



## *Trypanosoma cruzi*: Induction of benznidazole resistance *in vivo* and its modulation by *in vitro* culturing and mice infection

Fabiane Matos dos Santos<sup>a</sup>, Sérgio Caldas<sup>a</sup>, Stéfany Bruno de Assis Cáu<sup>b</sup>, Geovam Pereira Crepalde<sup>b</sup>, Marta de Lana<sup>a,c</sup>, George L.L. Machado-Coelho<sup>a,c</sup>, Vanja Maria Veloso<sup>a,b</sup>, Maria Terezinha Bahia<sup>a,b,\*</sup>

<sup>a</sup> Núcleo de Pesquisa em Ciências Biológicas, Universidade Federal de Ouro Preto, Morro do Cruzeiro, CEP 35400-000, Ouro Preto, Minas Gerais, Brazil

<sup>b</sup> Departamento de Ciências Biológicas, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Morro do Cruzeiro, CEP 35400-000, Ouro Preto, Minas Gerais, Brazil

<sup>c</sup> Escola de Farmácia, Universidade Federal de Ouro Preto, Morro do Cruzeiro, CEP 35400-000, Ouro Preto, Minas Gerais, Brazil

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### ABSTRACT

Through a continuous *in vivo* drug pressure protocol, using mice as experimental model, we induced benznidazole resistance in *Trypanosoma cruzi* stocks. Full resistance was obtained for four out of five *T. cruzi* stocks analyzed. However, the number of benznidazole doses (40–180), as well as the time (4–18 months) necessary to induce resistance varied among the different *T. cruzi* stocks. The resistance phenotype remained stable after *T. cruzi* stocks has been maintained by 12 passages in mice (six months) and in acellular culture for the same time. However, the maintenance of resistant parasite for 12 months in acellular culture induces a reduction in its level of benznidazole resistance, while no alteration was detected in parasite maintained for the same time in mice. The data showed the stability of the resistance acquired by drug pressure, but suggest the possibility of reversible changes in the resistance levels after maintenance for long time in acellular culture.

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### 1. Introduction

Chagas disease, caused by the flagellate parasite *Trypanosoma (Schizotripanum) cruzi*, is a major public health problem in Latin America. It is estimated that about 16–18 million people are infected with *T. cruzi* and other 40 million are at risk of acquiring the disease (WHO, 2005).

*Trypanosoma cruzi* is a heterogeneous population with two major phylogenetic groups (and several subgroups), which have been clearly defined (Tibayrenc et al., 1993; Anon, 1999). These two groups display completely different ecological, epidemiological and biological traits (Buscaglia and DiNoia, 2003) and the genetic variability found among parasite populations led to the hypothesis that *T. cruzi* undergo predominantly clonal evolution in nature with rare genetic exchange events (Brisse et al., 2000). This clonal evolution model postulated for *T. cruzi* predicts a correlation between the phylogenetic divergence of the clones and their biological properties (Villarreal et al., 2004; Toledo et al., 2002). In fact several reports have shown a link between genetic diversity of *T. cruzi* populations and its biological properties (Revollo et al., 1998; Tole-

do et al., 2002), including drug sensitivity/resistance phenotype (Toledo et al., 2004).

Drug resistance in *T. cruzi* has been previously reported and a variety of *T. cruzi* strains have shown to be inherently resistant to widely used anti-*T. cruzi* drugs, such as benznidazole (Bz) and nifurtimox (Filardi and Brener, 1987). Studies have shown that drug resistance may increase or be induced during maintenance of the parasite *in vivo* (Murta and Romanha, 1998) or *in vitro* (Buckner et al., 1998; Dvorak and Howe, 1977; Villarreal et al., 2005) under drug pressure. Recently, Caldas et al. (2008) demonstrated that the induction of Bz-resistance in *T. cruzi* stocks might also occur during long-term infection in vertebrate host in the absence of drug stress. It was also observed that when the same *T. cruzi* stocks were maintained by successive blood passages in mice a decrease or stability in drug resistance phenotype, as well as an increase in virulence occurred. One question that arises from these observations is whether Bz-resistance induced *in vivo* under drug pressure could also be modulated *in vitro* or during infection in mice. Moreover, until now there are no reports showing whether drug-resistant phenotypes are stable during *T. cruzi* life cycle. Addressing these questions would certainly help clarify whether propagation of chemoresistant parasite occurs in nature, especially considering the low cure indexes observed during Bz-treatment of chronically infected patients (Cançado, 2002; Ferreira, 1990; Lauria-Pires et al., 2000).

