New electrophoretic evidence of genetic variation and diploidy in *Trypanosoma cruzi*, the causative agent of Chagas' disease

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Abstract

By changing electrophoretic conditions additional genetic variability was observed in both previously and newly analysed enzymatic systems in *Trypanosoma cruzi*. Inferences on the quaternary structure of enzymes emerge from the electrophoretic patterns. The results of the present work are consistent with those obtained formerly in supporting both diploidy and lack of mating. These observations are relevant to the main question of the evolutionary origin of *T. cruzi* isozymic strains.

Introduction

Electrophoretic enzyme studies of *Trypanosoma cruzi* (Protozoa, Flagellates), the causative agent of Chagas' disease, have been initiated by Toyé (1974). More recently, Miles *et al.* (1977, 1978, 1980) have distinguished in Brazil three main isozymic strains, called 'zymodemes', according to phenotypic interpretation of zymograms. On the basis of their data, they have produced a classification of Brazilian strains using numerical taxonomy (Ready & Miles, 1980).

Previous electrophoretic work on *T. cruzi* allowed a genetic interpretation of Bolivian stock zymograms and provided evidence of both genome diploidy (Tibayrenc *et al.*, 1981a; see also Lanar *et al.*, 1981) and absence of mating (Tibayrenc *et al.*, 1981b).

Strain classification according to Nei's genetic distance and preliminary inferences on the evolutionary origin of the strains have been postulated (Tibayrenc & Miles, 1983; Tibayrenc *et al.*, 1983). In order to reveal possible additional genetic variability in *T. cruzi*, we have modified our usual electrophoresis conditions and surveyed new enzymatic systems. This paper reports the conclusions that emerge from these experiments.

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Material and methods

Origin of the stocks

Ten stocks originated from different hosts and collected in various South American countries were subjected to electrophoresis (Table 1). All Bolivian stocks were isolated from *Triatoma infestans* (Hemiptera, Reduviidae), the main vector of Chagas' disease in Bolivia, using a method described elesewhere (Tibayrenc *et al.*, 1982).

Sample preparation

Stocks are grown in LIT monophasic culture medium. They are centrifuged without washing. Cell forms are lysed in an equal volume of hypotonic enzyme stabilizer (Dithiothreitol, E aminocaproic acid and EDTA, each at 2 mM) and submerged during 20 min in ice. Pellets are stored at -70 °C until used for electrophoresis. Single culture tubes suffice to study the array of enzymes used.

Electrophoresis and enzyme staining

The following enzymes have been analysed: phos-

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Table 1. Origin of the stocks with reference to isozymic strains.

Stock	Origin	Originating host	Isozymic strains
C8cl1	Bolivia	*Triatoma infestans*	1
A82	French Guiana	*Didelphis marsupialis*	Closely related to 1
A98	French Guiana	*Didelphis marsupialis*	Closely related to 1
A99	French Guiana	*Didelphis marsupialis*	Closely related to 1
Sc43cl1	Bolivia	*T. infestans*	2
Tulahuen FRMA	Chile	?	2a
Jokl ITMAP240	Brazil	Man	2a
COM 18	Bolivia	*T. infestans*	2c
Esmeraldo Clone 3	Brazil	Man	Brazilian zymodeme 2 (Bz2)
CANIII Clone 1	Brazil	Man	Brazilian zymodeme 3 (Bz3)

phoglucomutase (E.C.2.7.5.1, PGM), glucose phosphate isomerase (E.C.5.3.1.9, GPI), malate dehydrogenase (oxaloacetate decarboxylating) (Nadp+) (E.C.1.1.1.40, ME), glutamate dehydrogenase Nadp+ (E.C.1.4.1.2, GDNadp+), glutamate dehydrogenase Nad+ (E.C.1.2.1.2, GDNad+), 6-phosphogluconate dehydrogenase (E.C.1.1.1.44, 6PGD) and two peptidases (E.C.3.4.1.1 or 13), peptidase A (substrate: leucyl-leucyl-leucine) and peptidase B (substrate: L-leucyl-L-alanine). All electrophoreses are carried out on cellulose acetate plates (Helena® Laboratories). The assay procedures are given in Tables 2 and 3. For some enzymes eletrophoretic conditions have been varied to obtain clear patterns and allow genetic interpretations.

