Trypanosoma cruzi high infectivity in vitro is related to cardiac lesions during long-term infection in *Beagle* dogs

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Trypanosoma cruzi *is a hemoflagelate parasite associated with heart dysfunctions causing serious problems in Central and South America.* Beagle *dogs develop the symptoms of Chagas disease in humans, and could be an important experimental model for better understanding the immunopathogenic mechanisms involved in the chagasic infection. In the present study we investigated the relation among biological factors inherent to the parasite (trypomastigote polymorphism and in vitro infectivity) and immunoglobulin production, inflammation, and fibrosis in the heart of* Beagle *dogs infected with either* T. cruzi *Y or Berenice-78 strains. In vitro infectivity of Vero cells as well as the extension of cardiac lesions in infected* Beagle *was higher for Y strain when compared to Berenice-78 strain. These data suggested that in vitro infectivity assays may correlate with pathogenicity in vivo. In fact, animals infected with Y strain, which shows prevalence of slender forms and high infectivity in vitro, presented cardiomegaly, inflammation, and fibrosis in heart area. Concerning the immunoglobulin production, no statistically significant difference was observed for IgA, IgM or IgG levels among* T. cruzi *infected animals. However, IgA together IgM levels have shown to be a good marker for the acute phase of Chagas disease.*

Key words: *Trypanosoma cruzi* - *Beagle* dogs - infectivity - cardiac lesions - Chagas disease - antibodies

Chagas disease is a serious public health problem which affects 13 million people in Central and South America (WHO 2005). This disease causes high morbidity, disabling the individuals for working, and generating expenses to the health system of developing countries.

The pathogenesis of chronic Chagas disease is not precisely understood and has been the subject of considerable controversy for several decades. Both host and parasite factors are certainly involved in this process. In relation to the vertebrate host, factors such as age, sex, and the immune response profile can modulate differences in the clinical manifestations of the disease (Dias 2000, Dutra et al. 2005). There are also evidences from the literature that point to inherent factors of the parasite, such as the biological and genetic variability among *Trypanosoma cruzi* populations, in the outcome of the disease (Marques-Araújo & Chiari 1988, Macedo et al. 2004). Biological characteristics of the parasite such as morphologic aspects and in vitro infectivity are likely to influence parasitemia and development of heart lesions in the vertebrate host. The determination of the real influence of these biological characteristics of different *T. cruzi* strains on cardiac lesions is then relevant for better understanding pathogenicity of chronic Chagas disease.

In this work we investigated the correlation of trypomastigote form infectivity in vitro with parasitaemia curves, humoral immune response, heart inflammation and fibrosis in *Beagle* dogs infected with either Y or Berenice-78 strains.

MATERIALS AND METHODS

T. cruzi strains - *T. cruzi* Y strain (*T. cruzi* II) isolated from an acute case of human Chagas disease (Silva & Nussenzweig 1953) and Berenice-78 strain (*T. cruzi* II), isolated by xenodiagnosis on 1978 (Lana & Chiari 1986) from the first reported Chagas disease patient, Berenice (Chagas 1909).

Morphological variations and infectivity of trypomastigotes forms of T. cruzi - Polymorphisms in blood trypomastigote forms of the two *T. cruzi* strains was determined through fresh blood examination. The blood was collected during the parasitaemia peak from Swiss mice infected with *T. cruzi* Y (7th day after infection) and Berenice-78 (14th day after infection) strains. Three microliters were collected from the mouse tail and 500 trypomastigotes counted. Parasites were classifying as slender, stout or very stout forms (Brener & Chiari 1963).

