp., Tokyo, Japan). An anesthetized monkey was positioned in left lateral decubitus and a transducer (5 MHz) was applied directly to the shaved thorax. The ventricular function was assessed in the M mode by calculating the fraction of ejection, in accordance with the guidelines provided by the Committee on M-mode Standardization of the American Society of Echocardiography and in the bi-dimensional mode by analyzing semiquantitatively the global systolic function, according to the procedures of Sahn and others27 and Amico and others.28

Two age-matched, uninfected, male animals (monkeys 81 and 94) maintained under the same experimental conditions as T. cruzi-infected animals and four age-matched, healthy, male rhesus monkeys (animals L17, L21, M31, and N31), obtained from the Primatology Department, were used as controls for the ECG, echocardiographic, and radiology examinations. Two analyses were performed on the infected and control animals at an interval of 16 months.

RESULTS

The present study aimed to characterize clinical alterations developed in rhesus monkeys infected for 15–19 years with the Colombian strain of T. cruzi. Periodical physical and clinical examinations revealed no other clinical infections or illnesses. The results of this cross-sectional study are demonstrated in Tables 1, 2, and 3.

General clinical aspects. Physical examinations showed that all monkeys were normal, except for an enlarged abdomen caused by excess adipose tissue. One of the infected animals (monkey 68) had unspcific artrosis and was humanely killed. No significant body weight or hematologic changes were detected in monkeys inoculated with T. cruzi compared with uninfected controls.

Parasite detection. Direct examination of blood samples failed to detect the presence of circulating T. cruzi trypomastigote forms. However, the presence of the parasite was demonstrated in all studied animals, using hemoculture, artificial xenodiagnosis and PCR (Table 1). Hemoculture was positive in two (28.5%) of seven animals, after ± 1.41 (mean ± SD)

<table>
<thead>
<tr>
<th>Monkey no.</th>
<th>Artificial xenodiagnosis</th>
<th>Hemoculture</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>64</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>68</td>
<td>Negative</td>
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</tr>
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<td>90</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>95</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>99</td>
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<td>Negative</td>
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</tr>
<tr>
<td>103</td>
<td>Positive</td>
<td>Positive</td>
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</tr>
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</table>
months of culture incubation. Artificial xenodiagnosis was positive in three (42.8%) of seven monkeys. Although T. infestans were also used for artificial xenodiagnosis, only P. megistus were infected (1.6%). Positive PCR amplification products with primers that annealed to T. cruzi kDNA were detected in six (100%) of six animals (Figure 1) that showed amplification of the β-globin gene. One animal (monkey 68) was negative by PCR analysis. The negative PCR result was probably due to the presence of inhibitors in the DNA sample of animal 68, as confirmed by the lack of amplification of the β-globin gene (Table 1). The PCR could not be repeated because this animal had been humanely killed.

**Specific IgG antibodies to T. cruzi.** Specific IgG antibodies to T. cruzi with titers ranging from 1:80 to 1:640 (IIF cut-off value ≥ 1:80) were detected (Figure 2) in all T. cruzi-infected animals at the first analysis. These results were confirmed using an ELISA, which showed the presence of antibodies to T. cruzi 18.05 ± 1.32 years after experimental infection. Interestingly, monkey 99, which was positive at the first analysis (ELISA positive, IIF titer = 1:80), was seronegative (ELISA negative, IIF: titer 1:40) at the second evaluation carried out 11 months later. This animal was found to be negative in three additional tests performed at two-week intervals.

**Radiologic studies.** No radiologic alterations were observed in T. cruzi-infected monkeys, either with regard to the CTI obtained from the chest radiograph or the diameter of the esophagus and colon revealed by contrast radiography of the gastrointestinal tract.