Results

Additional genetic variability

PGM

This enzyme appeared previously on the zymograms for isozymic strain 2c (IS 2c) from Bolivia and 'Zymodeme' 2 from Brazil, as one-banded. This single band was common to the two strains. Through various electrophoretic conditions, IS 2c reveals a two-banded pattern assumed to be derived from a heterozygote. None of the allozymes is shared with 'zymodeme' 2 (Fig. 1). This heterozygous pattern confirms the previously inferred monomeric structure of PGM in *T. cruzi* (Tibayrenc *et al.*, 1981a; Jeremiah *et al.*, 1982.)

GPI

In view of the very dark spot observed on the gels obtained under standard conditions (Tibayrenc & Miles, 1983) additional hidden heterogeneity might be expected within the Brazilian 'zymodeme' 2. In fact, this strain exhibits three distinct, equally spaced bands, of which the intermediate one is stained most intensively. This agrees with the dimeric structure admittedly assumed for *T. cruzi* GPI (Tibayrenc *et al.*, 1981a).

GDNadp+

This enzyme has been reported to be monomorphic in *T. cruzi* (Tibayrenc *et al.*, 1983) using the standard electrophoretic conditions. The use of different buffers allows the detection of mobility variation for GDNadp+ among the ten stocks surveyed here (Fig. 2).

Out of the 450 stocks investigated since the work on *T. cruzi* was initiated, only the A 99 stock has revealed a heterozygous pattern which clearly shows six well separated bands with staining intensity variation in different assay conditions (Fig. 2). This pattern may be considered to result from the expression of a pentameric enzyme. This result is not, however, in accordance with the interpretation of Walter and Ebert (1979), who inferred an hexameric structure for *T. cruzi* GDNadp+ from biochemical analysis.

As this enzyme is very active, some of the largest bands observed on zymograms could correspond to heterozygous patterns, especially if slight migrational differences between different alleles products are involved. Further electrophoreses through various

Table 2. Electrophoresis recipes. Each recipe gives 10 ml of staining solution to be mixed with 10 ml agarose 1.2% at 60 ° C in Petri dishes.

Enzyme	Tank buffer	Cell buffer	Voltage times	Distilled water	Staining buffer	Activator	Coenzyme	Linking enzyme	Substrate	Visualization method
PGM	A	A 1/4	200 v × 20 mn	2 ml	1:8 ml	$MgCl_2$ 1.0 M 250 μl	Nadp 5 mg	G6PD 20 μl	glucose 1P 20 mg	NBT 5 mg PMS 3 mg
GPI	A	A 1/10	200 v × 20 mn	2 ml	2:8 ml	$MgCl_2$ 1.0 M 250 μl	Nadp 5 mg	G6PD 20 μl	Fructose 6P 10 mg	NBT 5 mg PMS 3 mg
ME	A B	A 1/4 B 1/4	200 v × 20 mn	2 ml	3:8 ml	$MgCl_2$ 1.0 M 250 μl	Nadp 5 mg		Malate 1.0 M pH 7.0-0.6 ml (with malic acid)	NBT 5 mg PMS 3 mg
GDNadp	A B	A 1/4 B 1/4	200 v × 20 mn	-		-	Nadp 5 mg		Tris 0.05 M glutamate 0.1 M pH 7.0 10 ml (with glutamic acid)	NBT 5 mg PMS 3 mg
GDNad	A B	A 1/4 B 1/4	200 v × 20 mn	2 ml	3:8 ml	$MgCl_2$ 1.0 M 250 μl	Nad 5 mg		Tris 0.05 M glutamate 0.1 M pH 7.0 10 ml (with glutamic acid)	NBT 5 mg PMS 3 mg
PEP A	B	B 1/4	200 v × 20 mn	8.5 ml	4:1.5 ml	$MnCl_2$ 0.1 M 150 μl		L amino acid oxydase 0.5 mg Peroxidase 0.25 mg	L-leucyl- leucyl- leucine 5 mg	3 amino-9- ethylcarbazole 4 mg in ethanol 150 μl
PEP B	B	B 1/4	200 v × 20 mn	8.5 ml	4:1.5 ml	$MnCl_2$ 0.1 M 150 μl		L amino acid oxydase 0.5 mg Peroxidase 0.25 mg	L-leucyl- l-alanine 5 mg	3 amino-9- ethylcarbazole 4 mg in ethanol 150 μl

