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Drug-resistant epimastigotes of *Trypanosoma cruzi* and persistence of this phenotype after differentiation into amastigotes

Épimastigotes de Trypanosoma cruzi *chimiorésistants et conservation de ce phénotype après la différenciation en amastigotes*

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RÉSUMÉ

Des formes épimastigotes du parasite *Trypanosoma cruzi* ont été rendues résistantes au benznidazole en utilisant, *in vitro*, une pression médicamenteuse continue. Les parasites ont été sélectionnés en fonction de la caractérisation génétique antérieure de leurs loci isoenzymatiques. Tous les clones résistants sont capables de croître en culture à long terme en présence d'une concentration d'au moins 50 µM de benznidazole qui est la dose circulante de drogue chez un patient en cours de traitement. Le niveau le plus élevé de résistance atteint est 220 µM. Après la différenciation des formes épimastigotes en amastigotes, le niveau de résistance n'est pas altéré. La vitesse de croissance des formes résistantes d'épimastigotes et d'amastigotes est inférieure à celle des formes sauvages mais leur viabilité n'est pas affectée. Ces résultats peuvent avoir des conséquences à la fois pour entreprendre l'étude du phénotype résistant de clones du parasite *T. cruzi* mais également pour suivre le devenir de ce phénotype résistant au cours du cycle expérimental *in vivo*. ▲

Mots clés : Trypanosoma cruzi, *résistance aux drogues, benznidazole (benzyl-2-nitro-1-imidazole-acétamide), formes amastigotes, formes épimastigotes.*

ABSTRACT

In vitro *benznidazole resistance was induced in cloned* T. cruzi *epimastigotes using a continuous drug pressure protocol. Stocks were selected according to their previous genetic characterization by multilocus enzyme electrophoresis. All the resistant clones were able to grow in long term cultivation in the presence of at least 50 µM benznidazole, which is the drug plasma level during chemotherapy in man. The highest level of resistance achieved was 220 µM. After differentiation of the epimastigote into the amastigote forms, the drug-resistance level was not affected. In both, the resistant epimastigotes and the resistant amastigotes, growth curves exhibited a lower growth rate than the sensitive counterparts without affecting the viability of the parasites. These data could be significant in basic research, to study the drug-resistance phenotype on relevant chemoresistant clones of* T. cruzi, *and to follow this phenotype after* in vivo *cycles.* ▲

Key words: *Trypanosoma cruzi*, drug resistance, benznidazole (benzyl-2-nitro-1-imidazole-acetamide), epimastigote, amastigote.

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VERSION ABRÉGÉE

Le parasite *Trypanosoma cruzi* est l'agent de la maladie de Chagas dont les zones d'endémie sont localisées en Amérique centrale et en Amérique du Sud. Le benznidazole, drogue utilisée en thérapeutique humaine, présente une efficacité dépendante des souches parasitaires présentes. L'émergence de phénotypes résistants pose un problème thérapeutique aigu, aggravé par le faible nombre de drogues disponibles pour cette parasitose. Dans d'autres pathologies (cancers, paludisme, leishmanioses) l'obtention de modèles de résistance aux drogues a permis d'étudier ce phénotype et d'impliquer le rôle d'une glycoprotéine transmembranaire. Dans le cas de la maladie de Chagas, aucun mécanisme n'a été trouvé associé au phénotype résistant. La mise au point de modèles de résistance au benznidazole devrait nous permettre d'étudier les mécanismes associés à ce phénotype chimiorésistant. Ici, nous présentons le moyen d'obtenir des cultures d'épimastigotes de *Trypanosoma cruzi* capable de pousser en présence d'au moins 50 µM de benznidazole, qui est le taux plasmatique de cette drogue chez un malade en cours de traitement. Ces formes résistantes au benznidazole ont été obtenues en augmentant progressivement la concentration de drogue dans le milieu de culture. Seul le milieu LIT modifié permet d'obtenir et de stabiliser les formes devenues résistantes au benznidazole. Le niveau maximal de résistance obtenu varie en fonction des clones étudiés. Les épimastigotes du zymodème 12 peuvent être cultivés en présence d'une concentration molaire de 220 µM en benznidazole, alors que cette concentration est de 90 µM pour les parasites du zymodème 19 et de 70 µM pour les parasites du zymodème 39. La vitesse de croissance des parasites devenus résistants au benznidazole est significativement inférieure à celle des parasites non induits (39-51 %) mais la viabilité des parasites est identique entre les formes induites et non induites. Les formes épimastigotes, faciles à cultiver, sont les formes multiplicatives présentes chez l'insecte vecteur alors que les formes amastigotes sont présentes au sein des cellules humaines infectées. Nous avons suivi le devenir du phénotype résistant des formes épimastigotes après leur transformation *in vitro* en formes amastigotes cultivées en conditions axéniques. Nos résultats indiquent que des amastigotes issus d'épimastigotes résistants conservent leur phénotype résistant. La transformation n'altère pas le niveau de résistance des parasites, et ce pour les 4 zymodèmes étudiés.