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The trypomastigote infectivity was evaluated by parasite infection in Vero cells (American Type Culture Collection CLL-81). Subconfluent monolayers of Vero cells were obtained by plating cells at 1.25×10^4 cells in DMEM (GIBCO, Grand Island, New York, US) supplemented with 5% fetal bovine serum (Nutricell, Campinas, São Paulo, Brasil; inactivated at 56ºC, 30 min) 2.5% HEPES 1 M, pH 7.2, 1% of glutamina 2 mM/ml, 0.1% of mercaptoethanol 50 mM/ml, and 0.2% garamicin 200 g/ml (Shering-Plough, Rio de Janeiro, Brasil) in each well of a eight-well chamber slide (chamber slide, Nunc Inc. Illinois, US). Cells were incubated at 37ºC in a humidified atmosphere containing 5% CO_2 24 h prior to infection. Cell subconfluent monolayers were then infected with tissue culture trypomastigote forms of each Y and Berenice-78 strains (12.5×10^4 parasites/well) for 24 h at 37ºC in a humidified atmosphere containing 5% CO_2. After infection, chamber slides were washed twice with cold PBS in order to loose extracellular adhered parasites and cells were re-incubated with new DMEM media supplemented with 1% fetal bovine serum at 33ºC. Media was removed 48 h post infection and infected cells monolayers were fixed with Bouin for 15 min, washed 20 min in water, and stained with Giemsa stain for 16-18 h. After staining slides were differentiated in ethanol 70%, passed twice through acetone and xylene (3:7 and 7:3), and mounted in synthetic resin (Entellan, Merck). The percent of infected cells was determined by random examination of slides and counting a minimum of 500 cells under the microscope using high magnification (400 ×).

Experimental animals and infection - Fifteen *Beagle* dogs four months old from the kennel (Universidade Federal de Ouro Preto, UFOP, MG, Brasil) were fed a commercial ration and water was available ad libitum. Before the study, the animals were treated with antihelminthes and immunized against infectious diseases. All procedures and experimental protocols were conducted in accordance with the Cobea's instructions (Colégio Brasileiro de Experimentação Animal) for the use of animals in research, and approved by the ethics committee in animal experimentation of the UFOP. The animals were inoculated with 2000 blood trypomastigote forms of either *T. cruzi* Y or Berenice-78 strains per kg of body weight via peritoneal.

Parasitaemia - The parasitaemia was determined microscopically by examining daily collected fresh blood from the marginal ear vein of infected animals from day 5th post infection until parasites could no longer be detected (Brener 1962). The pre-patent period (PPP), parasitaemia curves (PC), patent period (PP), and the maximum parasitaemia peak (MPP) were determined.

Conventional serology antibodies (CSA/ELISA) - Serum samples were collected from blood of acute infected *Beagle* dogs at regular intervals of 15 days or 60 days for acute and chronic phases, respectively. The serum samples were stored at –79°C and ELISA assays were carried out according to Voller et al. (1976). *T. cruzi* Y strain epimastigote forms obtained from axenic cultures in LIT medium were used as antigen source and peroxidase-conjugate goat anti-dog immunoglobulins were used to determine IgA (1:2000), IgM (1:7500) and IgG (1:7500) (Bethyl Laboratories, Montgomery, US) levels. The cut-off was determined using the absorbance mean of ten non-infected animals plus two standard deviations.

Macroscopic evaluation - All animals were euthanized by injection with thionembutal (Abbott, São Paulo, Brasil) 0.5 ml/kg of body weight (0.03 g/ml of saline solution 0.8%) 100 weeks after infection. Necropsy was performed and a fragment of approximately $1.0 \times 1.0 \times 0.2$ cm from the middle of the right atrial wall of each dog was taken for histopathological analyses. Five age matching non-infected dogs were used as controls.

The cardiomegaly was determinated by the correlation index between heart weight and animal weight. The presence of cardiomegaly was determined when there was statistically significant difference in relation to non-infected age matching controls.

Histopathology and morphometric analysis - Tissue fragments were fixed in 10% buffered formalin solution, dehydrated, cleared, and embedded in paraffin. Blocks were cut into 4 µm-thick sections and stained by Hematoxylin-Eosin (H&E) for inflammation assessment or Masson's trichromic for fibrosis quantitative evaluation.

Thirty fields from each H&E or Masson's trichromic stained section were randomly chosen at a 40× magnification performing a total of 1.6×10^6 μm^2 analyzed myocardium area. Images were obtained through a JVC TK-1270/RGB microcamera and the KS300 software built in Kontron Elektronick/Carl Zeiss image analyzer. The inflammatory process was evaluated by the correlation index between the number of cells nucleus observed around myocardium muscle from non-infected and infected animals (Maltos et al. 2004). The fibrosis was determinated using the image segmentation function. All pixels with blue hues in Masson's trichrome section were selected to build a binary image and subsequently calculate the total area occupied by connective tissue in non-infected and *T. cruzi* infected dogs.