Table 3. Tank and staining buffers.

Tank buffers	Staining buffers
A = n° III of Shaw and Prasad (1970)	1 = Tris HCl 0.3 M pH 8.0 2 = Tris HCl 0.3 M pH 7.0
B = HR Helena[r] IS = 0.075 + 1 mM $MgCl_2$ (Lanham *et al.*, 1981)	3 = Tris HCl 0.3 M pH 7.4 (Lanham *et al.*, 1981) 4 = Disodium phosphate 0.1 M pH 7.4 (Miles *et al.*, 1980)

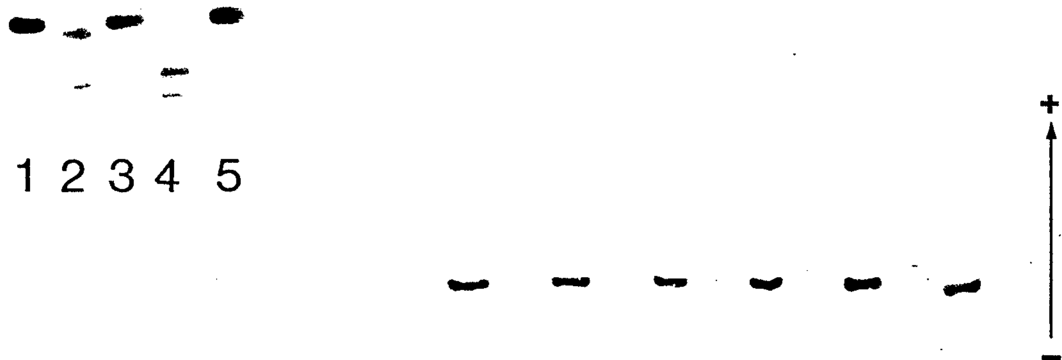

Fig. 1. Zymogram of PGM. Samples 1, 3, 5 = homozygous genotype 1/1 (isozymic strain 1); sample 2 = heterozygous genotype 2/5 (isozymic strain 2); sample 4 = heterozygous genotype 4/6 (isozymic strain 2c); The genotype of 'zymodeme' 2 (isozymic strain 2e) is 5/5 (not seen on the photograph).

Fig. 3. Zymogram of 6PGD. Homozygotes show one-banded patterns (genotype 3/3, IS 1) while heterozygotes display three bands, the middle one being more intensely stained (genotype 1/3, IS 2a).

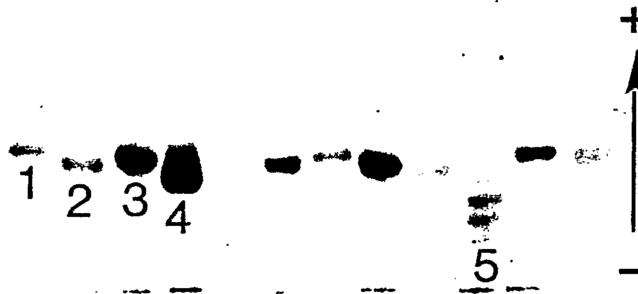

Fig. 2. Zymogram of GD Nadp+. Samples 1, 2, 3, 4 show clearly interstrain variation: 1 and 3 = isozymic strain 1, genotype 1/1; 2 = isozymic strain 2a, genotype 2/2; 4 = isozymic strain 2c, genotype 3/3. Samples 3 and 4, with large bands, would be perhaps heterozygotes. Sample 5 = a clearly six-banded pattern corresponding to heterozygote 1/5 (stock A99).

conditions will be necessary to establish whether the enzyme is a pentamer or hexamer.