Notre étude montre qu'il est possible d'induire une résistance au benznidazole avec différents clones ubiquistes du parasite *Trypanosoma cruzi*. De plus, le niveau de résistance que peuvent atteindre différents clones de ce parasite peut être largement supérieur à celui que pourrait produire un traitement thérapeutique.

L'obtention de parasites résistants au benznidazole nous permet d'envisager l'analyse des mécanismes biochimiques impliqués dans ce phénotype mais également d'étudier le devenir de ce phénotype résistant au cours de cycles *in vivo* sur un modèle expérimental. ▲

The human protozoan parasite *Trypanosoma cruzi* is the agent of Chagas'disease in Central and South America. It affects approximately 18 million people [1]. One of the main trypanocidal drugs used in human therapy is benznidazole which reaches a plasma level of 50 µM [2]. The efficiency of drugs seems to be variable according to the parasite strains [3, 4]. Drug resistance in human pathology such as cancer or tuberculosis also occurs in protozoan parasitic diseases [5, 6] such as malaria, leishmaniasis or trypanosomiasis. For parasitic diseases, the public health problem is threatening, due to the low number of available drugs. By using induced-resistant parasites in malaria [7] and leishmaniasis [8], it has been demonstrated that this resistance was associated with the presence of a multidrug-resistance (MDR) gene [8-12]. This gene codes for a membrane glycoprotein found in normal cells and is known as a membrane transport protein. It is also involved in the regulation of the drug efflux in mammalian cells [13, 14] as well as in parasitic protozoa [15]. A MDR-like gene has been recently detected in *T. cruzi* susceptible epimastigotes [16], but in the absence of any available drug-resistance model, the presence of this gene could not be associated with a functional drug-resistant phenotype. In order to further investigate the chemoresistant mechanisms induced by benznidazole, we have developed resistant *T. cruzi* on selected clones by steadily increasing doses of benznidazole. Different strains of *Trypanosoma cruzi* displayed different levels of resistance. The chemoresistant epimastigotes exhibited a longer doubling time than the sensitive parasites. Moreover, the resistant epimastigotes were *in vitro* transformed into metacyclic trypomastigotes and finally into amastigotes. No significant differences were observed in the resistance levels between the resistant epimastigotes and their amastigotes derived forms. These results suggest that the benznidazole molecule induced a chemoresistant phenotype which is retained after differentiation of epimastigotes into amastigotes.

Materials and methods

Parasite selection

According to the multilocus enzyme electrophoresis profile (MLEE) of *T. cruzi* based on the analysis of 15 enzyme loci [17] the following stocks were selected: Tehuantepec cl2 (enzyme genotype 12); 133-79 cl7 (enzyme genotype 19); Bug2148 cl1 and Bug2149 cl10 (enzyme genotype 39). Genotype numbers refer to Tibayrenc et Ayala [17]. The goal of this selection was to study a set of stocks representative of the whole genetic variability of the parasite.