Statistical analysis - The PPP, PC, PP, and MPP were compared by analysis of variance using square root transformation of the data Tukey test (Snedecor & Cochran 1989). All histological data were analyzed by Student's *t* test (GraphPad InStat software) among non-infected and *T. cruzi* Y or Berenice-78 strain infected animals. The regression analysis was used to compare infectivity, inflammation and fibrosis; where regression lines were compared by analysis of covariance (Snedecor & Cochran 1989). For all cases, differences were considered significant when $P < 0.05$.

RESULTS

Morphological variations correlated with infectivity of trypomastigotes forms of T. cruzi - The morphological evaluation of blood trypomastigote forms from *T. cruzi* Y strain showed 91% of slender forms, while

90% of Berenice-78 trypomastigotes were stout forms (Fig. 1). When we compared the infection of Vero cells with each strain we observed a higher infectivity of Y strain (27.7 ± 6.1%) in relation to Berenice-78 strain (1.2 ± 0.4%) (Figs 2, 3).

Strain of parasite as a determinant of parasitaemia profile - Parasitaemia curves generated from *Beagle* dogs infected with either *T. cruzi* Y or Berenice-78 strains showed different biological profiles as seen in Fig. 4. *T. cruzi* Y strain showed short PPP (12 days) and two distinct parasitaemia peaks, while Berenice-78 strain had PPP of 17 days, and three distinct parasitaemia peaks.

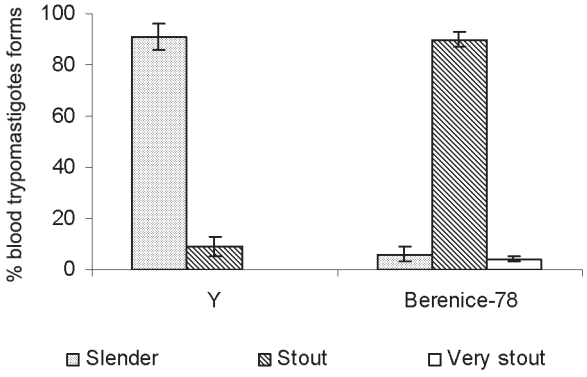

Fig. 1: morphological variations of *Trypanosoma cruzi* blood trypomastigotes during the parasitaemia peak in infected Swiss mice with Y (7th day of infection) and Berenice-78 (14th day after infection) strains.

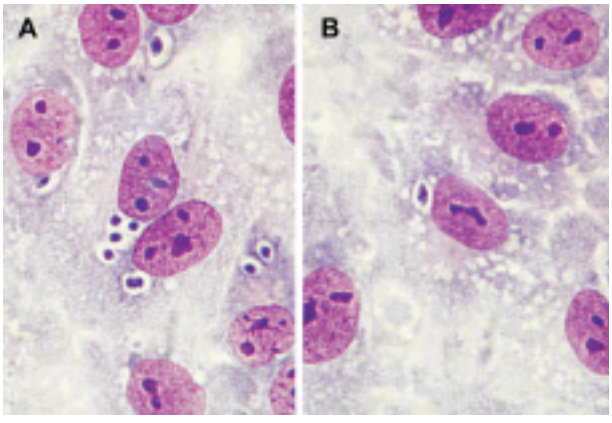

Fig. 2: Vero cells containing *Trypanosoma cruzi* amastigotes forms of Y (A) or Berenice-78 (B) strains 48 h after infection.

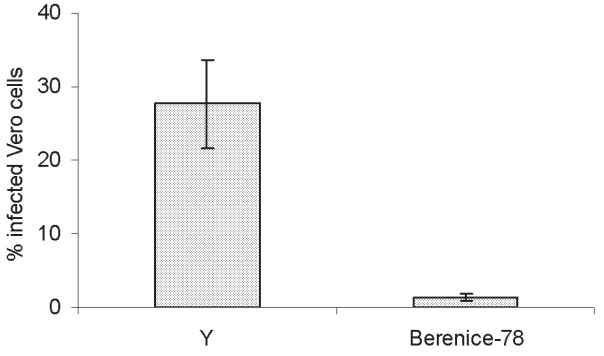

Fig. 3: percentage of Vero cells infected with *Trypanosoma cruzi* Y or Berenice-78 strains.