\* Corresponding author. Address: Departamento de Ciências Biológicas, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Campus Univer.—Morro do Cruzeiro, CEP 35400-000, Ouro Preto, Minas Gerais, Brazil. Fax: +55 31 3559 1689.

E-mail address: [mtbahia@nupeb.ufop.br](mailto:mtbahia@nupeb.ufop.br) (M.T. Bahia).

In this report, we used drug pressure to induce *in vivo* Bz-resistance in *T. cruzi* stocks isolated from dogs inoculated with Be-62 and Be-78 strains (both sensitive to Bz) 2–10 years ago. The number of Bz doses necessary for induction of full resistance in different *T. cruzi* stocks using mice as experimental model was also determined. The resistant parasites were analyzed to verify stability of resistance during its life cycle *in vivo* and *in vitro*, as well as evaluated the effect of benznidazole on the parasitemia levels.

## 2. Materials and methods

### 2.1. *T. cruzi* stocks

Berenice-62 and Berenice-78 strains, *T. cruzi* II (Cruz et al., 2006), were isolated from the patient Berenice in 1962 (Salgado et al., 1962) and 1978 (Lana and Chiari, 1986), respectively. Both strains were considered to be sensitive to Bz (Toledo et al., 1995).

Two *T. cruzi* stocks were obtained from different outbred dogs chronically infected with Be-62 (Be-62A and Be-62B) 10 years ago, and three were isolated from different dogs infected with the Berenice-78 strain after 2 (Be-78D), 7 (Be-78C) and 10 (Be-78E) years ago. Immediately after isolation, Bz sensitivity was determined using the mouse as experimental model. Mice inoculated with Be-62A and Be-62B were 50% and 60% resistant to Bz, while mice inoculated with Be-78C, Be-78D and Be-78E stocks were, respectively, 90%, 70% and 90% resistant to the drug. We have also verified that, contrary to the other stocks, Be-78D and Be-78E stocks changed to a drug sensitive phenotype after maintenance in mice for two years by successive blood passages (Caldas et al., 2008).

### 2.2. Mice infection and treatment with Bz

For each strain or *T. cruzi* stock, 16 Swiss outbred mice (30 days old, 18–20 g body weight) were infected by intraperitoneal route with  $5 \times 10^3$  blood trypomastigotes. Ten mice were submitted to treatment with benznidazole (*N*-benzyl-2-nitro-1-imidazolacetamide—Roche Company). Treatment consisted of oral administration of the drug (100 mg/kg of body weight) in a water suspension with 4% arabic gum (Sigma Chemical Co.) for 20 consecutive days. Six mice were kept as untreated infected controls. Treatment started after detection of parasitemia by fresh blood examination, approximately 10 days post-inoculation. All procedures and experimental protocols were performed according to COBEA (Brazilian College of Animal Experimentation) and behavior instructions for the use of animals in research.

### 2.3. Induction of resistance to benznidazole

For the induction of Bz-resistance, *T. cruzi* stocks were isolated from dogs by blood culture and cultivated in liver infusion tryptose (LIT) medium. Afterwards, parasites were inoculated in Swiss mice and maintained by successive cycles of treatment with Bz. In each cycle, 10 animals were treated for 20 consecutive days using the same therapeutic scheme described above. After treatment, animals that did not show parasitemia reactivation were immunosuppressed with cyclophosphamide (see Section 2.4.1). Parasites obtained after immunosuppression were inoculated in a new group of 10 mice. Successive treatment cycles (TC) were performed until fully Bz-resistant parasites were obtained for at least four successive TC.

