In vivo selection of a population of *Trypanosoma cruzi* and clones resistant to benznidazole

S. M. F. MURTA[1,2] *and* A. J. ROMANHA[1]*

[1] *Centro de Pesquisas 'René Rachou'–FIOCRUZ, Caixa Postal 1743, BH-MG, Brazil*
[2] *Departamento de Bioquímica e Imunologia, ICB-UFMG, Caixa Postal 846, BH-MG, Brazil*

(*Received 23 April 1997; revised 11 August 1997; accepted 11 August 1997*)

SUMMARY

A benznidazole-resistant population of *Trypanosoma cruzi*, Y strain, was selected after 25 successive passages (8 months) in mice treated with a single high drug dose. Initially, the resistant parasites produced a low parasitaemia level and low mortality rate in infected mice. Thereafter, the parasitaemia level and mortality rate increased to the same value obtained for mice infected with the wild-type strain. Long-term treatment with benznidazole (100 mg/kg/day) cured 71–80% of mice infected with the wild-type strain. No cure was observed in mice infected with the selected resistant parasite population. Treatment with 500 mg/kg of benznidazole at peak parasitaemia cleared all blood parasites from mice infected with wild-type parasites. No effect on parasitaemia level was observed in mice infected with the selected parasites. Benznidazole-resistant parasites showed cross-resistance to different drugs. Contrary to wild type, all clones analysed from the resistant *T. cruzi* population were resistant to benznidazole. Without drug pressure the resistance phenotype of clones was far more stable than that presented by the resistant population. This work demonstrates, for the first time, the *in vivo* selection of a population and clones of *T. cruzi* resistant to benznidazole, and makes available an experimental model for the study of mechanisms of drug resistance in *T. cruzi*.

Key words: experimental Chagas' disease, *Trypanosoma cruzi*, chemotherapy, drug resistance, *in vivo* selection, clones.

INTRODUCTION

Trypanosoma cruzi, the aetiological agent of Chagas' disease, is widely distributed on the American continent where 16–18 million inhabitants are infected (WHO, 1991). Chemotherapy of Chagas' disease is limited to the drugs benznidazole and nifurtimox, each of which presents low efficacy and severe side-effects (Rassi & Luquetti, 1992). Recent studies have shown that a bis-triazole derivative, D0870, was able to prevent death and induce parasitological cure during acute and chronic phases of 70–90% of infected mice (Urbina *et al*. 1996). Differences in the susceptibility of *T. cruzi* strains to drugs have been described by different authors (Hauschka, 1949; Bock, Gonnert & Haberkorn, 1969; Haberkorn & Gonnert, 1972; Brener, Costa & Chiari, 1976; Andrade, Magalhães & Pontes, 1985; Neal & Van Bueren, 1988). Filardi & Brener (1987) described the existence of strains that are naturally resistant and non-resistant to benznidazole and nifurtimox. Some of these strains were isolated from sylvatic vectors from an area where autochthonous human Chagas' disease does not exist. Natural resistance of *T. cruzi* to nitro-derivatives has recently been suggested as an important factor in explaining the low rates of cure detected in treated chagasic patients.

The multidrug resistance (MDR) found in tumoral cell lines and in protozoa like *Leishmania*, *Plasmodium* and *Entamoeba* is associated with a super-expression of a membrane P-glycoprotein (PGP), that functions as an ATP-dependent drug efflux pump to reduce the intracellular accumulation of cytotoxic compounds within the cell (Gottesman & Pastan, 1993; Ullman, 1995). Recently the MDR genes Tcpgp1 and Tcpgp2 were described in *T. cruzi* (Dallagiovanna, Castanys & Gamarro, 1994; Dallagiovanna, Gamarro & Castanys, 1996). However, in the absence of any available drug-resistance model, the presence of this gene could not be associated with a functional drug-resistance phenotype. Different authors using different protocols have obtained *in vitro T. cruzi*-resistant strains. Dvorak & Howe (1977) and Nozaki, Engel & Dvorak (1996) obtained nifurtimox-resistant strains and Nirdé, Larroque & Barnabé (1995) obtained benznidazole-resistant strains. However, comparative studies of drug susceptibility in strains of *T. cruzi in vitro* and *in vivo* demonstrated that there was no correlation between drug susceptibility *in vitro* and *in vivo* (Scott & Matthews, 1987; Neal & Van Bueren, 1988; Ribeiro-Rodrigues *et al*. 1995). Haberkorn & Gonnert (1972) were able to select an *in vivo* nitrofurazone-resistant strain of *T. cruzi*, but failed to select resistance for nifurtimox. In order to

* Corresponding address: Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas 'René Rachou'–FIOCRUZ, Av. Augusto de Lima, 1715, 30190-002, Caixa Postal 1743, Belo Horizonte, MG-Brazil. Tel: +55 031 2953566. Fax: +55 031 2953115. E-mail: romanha@netra.cpqrr.fiocruz.br

https://doi.org/10.1017/S0031182097002084 Published online by Cambridge University Press

investigate further the development of drug-resistance mechanisms, we have selected and biologically characterized an *in vivo* benznidazole-resistant population and clones of *T. cruzi* to serve as an experimental model for the study of mechanisms of drug resistance in this protozoan.

