Benznidazole alters the pattern of Cyclophosphamide-induced reactivation in experimental *Trypanosoma cruzi*-dependent lineage infection

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ABSTRACT

The factors involved in the reactivation of chronic Chagas disease infection are not clear enough and may be related to host immune unbalance and/or parasite genetic diversity. To evaluate the role of the *Trypanosoma cruzi* genetic background in the Chagas disease reactivation, we inoculated Cyclophosphamide-immunosupressed (CyI) Swiss mice with clonal stocks from *T. cruzi* I (Cuica cl1, P209 cl1, Gamba cl1, SP104 cl1), *T. cruzi* II (IVV cl4, MVB cl8) and *T. cruzi* (Bug2148 cl1, MN cl2) lineages. We used the parasitemia as the parameter for Chagas disease reactivation and observed that CyI animals infected with *T. cruzi* stocks showed no reactivation and those infected with *T. cruzi* II stocks showed only 5% of reactivation. In contrast, immunosuppressed mice infected with stocks from *T. cruzi* I lineage showed 77.5 and 51.25% reactivation of the infection when Cyclophosphamide treatment was performed 60 and 180 days after inoculation, respectively. Next, we evaluated the efficacy of the Benznidazole (Bz) pre-treatment in reducing or preventing the recurrence of the infection in these CyI animals. In general, the percentage of the parasite recurrence was not altered among the CyI mice that received the Bz pre-treatment during the acute phase of the infection. Interestingly, when pre-Bz treatment was performed during the chronic phase, we observed two different patterns of response: (i) an increased protection among the animals inoculated with the SP104 cl1 (genotype 19) and Cuica cl1 (genotype 20) stocks; (ii) an increased percentage of parasitemia reactivation among mice inoculated with Gamba cl1 (genotype 19) and P209 cl1 (genotype 20) *T. cruzi* stocks. Our results corroborate our hypothesis by showing that the *T. cruzi* genetic background in combination with specific Bz treatment has an important role in the Chagas disease reactivation in immunosuppressed animals.

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1. Introduction

Chagas disease is a zoonosis widely spread in Central and South America affecting 13 million people in 21 endemic countries (WHO, 2005). The acute phase or the early stage of the disease is characterized by an inoculation-infiltrated zone called a chagoma, regional adenopathy, and low fever (Gallerano et al., 2007) followed by a chronic phase in which most of the infected individuals remain asymptomatic characterized by subpatent parasitemia and positive serological tests. Although most individuals with chronic *Try-*panosoma cruzi infection remain in the asymptomatic form for all life, approximately 10–30% develop symptomatic chronic Chagas disease, which is characterized by cardiac and/or gastro-intestinal involvement (Dias, 1992).

New aspects of the pathology of Chagas disease have been recently described in immunocompromised patients. These patients may present unusual clinical manifestations such as cutaneous lesions, involvement of central nervous system and/or serious cardiac lesions related to the reactivation of infection (Gallerano et al., 2007; Ferreira et al., 1997). Reactivation of chronic Chagas disease does not occur spontaneously and has been associated with immunosuppression in patients who have undergone transplantation (Jost et al., 1977; Cantarovich et al., 1992), that were submitted to antineoplastic chemotherapy and/or corticotherapy and in those individuals with AIDS (Ferreira, 1999; Ferreira and Borges, 2002; Ferreira et al., 1997; Perez-Ramirez et al., 1999;

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Rivera et al., 2004). Interestingly, Chagas disease reactivation does not occur in all chagasic patients exposed to immunosuppressive agents (Arias et al., 2006; Lopez-Blanco et al., 1992; Barousse, 1980). Among patients with AIDS it was also observed an absence of reactivation (Pacheco et al., 1998) as well as an asymptomatic reactivation during the chronic Chagas disease which was detected by the increasing of blood parasitism (Sartori et al., 2002). Reactivation of the disease has been also demonstrated in experimental animals chronically infected with *T. cruzi* submitted to different immunosuppressive drugs which showed myocarditis and an increase of parasitemia and mortality (Guerra et al., 2001; Brener and Chiari, 1971).