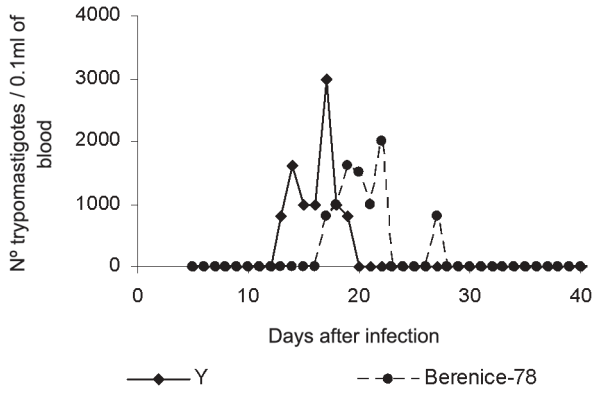

Fig. 4: parasitaemia curves of *Beagle* dogs infected with 2000 blood trypomastigote forms of either *Trypanosoma cruzi* Y or Berenice-78 strains.

Conventional serology antibodies (CSA/ELISA) did not show any differences among infected animals - All infected animals showed similar profiles of IgA, IgM, and IgG antibodies. IgA antibodies were detected in sera of all infected animals only during the acute phase of the infection, being detected in low levels among the 2nd to 15th week after infection (Fig. 5A). High levels of IgM antibodies in sera of infected animals were detected among the 2nd to 20th week post-infection (Fig. 5B). The IgG antibodies were detected after 4th week increasing up until the 10th week, and stabilizing all through the end of the experiment (100 weeks) (Fig. 5C). Unifected control did not show detectable levels of tested immunoglobulins.

TABLE

Cardiomegaly, inflammation, and fibrosis in infected *Beagle* dogs with Y and Berenice-78 *Trypanosoma cruzi* strains

T. cruzi strains	No. of dogs with cardiomegaly/total	No. of dogs with inflammation/total	No. of dogs with fibrosis/total
Y	4/5	4/5	4/5
Berenice-78	1/5	1/5	1/5

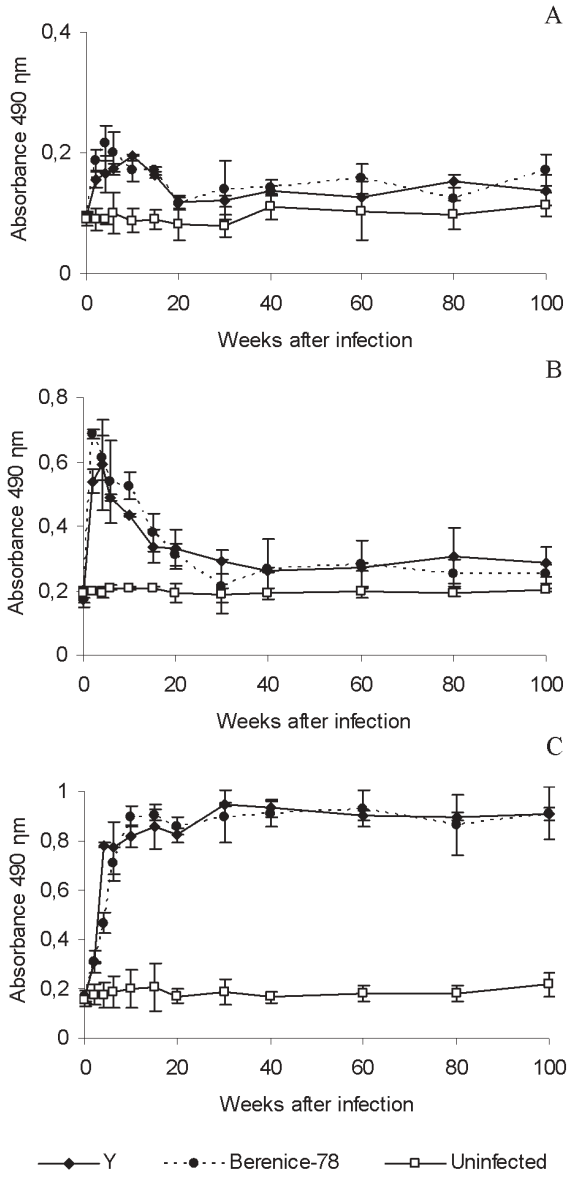

Fig. 5: *Trypanosoma cruzi* specific IgA (A), IgM (B), and IgG (C) antibodies in sera of *Beagle* dogs infected with either Y or Berenice-78 strains and uninfected animals.