To verify the stability of the phenotype, parasites isolated from the last TC were maintained by successive blood passages in non-treated mice or acellular culture in LIT medium for six months. After this, *T. cruzi* resistant stocks were inoculated into a new group

of animals and both drug resistance and biological properties (parasitemia, mortality and virulence) evaluated.

### 2.4. Assessment of parasitological cure

To assess parasitological cure, a battery of three independent tests, including fresh blood examination before, during and after cyclophosphamide immunosuppression, blood culture and polymerase chain reaction (PCR), was used. Animals were considered cured when all tests were persistently negative.

#### 2.4.1. Fresh blood examination and cyclophosphamide treatment

Parasitemia was evaluated from day 4 post-inoculation to day 10 post-treatment. After this period, animals that did not present parasitemia reactivation were immunosuppressed with cyclophosphamide (Genuxal®). The immunosuppression protocol consisted of three cycles of 50 mg of cyclophosphamide/kg of body weight, during four consecutive days, with an interval of 3 days between each cycle. Parasitemia of these animals was evaluated during and until 5 days after cyclophosphamide treatment.

#### 2.4.2. Blood culture

Thirty days after the end of cyclophosphamide treatment, mice that showed negative fresh blood test were bled by the orbital venous sinus and 0.4 mL of blood was collected. Blood was divided into two tubes containing 3 mL of LIT medium (Filardi and Brener, 1987). The tubes were incubated at 28 °C for 90 days and microscopically examined for detection of parasites 30, 60 and 90 days later.

#### 2.4.3. PCR assay

PCR was performed only in blood samples from animals that showed negative results in all parasitological tests. Mice were bled from the orbital venous sinus and 200 µL of blood were collected 30 days after the end of cyclophosphamide treatment. DNA extraction and PCR was performed according to Gomes et al. (1998) with some modifications. The primers used for the parasite minicircle amplification were: S35 5'-AAATAATGTACGGG(T/G)GAGATG CATGA-3' and S36 5'-GGGTTCCGATTGGGGTTGGTGT-3' (Ávila et al., 1990). Thirty-five amplification cycles were carried out in a Research Programmable Thermal Controller (MiniCycler). The cycles consisted of an initial denaturation for 5 min at 95 °C followed by 35 cycles of 1 min at 95 °C for denaturation, 1 min at 65 °C for primer annealing and 1 min at 72 °C for primer extension. All the stages were carried out in separate environments with reagents, materials and equipments exclusive to each area. Positive and negative, as well as control reagents, were also tested in parallel. Five microliters of the PCR products were analyzed by electrophoresis on a 6% polyacrylamide gel and visualized by silver staining (Santos et al., 1993).

### 2.5. Parasitemia curves

Groups of six outbred Swiss mice (18–21 g) were inoculated intraperitoneally with  $5 \times 10^3$  blood trypomastigotes from *T. cruzi* stocks before and after the first Bz-treatment and after the last TC. Parasitemia was followed daily after the 4th day of infection until parasite negativation. Fresh blood was collected from the mouse tail and the number of parasites quantified as described by Brener (1962). Parasitemia curves were plotted considering the average of the parasitemia obtained from six mice.

### 2.6. Statistical analysis

The parasitemia levels obtained from animals inoculated before and after the first Bz-treatment and the last TC were compared

using the Mann–Whitney tests (Snedecor and Cochran, 1989). Values of  $P < 0.05$  were considered significant.

### 3. Results

#### 3.1. Induction of *T. cruzi* benznidazole resistance in vivo

Bz-resistant *T. cruzi* parasites were obtained from four out of five *T. cruzi* stocks submitted to the long-term Bz-pressure protocol. The only stock where Bz-resistance was not induced was Be-62A stock. This stock showed 50% of resistance to Bz in the first TC, which was only evidenced by the PCR assay. Any animal showing parasitemia reactivation when treated with cyclophosphamide had its treatment interrupted.