6-PGD

Using modified techniques, 6-PGD zymograms exhibit one- two- or three-banded patterns, respectively associated with the previously described strains IS 1 (and related ones), IS 2 and IS 2a (Tibayrenc *et al.*, 1983). The three-banded pattern suggests a dimeric structure for this enzyme in accordance with the results of Jeremiah *et al.* (1982). Consequently the two-banded pattern is likely to be considered as a heterozygote for an active and a null allele. The inactive homodimer which shows occasionally some residual activity has the same mobility as the slowest band of the three-banded heterozygote (Fig. 3).

Genetic interpretation for new loci

Peptidases B

In previous studies the PEP A zymograms (substrate: L-leucyl-leucyl-leucine) consisted of multiple zones of activity stained more or less intensively, and whose genetic interpretation was not easy. Revelation of PEP B activity with L-leucyl-l-alanine as a substrate shows occasionally identical patterns to the PEP A systems due to the fact that the two loci exhibit overlapping substrate specificities. Nevertheless, PEP A is usually stained more faintly than PEP B on gels where the latter is expected (with L-leucyl-l-alanine as substrate). Consequently, parallel stainings of the same stocks with the two substrates allow assignment of the proteins to their respective loci (Figs 4 and 5).

GDNad+

Both GDNadp+ and GDNad+ are present in *T. cruzi* (Walter & Ebert, 1979). We have assayed GDNad+ in a previous study (Tibayrenc *et al.*, 1981a) and because of the diffuse pattern the genetic interpretation remained uncertain. In this work GDNadp+ and GDNad+ are stained on the same gel simply by adding the coenzyme NAD to the staining solution for GDNadp+. GDNad+ migrates faster than GDNadp+ and stains more faintly. Although no heterozygous patterns are observed, the single bands of different mobility among the various isozymic strains are attributed to different alleles at the same locus (IS 2: genotype 1/1; IS 2a, 2c and 'zymodeme' 2: genotype 2/2; IS 1; genotype 3/3).

Screening of new stocks

Malic enzyme (ME) was previously assayed for Bolivian stocks of *T. cruzi* and showed two very distant, well stained bands on the zymograms. These were therefore assumed to be encoded by two distinct loci (Tibayrenc *et al.*, 1981a). In the present study, ME usually displays a single-banded pattern at the two loci in all strains except for stock A98, which is three-banded for ME1 and stock A82, similarly three-banded for ME2 (Fig. 6). On the basis of the

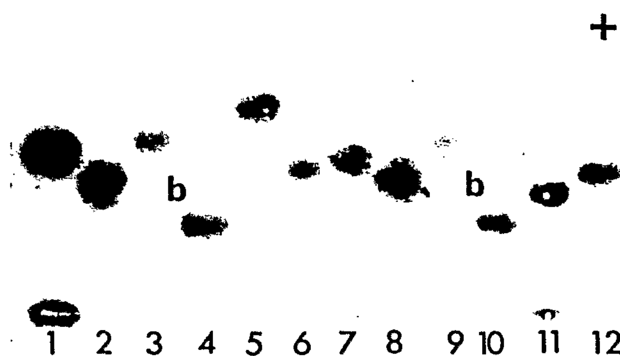

Fig. 4. Zymogram of PEP A. The 'b' fainter bands correspond to PEP B (compare with Fig. 5). Sample 1 = genotype 3/3 (IS 2a); samples 3 and 9 = genotype 2/2 (IS 2e); sample 5 = genotype 1/1 (IS 1); sample 11 = genotype 5/5 (IS 3); samples 2, 4, 6, 8, 10, 12 = other species of *Trypanosoma*.