MATERIALS AND METHODS

Drugs used

Benznidazole: *N*-benzyl-2-nitro-1-imidazolacetamide, sold under the name Rochagan (Roche Company); Nifurtimox: 3-methyl-4 (5′ nitrofurfurylidene-amino)-tetrahydro-4H-1, 4-thiazine-1,1-di-oxide, sold as Lampit (Bayer Company); MK-436: [3-(1-methyl-5-nitroimidazol-2-yl)3a, 4, 5, 6, 7, 7a-hexahydro-1,2-benzisoxazole] (Merck Sharp & Dohme) and Megazol: 2-amino-5-(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole (American Cyanamid Company). The drugs were dissolved in distilled water and given to mice by oral route through gavage.

Mice infections and parasitaemia curves

Blood of mice infected with the *T. cruzi* Y strain, isolated from a human acute case by Pereira da Silva & Nussenzweig (1953), was collected from orbital venous sinus (0·3–0·5 ml), diluted in 3·8% sodium citrate and inoculated intraperitoneally (i.p.) in normal mice. Groups of 10 male Swiss albino mice of 18–20 g were inoculated i.p. with 10^4 bloodstream forms of wild-type or the resistant *T. cruzi* that had been selected 7, 18 and 25 times in the presence of benznidazole and had their parasitaemia curve evaluated. For this evaluation the infected mice were not treated. The parasitaemia was followed from the 4th to the 13th day of infection by fresh blood collected from the mouse's tail and the number of parasites was estimated as described by Brener (1962). Curves were plotted using the mean and standard deviation of the parasitaemia of 10 mice. Mortality rate was expressed as a percentage of accumulated deaths within the period of 20 days after the inoculation.

Selection of benznidazole-resistant T. cruzi

The *in vivo* selection of a benznidazole-resistant population of *T. cruzi* was obtained through successive rapid treatments of mice infected with the *T. cruzi* Y strain. A group of 10 mice was inoculated i.p. with 5×10^4 bloodstream forms of *T. cruzi* and, at the peak of parasitaemia, they were submitted to a rapid treatment of 500 mg of benznidazole/kg of body weight, in a single oral dose. In this method of treatment, the drug dose administered is 5 times higher than the usual dose and yields a steep reduction in the number of bloodstream trypomastigotes (Filardi & Brener, 1984). Six hours after drug administration, the remaining parasites were inoculated into a new group of mice, that was thereafter submitted to the same procedure 25 times. As a control the wild-type strain was maintained in mice without treatment for the same number of passages as the selected population.

Long-term treatment

Groups of 10 male albino mice (18–20 g) were inoculated intraperitoneally with 10^4 *T. cruzi* blood forms. On the 4th day post-inoculation, the animals were examined to ensure that they were infected. Subsequently, the mice were submitted to long-term treatment with oral doses of 100 mg of benznidazole/kg, for 20 consecutive days.

Haemoculture

Haemoculture was used as the criterion of cure for treated mice. Thirty days after the end of long-term treatment, mice were bled from the orbital venous sinus and 0·4 ml of blood was collected and divided into 2 tubes containing 5 ml of LIT (Liver Infusion Tryptose) medium (Camargo, 1964). The tubes were incubated at 28 °C for 30–60 days and examined microscopically for the presence of parasites. If both haemocultures were negative, the mouse was considered cured.

T. cruzi *cloning*

The wild-type and resistant 25 × selected *T. cruzi* populations were cloned in BHI-LIT-agar-blood (BLAB) (Gomes, Marques de Araújo & Chiari, 1991). Eighty blood trypomastigotes, suspended in 100 μl of LIT, were homogeneously spread on the BLAB surface in Petri dishes, 8·5 cm in diameter, and incubated at 28 °C for 25–30 days. After this time, 30 colonies from each population were picked and transferred to LIT medium. Following growth, 10 clones from each population were inoculated in mice. These 10 clones, as well as the remaining 20 clones, were frozen in liquid nitrogen. Eight days post-infection, the mice were bled and 4×10^4 *T. cruzi* blood forms were inoculated in a new group of mice. At the peak of parasitaemia, they were submitted to rapid treatment to evaluate their susceptibility to benznidazole. In a parallel experiment to the cloning procedure above, the wild-type and resistant *T. cruzi* parental populations were maintained in LIT medium and inoculated into mice at the same time as the above clones.