The clinical manifestations and variations in the immune response observed during chagasic infection are not well understood but are believed to be associated with the host or parasite genetic variability. Several studies involving *T. cruzi* infection have confirmed that genetic diversity is correlated with intrinsic characteristics of the parasite such as virulence, drug resistance, parasitemia, tropism toward specific organs, pathological alterations, capacity to induce host mortality and pattern of humoral immune response (de Diego et al., 1998; Toledo et al., 2002, 2003; Mejia and Triana, 2005; dos Santos et al., 2009). It is reasonable to suppose that the host immunological conditions, associated or not with the reactivation of the infection and parasite strain may have an important role during the course of the disease.

Benznidazole and Nifurtimox have proved to be effective for the treatment of the reactivated form of the human and experimental Chagas disease (Rezende et al., 2006) leading to the disappearance of clinical signs and symptoms of the disease, but without parasitological cure (Ferreira, 1999). However, it is not well defined whether the prophylactic pre- or post-immunosuppression using Benznidazole is truly effective to prevent the reactivation of the Chagas disease.

The process of reactivation opens an interesting field to investigate the influence of the *T. cruzi* genetic diversity in the experimental Chagas disease under immunosuppression circumstances. In the present work, the relationship between *T. cruzi* phylogenetic divergence and Chagas disease reactivation has been investigated through the detection of circulating blood trypomastigote forms of Cyclophosphamide immunosuppressed mice during the chronic phase of infection. Here, we also report that the efficacy of prophylactic Benznidazole therapy in preventing the reactivation of Chagas disease in experimental immunosuppression circumstances appears to be *T. cruzi* stock dependent.

2. Material and methods

2.1. T. cruzi stocks

The eight standard *T. cruzi* clones of the major clonets or principal genotypes representative of the widespread clonal genotypes (Tibayrenc and Ayala, 1988) used in this study were: Gamba cl1 and SP104 cl1 of genotype 19 and Cuica cl1 and P209 cl1 of genotype 20 both of which genotypes belong to the *T. cruzi* I lineage; IVV cl4 and MVB cl8 of genotype 32 belonging to *T. cruzi* II lineage and Bug 2148 cl1 and MN cl2 of hybrid genotype 39 classified as belonging to the *T. cruzi* lineage. Genotype 32 (*T. cruzi* II) and genotype 39 (*T. cruzi*) are closely related to each other, but distantly related to genotypes 19 and 20 (*T. cruzi* I). These clones were obtained from different geographical areas in Latin America and from various hosts of the domestic and wild transmission cycles. All stocks were cloned by micromanipulation and characterized by isoenzyme profile and randomly amplified by polymorphic DNA analyses (Tibayrenc et al., 1993; Barnabé et al., 2000). All experiments were carried out in full

accordance with the principles defined by the Brazilian School of Animal Experimentation (COBEA) and the animal procedures were approved by the Ethical Committee on Research of the Universidade Federal de Ouro Preto (MG, Brazil).

2.2. Experimental designs and infection procedure

A total of 240 female Swiss mice (18–20 g each) were obtained from the Animal Facility at the Universidade Federal de Ouro Preto, Minas Gerais, Brazil, and maintained in a temperature-controlled room with access to water and food *ad libitum*. Mice were randomly divided into eight groups of 30 individuals, each of which was inoculated via intraperitoneal injection with 10,000 blood trypomastigote forms of *T. cruzi* clones. Inoculum were obtained from Swiss mice that had been previously infected with a large number of metacyclic trypomastigote forms obtained from late stationary phase cultures on liver infusion tryptose (LIT) medium. The number of parasites in each inoculum was determined according to the method of Toledo et al. (2003).

2.3. Cyclophosphamide immunosuppression (CyI) and parasite detection

Each group of 30 animals previously described was subdivided into three subgroups of 10 animals. Animals from subgroups 1 and subgroups 2 received Cyclophosphamide (Cy) (Genuxal, Baxter Oncology, Frankfurt) after 60 or 180 days of inoculation, respectively. These periods for immunosuppression were determined considering that the chronic phase of Chagas disease starts when parasitemia falls to undetectable levels and when the general symptoms and clinical manifestations of acute phase disappear. These parasitological and clinical changes usually take place 4–8 weeks after infection (WHO, 2002). Animals included in subgroups 3 did not receive any medication.

Cy was dissolved in sterile PBS and administered according to therapeutic scheme previously described by Caldas et al. (2008b), which consisted of three cycles of 50 mg of Cy/kg of body weight, for four consecutive days, with intervals of 3 days between each cycle.

Parasitemia of all animals was evaluated from the 4th to the 60th day of infection. It was also performed before, during, and until 10 days after the Cy treatment by fresh blood collected from the mouse's tail and the number of parasites was estimated as described by Brener (1962).

