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BIOCHEMICAL BEHAVIOR OF *TRYPANOSOMA CRUZI* STRAINS ISOLATED FROM MICE SUBMITTED TO SPECIFIC CHEMOTHERAPY

Jésila Pinto M. Marretto and Sonia G. Andrade

To investigate the influence of chemotherapy on the biochemical behavior of Trypanosoma cruzi *strains, three groups of mice were infected with one of three strains of* T. cruzi *of different biological and isoenzymic patterns (Peruvian, 21SF and Colombian strains). Each group was subdivided into subgroups: 1 - treated with nifurtimox; 2 - treated with benznidazole and 3 - untreated infected controls. At the end of treatment, that lasted for 90 days, xenodiagnosis, subinoculation of blood into new born mice and haemoculture were performed as tests of cure. From the positive tests, 22 samples of* T. cruzi *were isolated from all subgroups. Electrophoretic analysis of the isoenzymes PGM, GPI, ALAT and ASAT failed to show any difference between parasite strains isolated from treated and untreated mice, which indicates that no detectable clonal selection or parasite genetic markers alterations concerning the isoenzymes analysed have been determined by treatment with drugs of recognized antiparasitic effect, suggesting stability of the phenotypic characteristics of the three biological types of* T. cruzi *strains.*

Key-words: Trypanosoma cruzi *strains. Isoenzymes. Chemotherapy.*

During experimental chemotherapy of *Trypanosoma cruzi* infection in mice, parasite strains show different susceptibility to the drugs now in clinical use, benznidazole and nifurtimox[3 5 10]. There is good correlation between these responses and the biological behavior of the *T. cruzi* strain[6]. However, a rapid decrease of parasitemia occurs during treatment, even in the presence of the most resistant strains, indicating that only a few parasites are really resistant to the drugs. A selection of resistant clones, that could differ in some way from the original strain has been suggested to explain the persistence of parasites in the vertebrate host after prolonged treatment[5]. Although previous investigations have stressed the stability of biological and biochemical patterns of laboratory strains[2 20 21], clonal analysis reveals homogeneous and heterogeneous populations[11 19]. Therefore, selected clones could persist after chemotherapy.

The present investigation was planned to identify possible biochemical differences between strains isolated from treated mice, that could be traced to alterations of the parasite genetic markers. The investigation on the possibility of a selection of different clones by the action of drugs seems important not only for drug therapy, but also to test the concept of stability of the biochemical patterns of *T. cruzi* strains.

MATERIAL AND METHODS

Three groups of 100 Swiss mice, weighing 15 to 20g were intraperitoneally infected with blood forms of *T. cruzi*. The strains used to infect each group were respectively: Peruvian strain[27]; 21 SF strain[7]; Colombian strain[17]. The three strains are prototypes for the Types I, II and III respectively, as previously described[1]. Each experimental group was divided into 3 subgroups: a) treated with nifurtimox (tetrahydro-3-methyl-4 (nitrofurfuryldieneamino-1,4 thiazine-1,1-dioxide), b) treated with benznidazole (N-benzyl-2- (2 nitro-imidazol-1-yl)acetamide), c) untreated controls. Schedules of treatment were as previously described[6]; briefly : nifurtimox - 4 doses of 200mg/kg b.w. followed by daily doses of 50mg/kg b.w. orally administered, during 90 days; benznidazole - 100mg/kg b.w./day, given orally, during 90 days.

Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Universidade Federal da Bahia, Salvador, BA.
Address to: Drª Sonia G. Andrade. Centro de Pesquisas Gonçalo Moniz/FIOCRUZ/UFBA. R. Valdemar Falcão 121 Brotas, 40295-001 Salvador, BA, Brasil.
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Parasitemia and mortality were evaluated during the experiment. Table 1 shows general data about the experimental groups, such as the number of animals, inocula, treatment and cure rates for each group. Surviving animals belonging to the treated groups were evaluated three months after the end of treatment by the following parasitological cure tests: direct parasitemia (peripheral blood examination under cover slides 22x22 in 50 microscopic fields, 400X); haemoculture in Warren's medium; xenodiagnosis with five 4th - 5th stage *Rhodnius prolixus* nymphs and subinoculation of blood into newborn mice (0.1mg, intraperitoneally).