RESULTS

Selection of benznidazole-resistant T. cruzi

The benznidazole-resistant *T. cruzi* population was selected after 25 successive rapid treatments (8 months) with benznidazole. The *T. cruzi* Y strain is

https://doi.org/10.1017/S0031182097002084 Published online by Cambridge University Press

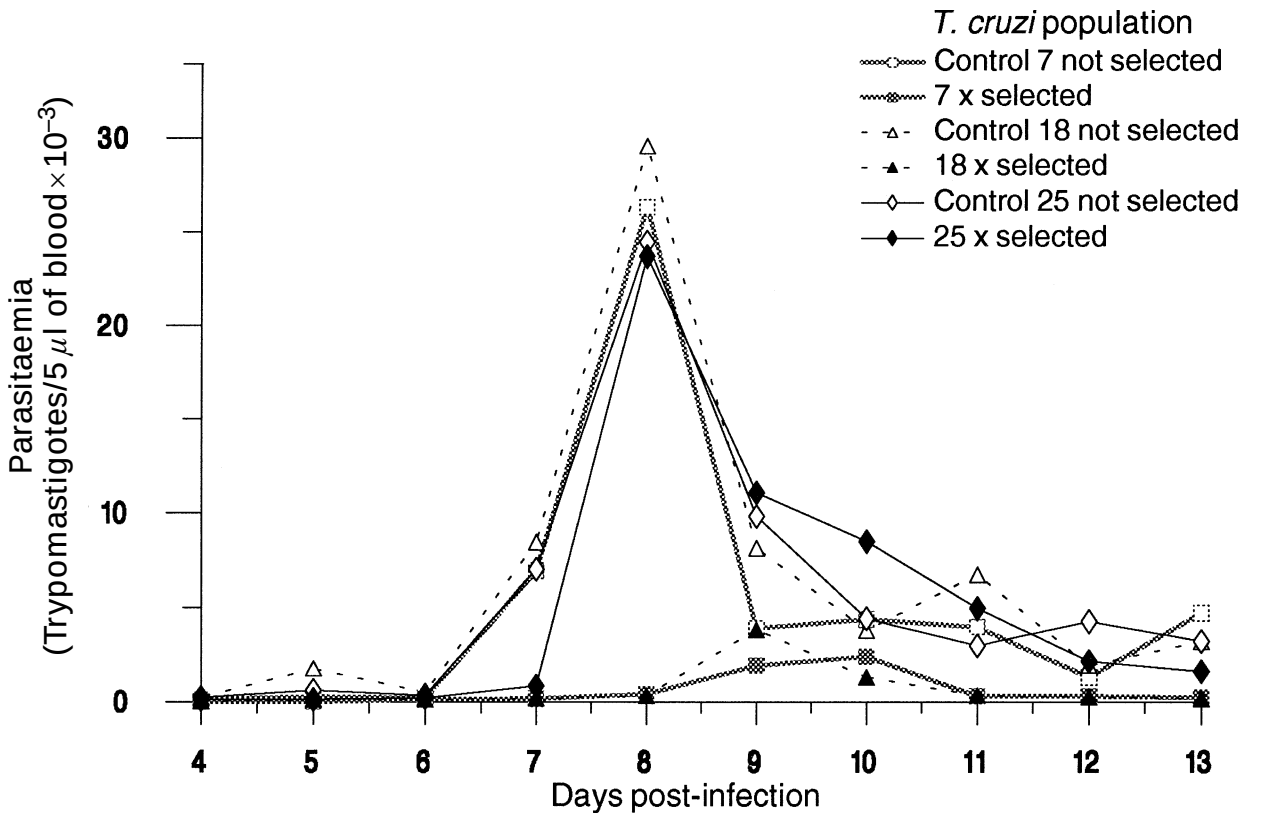

Fig. 1. Parasitaemia curves of *Trypanosoma cruzi* populations not selected and selected after 7, 18 and 25 rounds of rapid treatment with benznidazole. Each curve represents the mean of 2 distinct experiments ($n = 10$ mice/experiment). The mice were infected i.p. with 10^4 blood trypomastigotes. The maximum standard deviation of the mean was 23 %.