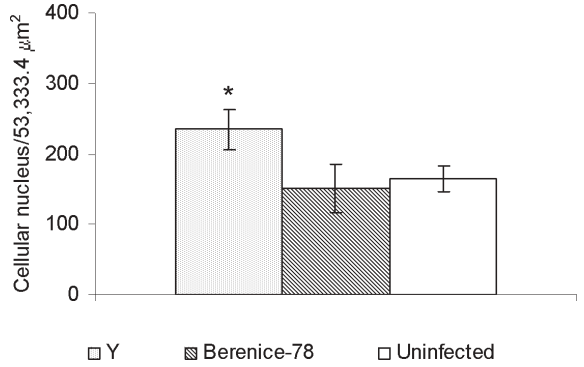

Fig. 6: no. of cellular nucleus in right atrium of *Beagle* dogs infected with 2000 blood trypomastigote forms of *Trypanosoma cruzi* Y or Berenice-78 strains, and uninfected dogs (* $P < 0.05$ significant difference, when comparing Y infected animals to Berenice-78 and uninfected animals).

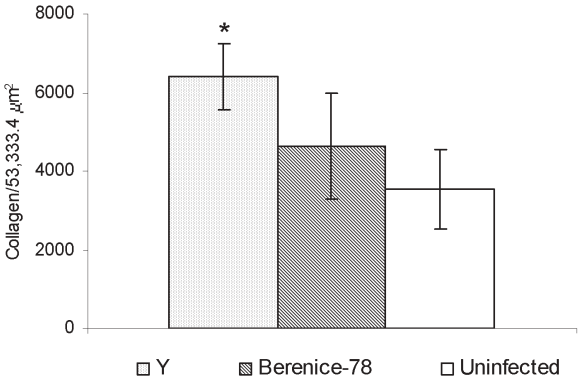

Fig. 7: collagen in right atrium of *Beagle* dogs infected with 2000 blood trypomastigote forms of *Trypanosoma cruzi* Y or Berenice-78 strains, and uninfected dogs (* $P < 0.05$ significant difference, when comparing Y infected animals to uninfected animals).