**Table 2**

<table>
<thead>
<tr>
<th>Monkey no</th>
<th>Infected</th>
<th>First analysis</th>
<th>Chronic phase (years after infection)</th>
<th>Second analysis</th>
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<tbody>
<tr>
<td>42</td>
<td>T wave abnormal (18.92)</td>
<td>Normal (19.08)</td>
<td></td>
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<tr>
<td>64</td>
<td>T wave inversion (18.67)</td>
<td>Normal (19.83)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>Ventricular extrasystoles, T wave inversion (15.83)</td>
<td>Atrial extrasystoles (17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>ILBBB, T wave inversion (15.83)</td>
<td>ILBBB, T wave inversion, abnormal ventricular conduction (17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99</td>
<td>Normal (15.83)</td>
<td>Normal (17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>Normal (15.83)</td>
<td>Incomplete AV block, T wave inversion (17)</td>
<td></td>
<td></td>
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<tr>
<td>Noninfected</td>
<td>T wave inversion</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>81</td>
<td>Normal</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>Normal</td>
<td>Normal</td>
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<td>64</td>
<td>Normal</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99</td>
<td>Normal</td>
<td>T wave inversion</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ILBBB = Incomplete left bundle branch block; AV = atrioventricular.

**Electrocardiographic and echocardiographic studies.** The ECG alterations found in rhesus monkeys chronically infected with T. cruzi are summarized in Table 2. Five (83.3%) of six infected animals showed T wave alterations. These alterations were also found in two (33.3%) of six sex- and age-matched controls. The T wave abnormalities seen in T. cruzi-infected rhesus monkeys 42 and 64 and in control monkeys 81 and N31 appeared similar to those produced by anesthesia. The electrocardiographic patterns of one infected monkey (monkey 99) were normal. Interestingly, three of six T. cruzi-infected animals showed significant electrocardiographic abnormalities. In one infected animal (monkey 90), multiformal ventricular extrasystoles and T wave inversion were observed at the first examination, and atrial extrasystoles were observed at the second analysis. In the second animal (monkey 95), incomplete left bundle branch block (ILBBB) was seen at both examinations, with more accentuated T wave inversions.

**Figure 1.** Representative results of polymerase chain reaction (PCR) amplification of variable regions of the Trypanosoma cruzi minicircle molecule from blood samples. The 330-basepair (bp) band is the expected T. cruzi-specific product. Molecular weight markers (100-bp ladder) are shown in lanes 1 and 13. Lanes 2, 3, 6, 8, 9, and 10 contain positive samples from infected monkeys (42, 64, 90, 95, 99, and 103, respectively). Lane 4 contains a negative sample from an infected monkey (68). Lanes 5 and 7 contain negative control samples from uninfected monkeys. Lane 11 contains a negative control in which no DNA was added to the PCR. Lane 12 contains a positive control from a confirmed chagasic human patient.
sion at the first examination (Figure 3). First-degree atrioventricular (AV) conduction disturbance and T wave inversion were observed in monkey 103 at the second examination. Only one of six infected monkeys (monkey 95) showed an echocardiographic abnormality, asynchronic interventricular septum motility (Figure 4), with a decrease in the ejection fraction.

**DISCUSSION**

To our knowledge, this is the first report describing a well-characterized chronic phase of *T. cruzi* infection in a non-human primate. The main finding of our study was the presence of cardiomyopathy characterized by abnormal electrocardiographic and echocardiographic patterns in non-human primates compatible with ongoing Chagas’ disease.

Due to the infrequency of clinical signs in the chronic phase, *T. cruzi* infection is difficult to identify without specific parasitologic and serologic tests. Circulating *T. cruzi* was demonstrated by artificial xenodiagnosis in three infected monkeys and by hemoculture in two. Our findings are similar to those observed in humans and in other non-human primates during chronic Chagas’ disease. Failure to isolate the parasite from seropositive monkeys was attributed to the scarcity of parasites in the bloodstream, as is commonly observed in the chronic human disease.