As indicated in Fig. 1 the other *T. cruzi* stocks evaluated were totally resistant after 2–9 successive cycles of Bz-treatment. However, the number of TC necessary to obtain fully resistant parasites varied among the stocks.

Be-62B showed full resistance to Bz after the 5th TC. In the 6th TC all treated animals started to present parasitemia reactivation after cyclophosphamide immunosuppression, indicating an increase in Bz-resistance (Fig. 1A).

Be-78C was completely resistant to Bz in the 2nd TC. Natural reactivation of the parasitemia after treatment was observed for 10%, 100%, 40%, 90% and 100% of the animals after the 1st, 2nd, 3rd, 4th and 5th TC, respectively (Fig. 1B). Moreover, during the 10th TC, Bz was not effective for suppression of parasitemia even during the treatment (Fig. 2). The data show the increase of Bz-resistance throughout the TC.

Differently, Be-78D stock was completely resistant to Bz only after 9 TC. The cure index of mice after Bz-treatment varied from 60% to 100% until the 9th TC, becoming stable afterwards. The lower level of resistance showed by Be-78D in relation to Be-78C could also be observed by the absence of animals with natural parasite-

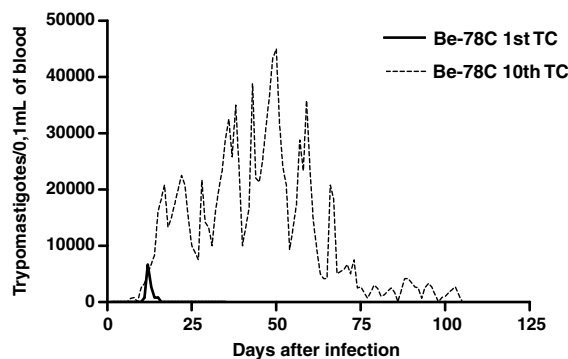


Fig. 2. Parasitemia curve of Be-78C stock in mice treated with benznidazole during the first and 10th treatment cycle.

mia reactivation after treatment. This stock reached resistance stability in 100% of the animals after the 9th TC. This was always observed after immunosuppression treatment, and sometimes also by blood culture (Fig. 1C). The same fluctuation was observed with Be-78E when animals infected with this stock started to show 0% of cure in the 6th TC presenting 100% parasitemia reactivation induced by cyclophosphamide immunosuppression and blood culture detection (Fig. 1D).

#### 3.2. Stability of resistance phenotype

In order to evaluate how stable were the resistant stocks, they were successively passed 12 times in mice (for six months) without drug treatment, as well as in acellular culture for the same period of time. Afterwards the parasites were again inoculated in mice to receive a new Bz-treatment.