Drug pressure protocol

Trypanosoma cruzi epimastigotes were grown at 28°C in a culture medium, consisting of 60% LIT [18], 30% TC 100 insect medium (Gibco, BRL) and 10% decomplemented foetal calf serum in a 25 cm^2 tissue culture flask (TPP). The concentration of parasites ranged from 2×10^4 to 2×10^6 parasites ml^{-1}. The drug-pressure protocol was adapted to each clone. To assess the doubling time, the exponential growth phase and the plateau phase of the cultures, kinetics were performed. The drug pressure was achieved with benznidazole in culture medium starting from 5×10^{-7} M. Benznidazole was dissolved in dimethyl sulfoxide (DMSO)

to 0.4 M, then diluted with methanol to 5×10^{-2} M and finally diluted with culture medium. Epimastigotes were cultivated in the presence of the desired drug level for 10 generations before the drug level was increased. Before the drug level was raised, the epimastigote population was checked to verify if it had the same mortality percentage as the sensitive counterparts. Only epimastigote population that had the same percentage were used at higher levels. Drug addition was in 5 µM increments. If this step induced a mortality higher than 40%, the increment was lowered to 2 µM for the second assay and to 1 µM for the third one. The optimum culture volume was 5 ml per tissue culture flask in an upright position. For drug concentration above 70 µM, the tissue culture flask was put in a horizontal position to enhance the medium-air interface.

Benznidazole assays

For each step during the drug-pressure protocol, the accurate drug concentration was verified on a medium aliquot by high pressure liquid chromatography analysis. Isocratic conditions were run using 40% methanol in water as mobile phase and a microbondapack phenyl column (Waters) as stationary phase. The pump (Waters M 660) delivered a 1 ml. min^{-1} continuous flow and molecules were detected on a photodiode array (Waters, PDA 990). Benznidazole quantification was performed on the basis of the peak height as compared to standard curve.

Transformation of epimastigotes into amastigotes

Transformation of *T. cruzi* epimastigote into amastigotes were performed using infection of Vero cells [19] after differentiation into trypomastigotes [20]. Amastigotes were grown in incubator at 37° C under 5% CO_2 in the presence of 20% foetal calf serum.

Data management

The mean of 3 independent experiments run in triplicate are presented in the results. LD_{50} was calculated by a non-linear regression using the Gompertz model and standard deviation were done with Systat software (Systat inc.).

Results

Using the described experimental procedure, we obtained drug-resistant epimastigotes which were able to grow in the presence of benznidazole levels from 70 µM to 220 µM *(Fig. 1)* depending upon the clone used. The lowest resistance level observed was 70 µM for Bug 2148 and Bug 2149 clones. A higher resistance level of 90 µM was found for 133-79 clones, and up to 220 µM was observed for the Tehuantepec clone. The corresponding doses which killed 50% of the resistant parasite (LD_{50}) were 80 ± 4 µM, 75 ± 3 µM, 95 ± 5 µM and 259 ± 11 µM respectively. The LD_{50} of the wild type clones was below 11 µM for all clones. During the drug-resistance acquisition, the parasite growth rate was reduced by 39 to 51% depending upon the clones as shown in *Figure 2*. The curves also showed that the lysis of the parasites rapidly occurred after they have

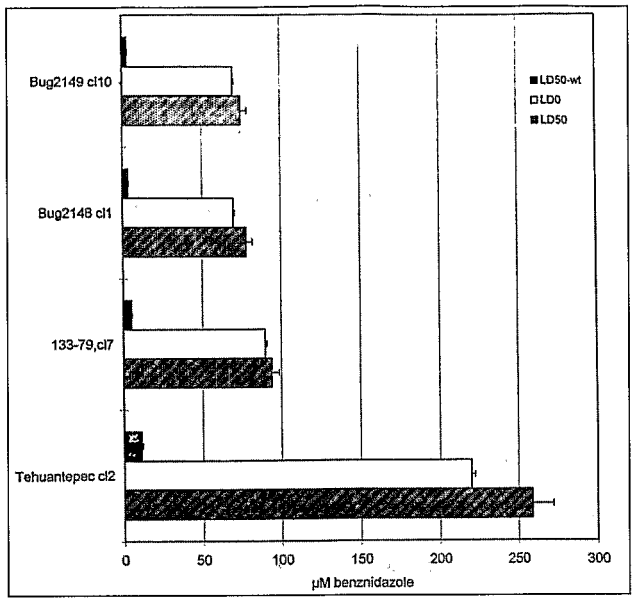

Figure 1. ***Resistance levels in* T. cruzi *epimastigotes.*** *The natural drug sensitivity (LD50-wt) of epimastigotes (dark bars) was compared to the level of drug tolerated by the resistant mutants at steady state (LD0) (hatched bars) and their corresponding LD50 (open bars). Error bars represent SD.*