2.4. Prophylactic pre-immunosuppression treatment with Benznidazole

The efficacy of the prophylactic treatment with Benznidazole (Bz) (*N*-benzyl-2-nitro-1-imidazolacetamide – Roche Company) was evaluated in mice infected with *T. cruzi* stocks that induced the reactivation of acute infection after CyI. For this, 50 animals were inoculated with each *T. cruzi* stock which was further subdivided into three subgroups of 10 or 20 animals. Subgroup 1 (20 mice) were treated orally (by gavage) during the acute phase on the 10th day after infection while subgroup 2 (20 mice) were treated during the chronic phase on the 120th after infection. Animals were treated with 100 mg of Bz per kg of body weight suspended in 4% gum Arabic for 20 consecutive days. Subgroups 3 (10 mice) consisted of non-treated animals that had their parasitemia curve evaluated daily. Thirty days after the Bz treatment ended, 10 animals of the subgroups 1 and 2 received the Cy. Parasitemia of these animals was evaluated daily during the immunosuppression cycles as well as for the following 10 days after the end of the CyI.

Table 1
Mean values and standard deviations of biological parameters under study in mice infected with *Trypanosoma cruzi*.

T. cruzi lineage	*T. cruzi* stock	Parameters – mean ± standard deviation		
		PPP	Parasitemia peak	Day of peak
T. cruzi I	Gamba cl1	14.5 ± 5.2	91.3 ± 40.1	35
T. cruzi I	SP104 cl1	14.3 ± 0.6	1.3 ± 1.4	16
T. cruzi I	Cuica cl1	11.5 ± 1.2	512.5 ± 240.6	30
T. cruzi I	P209 cl1	7.8 ± 1.2	5207.5 ± 2473.9	30
T. cruzi II	IVV cl4	10.2 ± 0.4	7.7 ± 2.6	12
T. cruzi II	MVB cl8	7.0 ± 0.75	4.6 ± 1.0	8
T. cruzi	Bug 2148 cl1	9.4 ± 1.9	5.5 ± 2.7	11
T. cruzi	MN cl2	7.7 ± 1.9	8.2 ± 3.4	10

PPP – pre-patent period (days). Parasitemia peak – number of trypomastigote forms × 1000/0.1 mL of blood.

2.5. Statistical analysis

The parasitemia level and patent period were compared using the non-parametric Mann–Whitney test, according to Snedecor and Cochran (1989). χ^2-Test was used to evaluate the association between percentages of the parasitemia reactivation among the different *T. cruzi* stocks. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Reactivation in Cyclophosphamide immunosuppressed animals depends on the T. cruzi major lineages

First we evaluated the biological parameters such as pre-patent period and parasitemia levels in mice infected with different *T. cruzi* lineages. In general, all animals inoculated with *T. cruzi* I lineage exhibited increased pre-patent period and parasitemia levels compared with animals inoculated with parasites included in *T. cruzi* II and *T. cruzi* lineage (Table 1), except to those animals inoculated with SP104 cl *T. cruzi* stock which parameters were more similar to *T. cruzi* II and *T. cruzi* lineages.

Next, we evaluated the influence of the *T. cruzi* lineages in the experimental Chagas disease reactivation under immunosuppression circumstances. With the exception of the one mouse infected with IVV cl4 stock, the animals infected with all other *T. cruzi* II and *T. cruzi* genotypes did not show parasites in peripheral blood after CyI. Conversely, we observed higher percentages of reactivation of blood parasitism in CyI animals inoculated with stocks from *T. cruzi* I lineage (Fig. 1). However, the percentage of reactivation varied depending on the period which Cy treatment was performed. We detected 77.5% of reactivation in animals immunosuppressed 60
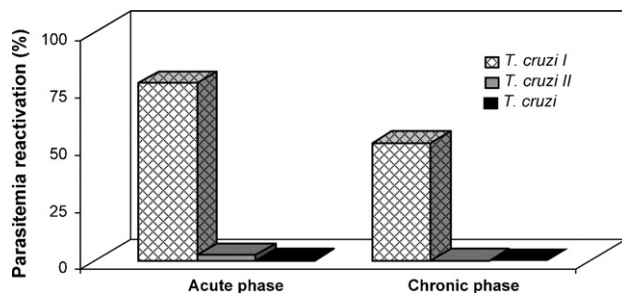

