

Changes in the isoenzyme and kinetoplast DNA patterns of *Trypanosoma cruzi* strains induced by maintenance in mice

Mariângela Carneiro¹, E. Chiari¹, A.M. Gonçalves², A.A. da Silva Pereira¹, C.M. Morel² and A.J. Romanha³

¹Departamento de Parasitologia e de Bioquímica, ICB-UFMG, Belo Horizonte, Brazil, ²Departamento de Bioquímica e Biologia Molecular, IOC-FIOCRUZ, Rio de Janeiro, Brazil, and ³Centro de Pesquisas René Rachou, FIOCRUZ, Belo Horizonte, Brazil

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Culture forms of thirteen *Trypanosoma cruzi* strains from 4 zymodemes and 9 schizodemes were inoculated and kept by successive passages in C3H mice. The strains were initially from the following zymodemes: 3 from A, 3 from B, 4 from C and 2 from D and 1 from AB mixed zymodemes. After approximately 18 months maintenance the parasites were isolated by hemoculture and again typed according to their isoenzyme and kinetoplast DNA patterns. The zymodeme A strains kept their initial patterns; from the 3 zymodeme B strains, two kept the initial patterns and one changed to zymodeme A; from the 4 zymodeme C, two kept the initial pattern and two changed to zymodeme B; from the 2 zymodeme D strains, one kept the initial pattern and one changed to zymodeme A. The strain from AB mixed zymodeme was reduced to zymodeme A. The zymodeme changes were accompanied by schizodeme changes. Although not simultaneously, in one *T. cruzi* strain the parasitemia change was followed by zymodeme and schizodeme changes. The results showed that prolonged maintenance of *T. cruzi* in mice by successive passages alters the isoenzyme and k-DNA patterns of some strains and that these alterations tend to move towards zymodeme A, suggesting a selective effect of mice over these *T. cruzi* populations.

Key words: *Trypanosoma cruzi*; Zymodeme; Schizodeme.

Introduction

Intraspecific variations in *T. cruzi* strains have long been observed based on their behaviour in vertebrate and invertebrate hosts as well as in culture (Brener, 1977, 1985). Characterization in such models is, however, strongly influenced by a number of extrinsic factors and therefore the biological methods of characterization available have proven to be insufficient to detect parasite variation. On the other hand, biochemical methods have been successfully introduced for the intrinsic characterization of parasites. Among these methods, isoenzyme and kinetoplast DNA (k-DNA) patterns have been much used (Miles et al., 1980, 1981; Romanha et al., 1979a,b; Morel et al., 1980; Gonçalves et al., 1984b; Tibayrenc and Ayala, 1988). Using

Correspondence address: M. Carneiro, Departamento de Parasitologia, ICB-UFMG, Av. Antonio Carlos, 6627, C.P. 2486, 30161 Belo Horizonte, MG, Brazil

isoenzyme patterns Romanha et al. (1982) typed *T. cruzi* isolates from 69 chronic chagasic patients, from the endemic region, of Bambui, in Southeast Brazil, into four groups, zymodemes; A, B, C and D. The same isolates were further typed according to the electrophoretic pattern obtained after digestion of their k-DNAs by restriction endonucleases, and grouped into schizodemes (Morel et al., 1980). The number of schizodemes was higher than that observed for zymodemes.

In the present work we determined the influence of prolonged serial passages of *T. cruzi* in mice on their isoenzyme and k-DNA patterns.

Material and Methods

T. Cruzi strains

The *T. cruzi* strains were isolated by hemoculture from patients in the chronic phase of Chagas' disease, from the endemic region of Bambui (Chiari et al., 1979). The isoenzyme and k-DNA classifications of the isolates were made previously by Romanha et al. (1982) and Gonçalves et al. (1984b) respectively. After classification, the isolates were cryopreserved in liquid nitrogen. Thirteen out of 69 strains belonging to four zymodemes were chosen randomly, thawed and transferred to LIT (Liver Infusion Tryptose) medium (Camargo, 1964). The distribution of the 13 strains according to the zymodeme is shown in Table 1. The strains were established and large numbers grown in LIT medium at 28°C. A 90 ml culture containing approximately 2.5×10^7 parasites/ml was washed and harvested by centrifugation. Pellets from 30 and 60 ml cultures were kept at -70°C until used for isoenzyme and k-DNA determination, respectively. In order to obtain high percentages of infective trypomastigotes, flagellates from LIT medium were transferred to M-16 medium (Chiari et al., 1980). On the 6th day of culture in M-16, parasite suspensions containing 10^7 metacyclic trypomastigotes of each strain were inoculated intraperitoneally and maintained in C3H isogenic mice.