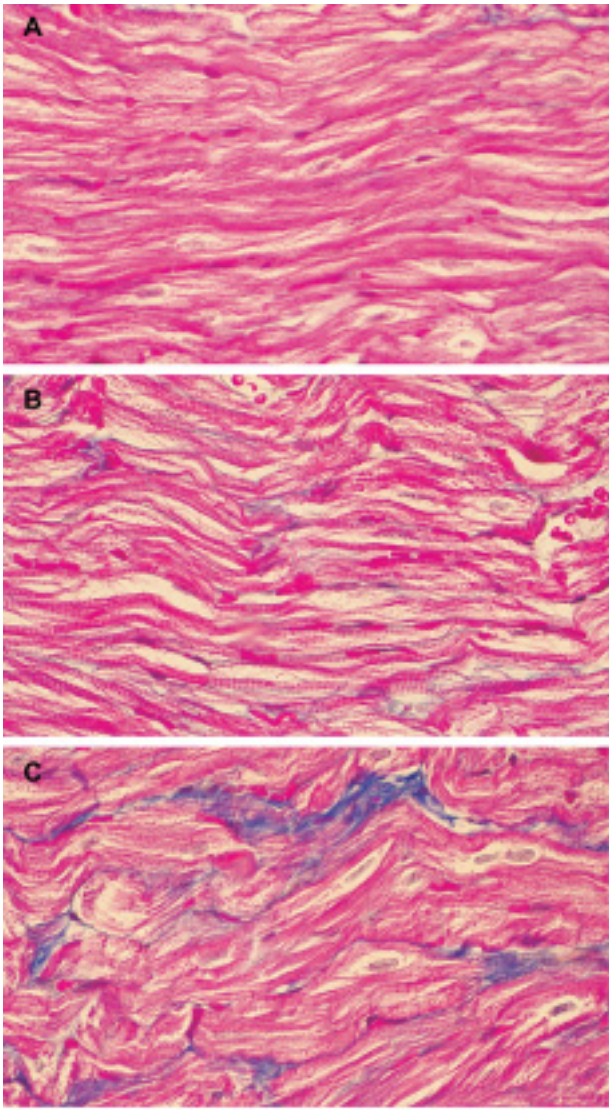

Fig. 8: Masson trichromic stained right atrium from uninfected control animals (A) and *Beagle* dogs infected with 2000 blood trypomastigote forms of either *Trypanosoma cruzi* Berenice-78 strain showing discreet fibrosis (B) or Y strain showing intense fibrosis (C).

Cardiomegaly induced by infection is T. cruzi-strain specific - Cardiomegaly is one of the symptoms of heart dysfunction in chronic Chagas disease. Only 20% of Berenice-78 strain infected animals showed cardiomegaly, right ventricle flaccidity, inflammation, and fibrosis, contrary to what was observed for Y strain infected dogs, where 80% of animals presented these alterations (Table).

Quantitative analysis of inflammation (Fig. 6) and fibrosis (Figs 7, 8) in right atrial wall showed severe and mild alterations in Y and Berenice-78 strains infected dogs, respectively. The number of cellular nucleus and collagen per 53,333.4 μm^2 of heart tissue was 235 ± 29 and 6399 ± 835 for Y, 151 ± 34, and 4641 ± 1357 for Berenice-78 strain infected animals, while non-infected dogs showed 165 ± 18 and 3541 ± 1003, respectively.

These results show that animals infected with Y strain present an increase of the heart area and a more intense inflammatory process when compared to Berenice-78 infected and non-infected dogs ($P < 0.05$). Also intense fibrosis was demonstrated in animals infected with Y strain when compared to non-infected controls ($P < 0.05$).

DISCUSSION

Beagle dogs infected with Y and Berenice-78 strains have shown a correlation among *T. cruzi* trypomastigote polymorphism, infectivity in vitro, and cardiac lesions.

The morphological shape of blood trypomastigotes from different *T. cruzi* strains had already been described by Chagas (1909) and confirmed for several other authors (Brener & Chiari 1963, Lana & Chiari 1986, Marques-Araújo & Chiari 1988). Our results are in accordance to those described in the literature for Y strain (Brener & Chiari 1963, Marques-Araújo & Chiari 1988). However *T. cruzi* Berenice-78 strain infectivity in vitro and blood trypomastigote polymorphism were reported by the first time.

High infectivity in vitro was observed for Y strain (27.7%) compared to very low infection rates observed for Berenice-78 strain (1.3%). Previous data have correlated morphological aspects of *T. cruzi* blood trypomastigote morphological variations with in vitro infectivity of the parasite (Bertelli et al. 1977, Bertelli & Brener 1980). High in vitro infection rates were observed for Y strain in contrast to very low infectivity of CL strain (Bertelli et al. 1977, Bertelli & Brener 1980). In this work we correlated blood trypomastigote morphological polymorphism with in vivo infectivity using *Beagle* dogs infected with either *T. cruzi* Y strain (high percentage of slender form) or Berenice-78 strain (high percentage of stout form). Infection with Y strain produced higher parasitaemia and shorter PPP in comparison with Berenice-78 strain. The influence of those biological aspects in vivo infections had been previously described for mice (Marques-Araújo & Chiari 1988, Veloso et al. 2005) and mongrel dogs using *T. cruzi* Y and Berenice-78 strains (Guedes 2001, Bahia et al. 2002). Also blood stream forms of the Y and CL strains had been shown to diversely interact in vitro with anti-sera from chronically infected mice (Krettli & Brener 1976). Experimental infections with these *T. cruzi* strains exemplify extremes of different morphological, biological, and immunological characteristics. Together these data strongly suggested that slender forms are more infective than stout forms for cellular culture as well as host tissues, and frequently produce more host tissue damage (Schmatz et al. 1983, Penin et al. 1997).