Table 1. Percentage cure induced by benznidazole after long-term treatment of mice infected with wild-type or resistant *Trypanosoma cruzi* populations selected 9 and 25 times

T. cruzi population infecting mice	Positive haemocultures	Percentage cure
Wild-type – control[a]	4/14 (28·6 %)	71·4
Resistant 9 × selected	9/9 (100 %)	0
Wild-type – control[b]	2/10 (20 %)	80
Resistant 25 × selected	10/10 (100 %)	0

[a] and [b], *T. cruzi* wild-type passed respectively 9 and 25 times in mice without rapid treatment.

considered to have medium resistance to benznidazole. Mice infected with this strain showed 50 % cure rate, after treatment either with benznidazole or nifurtimox (Filardi & Brener, 1987). At the 7th and 18th round of parasite selection, the parasitaemia peak was very low and occurred 9–10 days post-infection compared to the wild-type, which reached parasitaemias approximately 10 times higher and peaked 8 days post-infection. In contrast, by the 25th round of selection the parasitaemia peak was re-established on the 8th day post-infection and reached the same parasitaemia observed for the wild-type strain (Fig. 1). The mortality rate of mice infected with either the wild-type or the resistant *T. cruzi* that had been selected 7, 18 and 25 times in the presence of benznidazole was evaluated. On the 20th day of infection the mortality rate was 100 % for the mice infected with wild-type parasites. In contrast, the mortality rate was 0, 10 and 100 % for the mice infected with the parasites selected 7, 18 and 25 times, respectively. Furthermore, the process of resistance selection could be roughly followed by the progressive increase in parasitaemias following each round of rapid treatment.

Parasitological cure

Table 1 shows the percentage cure induced by benznidazole after long-term treatment of different groups of mice infected with wild-type or resistant parasites that had been submitted to 9 and 25 rounds of selection. The percentage cure in the wild-type infected mice group, was 71·4 and 80 %. In contrast, mice infected with resistant parasites presented 0 % cure for populations selected 9 or 25 times.

The effect of benznidazole on the reduction of parasitaemia

The parasitaemia of mice infected with the wild-type or resistant 25 × selected *T. cruzi* was evaluated at

https://doi.org/10.1017/S0031182097002084 Published online by Cambridge University Press

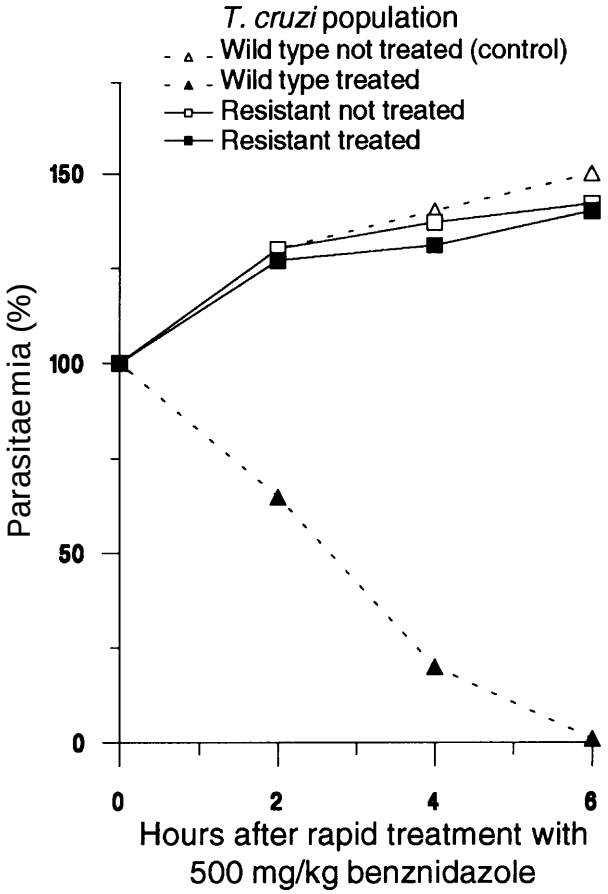

Fig. 2. Parasitaemia changes after a single high-dose treatment with benznidazole in mice infected with the wild-type or resistant 25 × selected *Trypanosoma cruzi*.

different points after a single rapid treatment (Fig. 2). A reduction of 99 % was observed for the wild-type *T. cruzi* strain treated with benznidazole after 6 h of treatment, whereas no reduction was detected for the wild-type non-treated (control) or the resistant parasites treated and non-treated.

Cross-resistance to different drugs

Figure 3 shows the effect of different drugs on the parasite resistance as measured by the percentage of parasites remaining after rapid treatment. In this experiment, the drugs benznidazole, nifurtimox, MK-436 and megazol were used at a single dose of 500 mg/kg in mice. After 7 h of treatment, increases in parasitaemia of 150 and 120 % respectively were observed in wild-type and resistant 25 × selected parasite non-treated controls. The drugs benznidazole, nifurtimox, MK-436 and megazol were quite active, reducing the number of circulating wild-type parasites by 95–100 %. In the animals inoculated with resistant parasites, subsequently treated with benznidazole or nifurtimox, increases in the parasitaemia of 110 and 86 % respectively were observed. Megazol and MK-436 had some effect on the parasitaemias, preventing any increase during the 7 h observation.