T. cruzi maintenance in mice

The strains presenting patent parasitemia in mice were maintained throughout successive blood passages by i.p. injections at regular intervals. According to the parasitemia peak of each strain the passage intervals ranged from 7 to 20 days. Mice with subpatent parasitemia were submitted to hemocultures. The positive hemocultures were inoculated into mice and the parasites then maintained by alternated mouse-hemoculture passages.

Isolation of T. cruzi strains from mice

After a period of maintenance in mice of approximately 18 months, the strains were isolated by hemoculture in LIT medium. Table 1 shows the kind of maintenance, the parasitemia type, and the number of passages to which each strain has been submitted. The positive hemocultures were established and bulk cultures grown in LIT medium. The parasite culture forms were harvested and washed 3 times by centrifugation, $2000 \times g$, 4°C , 10 min, in KRT (Krebs Ringer Tris, pH 7.3) buffer.

The parasite pellet was stored at -70°C until used for isoenzyme and k-DNA pattern determination.

The isoenzyme pattern

The culture form pellet stored at -70°C was thawed at room temperature and submitted to osmotic lysis in an enzyme stabilizer (2.0 mM dithiothreitol, 2.0 mM ϵ -aminocaproic acid and 2 mM Na_2 -EDTA, pH 7.0) at 4°C , at the volume ratio 1:1. The lysate was centrifuged at $15000\times g$, 1 h at 4°C . The supernatant, called enzymatic extract, was cryopreserved in liquid nitrogen as small drops of approx. 15 μl . The isoenzymes were separated by refrigerated horizontal thin-layer starch gel electrophoresis. The enzymes studied were: alanine aminotransferase (ALAT) [EC 2.6.1.2], aspartate aminotransferase (ASAT) [EC 2.6.1.1], malic enzyme (ME) [EC 1.1.1.40], 6-phosphogluconate dehydrogenase (6PGD) [EC 1.1.1.44], phosphoglycomutase (PGM) [EC 2.7.5.1], glucose-6-phosphate dehydrogenase (G6PD) [EC 1.1.1.49], glucose phosphate isomerase (GPI) [EC 5.3.1.9] and malate dehydrogenase (MDH) [EC 1.1.1.37]. The staining system and electrical conditions for ALAT, ASAT, PGM and GPI were those of Romanha et al. (1979a), for G6PD and ME those of Miles et al. (1980). For 6PGD, the electrode buffer was 150 mM Tris-maleate, pH 7.4, which was diluted ten times in water for the gel. Electrophoresis was carried out for 3 h at constant voltage, 20 V/cm. For MDH, the electrode buffer was 75 mM sodium citrate, pH 6.0, which was diluted ten times in water for the gel. Electrophoresis was carried out for 2.5 h at constant voltage, 25 V/cm. The staining solutions for both enzymes were as according to Harris and Hopkinson (1976).

k-DNA pattern

The washed culture form pellet stored at -70°C was thawed at room temperature and submitted to k-DNA extraction according to Gonçalves et al. (1984a). The k-DNA was digested by the enzyme EcoRI and the fragments separated by vertical electrophoresis in acrylamide gel. The electrophoretic pattern was visualized by ethidium bromide fluorescence (Gonçalves et al., 1984a).

Results

Parasitemia

Whatever the initial zymodeme of the *T. cruzi* strains, the parasitemia kept the same pattern after prolonged maintenance in mice. The only exception was the 222 strain which changed its parasitemia from subpatent to patent after eight passages in mice (Table 1).

Isoenzyme patterns

The isoenzyme pattern of 5 out of 13 *T. cruzi* strains maintained in mice by serial blood passages for about 18 months changed when compared with their initial patterns. Isoenzyme pattern changes occurred at the same time in 6 out of 8 enzymes

TABLE 1

Parasitemia and zymodemes of *Trypanosoma cruzi* strains before and after maintenance in C3H mice

Strain	Maintenance	Passage number	Parasitemia ^a		Zymodeme	
			Initial	Final	Initial	Final
138	Blood	34	P	P	A	A
229	Blood	44	P	P	A	A
239	Blood	26	P	P	A	A
84	Blood	11	P	P	AB	A
144	Blood	27	P	P	B	B
147	Blood	48	P	P	B	B
167	Blood	48	P	P	B	A
182	Alternate ^b	8	S	S	C	B
222	Alternate	8	S	P	C	C
222p	Blood	9	P	P	C	B
231	Alternate	3	S	S	C	C
254	Alternate	3	S	S	C	C
150	Blood	32	P	P	D	D
207	Blood	21	P	P	D	A