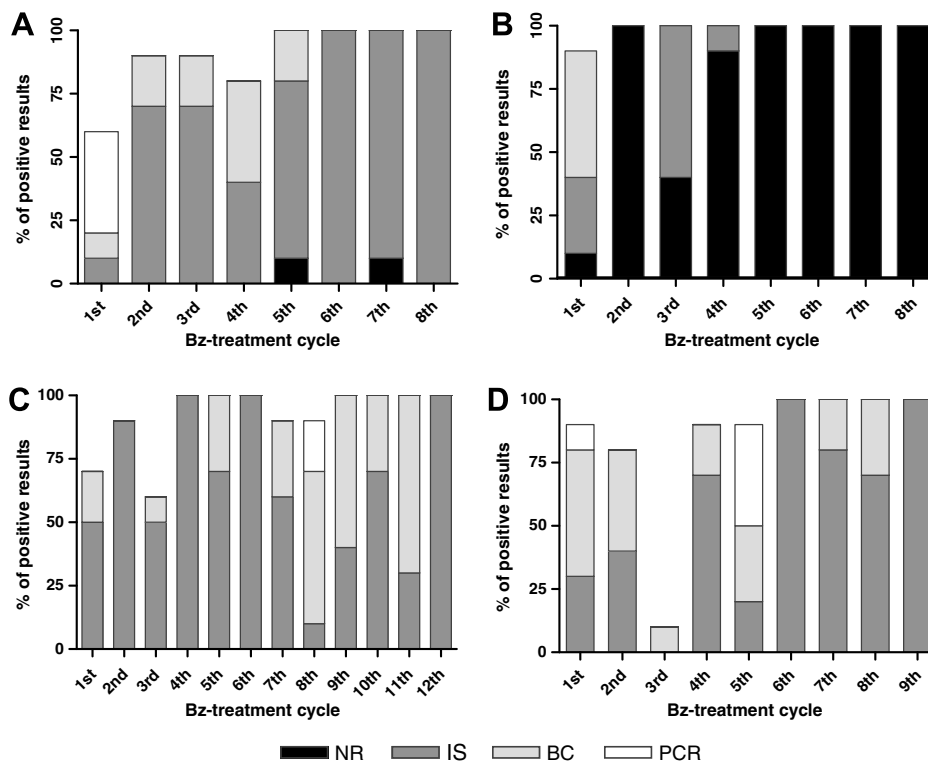


Fig. 1. Cure percentage during successive cycles of benznidazole treatment in mice inoculated with Be-62B (A), Be-78C (B), Be-78D (C) and Be-78E (D) stocks, detected by fresh blood examination with natural reactivation (NR) and after cyclophosphamide immunosuppression (IS), by blood culture (BC) and PCR.

Animals inoculated with the resistant stocks maintained for 12 successive blood passages in mice showed no reduction of the cure rate. However, a decrease in the number of animals that presented parasitemia reactivation after immunosuppression was also observed (Table 1). Similar results were obtained for animals infected with parasites maintained for six months in acellular culture. Only a small reduction in the index of cure was detected in mice inoculated with Be-78E stock. However, as shown in the Table 1, for the detection of the therapeutic failure in animals inoculated with parasites after *in vitro* culturing it was usually necessary the use of more sensitive methods, as blood culture and PCR. These data indicate a great reduction of parasitemia induced by Bz-treatment in resistant *T. cruzi* stocks after maintenance in culture.

To confirm these results, the most resistant *T. cruzi* stock, Be-78C, was maintained for 24 blood passages in mice (12 months) without drug treatment, as well as for 12 months in acellular culture. Parasites were then reinoculated in mice and submitted to Bz-treatment. Fifty percent of reduction in Bz-resistance was detected only among mice inoculated with Be-78C maintained for 12 months in acellular culture (Table 1). Nevertheless, all treated animals showed parasitemia suppression during the period of treatment. Natural reactivation of parasitemia was not observed in any of the treated mice, which indicated a decrease in the level of drug resistance for this stock.

### 3.3. The effect of benznidazole on parasitemia

Parasitemia curves obtained from infected mice before and after the first and the last TC with Bz are shown in Fig. 3. A significant increase in parasitemia levels in mice inoculated with Be-62B ( $P = 0.036$ ) and Be-78C ( $P = 0.008$ ) after the first TC when compared with the parasitemia level detected before the start of TC was observed. Among animals inoculated with the *T. cruzi* stock Be-78D no significant differences in the parasitemia levels was observed during the TC ( $P = 0.29, 0.26$  and  $0.9$ ). On the other hand a reduction in parasitemia was observed among mice inoculated with blood trypomastigotes of Be-78E before the Bz-treatment ( $P = 0.027$ ) and also with those inoculated with parasites from the 1st TC ( $P = 0.006$ ) and the last TC. Concerning infectivity, pre-patent period and mortality rates, no significant differences were observed among parasites obtained before the TC and after the first or last TC.

## 4. Discussion

The major obstacle to treatment and prevention of parasitic diseases in human has been and continues to be the drug resistance. The development of models of chemoresistance has strongly facilitated studies related to the molecular basis of drug resistance/susceptibility.