Fig. 1. Parasitemia reactivation, induced by Cyclophosphamide immunosuppression performed 60 (acute phase) and 180 (chronic phase) days after inoculation, in blood of mice infected with *Trypanosoma cruzi* stocks included in *T. cruzi* I (Gamba cl1, SP104 cl1, Cuica cl1 and P209 cl1), *T. cruzi* II (MVB cl8 and IVV cl4) and *T. cruzi* (Bug2148 cl1 and Mn cl2) lineage.
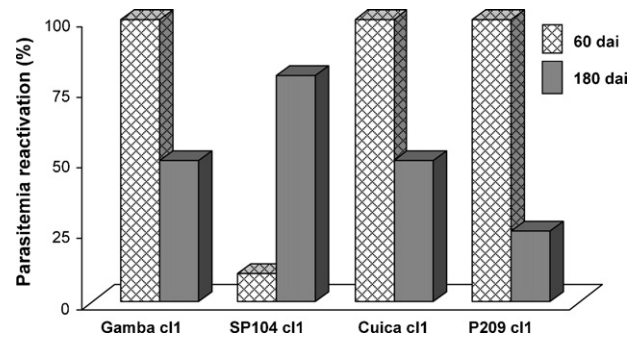

Fig. 2. Parasitemia reactivation of mice inoculated with 10,000 blood trypomastigotes of Gamba cl1, SP104 cl1, Cuica cl1 and P209 cl1 *T. cruzi* stocks (*T. cruzi* I lineage) induced by Cyclophosphamide immunosuppression performed 60 (acute phase) and 180 (chronic phase) days after inoculation (dai).

days after the inoculation and 51.25% in those immunosuppressed 180 days after infection.

3.2. Reactivation and parasitemia levels in Cyclophosphamide immunosuppressed animals are not correlated to T. cruzi genotype

In order to evaluate whether the reactivation pattern was also correlated with the parasite genetic background, we re-analysed our results according to the *T. cruzi* genotypes. Among the four stocks belonging to genotype 19 and 20, three of them, Gamba cl1 cl1 (genotype 19), Cuica cl1 and P209 cl1 (genotype 20) induced 100% of the reactivation in animals immunosuppressed 60 days after inoculation. On the other hand, we detected only 10% of reactivation of the parasitemia in the CyI animals inoculated with SP104 cl1 (genotype 19). Particularly, the parasitemia levels detected after the immunosuppression were 2.5 times higher for those animals infected with Gamba cl1 (genotype 19) and 1.5 times higher for those animals infected with P209 cl1 (genotype 20) than the levels identified during the acute phase. While animals infected with Cuica cl1 (genotype 20) showed a reduction in the parasitemia levels to half of the original level during the acute phase (Fig. 2 and Table 2).

Interestingly, the CyI performed 180 days after the parasite inoculation induced a significantly smaller reactivation and parasitemia levels compared to those animals that received Cy 60 days after inoculation ($p < 0.05$) (Fig. 2 and Table 2). An exception was observed in the animals inoculated with SP104 cl1 in which the percentage of those presenting parasitemia reactivation was more elevated when CyI was performed 180 days after inoculation (Fig. 2). It is worth mentioning that the levels of parasitemia were either lower or similar to those detected during the acute phase of the infection (Tables 1 and 2). Our data indicate that the intensity of the reactivation of the parasitemia induced by the CyI is related to the *T. cruzi* genetic lineage but the reactivation pattern is variable among animals infected with parasite stocks even from the same genotype.

3.3. Benznidazole-treatment protection against parasitemia reactivation in Cyclophosphamide immunosuppressed animals does not depend on the T. cruzi genotype

Next we evaluated the efficacy of the pre-treatment with Bz in preventing the reactivation in CyI animals infected with parasite stocks belonging to *T. cruzi* I lineage. Administration of Bz during the acute phase (10 days after inoculation) was not effective in preventing the reactivation of the parasitemia induced by Cy. Similar rates of the parasitemia reactivation were detected among Bz-treated and non-treated animals infected with Gamba

Table 2
Parasitemia reactivation induced by Cyclophosphamide immunosuppression in animals infected with clonal stocks Gamba cl1, SP104 cl1, Cuica cl1 and P209 cl1 (*T. cruzi* I) treated with Benznidazole or vehicle (untreated group).