reached the plateau. This figure shows that the maximum cell density was lower for the resistant parasites than for the wild types. However, no differences on the percentage mortality were observed between sensitive and resistant clones (5-11%). Neither LIT medium alone, nor other chemically defined media used for *T. cruzi* cultures have previously allowed the induction of such high drug-resistance levels. Indeed, parasites under benznidazole pressure protocol began to quickly die after about 6 weeks of cultivation in these media. To verify the genotype stability of the epimastigotes, we have performed an isoenzyme analysis on 22 gene loci, before and after the drug pressure. No changes were observed in the isoenzymatic pattern of the drug-resistant clones as compared to sensitive ones. However, band staining intensity can be modulated for some isoenzymes but not the overall pattern (data not shown). The parasites were still drug resistant after 6 months without drug pressure, and this was not affected by cryopreservation in liquid nitrogen (data not shown).

Figure 3 shows the maximum tolerated benznidazole concentration in which resistant amastigotes could propagate with the same mortality percentage as the controls (LD0). No significant differences could be observed in the resistance levels between the resistant epimastigote forms and the amastigotes obtained after differentiation of the resistant epimastigotes. The lowest resistance levels were for amastigotes belonging to Bug 2148 (70 ± 3 µM) and for Bug 2149 (70 ± 5 µM) clones. Amastigotes from 133-79 clone displayed a higher resistance level (90 ± 7 µM), but Tehuantepec remained the most resistant amastigote clone (221 ± 16 µM). Like the epimastigote forms, a reduced growth rate (48-61%) was associated with the resistant