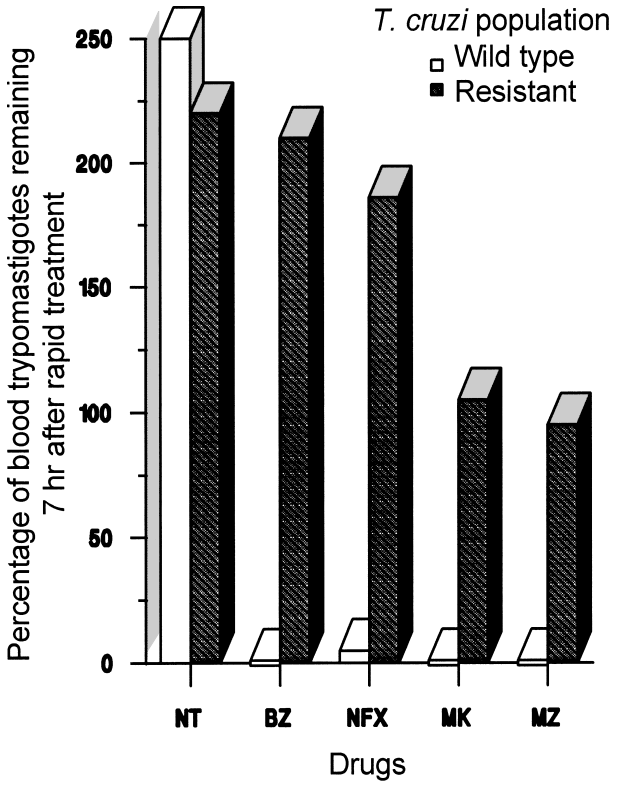

Fig. 3. Effects of different drugs on mice infected with wild-type and resistant *Trypanosoma cruzi* populations. NT, non-treated; BZ, benznidazole; NFX, nifurtimox; MK, MK-436; MZ, megazol.

Resistance of T. cruzi *clones to rapid treatment*

The reduction in parasitaemia after rapid treatment of a group of 6 mice infected with wild-type or resistant 25 × selected *T. cruzi* clones varied. The group of mice infected with 10 clones from wild-type *T. cruzi* had the parasitaemia reduced by 92–99 %. However, the group of mice infected with 10 clones from resistant *T. cruzi* presented almost no reduction in parasitaemia. Similarly, a reduction of 98 % in parasitaemia was observed in the wild-type parental population, whereas no reduction was detected in mice infected with the resistant parental population.

The stability of the resistance

In order to evaluate the stability of the resistance the resistant *T. cruzi* population and a single resistant clone were passed 9 times in mice without drug treatment. At the 3rd, 6th and 9th passages, during the peak of parasitaemia, the mice were subjected to rapid treatment and the percentage of parasites remaining after 7 h was measured (Fig. 4). The mice infected with the resistant clone showed no reduction in parasitaemia, whereas the mice infected with the wild-type population showed a reduction in parasitaemia of 90–100 %. Until the 3rd passage of the resistant parasite population in mice, no difference in the level of parasitaemia was observed. However, after the 6th and 9th passages, reductions of 11 and

https://doi.org/10.1017/S0031182097002084 Published online by Cambridge University Press

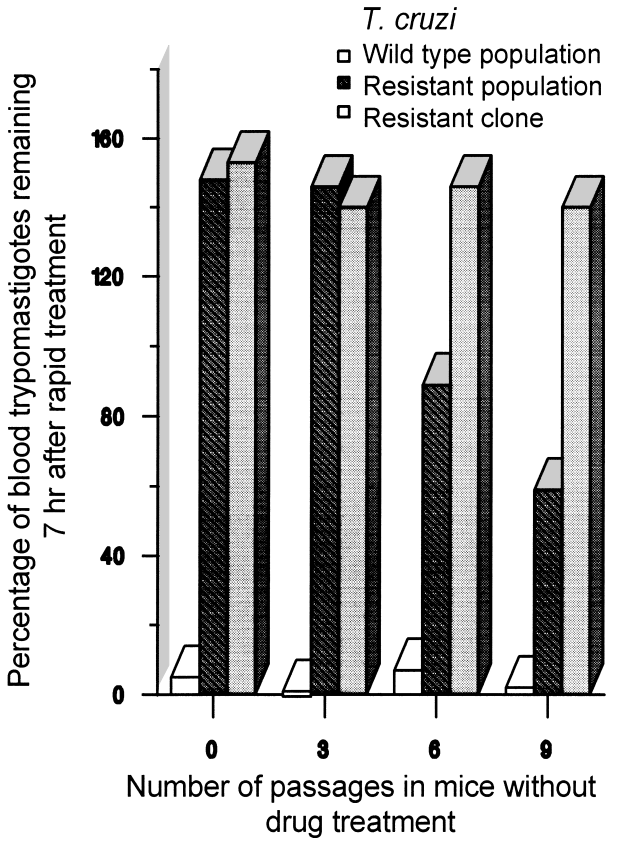

Fig. 4. Stability of benznidazole resistance after passage of the resistant parasites in mice without drug treatment.