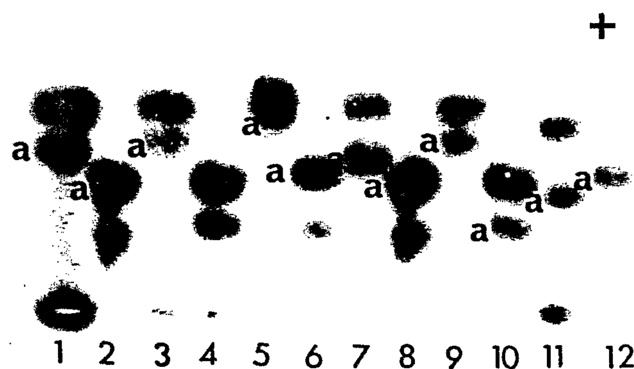

Fig. 5. Zymogram of PEP B. The same stocks are run for PEP A and B. Despite their overlapping substrate specificities comparison with Figure 4 allows correct assignment of the proteins to PEP A and PEP B loci. 'a' = specific PEP A bands. PEP B genotypes are: 1/1 (samples 1: IS 2a, 3 and 9: IS 2e, 5: IS 1) and 2/2 (sample 11: IS 3).

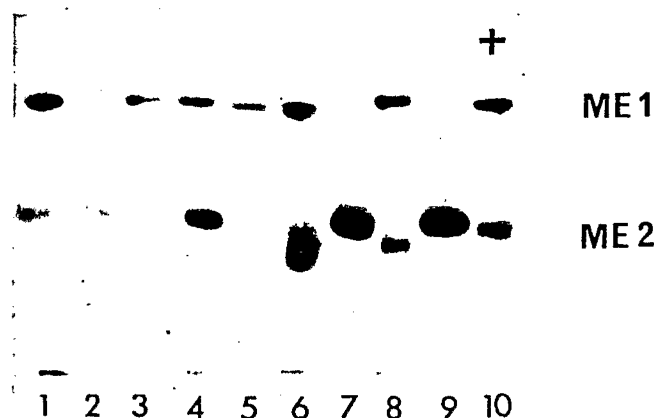

Fig. 6. Zymogram of ME. Both ME1 (the faster locus) and ME2 (the slower one) show variability. ME1: samples 1, 3, 4, 5, 6, 10 = homozygote 2/2; sample 2 (stock A98) = three-banded heterozygote 2/4; sample 8 = homozygote 1/1 (IS 2a); samples 7 and 9 = other species of *Trypanosoma* (not revealed for ME1). ME2: samples 1, 2, 3, 4, 10 = homozygote 2/2; samples 5 (stock A82), and perhaps 6 (another guyanese stock) = 2/5; sample 8 = heterozygote homozygote 3/3 (IS 2a). 1, 3, 4, 10 refer to IS 1e.

zymograms we assume these strains to be heterozygous for the three banded patterns. The observed patterns are consistent with a dimeric structure of the enzyme as was inferred by Jeremiah *et al.* (1982) on the basis of biochemical studies for one of the two enzymes.

Occurrence of natural mixed stocks

Owing to our improved electrophoresis techniques some complex patterns obtained for stocks isolated from a single insect vector are clearly derived from the juxtaposition of the usual patterns observed in different single strains. For example, various patterns of GPI are reliably explained by the mixture of several strains (Fig. 7). The two-banded pattern observed (Fig. 7, n° 6 and 10) consists of two allozymes produced by homozygous genotypes 4/4 (IS 1) and 2/2 (IS 2c) while three bands would be expected in a heterozygote for a dimeric enzyme. The frequency of such natural mixtures can be estimated to be about ten percent in domestic transmission cycles (in prep.). The stock mixtures may also explain some complex patterns noticed by Zillmann and Ebert (1983).