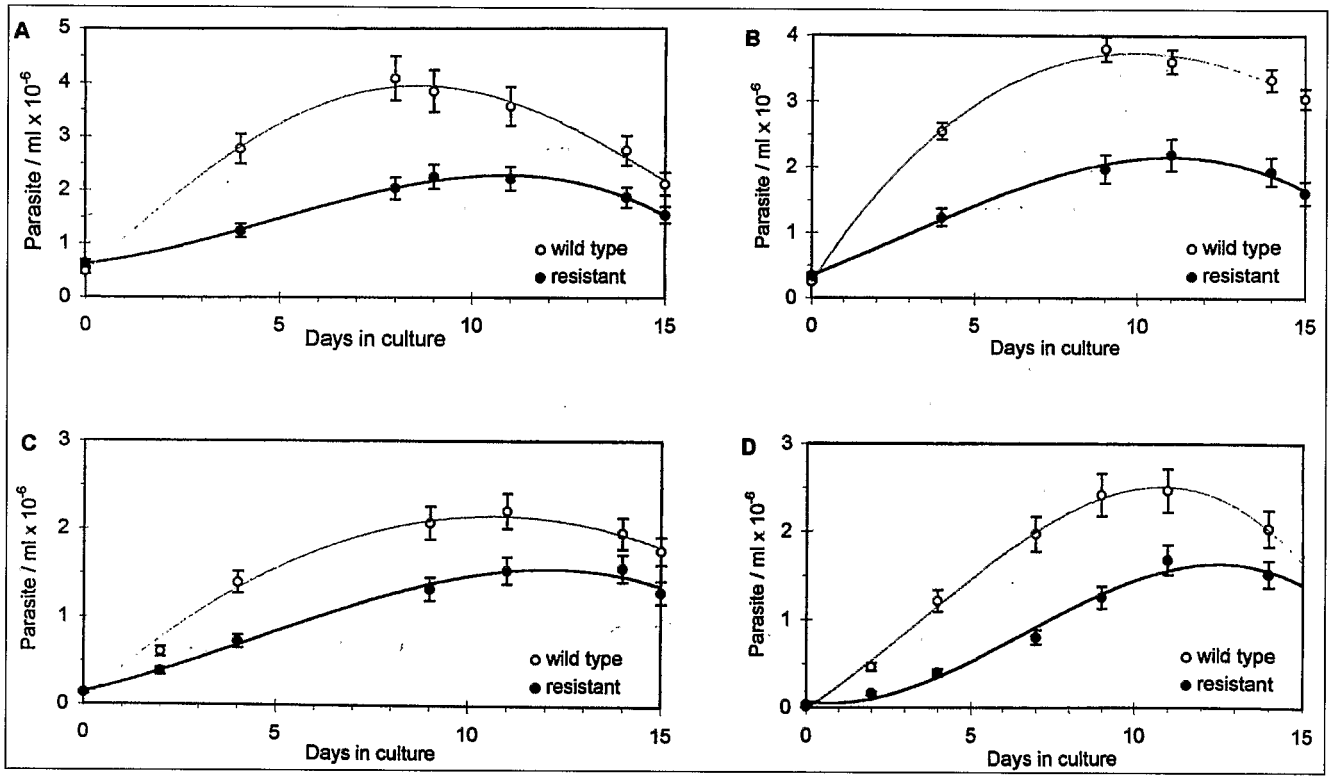

Figure 2. ***Growth rate for the wild type (open circle) and the drug-resistant (closed circle) epimastigotes.*** *(A) Tehuantepec clone. (B) 133-79, Cl3 clone. (C) Bug 2148 clone. (D) Bug 2149 clone.*

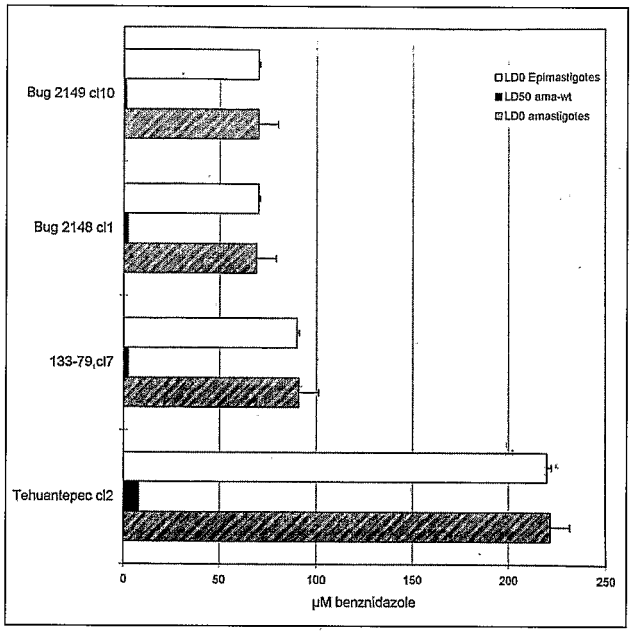

Figure 3. ***Resistance of amastigotes derived from drug-resistant epimastigote forms.*** *The level of drug tolerated by the epimastigotes at steady state (LD0 – hatched bars) were compared to the level of resistance of wild type amastigotes (LD50 ama-wt – dark bars) and to the derived amastigotes at steady state (LD0 amastigotes – open bars). Error bars represent SD.*

phenotype of the amastigote forms as compared to the non resistant amastigotes of the same stocks *(Fig. 4).*

Discussion

The resistant clones obtained in the present study were able to grow in the presence of drug levels as high as those generated during chemotherapy. This shows that it is possible to obtain high levels of resistance to benznidazole using an appropriate medium. The culture must be stabilized for 10 generations before proceeding to the next higher drug level.

Tolerance indicates an innate resistance of a parasite population to a drug. Tolerance studies have been recently reported for *T. cruzi* and benznidazole [21]. They indicate that in some natural *T. cruzi* population cultivated in LIT medium an innate resistance to benznidazole from 0.9 up to 6.9 µM can be observed. The results presented herein show that the tolerance is higher for parasites cultivated in LIT/TC100 medium than for parasites cultivated in LIT alone.