In the present work we have shown that Bz-resistance of *T. cruzi* stocks can be induced *in vivo* through maintenance of the parasite in constant drug pressure (four out of five parasite populations evaluated). However, the minimum necessary number of TC for induction of resistance was variable among the different *T. cruzi* stocks used. Be-78C showed full resistance after 2 TC (40 doses of Bz), while 9 cycles (180 doses of Bz) were necessary for Be-78D. Intermediate number of TC (5–6) was necessary for the induction of resistance in Be-62B and Be-78E stocks, respectively. Interestingly, it was not possible to induce Bz-resistance in the Be-62A stock. Any of the animals infected with this stock showed parasitemia reactivation after treatment, being the therapeutic failure detected only by PCR test.

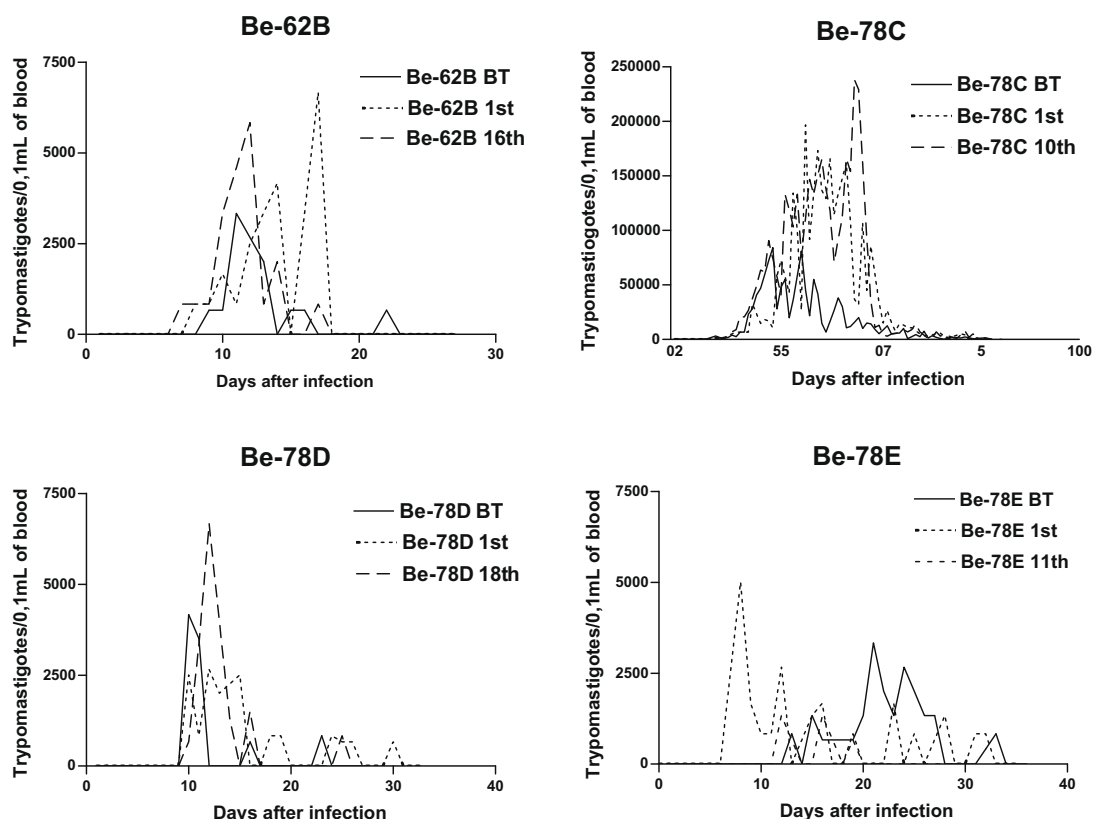
These results support the hypothesis that each *T. cruzi* stock acts independently of its genetic cluster when submitted to a drug stress since all of them belonged originally to *T. cruzi* II group, sublineages IIb (Cruz et al., 2006; Brisse et al., 2000). This hypothesis might be corroborated by the important differences found in drug resistance levels during the induction of Bz-resistance among the stocks, as follows: (1) all animals infected with Be-78C showed natural parasitemia reactivation in the 5th TC and after the 10th TC no parasitemia suppression was observed in these animals even during the treatment; (2) mice inoculated with Be-62B showed post-treatment parasitemia reactivation only after cyclophosphamide immunosuppression being 100% of them observed only after the 5th TC; (3) animals infected with Be-78D and Be-78E did not show natural parasitemia reactivation after Bz-treatment, and the reactivation of parasitemia induced by cyclophosphamide was detected in 100% of the animals during the last TC, in both stocks.