In the present study, only *P. megistus* bugs were infected by artificial xenodiagnosis, which corroborates a remarkable difference between vector species observed in previous studies. However, when *P. megistus*, *T. infestans*, and *Rhodnius prolixus* were used for xenodiagnosis of the Peru strain of *T. cruzi* in rhesus monkeys, only the two former species were suitable for the diagnosis of this particular strain. Thus, our result may reflect interaction between the invertebrate host and the Colombian strain in the chronic phase of the experimental infection when *T. cruzi* blood forms were scarce, since *T. infestans* were infected by xenodiagnosis during the acute phase.

The performance of the PCR far exceeded that of artificial xenodiagnosis or hemoculture, and may become the gold standard technique for parasite detection in the chronic phase of Chagas’ disease in rhesus monkey, as previously demonstrated for human patients, since the sensitivity of the amplification process is believed to be sufficient to detect a single parasite in 20 mL of peripheral blood. Our results suggest the persistence of the parasite, although scarce in circulating blood, during long-lasting infection of rhesus monkeys with the Colombian strain of *T. cruzi*.

Rhesus monkeys can live up to 40 years in captivity. Our results show that these animals can support a long-lasting sub-clinical *T. cruzi* infection with scarce parasitemia. Cellular and humoral immune responses are crucial to control parasitemia and parasitism during acute and chronic *T. cruzi* infection. Our results show that circulating antibodies to *T. cruzi* persisted throughout the chronic phase of infection and were not related to the presence of circulating parasites. Furthermore, profiles of humoral immune responses in *T. cruzi*-infected monkeys during acute and chronic infection were demonstrated to be similar to those observed in humans and *Cebus* monkeys. The limitations of serologic testing in the diagnosis of chronic Chagas’ disease have long been recognized as the result of variations of antibody levels related to oscillatory periods of parasitemia. We showed that in a *T. cruzi*-infected rhesus monkey (monkey 99), the results of serologic analysis for IgG antibodies to *T. cruzi* became negative at the second evaluation carried out 11 months after the first analysis, when circulating parasites were not detected. Conversely, monkey 90 showed high levels of IgG antibody to...
T. cruzi, but with negative parasitemia. However, both animals showed positive PCR results. Although these findings may be controversial, recent experimental data show that the detection of kinetoplast DNA (kDNA) by a PCR reflects the persistence of infection since T. cruzi kDNA was detected only for two days after injection into mice and not thereafter.50 Furthermore, the detection of T. cruzi in monkey 99 by the PCR confirms the importance of this method for diagnosis, especially during the chronic phase.

The Colombian strain of T. cruzi has a peculiar capacity to reproduce several histopathologic aspects of the chronic chagasic cardiomyopathy in mice.16,51–53 Rhesus monkeys acutely infected with the Colombian strain also showed aggressive cardiomyopathy.13,14 In addition, the histopathologic analysis of the chronically infected monkeys that were killed (monkeys 42 and 68) confirms the presence of mild myocarditis in absence of significant ECG abnormalities (Carvalho CME and others, unpublished data). Interestingly, we have shown that rhesus monkeys chronically infected with the Colombian strain developed electrocardiographic alterations similar to those observed in chronic chagasic patients.54–64

Electrocardiographic abnormalities, suggesting the presence of acute T. cruzi-elicited myocarditis, have been described in Cebus apella and Saimiri sciureus.44,65–70 In fact, our animals presented several ECG abnormalities attributed to the presence of the parasite in the cardiac tissue from the fourth week of infection up to the 12th week of the acute phase.13 Moreover, after 17 years of infection, the ECG abnormalities in monkeys 90, 95, and 103 may have been due to T. cruzi-elicited myocardial damage, while the alterations observed in monkeys 42 and 64 were transitory and isolated, consequently without diagnostic significance, suggesting that these animals had the indeterminate form of chronic Chagas’ disease.55 This was further supported by the histopathologic finding of mild myocarditis in monkey 42 (Carvalho CME and others, unpublished data).