Biochemical characterization. The electrophoretic patterns of isoenzymes were determined for several samples of each strain, individually isolated from treated non-cured mice and from untreated controls either directly from the blood of positive animals or after haemoculture; from the blood of mice used for subinoculation or from xenodiagnosis. In either case, the samples were inoculated into mice and then isolated by haemoculture into Warren medium for the obtaining of culture forms and enzymic extracts.

Twenty two samples of *T. cruzi* were isolated from treated animals as follows: 1st - **Peruvian strain** (seven samples): 1 sample from mouse treated with nifurtimox; 1 from mouse used for subinoculation of the blood of animal treated with benznidazole; 1 from xenodiagnosis of mouse treated with benznidazole; 4 from the peripheral blood of mice treated with benznidazole. 2nd - **21 SF strain** (2 samples):, each one originating from one mouse treated with nifurtimox, after subinoculation into newborn mice. 3rd - **Colombian strain** (thirteen samples): 4 samples individually isolated from the blood of mouse treated with nifurtimox; 1 sample from xenodiagnosis of mouse treated with nifurtimox; 8 samples from the blood of mouse treated with benznidazole.

Controls. Three samples of each strain obtained from untreated mice were also maintained in culture for isoenzymic study.

Cultivation in Warren medium was performed at 28°C. Parasites were washed with KRT buffer solution and enzymic extracts were obtained according to Godfrey and Kilgour[18], and maintained in liquid nitrogen as "pearls" according to Miles et al[25].

Isoenzymes tested: alanine aminotransferase (ALAT) -E.C. 2.6.1.2; aspartate aminotransferase (ASAT) -E.C.2.6.1.1.; glucose phosphate isomerase (GPI) - E.C.5.3.1.9.; phosphoglucomutase (PGM) - E.C.2.7.5.1.

Thin-layer starch-gel electrophoresis was performed by application of 30V/cm, during 90 minutes for ALAT and 60 minutes for ASAT and of 20V/cm during 150 minutes for GPI and 120 minutes

Table 1 - General data on mice infected with Trypanosoma cruzi, *treated with benznidazole and nifurtimox. Experimental groups.*

T. cruzi strains (Type)	Inoculum (nº trip.)	Experimental groups of treatment	Nº mice	Mortality indices (%)	Cure rates (%)
Peruvian	5×10^4	Treat. benz*	40	30	45.4
(I)		Treat. nifurt**	40	15	37.7
		Untreated	20	100	-
21 SF	1×10^5	Treat. benz	40	30	100.0
(II)		Treat. nifurt	40	12	75.0
		Untreated	20	100	-
Colombian	1×10^5	Treat. benz	40	65	30.0
(III)		Treat. nifurt	40	65	0.0
		Untreated	20	100	-

* Benz = benznidazole - 4 doses of 200mg/kg b.w. followed by daily doses of 50mg/kg b.w. during 90 days.

** Nifurt = nifurtimox - Daily doses of 100mg/kg b.w. during 90 days.

for PGM. The enzymes ALAT and ASAT were developed with phosphate buffer solution 0.1M and beta NAD, and examined by ultra-violet light; for the enzymes GPI and PGM, TRIS/HCl buffer solution 0.3M and NADP were used besides the MTT (dimethilthiazole 2-yl 2-5 diphenil tetrazolium bromide) 0.36mM, agar gel 0.6% and phenazine metasulfate, 0.03mM.

RESULTS

Response to chemotherapy - The cure rates for each strain of mice treated either with nifurtimox or benznidazole are shown in Table 1. It was of 37.7% for nifurtimox and 45.4% for benznidazole in mice infected with Type I strain; Type II strain disclosed 75% and 100% of cure rates respectively, for treatment with nifurtimox and benznidazole; Type III strain showed 0% and 30% of cure when treated respectively with nifurtimox and benznidazole.

Biochemical analysis -The electrophoretic profiles revealed for the isoenzymes ALAT, ASAT, PGM and GPI with the Peruvian strain corresponded to the zymodeme Z2a, according to Tybayrenc et al[30]. With the 21 SF strain and the Colombian strain these isoenzymes corresponded to Z2 and Z1, respectively, according with Miles et al[24]. No differences could be detected in the isoenzymic profiles for ALAT, ASAT, PGM and GPI of the strains, in the several samples isolated from treated mice when compared with the control non-treated strains. Examples of the isoenzymic electrophoretic patterns of the three strains for the different enzymes are shown in Figures 1 to 5.