^aParasitemia: P = patent and S = subpatent.^bAlternate = cyclic mouse-hemoculture passages.

studied. The three strains initially classified as zymodeme A remaining unchanged during this period (Table 1 and Fig. 1a). The strain 84 classified initially as mixed zymodeme AB was clarified as zymodeme A after this time of passage. The strains 144 and 147 (classified initially as zymodeme B) maintained the same zymodeme whereas strain 167, also classified as zymodeme B, changed to A (Figs. 1a and 2a). In order to detect the time the isoenzyme pattern may change, the 167 strain zymodeme was determined after each successive passage. For this particular strain the isoenzyme pattern changed from zymodeme B to A after the first passage in mice (results not shown).

Strains 222, 231 and 254, classified initially as zymodeme C, after being maintained in mice by alternate passages, showed the same initial zymodeme (Table 1 and Figs. 1b and 2b). Strain 182, after eight alternate passages, changed from zymodeme C to B. On the other hand, strain 222, after eight alternate passages, kept the zymodeme unchanged but changed the parasitemia from subpatent to patent. Due to this parasitemia change, strain 222 (now called 222p), was maintained in mice thereafter by successive blood passages. Thus, after nine successive blood passages the parasites changed from zymodeme C to B (Table 1, Figs. 1b, 2b).

Strain 150 classified initially as zymodeme D, a mixture of zymodemes A and C, after being maintained 18 months in mice kept the same zymodeme whereas strain 207 changed its zymodeme from zymodeme D to A (Table 1, Figs. 1b, 2b).

k-DNA patterns

Figs. 3 and 4 show the *k*-DNA patterns of *T. cruzi* strains before and after maintenance in mice. Except for 167, 182, 222p and 207, the other strains kept their

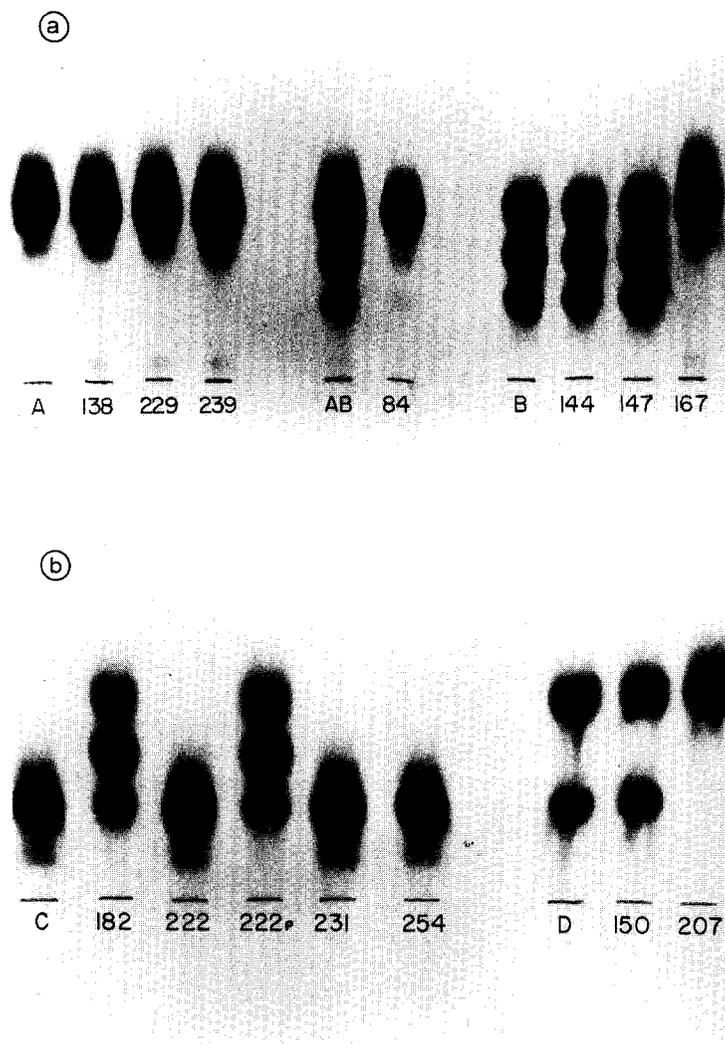


Fig. 1. GPI enzyme patterns of *T. cruzi* strains before and after maintenance in C3H mice: (a) *T. cruzi* strains from zymodemes A, AB and B; (b) *T. cruzi* strains from zymodemes C and D. A, AB, B, C and D are the patterns presented before mouse passage and the numbers the patterns presented by the coded strains after maintenance in mice.