41 % respectively in the parasitaemias were detected. In order to study better the resistance phenotype of the clones, 2 other resistant clones and 1 sensitive clone were passed 20 times through normal mice without treatment. The mice infected with the resistant clones showed no reduction in parasitaemia whereas in the mice infected with the sensitive clone the parasitaemia was reduced almost completely after rapid treatment. The stability of the resistance was also evaluated when the parasites were maintained in culture without drug. The mice infected with the resistant parasites maintained in LIT medium during 6 months showed no reduction in parasitaemia, after rapid treatment.

DISCUSSION

In contrast to the parasites *Plasmodium*, *Leishmania* and *Entamoeba*, relatively few reports have been published concerning the drug resistance phenomenon in *T. cruzi*. Andrade, Andrade & Figueira (1977) and Andrade & Figueira (1977) showed that the resistance of the Colombiana strain to the drugs nifurtimox and benznidazole increased when the parasites were isolated from mice previously treated with these same drugs. Neal & Van Bueren (1988) observed a small decrease in sensitivity *in vitro* by a non-responsive strain of *T. cruzi* after re-isolation from treated mice. Although a nifurtimox-resistant strain of *T. cruzi* was readily produced *in vitro* (Dvorak & Howe, 1977; Nozaki *et al.* 1996) the induction of *in vivo* resistance to this same drug failed, even after 25 months of continuous passage in drug-treated mice (Haberkorn & Gonnert, 1972). On the other hand, Haberkorn & Gonnert (1972) were able to select an *in vivo* nitrofurazone-resistant strain of *T. cruzi* after 20 passages in 8·5 months. In this study we selected, *in vivo*, a benznidazole-resistant population from the Y strain of *T. cruzi*. Our selection protocol was based on successive single, high-dose treatments. This protocol allows us to determine in 4–6 h and *in vivo* the sensitivity of *T. cruzi* strains to drugs. The drug is used at a high concentration (500 mg/kg), approximately 50 times higher than that administered in human chemotherapy and 5 times higher than that used for treatment in mice. The rationale for this method was based on the pharmacokinetics of benznidazole which, in humans, reaches a maximum concentration in the blood 3–4 h after oral administration (Raaflaub & Ziegler, 1979). Data generated by this method show a good correlation with those obtained by long-term treatment (Filardi & Brener, 1984). At the beginning of the selection the resistant parasites showed biological features distinct from the parental strain such as low peaks of parasitaemia and a low mortality rate, but further in the selection process, both features reverted, reaching similar values as seen in the wild-type strain. These data demonstrate the heterogeneity present in the Y strain and suggest that the initial treatment eliminated the sensitive parasites, preserving the resistant ones, which multiplied and came to dominate the population. An alternative hypothesis to a heterogeneous starting population is that drug-resistant mutants arise and eventually predominate in the population. Nine consecutive rapid treatments were sufficient to select a benznidazole-resistant *T. cruzi* population. However, the resistant phenotype of the population selected 9 times was unstable. After 3 passages in mice, in the absence of drug, the population became drug sensitive again. Both long-term treatment and rapid treatment confirmed the resistance of the *T. cruzi* population selected *in vivo*. Furthermore, some resistance was also observed *in vitro*. The resistance of the selected population was double the wild-type population for benznidazole and nifurtimox.

In the cross-resistance study, the drugs benznidazole, nifurtimox, MK-436 and megazol were used. Currently, benznidazole and nifurtimox are the drugs used for treatment of human Chagas' disease; however, they have low efficacy. MK-436 is highly active for the treatment of chronically infected mice (Andrade, Silva & Santiago, 1989). Megazol is an extremely active compound that displays a marked curative effect in mice infected with the Colombiana strain, which is highly resistant to benznidazole and nifurtimox (Filardi & Brener, 1982). The selected

benznidazole-resistant parasites exhibited resistance to all drugs tested regardless of their chemical group.

