Trypanosoma cruzi strains isolated from human, vector, and animal reservoir in the same endemic region in Mexico and typed as *T. cruzi* I, discrete typing unit 1 exhibit considerable biological diversity

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In this study, three strains of Trypanosoma cruzi *were isolated at the same time and in the same endemic region in Mexico from a human patient with chronic chagasic cardiomyopathy (RyC-H); vector* (Triatoma barberi) *(RyC-V); and rodent reservoir* (Peromyscus peromyscus) *(RyC-R). The three strains were characterized by multilocus enzyme electrophoresis, random amplified polymorphic DNA, and by pathological profiles in experimental animals (biodemes). Based on the analysis of genetic markers the three parasite strains were typed as belonging to* T. cruzi *I major group, discrete typing unit 1. The pathological profile of RyC-H and RyC-V strains indicated medium virulence and low mortality and, accordingly, the strains should be considered as belonging to biodeme Type III. On the other hand, the parasites from RyC-R strain induced more severe inflammatory processes and high mortality (> 40%) and were considered as belonging to biodeme Type II. The relationship between genotypes and biological characteristics in* T. cruzi *strains is still debated and not clearly understood. An expert committee recommended in 1999 that Biodeme Type III would correspond to* T. cruzi *I group, whereas Biodeme Type II, to* T. cruzi *II group. Our findings suggest that, at least for Mexican isolates, this correlation does not stand and that biological characteristics such as pathogenicity and virulence could be determined by factors different from those identified in the genotypic characterization*

Key words: *Trypanosoma cruzi* - genetic characterization - major groups - discrete typing units - pathogenic profile - biodemes

Trypanosoma cruzi is the etiologic agent of Chagas disease affecting approximately 20 million people in Central and South America (Moncayo 1997). *T. cruzi* is a parasitic protozoan that consists of a heterogeneous population composed of a pool of strains circulating in both the domestic and sylvatic cycles in humans, vectors, and animal reservoirs (Souto et al. 1996).

In Chagas disease, different clinical pictures predominate in different areas, e.g., in Brazil, the asymptomatic or indeterminate form is the most common (60-70%), followed by the cardiac and digestive forms (20-30 and 8-10%, respectively); however, in central Brazil and Chile, the digestive form of Chagas disease predominates, whereas it is practically non-existent in Venezuela and Central America (Luquetti et al. 1986, Dias 1992). The reason for this geographical heterogeneity and why different patients develop different clinical forms poses the question of its relationship to the genetic heterogeneity of *T. cruzi* populations in different areas.

Population genetics analysis has shown that *T. cruzi* presents a typical clonal population structure (Tibayrenc et al. 1986). It was later proposed that *T. cruzi* natural clones are distributed into two major phylogenetic lineages (Tibayrenc 1995, Souto et al. 1996), which were named by consensus as *T. cruzi* I and *T. cruzi* II (Anonymous 1999). Recently, it has been proposed that populations of *T. cruzi* can be characterized by various specific isoenzyme markers, suitable for routine identification, and named discrete typing units (DTUs). This represents a genetic subdivision from the two main phylogenetic lineages. It was suggested that different DTUs correspond to distinct epidemiological pictures (Barnabé et al. 2000).

T. cruzi multiclonal populations differs in genetic and biological characteristics and in their behavior in the vertebrate host (Andrade & Magalhaes 1997). Previous studies of the biological and pathogenic characteristics of *T. cruzi* natural strains obtained in different regions by histopathological profile in experimental animals have disclosed the possibility of grouping different patterns of pathogenic behavior in types or biodemes, e.g., Type I, Type II, and Type III (Andrade 1974). This biological be-

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havior in animal models has indeed shown a correlation between *T. cruzi* natural strain behavior and the genetic variability in different geographical areas (Andrade & Magalhaes 1997). Biodeme Type III would correspond to *T. cruzi* major I group, whereas Biodeme Type II, to *T. cruzi* II major group (Anonymous 1999).

Genetic studies are important to clarify the intraspecific heterogeneity of the parasite; however, the ability to study the biological behavior and the host-parasite relationship is crucial for the evaluation of the impact of the genetic diversity of the parasite regarding its relevant medical properties such as pathogenicity and virulence.