DISCUSSION

The strains of *T.cruzi* are not necessarily homogeneous populations and different clones can be disclosed that differ in their antigenic composition[11], virulence[28], isoenzymic patterns[16 19 31] or the patterns of kinetoplast DNA[26]. The clonal structure of *T. cruzi* strains has been postulated by Tibayrenc et al[31], by identification of 43 genetically different clones from 121 strains isolated from different geographical areas and identified by isoenzymic profiles and dendogram analysis of genetical distances. Since the description of three different zymodemes in Brazil by Miles[25], intra-zymodeme variability has been detected by the genetic distances or expressed by different alleles in

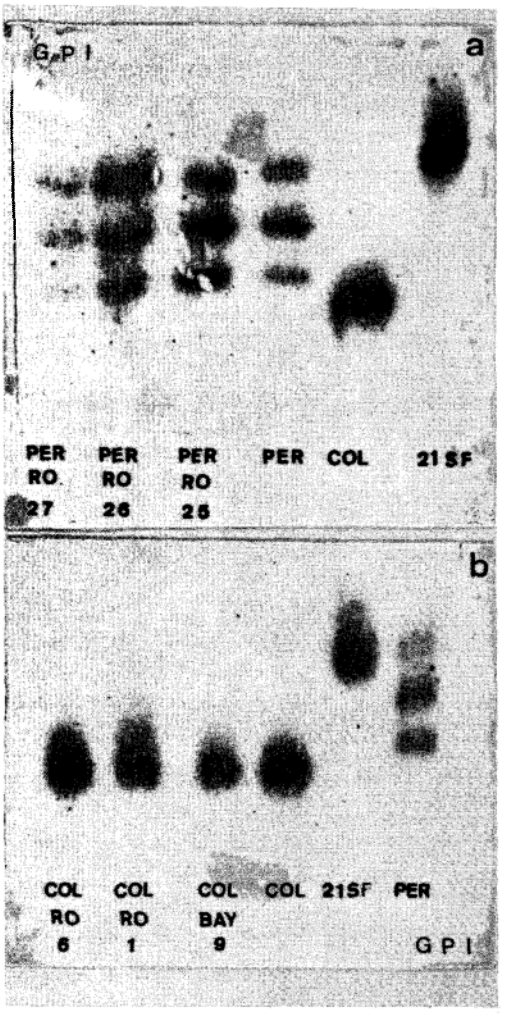

*Figure 1 - **GPI** isoenzyme: **a)** the Peruvian strain isolated from untreated controls (PER) or from animals treated with benznidazole (RO), disclosed identical electrophoretic pattern, belonging to zymodeme 2a (Z2a); **b)** the Colombian strain isolated from mice treated with benznidazole (RO) or nifurtimox (BAY) showed the same electrophoretic pattern as the strain isolated from untreated control corresponding to zymodeme 1 (Z1). In each plate, control strains are included (in **a**, Col (Z1) and 21 SF (Z2); in **b**, PER (Z2a) and 21SF (Z2).*

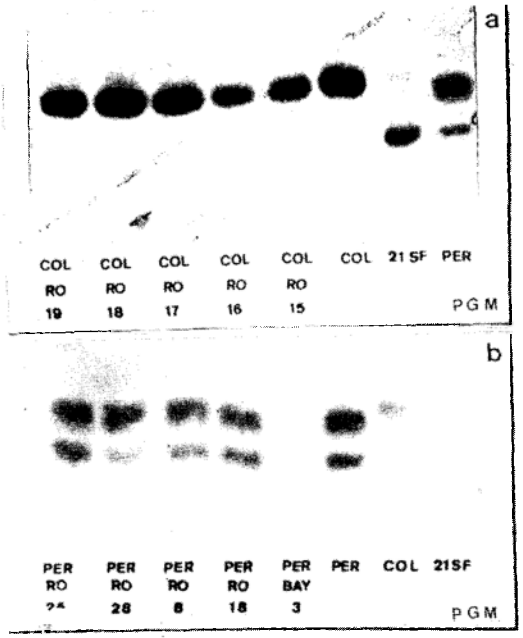

Figure 2 - ***PGM*** *- the isoenzymic patterns of zymodemes Z1 (COL), Z2 (21 SF) and Z2a (PER), are shown.* ***a)*** *The Colombian strain isolated from mice treated with benznidazole (RO) maintained the same pattern disclosed by the untreated strain (COL) corresponding to Z1;* ***b)*** *the electrophoretic patterns for the Peruvian strain isolated from treated mice (RO and BAY) are similar to those disclosed by the strain from untreated control (PER).*