The ability of protozoan parasites to develop resistance to drugs probably demonstrates their genetic adaptability. Probably drug stress leads to the selection of appropriate strategies to escape the lethal effects of the tripanocida compound. Drug resistance has a strong impact on Chagas disease chemotherapy increasing therapeutic failure and limiting the options of treatment. The occurrence of natural resistance to different drugs has been de-

**Table 1**  
Determination of the stability of benznidazole resistance after passage of resistant parasites in mice and acellular culture without drug treatment

<i>T. cruzi</i> stock	Parasitemia reactivation		Blood culture (%)	PCR (%)	Total + results (%)
	Before IS (%)	After IS (%)			
R-Be-62B	0	100	ND	ND	100
R-Be-62B/6M in AC	0	60	30	10	100
R-Be-62B/6M in BP	10	50	40	NR	100
R-Be-78C	100	ND	ND	ND	100
R-Be-78C/6M in AC	20	80	ND	ND	100
R-Be-78C/6M in BP	100	ND	ND	ND	100
R-Be-78C/12M in AC	0	20	0	30	50
R-Be-78C/12M in BP	0	100	ND	ND	100
R-Be-78D	0	100	ND	ND	100
R-Be-78D/6M in AC	0	60	40	ND	100
R-Be-78D/6M in BP	0	80	10	10	100
R-Be-78E	0	100	ND	ND	100
R-Be-78E/6M in AC	0	10	60	10	80
R-Be-78E/6M in BP	0	60	40	0	100

BC, blood culture; IS, immunosuppression; R-Be-62B, resistant Be-62B stock; R-Be-78C, resistant Be-78C stock; R-Be-78D, resistant Be-78D stock; R-Be-78E, resistant Be-78E stock; 6M in AC, maintained for six months in acellular culture; 12M in AC, maintained for 12 months in acellular culture; 6M in BP, maintained for six months in blood passage in mice; 12M in BP, maintained for 12 months in blood passage in mice; ND, no done.



**Fig. 3.** Parasitemia curves of *Trypanosoma cruzi* stocks in mice inoculated with blood trypomastigotes obtained from mice before the first treatment cycle (BT), as well as after the first and the last Bz-treatment cycle. The mice were infected with  $5 \times 10^3$  trypomastigotes of the Be-62B, Be-78C, Be-78D and Be-78E stocks.

scribed for *T. cruzi* populations (Filardi and Brener, 1987; Guedes et al., 2004). More recently, the induction of Bz-resistance *in vitro* or selection of resistant parasites *in vivo* after drug pressure has been described (Buckner et al., 1998; Dvorak and Howe, 1977; Villarreal et al., 2005; Murta and Romanha, 1998). Caldas et al. (2008) also showed that *T. cruzi* stocks, totally susceptible to Bz, became 50–90% resistant to Bz after long-term infection in dogs, even in the absence of drug pressure, representing in this case examples of natural drug resistance.

Alternative models to study the induction of *T. cruzi* drug resistance are important. According to Murta et al. (2006) the identification of genes that are differentially expressed in drug-resistant and drug susceptible *T. cruzi* populations could increase our understanding of the molecular basis of drug resistance in this parasite and most likely will lead the discovery of new targets for chemotherapy. However, Villarreal and co-workers (2004) during their studies about differential gene expression of distinct *T. cruzi* stocks after Bz-resistance induced *in vitro* did not find a link between drug resistance and genetic clustering by MLEE or RAPD.