k-DNA patterns constant. Since the initial k-DNA pattern of the 239 strain was not determined it is not possible to say whether it had changed or not after maintenance in mice. All k-DNA pattern changes were towards simpler patterns with less bands and better resolution. A typical example of this type of change is that of strain 207 which showed a final simple pattern resulting from the disappearance of a number of bands present in the more complex initial pattern. The final patterns were all different among the strains.

As with their isoenzyme patterns, strains 222, 231 and 254 kept the same initial k-DNA patterns (Fig. 4). Strain 182, after eight alternate passages, changed its k-DNA

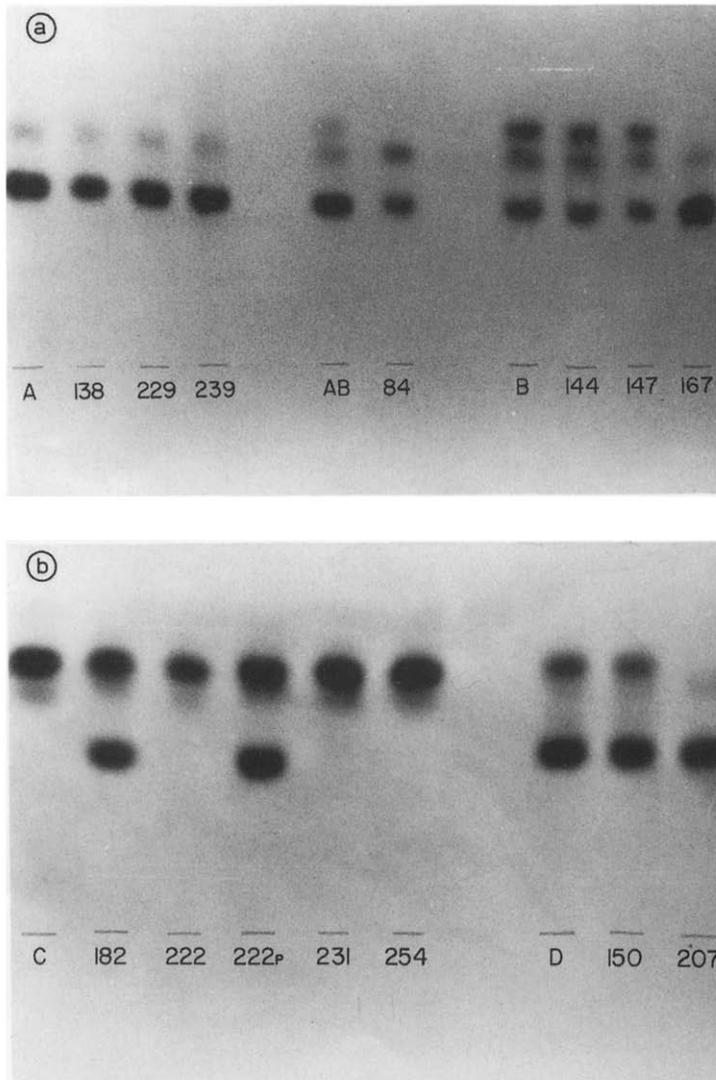


Fig. 2. PGM enzyme patterns of *T. cruzi* strains before and after maintenance in C3H mice: (a) *T. cruzi* strains from zymodemes A, AB and B; (b) *T. cruzi* strains from zymodemes C and D. A, AB, B, C and D are the patterns presented before mouse passage and the numbers the patterns presented by the coded strains after maintenance in mice.

pattern without changing parasitemia. On the other hand, strain 222 after eight alternate passages kept the k-DNA pattern unchanged and changed the parasitemia (222p). Furthermore, after nine blood passages strain 222p also changed its k-DNA pattern (Table 1, Fig. 4).

Again like the isoenzyme pattern, strain 150 kept the k-DNA pattern constant while strain 207 changed after maintenance in mice (Figs. 1b, 2b and 4).