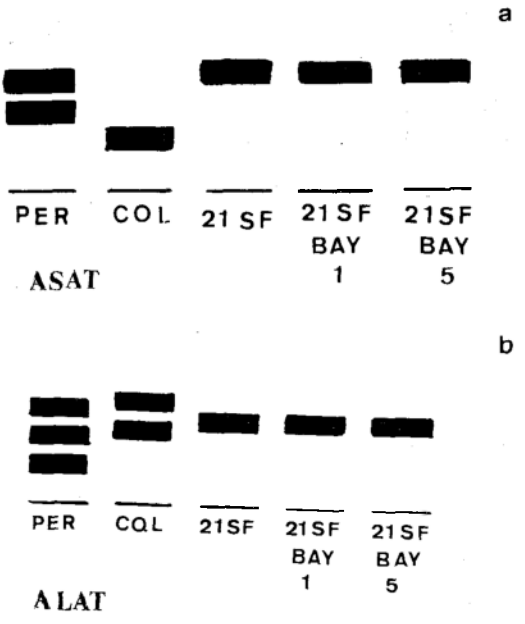

Figure 3 - ***ASAT*** *and* ***ALAT*** *- The 21 SF strain (Z2) maintained the electrophoretic profiles for both isoenzymes when isolated from mice treated with nifurtimox (BAY) as compared to control (21 SF). The profiles of the Z2a (PER) and Z1 (COL) are also shown.*

the same zymodeme[8 9 15 23 29 32]. However, the stability of biological characters of strains maintained in laboratory, under well controlled conditions, suggests that there is an equilibrium of the different populations within one strain, even after modifications of environmental conditions as passages into axenic cultures, cryopreservation in liquid nitrogen or passages in triatominae[20 21]. Although changes in zymodemes profiles and schizodeme patterns after passages of strains in C3H mice has been registered[12], the stability of the isoenzymic characters has also been shown for the Y strain cryopreserved for 6 and 7 years or after being isolated from treated mice[13 14].

The drugs used in the present study have a known intracellular action against *T. cruzi*. The strains differed in its susceptibility. The Type II strain (21 SF), biochemically characterized as zymodeme 2 according to Miles et al[24], showed a high susceptibility, with cure rates of 75% with nifurtimox and 100% with benznidazole. The most resistant one was the Colombian (Type III), corresponding to zymodeme 1, showing 0% of cure with nifurtimox and 30.7% with benznidazole. Type I strain (Peruvian), which isoenzymic profile can be compared to Z2a described by Tibayrenc et al[30], showed cure-rates of 37.7% with nifurtimox, and 45.4% with benznidazole. As could be seen in the present study, a percentage of mice maintained the infection after treatment with one of the two drugs or with both of them. Parasites in these non-cured mice could be considered as most resistant; in previous papers it was demonstrated that a higher resistance to the same drugs or a cross resistance to both nifurtimox or benznidazole could be detected in the strains isolated from treated uncured mice[3 4]. The possibility of genomic alterations or clonal

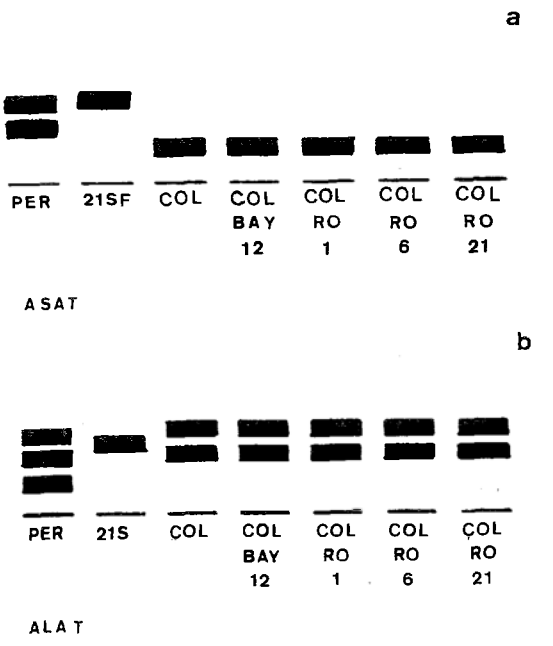

*Figure 4 - **ASAT** and **ALAT** - The Colombian strain isolated from mice treated either with nifurtimox (BAY) or benznidazole (RO) showed the same isoenzymic pattern (Z1) as the control strain (COL), isolated from untreated mice. The profiles of the Z2 (21SF) and Z2a (PER) are shown.*