Other investigators have reported electrocardiographic alterations in non-human primates in the chronic phase of Chagas’ disease, including rhesus and Cebus monkeys.26,42,66,67,71 Szarfman and others had observed ECG abnormalities suggestive of myocardial damage in a female rhesus monkey infected with T. cruzi 29 years earlier.42 Multiform ventricular

**FIGURE 3.** Electrocardiographic analysis of monkey 95 demonstrating incomplete left bundle branch block. A. First analysis. B. Second analysis. The **arrows** indicate T-wave inversion.
extrasystoles and T wave inversion found in the monkey 90 have also been recorded in other animal models for chronic T. cruzi-induced heart disease, including rabbits, mice, and dogs.72–74 Importantly, these alterations are common in chronic Chagas’ heart disease.65,67,85 The first-degree AV conduction disturbance and T wave abnormalities were also observed in one of the T. cruzi-infected monkeys (monkey 103). Furthermore, the first-degree AV block suggests damage to the atroventricular node or to the autonomic nervous tissue supplying these regions that occurs mainly during the early stages of chronic Chagas’ disease.62,76–78 Although first-degree AV block, T wave inversion, and extrasystoles may be considered unpecific alterations, our findings showing associations of these abnormalities may suggest a role for T. cruzi infection in the genesis of these dysfunctions, since these associations were absence in all control animals.75

In monkey 95, ILBBB with T wave inversion was observed. Right bundle branch block is the most frequent conduction disturbance in chronic Chagas’ heart disease, and has a high prevalence (38.8–55.7%). In contrast, LBBB has a low prevalence (0.5–9.6%); however, left branch alterations are expected to appear when the injuries are much more severe.54,55,58,61,62,64,75,78 If one takes these into account, our findings suggest that the monkey 95 is developing significant T. cruzi-elicited heart disease. Unfortunately, since this study was a cross-sectional rather than longitudinal, the timing of the last normal ECG is unknown in this animal.

Echocardiography is a noninvasive tool frequently used for clinical diagnosis in chronic Chagas’ disease that makes possible a direct evaluation of the presence, type, and extension of the myocardial involvement.59–82 It represents a more sensitive methodology in assessing cardiac performance than an ECG or chest radiographs. The asynchronous movement of the interventricular septum, with a decrease in the ejection fraction and an increase in the systolic diameter, were observed in our study (monkey 95). This condition has also been observed in T. cruzi-infected dogs and Cebus monkeys.26,74 In humans, the most typical echocardiographic findings are apical left ventricular aneurysm and/or posterior basal akinesia or hypokinesia with preserved septal contraction. In cases of advanced cardiomyopathy with cardiac failure, biventricular dilatation occurs without hypertrophy.81,83 In our study, this asynchronous movement of the interventricular septum is attributed to the ILBBB observed in the same animal. Importantly, Casado and others have suggested that during late-stage disease, when several significant ECG abnormalities are detected, there is an increase in the left ventricular volume and a decrease in the ejection fraction, as observed in the records of monkey 95.84

Relative to humans, infected rhesus monkeys seem to develop the chronic phase of Chagas’ heart disease, with a long asymptomatic evolution. Although the ECG and echocardiography abnormal patterns reported here are not frequently observed in human chronic Chagas’ disease, they are highly relevant when detected. In fact, studies carried out in Brazil have demonstrated a low incidence of LBBB; however, this abnormality has been more frequently described in chagasic patients and individuals with cardiomyopathies of obscure origin from Colombia, the origin of the T. cruzi strain used here, and in mammalian reservoirs from Panama.16,56,61,85–86 In addition, the low morbidity of Chagas’ disease in Colombia is postulated to be due to the genotype of the circulating parasites.56,87 Thus, one cannot exclude the possibility that the particular findings observed in our group of T. cruzi-infected rhesus monkeys are due mainly to the Colombian strain of the parasite used in our study.

In conclusion, the findings reported here support the validity of rhesus monkeys as an experimental model for acute, indeterminate, and cardiac chronic Chagas’ disease. These findings will also contribute to a better understanding of the parasite/host interactions and the physiopathogenesis of this parasitic disease, and can be used to evaluate new T. cruzi-specific chemotherapy and identify putative markers for disease progression.

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