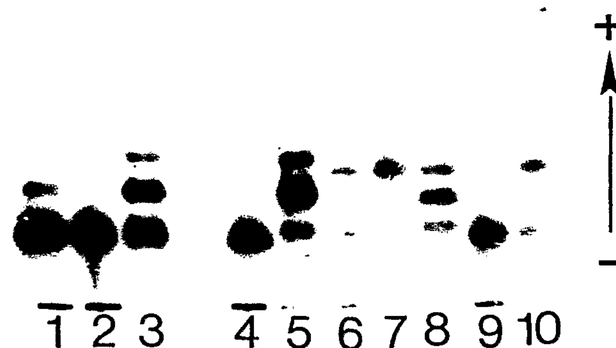

Fig. 7. Zymogram of GPI. 2, 4, 9 = homozygous genotype 4 4 (IS 1); 5 = heterozygous genotype 1 3 (IS 2); 1 and 3 = mixed stocks IS 1 + IS 2; 7 = homozygous genotype 2 2 (IS 2c); 8 = heterozygous genotypes 2 3 (IS 2a); 6 and 10 = mixed stocks IS 1 + IS 2c.

Discussion

Owing to the better resolution given by improved conditions of electrophoresis and the additional loci studied, we have been able to detect more variability than had been formerly detected. The new results concern the occurrence of heterozygous patterns (e.g. GDNadp+, GPI and PGM) or allelic differences (for loci GDNadp+, GDNad+, PEP A, PEP B). The screening of additional stocks (ME, GDNadp+) also supports more genetic variability in *T. cruzi*. To date 13 loci out of the 14 studied were shown to be variable (in prep.). On the other hand, the fact that 50% of the loci exhibit heterozygous patterns strongly supports the hypothesis of diploidy in all *T. cruzi* isozymic strains. Accordingly, the alternative explanations put forward in a previous paper (Tibayrenc *et al.*, 1981a) should be ruled out.

The present work provides, in addition, proof to the hypothesis of the absence of segregation formerly hypothesized in *T. cruzi* (Tibayrenc *et al.*, 1981b). The assumption rests on the absence of apparent homozygous as well as heterozygous genotypes that would be expected to result from segregation.

In view of the numerous alleles detected at the variable loci, one could expect to find a large number of genotypes. This is not the case because of the relatively constant allele association at different loci. The allele associations found at different loci can reliably be separated into three major categories, two of them showing secondary categories (Tibayrenc *et al.*, 1984). Out of the 10^{12} expected genotypic combinations only 13 (Tibayrenc *et al.*, 1984) have been observed so far among more than 400 stocks studied. This departs widely from the theoretical number of genotypes that should be expected for the 13 loci considered in a panmictic population, even though a number of these are highly improbable or could be selected against. The number of stocks and loci analysed suffices to prove that there is no genetic recombination.

Furthermore, some of the allele associations observed are spread over a wide geographic area. For example, isozymic strain 1 occurs in Bolivia and French Guyana as well. Seemingly, isozymic strain 2a occurs in Bolivia, Chile and Brazil. Conceivably, such a wide geographic range should be only consistent with asexual reproduction.

Finally, it is worth emphasizing the situation found in some natural stocks isolated from a single *Triatoma*. The distinctive genotype associations allow assignment of stocks with complex enzyme patterns to represent a mixture of well known strains. The lack of heterozygotes between two different alleles at a single locus, as evidenced for the dimeric enzyme GPI, shows clearly that in spite of mating opportunity within the vector insect, the strains retain their genetic individuality.

Even if they cannot be detected, one can also infer that mixtures of stocks with the same isozymic profile are also possible. Such a situation shows clearly that hypothetical mating types (with either different or identical isozymic profiles) have the opportunity of mating. Nevertheless, the lack of segregation shows clearly that any sexuality can be ruled out.

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