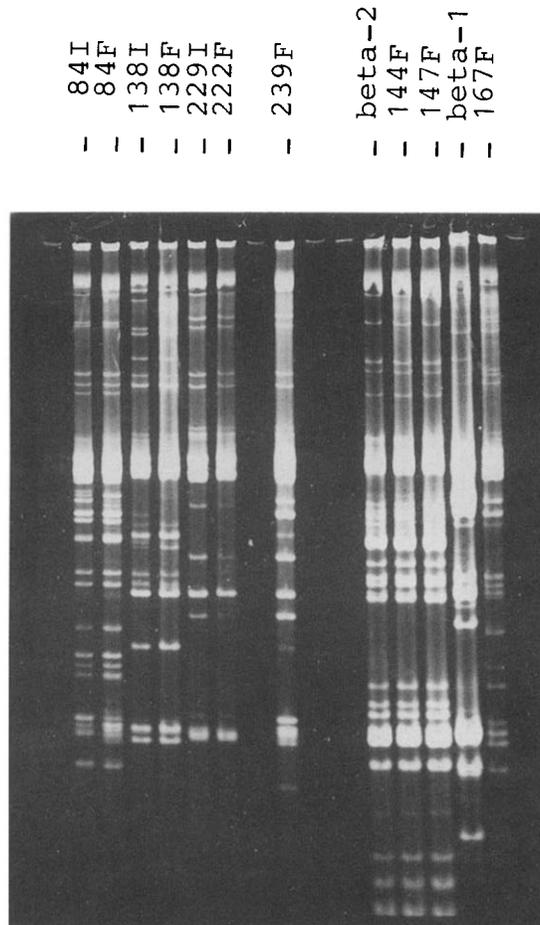


Fig. 3. k-DNA patterns of *T. cruzi* strains before and after maintenance in C3H mice. The k-DNA was previously digested by EcoRI restriction enzyme. I, β -1 and β -2 are the patterns presented before mouse passage and F after mouse passage.

Discussion

The maintenance of *T. cruzi* strains in C3H mice for 18 months changed the isoenzyme and k-DNA patterns of some strains. Five out of 13 strains had their zymodeme as well as schizodeme changed, and schizodeme changes occurred simultaneously with the zymodeme. Two different hypotheses can be considered to explain such changes: (1) the modification of the patterns observed are due to regulatory phenomena within a given genotype. In other words, the markers employed are not reliable as genetic markers and can be modified by environmental factors. Such a hypothesis was proposed by Mirelman (1987) to explain zymodeme changes in *Entamoeba histolytica*; (2) the markers are reliable genetic markers, and the changes are due to the fact that the original populations were in fact mixed stocks of two or more different genotypes. Some minor modifications would be accepted for a regulatory phenomena, but a total shift involving seven loci is hardly conceivable.

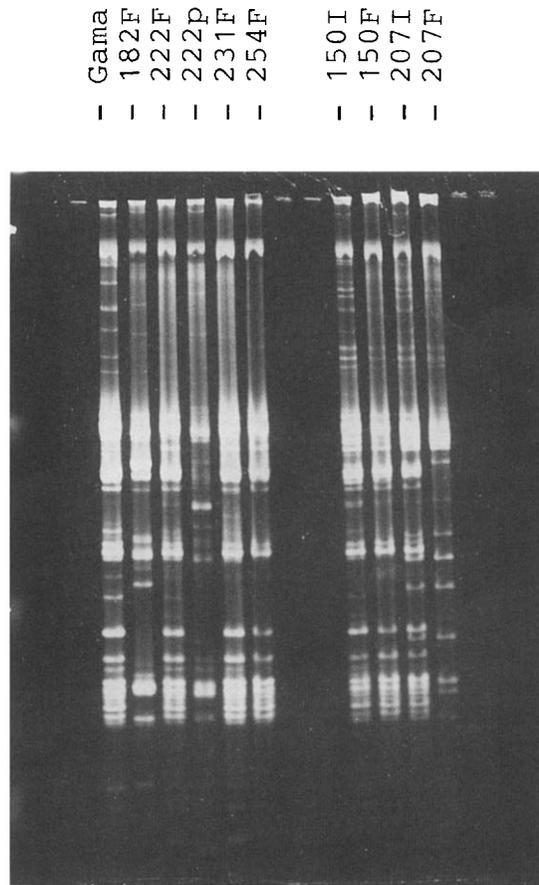


Fig. 4. k-DNA patterns of *T. cruzi* strains before and after maintenance in C3H mice. The k-DNA was previously digested by EcoRI restriction enzyme. γ and I are the patterns presented before mouse passage and F after maintenance in mice.