The utilization of *T. cruzi* clones is essential for understanding the constitution of parasite populations, and to obtain reproducible and less variable results (Postan, McDaniel & Dvorak, 1986; Finley & Dvorak, 1987). In this work we have studied the constitution of a population of *T. cruzi* resistant to benznidazole and the stability of the resistance after cloning. All 10 clones derived from the resistant population were highly resistant to rapid treatment with benznidazole, whereas the clones from the wild-type population were very sensitive. These data suggest that the resistance of the strain seems to be related to the sensitive/resistant clone ratio in the population.

To the best of our knowledge no author has described *in vivo* stability of the drug-resistance phenotype in *T. cruzi*. However, different levels of *in vitro* stability of drug resistance in *T. cruzi* were described by Dvorak & Howe (1977), Nirdé *et al.* (1995) and Nozaki *et al.* (1996). As expected, our data show that the resistance phenotype of the clones was far more stable than that of the population. The resistance of the cloned population kept for at least 20 passages in mice without treatment. The resistance phenotype of the selected parental population was stable after 6 months of maintenance in culture medium and after 1 passage in *Triatoma infestans*.

In this work, the model for resistance in *T. cruzi* was based on the selection of naturally occurring resistance to benznidazole, rather than in artificial induction. There is a substantial literature on drug resistance mechanisms in *Leishmania* spp., based on models produced from the artificial induction of resistance in promastigote stages. However, there is essentially no information on the biochemical mechanisms that underlie drug resistance in field isolates of this parasite. Also, in malaria, research on the connection between mdr gene amplification and resistance to chloroquine in *Plasmodium falciparum* has not been borne out by examination of clinical populations (Ullman, 1995). Therefore, drug-resistant *T. cruzi* populations and clones obtained by *in vivo* models are representative of the natural resistance. Probably, the mechanism of drug resistance in these parasites more closely reflects the situation in field infections. We now have available a well-characterized set of drug-resistant, and sensitive, *T. cruzi* populations and clones that will be useful for understanding the mechanisms of drug resistance in *T. cruzi*.

Professor Zigman Brener is thanked for critical reading of the manuscript, Wagner Gouvêa dos Santos for suggestions during the development of the work and Jessica Kissinger for the English corrections and suggestions. This work was supported by CNPq, PRONEX nº 2704 and PAPES/FIOCRUZ.

REFERENCES

ANDRADE, S. G., ANDRADE, Z. A. & FIGUEIRA, R. M. (1977). Estudo experimental sobre a resistência de uma cepa do *Trypanosoma cruzi* ao Bay 2502. *Revista do Instituto de Medicina Tropical de São Paulo* **19**, 124–129.

ANDRADE, S. G. & FIGUEIRA, R. M. (1977). Estudo experimental sobre a ação terapêutica da droga Ro 7-1051 na infecção por diferentes cepas do *T. cruzi*. *Revista do Instituto de Medicina Tropical de São Paulo* **19**, 335–341.

ANDRADE, S., MAGALHÃES, J. B. & PONTES, A. L. (1985). Evaluation of chemotherapy with benznidazole and nifurtimox in mice infected with *Trypanosoma cruzi* strains of different types. *Bulletin of the World Health Organization* **63**, 721–726.

ANDRADE, S. G., SILVA, R. C. & SANTIAGO, C. M. G. (1989). Treatment of chronic experimental *Trypanosoma cruzi* infections in mice with MK-436, a 2-substituted 5-nitroimidazole. *Bulletin of the World Health Organization* **67**, 509–514.

BOCK, M., GONNERT, R. & HABERKORN, A. (1969). Studies with Bay 2502 on animals. *Boletín Chileno de Parasitología* **24**, 13–19.

BRENER, Z. (1962). Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi*. *Revista do Instituto de Medicina Tropical de São Paulo* **4**, 389–396.

BRENER, Z., COSTA, A. G. & CHIARI, E. (1976). Differences in the susceptibility of *Trypanosoma cruzi* strains to active chemotherapeutic agents. *Revista do Instituto de Medicina Tropical de São Paulo* **18**, 450–455.

CAMARGO, E. P. (1964). Growth and differentiation in *Trypanosoma cruzi*. I. Origin of metacyclic trypanosomes in liquid media. *Revista do Instituto de Medicina Tropical de São Paulo* **6**, 93–100.

DALLAGIOVANNA, B., CASTANYS, S. & GAMARRO, F. (1994). *Trypanosoma cruzi*: sequence of the ATP-binding site of a P-glycoprotein gene. *Experimental Parasitology* **79**, 63–67.

DALLAGIOVANNA, B., GAMARRO, F. & CASTANYS, S. (1996). Molecular characterization of a P-glycoprotein-related tcpgp2 gene in *Trypanosoma cruzi*. *Molecular and Biochemical Parasitology* **75**, 145–157.

DVORAK, J. A. & HOWE, C. L. (1977). The effects of Lampit (Bayer 2402) on the interaction of *Trypanosoma cruzi* with vertebrate cells *in vitro*. *American Journal of Tropical Medicine and Hygiene* **26**, 58–63.