Parasite clonal stocks	Experimental group	Higher parasitemia after CyI	
		Days after inoculation	
		60 days	180 days
Gamba cl1	C	ND	ND
	CyI	229.0 ± 222.6	4.0 ± 0.0
	Bz	ND	ND
	Bz-CyI	621.9 ± 538.3	2.0 ± 0.0
SP104 cl1	C	ND	ND
	CyI	5.0 ± 0.0	5.0 ± 2.5
	Bz	ND	ND
	Bz-CyI	ND	ND
Cuica cl1	C	ND	ND
	CyI	108.2 ± 94.2	4.5 ± 0.7
	Bz	ND	ND
	Bz-CyI	284.6 ± 283.7	4.3 ± 5.8
P209 cl1	C	ND	ND
	CyI	706.8 ± 489.7	7.5 ± 3.5
	Bz	ND	ND
	Bz-CyI	8966.1 ± 6753.9	24.4 ± 26.5

C – infected, but untreated mice. CyI – infected and submitted to Cyclophosphamide immunosuppression. Bz – infected and treated with Benznidazole. Bz-CyI – infected, treated with Benznidazole and submitted to CyI. Higher parasitemia – number of trypomastigote forms × 1000/0.1 mL of blood. ND – not detected.

cl1 (88.9%), Cuica cl1 (100%) and P209 (100%) stocks (Fig. 3). However, Bz treatment during acute phase was effective in preventing the parasitemia reactivation in the animals infected with the SP104 cl1 stock (Table 2 and Fig. 3).

Interestingly, the Bz treatment performed during the chronic phase (120 days after inoculation) induced two patterns of response that were not correlated to the parasite genotype. The first pattern was detected in CyI mice infected with SP104 cl1 (genotype 19) and Cuica cl1 (genotype 20) stocks. In these animals, we observed a reduction of the parasitemia reactivation in Bz-treated immunosuppressed animals (Fig. 3) compared to non-treated immunosuppressed animals (Fig. 2). In other words, the Bz-treatment provided protection against parasitemia reactivation. The second pattern was detected in animals infected with Gamba cl1 (genotype 19) and P209 cl1 (genotype 20) stocks. In these animals, the percentage of reactivation was higher in Bz-treated CyI animals than in those non-treated and CyI. Bz-treated animals before CyI showed a parasitemia reactivation of 60% for Gamba cl1 and 55.5% for P209 cl1 (Fig. 3) in contrast to 50 and 25% parasitemia reactivation in non-treated immunosuppressed mice
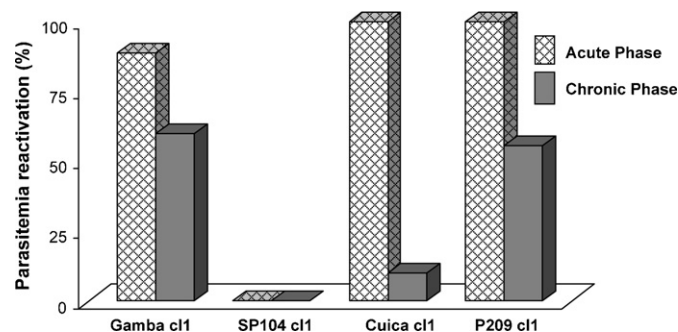

Fig. 3. Parasitemia reactivation induced by Cyclophosphamide immunosuppression in mice inoculated with 10,000 blood trypomastigotes of Gamba cl1, SP104 cl1, Cuica cl1 and P209 cl1 *T. cruzi* stocks after Benznidazole treatment performed during the acute or chronic phase of the experimental infection.

infected respectively with the same *T. cruzi* stocks (Fig. 2). In addition, the levels of parasitemia detected in peripheral blood of these animals were significantly higher in relation to those that did not receive Bz (Table 2).

Taking all together, our results indicate that the efficacy of the Bz treatment in preventing the reactivation of the Chagas disease in experimental immunosuppression conditions depends on the parasite genetic background but not to any specific *T. cruzi* genotype.

4. Discussion

Chagas disease reactivation is a process observed in immunocompromised host which may present acute and severe illnesses, in particular meningoencephalitis and myocarditis (Sartori et al., 2002; Ferreira et al., 1997). These manifestations are usually associated with the detection of *T. cruzi* trypomastigote forms by direct microscopic examination of blood (Silva et al., 1999; Sartori et al., 2002), cerebrospinal (Silva et al., 1999) or other body fluids (Ferreira et al., 1997). High levels of blood parasites can precede clinical manifestations (Sartori et al., 1995) or may be a later finding (Sartori et al., 1995) during the reactivation process, but they are found consistently (Sartori et al., 2002). According to Sartori et al. (2002) detection of *T. cruzi* by direct microscopic examination characterizes the reactivation of Chagas disease even without clinical manifestations.