The fact that schizodeme changes were parallel to isoenzyme modifications strongly favors the second hypothesis. To confirm either hypothesis, clones should be obtained and the experimental protocol repeated with them. Only strain 84 changed its zymodeme without changing its schizodeme. This may be due to the fact that by the time the strain was in the AB zymodeme it had already changed its schizodeme. All changes were towards zymodeme A, suggesting that mice might select zymodeme A *T. cruzi* populations.

Lana et al. (1981) reported isoenzyme pattern stability for the *T. cruzi* Berenice strain during mouse passage. They also reported the isoenzyme stability of this strain isolated from a patient twice over a six year interval. Romanha (1982) showed that the maintenance of the Y and CL strains in exponential growth in liquid medium induced isoenzyme pattern changes towards zymodeme C. Taken together these data would suggest that zymodeme A strains develop, or are better adapted to, the vertebrate host while zymodeme C strains are better adapted to culture medium. Reinforcing this suggestion are the data from *T. cruzi* strains 84 and 207. They were initially mixtures of zymodemes A and B (zymodeme AB), strain 84, and A and C

(zymodeme D), strain 207, and after maintenance in mice were characterized as zymodeme A. On the other hand, the permanence of mixed stocks represented by zymodeme D strain 150, over 18 months of animal maintenance is an interesting result, suggesting that elimination or overdominance of one genotype by another is not a constant fact, and that two radically dissimilar genotypes can coexist for a long period of successive passages in mice.

The adaptation of strains from different zymodemes to mice is also suggested when parasitemia is considered. Strains from zymodeme A presented patent parasitemia while those from zymodeme C presented subpatent parasitemia. Since a continuous blood passage would put at risk the maintenance of subpatent parasitemia strains (zymodeme C) we alternated mouse infections with hemoculture. Strain selection in mice was also reported by Deane et al. (1984) who observed that the schedule of parasite inoculation and isolation favored the emergence of one or other strain when mice were double infected.

The better adaptation of zymodeme A *T. cruzi* strain to the vertebrate host is also suggested by the predominance of this zymodeme in isolates from chronic chagasic patients in Brazil. Romanha (1982) found 54% of A zymodemes in chronic patients from Bambui city, Minas Gerais State, Brazil; Schlemper Jr. (1982) at Iguatama and Virgem da Lapa cities in the same State found 100% and Miles et al. (1981) in east and central and Luquetti et al. (1986) in a central region of Brazil found almost only zymodeme 2 (= zymodeme A) in chronic patients. In Bolivia, Tibayrenc and Desjaux (1983) and Tibayrenc et al. (1986) found that *T. cruzi* zymodeme predominance was more related to different areas and/or climatic factors than to a specific host.

A direct and immediate relationship between zymodeme changes and parasitemia had not been detected. Some strains changed their zymodeme without changing their parasitemia and vice-versa. However, strain 222 changed its zymodeme a few passages after its change in parasitemia, showing for this strain a direct but not immediate relationship. Since we did not follow the isoenzyme pattern of strain 222 after each murine passage we can not say precisely how many passages were needed for the zymodeme to become changed after the parasitemia had changed. What can be said is that at the end of nine successive blood passages the zymodeme had also changed (Table 1, Figs. 1b, 2b). Conversely, strain 182 showed zymodeme change without changing parasitemia. Strain 167 was the only one we followed to detect the passage the zymodeme and schizodeme would change. Zymodemes and schizodemes changed just after the first mouse passage, then kept constant thereafter up to the 48th passage when it was last classified. No parasitemia change was observed for this strain. On the other hand, strain 147, which had the same initial zymodeme as 167 and had also been passaged in mice the same number of times, did not change either its zymodeme or schizodeme.

Gonçalves et al. (1984b) showed that the k-DNA pattern divides *T. cruzi* strains in more groups than the isoenzymes at least for an isoenzyme range comparable to the one we used here. Therefore the 13 strains we studied were grouped into four zymodeme and nine schizodemes. Further, the strains from zymodeme B were divided into two schizodemes; β -1 and β -2. According to Carneiro et al. (1989), only the β -2 strains are lethal for C3H mice suggesting a relationship between schizodeme and lethality. Conversely Sanchez et al. (1984) found no correlation between *T. cruzi* k-DNA minicircle structure and mice lethality.

The isoenzyme and k-DNA patterns have again been seen as good *T. cruzi*

population markers and that mice, the most used experimental host, can selectively favor *T. cruzi* populations.

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