FILARDI, L. S. & BRENER, Z. (1982). A nitroimidazole–thiadiazole derivative with curative action in experimental *Trypanosoma cruzi* infections. *Annals of Tropical Medicine and Parasitology* **76**, 293–297.

FILARDI, L. S. & BRENER, Z. (1984). A rapid method for testing *in vivo* the susceptibility of different strains of *Trypanosoma cruzi* to active chemotherapeutic agents. *Memórias do Instituto Oswaldo Cruz* **79**, 221–225.

FILARDI, L. S. & BRENER, Z. (1987). Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas' disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**, 755–759.

FINLEY, R. W. & DVORAK, J. A. (1987). *Trypanosoma cruzi*: analysis of the population dynamics of heterogeneous mixtures. *Journal of Protozoology* **34**, 409–415.

GOMES, M. L., MARQUES DE ARAÚJO, S. & CHIARI, E. (1991). *Trypanosoma cruzi*: growth of clones on solid medium using culture and blood forms. *Memórias do Instituto Oswaldo Cruz* **86**, 131–132.

GOTTESMAN, M. M. & PASTAN, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual Review of Biochemistry* **62**, 385–427.

HABERKORN, A. & GONNERT, R. (1972). Animal experimental investigations in the activity of nifurtimox against *Trypanosoma cruzi*. *Arzneimittel-Forschung* **22**, 1570–1580.

HAUSCHKA, T. S. (1949). Persistence of strain-specific behavior in two strains of *Trypanosoma cruzi* after prolonged transfer through inbred mice. *Journal of Parasitology* **35**, 593–599.

NEAL, R. A. & VAN BUEREN, J. (1988). Comparative studies of drug susceptibility of five strains of *Trypanosoma cruzi in vivo* and *in vitro*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **82**, 709–714.

NIRDÉ, P., LARROQUE, C. & BARNABÉ, C. (1995). Drug-resistant epimastigotes of *Trypanosoma cruzi* and persistence of this phenotype after differentiation into amastigotes. *Medical Sciences* **318**, 1239–1244.

NOZAKI, T., ENGEL, J. & DVORAK, J. (1996). Cellular and molecular biological analyses of nifurtimox reistance in *Trypanosoma cruzi*. *American Journal of Tropical Medicine and Hygiene* **55**, 111–117.

PEREIRA DA SILVA, L. H. & NUSSENZWEIG, V. (1953). Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. *Folia Clinica et Biologica* **20**, 191–208.

POSTAN, M., MCDANIEL, J. P. & DVORAK, J. A. (1986). *Trypanosoma cruzi*: constancy of clone pathogenicity for inbred mice during long-term *in vitro* maintenance. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80**, 659–662.

RAAFLAUB, J. & ZIEGLER, W. H. (1979). Single-dose pharmacokinetics of the trypanosomicide benznidazole in man. *Arzneimittel-Forschung* **29**, 1611–1614.

RASSI, A. & LUQUETTI, A. O. (1992). Therapy of Chagas disease. In *Chagas Disease* (*American Trypanosomiasis*) *: its Impact on Transfusion and Clinical Medicine* (ed. Wendel, S., Brener, Z., Camargo, M. E. & Rassi, A.), pp. 237–247. ISBT Brazil 1992, São Paulo, Brazil.

RIBEIRO-RODRIGUES, R., DOS SANTOS, W., OLIVEIRA, A. B., SNIECKUS, V., ZANI, C. & ROMANHA, A. (1995). Growth inhibitory effect of nafthofuran and nafthofuranquinone derivatives on *Trypanosoma cruzi* epimastigotes. *Bioorganic and Medicinal Chemistry Letters* **5**, 1509–1512.

SCOTT, V. R. & MATTHEWS, T. R. (1987). The efficacy of an n-substituted imidazole, RS-49676, against a *Trypanosoma cruzi* infection in mice. *American Journal of Tropical Medicine and Hygiene* **37**, 308–313.

ULLMAN, B. (1995). Multidrug resistance and P-glycoprotein in parasitic Protozoa. *Journal of Bioenergetics and Biomembranes* **27**, 77–84.

URBINA, J., PAYARES, G., MOLINA, J., SANOJA, C., LIENDO, A., LAZARDI, K., PIRAS, M., PIRAS, R., PEREZ, N., WINCKER, P. & RYLEY, J. (1996). Cure of short- and long-term experimental Chagas' disease using D0870. *Science* **273**, 969–971.

WORLD HEALTH ORGANIZATION (1991). Control of Chagas disease. *WHO Technical Report Series* No. 811. WHO, Geneva.