Besides biological characteristics of *T. cruzi* strains, aspects related to the human immune response are subject of extensive study in the chagasic infection. Immune aspects from the host most certainly play a role on mechanisms involved in the resistance or pathogenesis of Chagas disease (Morgan et al. 1996, Cordeiro et al. 2001). Until today, the pathogenesis of the chronic phase is not fully understood, in part by the difficulty in finding the ideal animal model and by the lack of correlation between the host immune response and heart damage. Since chronic canine chagasic cardiomyopathy has been shown to resemble the human disease (Lana & Chiari 1986, Lana et al. 1992) we decided to investigate the correlation between the production of different immunoglobulins isotypes and pathogenesis of Chagas disease.

No differences in the levels of IgA, IgM, and IgG specific antibodies were observed for *T. cruzi* Y strain and Berenice-78 strain infected animals. However the immunoglobulin isotype was specifically related to the disease phase. Previous studies with acutely infected chagasic patients had shown the presence of IgA, IgM, and IgG antibodies. Also it has been shown that IgM and IgG are detected in the beginning of the chronic phase, but only IgG is present along the chronic phase (Magnani et al. 1973, Camargo & Amato Neto 1974, Scott & Goss-Sampson 1984, Ouaissi et al. 1991, Grauert et al. 1993, Medrano-Mercado et al. 1996). Concordant results were observed in experimentally infected dogs with *T. cruzi* (Andrade et al. 1981, Bambirra et al. 1984, Barr et al. 1991, Guedes et al. 2002, 2004). Nevertheless, differences in antibody isotype production in relation to clinical forms were not reported by these authors. In our results we showed that both *T. cruzi* Y and Berenice-78 infected dogs presented high levels of IgM production in the acute phase, but not in the chronic phase. IgA was also present during the acute phase although in lower levels. Besides IgA antibody levels decreased (15 weeks after infection) earlier than the IgM isotype (20 weeks after infection). Since IgM antibodies have been detected for longer periods in patients (Ouaissi et al. 1991) and in the canine model during the initial chronic phase of the infection (Lana et al. 1992), we suggest here that the association of IgA and IgM antibody levels are certainly useful specific markers for the acute chagasic infection. As expected IgG isotype levels were higher during the chronic phase. While IgA antibodies may aim in congenital Chagas disease diagnosis achievement, as used in acute toxoplasmosis diagnosis (Signorell et al. 2006, Schmidt et al. 2006).

In parallel to humoral response, we also investigated the effects of these *T. cruzi* strains on cardiac damage. The cardiomegaly, inflammation, and fibrosis were associated to the presence of slender forms and high infectivity in vitro of *T. cruzi* strains. These abnormalities can contribute to the appearance of heart disfunction.

Eighty percent of animals infected with Y strain presented cardiac alterations in contrast to only 20% of Berenice-78 strain infected dogs. The Berenice-78 *T. cruzi* strain was isolated from a chagasic patient with the indeterminate form of the disease (Salgado et al. 1962). Using genetic homogeneous *Beagle* dogs as experimental models we observed great similarity with the human disease. Parasites isolated from patients with the indeterminate form of the disease produced mild symptoms in *Beagle* dogs showing a correlation between parasite variability and the clinical/pathological manifestations. Mongrel dogs infected with Berenice-78 *T. cruzi* strain, on the other hand, showed severe cardiac disease (Bahia et al. 2002), suggesting that mongrel are more susceptible than *Beagle* dogs to *T. cruzi* infection.

Our results also showed that intra-fascicular inflammatory focuses continues to develop slowly and progressively along the years, and are modulated by ineffective immune responses that lead to chronic fibrosis myocarditis. This finding was observed mainly for Y strain infected animals, which presented high parasitaemia, high in vitro infectivity, and high percentage of slender blood stream forms in accordance with several authors (Tafuri 1970, Lopes et al. 1970). Based on this study, we conclude that *Beagle* dogs reproduce the human Chagas disease and are an important experimental model for better understanding the immunopathogenic mechanisms involved in the infection with *T. cruzi*. Besides, these animals are genetically homogeneous making it possible to obtain reproducible results.

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