Acquisition of the drug-resistant phenotype is associated with a lower growth rate of the epimastigote. However, the percentage mortality was identical between the resistant and the sensitive parasites. Unsuccessful attempts have been made to significantly improve the growth rate of the resistant epimastigotes (*i.e.* variations of the parasite inocu-

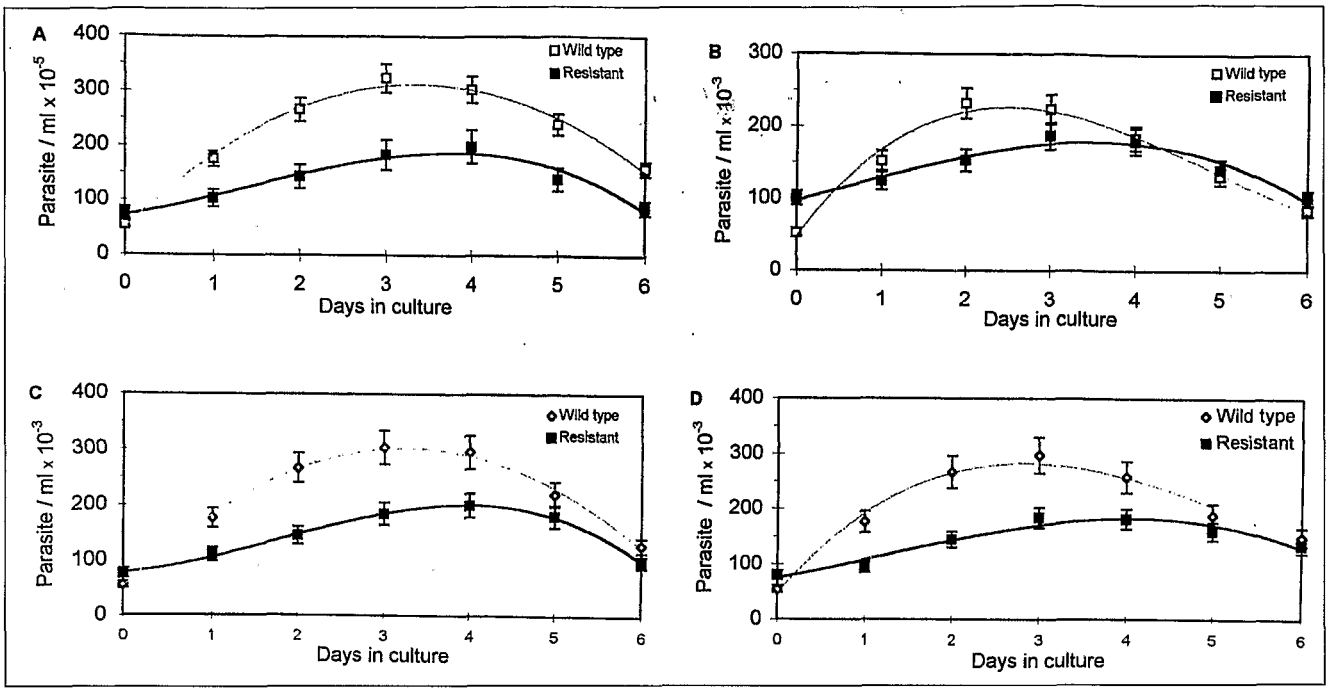

Figure. 4. ***Growth rate for the wild type (open squares) and the drug-resistant (closed squares) amastigotes.*** *(A) Tehuantepec clone. (B) 133-79, Cl3 clone. (C) Bug 2148 clone. (D) Bug 2149 clone.*

lum, modifications of the culture medium composition, changes in the flask geometry).

The stocks were selected according to the main ubiquitous genotypes found in the natural population of *T. cruzi* [17] and hence, might allow more complete studies on different mechanisms involved in the chemoresistant phenotype of this species. The hypothesis of the clonal mode of propagation for the kinetoplastid *Trypanosoma cruzi* [17] certainly has biological implication on its biological properties. The observed resistance level might be linked to *T. cruzi* clonal variability, a point that needs to be explored on a more diversified stock sample. Particular attention should be given to the fact that after differentiation of the epimastigote stage into the amastigote, the drug-resistant phenotype was preserved. This fact might be significant in the transmission of drug-resistant strains of *T. cruzi* by the triatomine vector or by blood transfusion. This hypothesis deserves further investigation. The follow up of this resistant phenotype after several *in vivo* cycles involving the insect vector and mice as host, is presently in progress. ▼

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