Using the parasitemia as parameter of Chagas disease reactivation we demonstrated that the percentage of parasitemia reactivation induced by CyI was related to the *T. cruzi* major lineage. This pattern of parasitemia recrudescence CyI was detected only in animals infected with parasites of *T. cruzi* I. The higher percentages of reactivation were observed in parasite stocks belonging to genotypes 19 and 20. This fact can be explained by the genetic proximity of these two genotypes of the *T. cruzi* (Tibayrenc and Ayala, 1988). According to Hudson et al. (1983) the clonal genotypes 19, 20, 39 and 32 present different phylogenetic relationships, as 20/19 and 39/32 being more related among themselves. The genotypes 19 and 20 differ in few characteristics, such as the isoenzymatic 6PGDH locus, in which genotype 20 is heterozygote and genotype 19 is homozygote. Our results showed that reactivation of experimental *T. cruzi* infection may be influenced by the intrinsic characteristics of *T. cruzi* stocks explaining, at least partially, the fact of the recrudescence of the Chagas disease does not occur in all chagasic patients exposed to immunosuppressive agents (Arias et al., 2006).

However, the parasitemia reactivation and the levels of circulating parasites after CyI varied among *T. cruzi* stocks even when belonging from the same genotype (19 and 20 genotypes). These results suggest that it is necessary to take into account the lesser phylogenetic subdivisions due to the heterogeneous behavior of stocks from the same genotype (Toledo et al., 2003).

Together, these data reinforce the association between the reactivation of latent infections induced by *T. cruzi* with certain stocks of the parasite, and also support the hypothesis that therapeutic response during these reactivation episodes in immunosuppressed individuals could be dependent of the parasite population. The data obtained with the Bz-treatment before CyI support partially this hypothesis. The efficacy of the pre-treatment in reducing or preventing the recurrence of the infection was related to the time of infection and also to the population of the *T. cruzi*. The percentage of the circulating parasite recurrence after CyI was not altered among animals that received Bz during acute phase of the infection. On the other hand, the Bz treatment performed during chronic phase was able to induce two different patterns of response: (i) protection in the animals inoculated with the SP104 cl1 stock (genotype 19) and Cuica cl1 stock (genotype 20) and (ii) increase of the parasitemia reactivation in animals inoculated with Gamba cl1 (genotype 19) and P209 cl1 (genotypes 20) *T. cruzi* stocks. In addition, the para-

sitemia levels in these animals were significantly higher in relation to those detect after CyI without Bz treatment.

Interestingly, the efficacy of Bz in reducing the recrudescence of chronic Chagas disease was related to the T. cruzi stock, but not a specific genotype of the parasite. These results were also observed by Toledo et al. (2004) and Caldas et al. (2008a) that showed that Bz treatment was not able to reduce both tissue parasitism and inflammation in mice infected with all T. cruzi stocks. In addition, Toledo et al. (2004) showed that the Bz-treated mice infected with stocks from genotype 39 displayed a significantly higher parasitism during the acute phase comparatively the non-treated group, demonstrating a negative effect of the treatment on the course of infection.

The use of trypanosomicidal drugs in animals with reactivated infection reduced the magnitude of the disease manifestations and prevented further reactivation, suggesting their relevance for clinical practice (Meckert et al., 1988). Bz administered at the recommended dose for 60 days was found to be effective in the treatment of the reactivation, leading the disappearance of signs and symptoms of the disease, but without cure of the parasitosis (Ferreira and Borges, 2002). The evaluation of the prophylactic pre-treatment prior immunosuppression is important, especially in pre-transplant, because the Bz treatment may be effective in the reduction of the parasitism and controlling the Chagas disease reactivation.

Considering that T. cruzi stocks react differently to the immunosuppressive effect of Cy, it is reasonable to suppose that immunological conditions of the host induced by diverse immunosuppressive drugs associated with particular parasite stock may have an important role in the reactivation of T. cruzi infection. New investigations are welcome to the best understanding of the interaction among immunosuppressive drugs and evolution of experimental chronic Chagas disease.

Acknowledgements

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