In this scenario we believe that the Bz-sensitive parental *T. cruzi* populations, Be-62 and Be-78 (Filardi and Brener, 1987; Toledo et al., 1995; Veloso et al., 2001), and their isolates obtained from long-term infected dogs (Be-62A and B, Be-78C, D and E, presenting Bz-resistance levels varying from 50% to 90%) together with these same stocks after *in vivo*-induced Bz-resistance, provide an important model for the study of differential gene expression in sensitive and resistant *T. cruzi* stocks.

Based on these results we could speculate that probably several different pathways or mechanisms involved in *T. cruzi* Bz-resistance exist induced either by long-term infection of the vertebrate host or drug pressure. According to Villarreal et al. (2005) the mechanisms involved in natural drug sensitivity/resistance are different from those involved in induced chemoresistance.

Drug resistance stability was observed in Be-62B and Be-78C, D and E after 12 blood passages in mice and six months of maintenance in acellular culture without drug pressure. On the other hand, Murta and Romanha (1998) showed that Bz-resistance *in vivo*, induced by rapid treatment of Y strain infected mice with 500 mg/kg of the drug at parasitemia peak, was very unstable. The authors verified that after three passages in mice in the absence of drug pressure the population became again drug sensitive. These data support the hypothesis that the stability of the resistance phenotype may be related with the protocol used to resistance induction, in other words, the use of a rapid treatment protocol (Murta and Romanha, 1998) or the long-term treatment used in this study.

However, the more resistant *T. cruzi* stock, Be-78C, showed a reduction in its level of resistance to Bz after 12 months of maintenance in acellular culture, while no alteration was detected when this same stock was maintained during the same time by successive blood passages in mice. These data suggest the possibility of reversible changes in the resistance phenotype (acquired after maintenance under drug pressure) after *T. cruzi* stocks are kept for a long time in acellular culture *in vitro*. This hypothesis is in according with other authors that demonstrated different levels of stability of *T. cruzi* drug resistance *in vitro* (Dvorak and Howe, 1977; Nirdê et al., 1995; Nozaki et al., 1996). It is possible that the laboratory methods for parasite manipulation might favor the amplification of one subpopulation throughout regular maintenance of acellular culture or by passages in mice. In addition, particular attention should be given to the fact that we have shown a significant decrease in induced Bz-resistance when trypomastigotes were differentiated into epimastigotes. Similar results were observed in *Leishmania* by Sereno and Lemesre (1997) that showed significant decrease of pentamidine induced resistance *in vitro* when amastigotes differentiated in promastigotes. These data



strongly suggest that the resistance acquired by maintenance under drug pressure could probably be lost during the propagation of the parasite in the insect vector during its life cycle.

The induction in *T. cruzi* stocks of Bz-resistance *in vivo* was associated, most of the time, with an increase in their growth rate in mice. These results are different from those obtained by Villarreal et al. (2005), where the induction of resistance *in vitro* was associated with a decrease in parasite growth rate in culture. Sereno and Lemesre (1997) also showed in *Leishmania* model that the acquisition of resistance *in vitro* was associated with lower growth rate of the amastigotes without interference in parasite viability. Therefore our results support the hypothesis that the mechanisms involved in chemoresistance induction *in vivo* are different from those involved in resistance induction *in vitro*. On the other hand, it has been shown that maintenance of *T. cruzi* stock for several blood passages in mice induces increase of parasitemia (Brener and Chiari, 1963; Carneiro et al., 1990). In this case, the alternative hypothesis is that the increase of parasitemia observed during the induction of drug resistance might not be associated with the mechanism of resistance itself, but to the particular adaptability of each stock to the new host.

The results here obtained show that the acquired drug resistance seems to be a complex and multi-stage process involving not only biochemical mechanisms but also other elements such as the host immune system. As shown, the resistant phenotype was retained for a long time after *in vitro* culturing and mice infection without drug pressure. This might mean that the therapeutic failure commonly observed in patients treated with Bz during the chronic phase of the disease could lead to the selection of highly resistant parasites. Those parasites could then be transmitted from host to host in endemic areas where the vectorial transmission was not controlled.

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