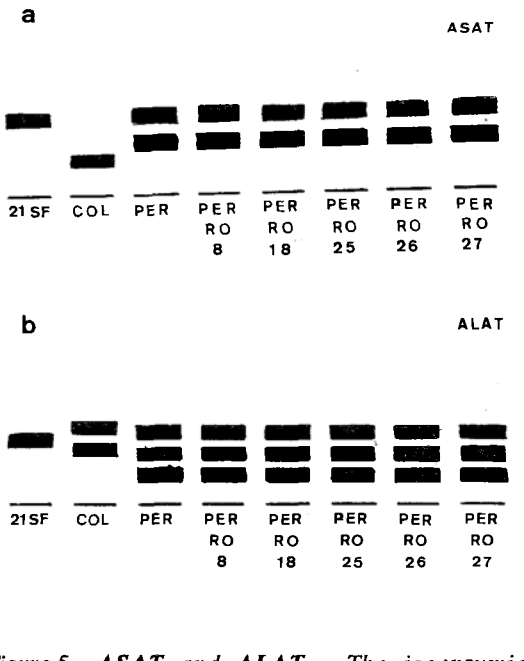

*Figure 5 - **ASAT** and **ALAT** - The isoenzymic electrophoretic profiles of the Peruvian strain (Z2a) for both isoenzymes are maintained for the strain isolated from mice treated with benznidazole (RO-PER) as compared with the control strain isolated from untreated mice (PER). The profiles of the Z1 (COL) and Z2 (21 SF) strains are also shown.*

selection was then evident. However, the investigation of the post-treatment biochemical behavior of the strains here studied did not reveal consistent differences that could be correlated with clone selection. Biochemical analysis confirmed that no phenotypic differences, at least for the enzymes used for the present study: GPI, PGM, ALAT and ASAT, have been expressed by the strains submitted to chemotherapeutic agents. Results of the present investigation suggest a stability of biochemical characteristics of the strain types studied. Recent observations by McDaniel and Dvorak[22] have shown that single cell derived clones of the Y strain differed in its DNA content eventhough the isoenzyme and schizodeme profiles were maintained. Investigations on the genotypic characteristics of the strains here studied by the analysis of the genomic and kinetoplast DNA could further clarify these aspects.

RESUMO

Com o objetivo de investigar a influência da quimioterapia no padrão bioquímico de diferentes cepas do Trypanosoma cruzi, *três grupos de camundongos foram infectados respectivamente com as cepas Peruana, 21 SF e Colombiana, que correspondem a diferentes padrões biológicos e isoenzimáticos . Cada grupo foi subdividido em subgrupos: 1 - tratados com nifurtimox; 2 - tratados com benzonidazol ; 3 - controles infectados não tratados. Ao final do tratamento que durou 90 dias, os animais foram submetidos a testes parasitológicos de cura: xenodiagnóstico, subinoculação do sangue em camundongos recém-nascidos e hemocultura em meio Warren. A partir da positivação destes testes, foram isoladas 22 amostras do* T. cruzi *dos três subgrupos. A análise eletroforética dos extratos enzimáticos obtidos após cultura, para as enzimas PGM, GPI, ALAT e ASAT demonstrou identidade dos padrães enzimáticos entre os*

parasitos de uma mesma cepa isolados de animais tratados ou não tratados. Conclui-se que não houve seleção clonal ou alterações genéticas dos parasitos, detectáveis pelas isoenzimas examinadas, dependentes da ação de drogas de reconhecida ação antiparasitária. Isto sugere uma estabilidade dos caracteres fenotípicos dos três tipos biológicos de cepas do T. cruzi.

Palavras-chaves: Cepas do Trypanosoma cruzi. *Isoenzimas. Quimioterapia.*

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