In this work we attempt to understand the genetic structure, pathogenic pattern, and epidemiological and clinical implications of *T. cruzi* parasites from an endemic area in Mexico. Mexican strains of *T. cruzi* isolated from human, vector, and animal reservoir at the same time and in the same endemic area were genetically characterized by multilocus enzyme electrophoresis (MLEE), random amplified polymorphic DNA (RAPD), and pathological profiles by biological in vitro and in vivo analysis (biodemes).

MATERIALS AND METHODS

Animals

Mice - A total of 48 BALB/c female mice weighing 25-30 g were used at 10 to 12 weeks of age for each isolate. Mice were maintained in a temperature-controlled environment and provided with a balanced mouse ration and water ad libitum. All animals were maintained in the same facility for a minimum of two weeks before infection.

Parasites - Three *T. cruzi* isolates were obtained, respectively, from feces of adult *Triatoma barberi* (RyC-V), peripheral blood of *Peromyscus peromyscus* (RyC-R), a rodent from the Muridae family, and by xenodiagnostics of human hosts with chronic Chagas disease (RyC-H). The strains were isolated during epidemiological studies in Puebla, Mexico. Isolates were maintained by serial intraperitoneal (ip) passages in 10-week-old BALB/c mice and were grown axenically as epimastigote forms (culture forms) at 28°C in liver infusion-tryptose medium (LIT) (Difco Laboratories, Detroit, MI) supplemented with 10% heat-inactived fetal calf serum (Gibco, Grand Island, NY).

Genetic characterization

MLEE - Methods for isolating, growing in LIT medium, harvesting, and storing the stocks have been previously described (Tibayrenc & Le Ray 1984). The conditions for electrophoresis on cellulose acetate were performed as described by Ben Abde-rrazak et al. (1993) with slight modifications. Twenty enzyme systems were assayed (Barnabé et al. 2000), namely, aconitase (EC 4.2.1.3, ACON), alanine aminotransferase (EC 2.6.1.2, ALAT), diaphorase (EC 1.6.99.2, DIA), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, GAPD), glutamate dehydrogenase NAD^+ (EC 1.4.1.2, GDH-NAD^+), glutamate dehydrogenase $NADP^+$ (EC 1.4.1.4. GDH-$NADP^+$), aspartate amino transferase (EC 2.6.1.1, GOT), glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PD), glucose-6-phosphate isomerase (EC 5.3.1.9, GPI), isocitrate dehydrogenase (EC 1.1.1.42, IDH), leucine aminopeptidase (cytosol aminopeptidase) (EC 3.4.11.1, LAP), malate dehydrogenase (EC 1.1.1.37, MDH), malate dehydrogenase (oxaloacetate decarboxylating, $NADP^+$) or malic enzyme (EC 1.1.1.40, ME), mannose-phosphate isomerase (EC 5.3.1.8, MPI), nucleoside hydrolase (EC 2.4.2.1, NHi); substrate: inosine, peptidase 1 (EC 3.4.22.3) (formerly EC 3.4.4.12), (PEP-1); substrate: leucyl-leucyl-leucine, peptidase 2 (EC 3.4.22.4) (formerly EC 3.3.3.24), PEP-2; substrate: leucyl-L-alanine), 6-phosphoglucomutase dehydrogenase (EC 1.1.1.44, 6PGD), phosphoglucomutase (EC 5.4.2.2) (formerly EC 2.7.5.1.), PGM), and superoxide dismutase (EC 1.15.1.1, SOD).

RAPD - was performed according to Tibayrenc et al. (1993) with slight modifications. Ten different decamer primers from the A-kit of Operon Technologies were used.

Biological characterization

Growth kinetics of epimastigote forms - A total of 25×10^6 parasites was inoculated in a final volume of 50 ml of LIT medium. Cultures were incubated at 27ºC. Kinetics were followed for 24 days. The culture concentration was estimated three times every two days by counting in a Thomas chamber. Analysis of growth kinetics takes into account the doubling time, estimated in hours, calculated from the log phase and the parasite concentration in 10^{-6} parasites per ml (10^{-6} p/ml) and the end of the log phase.

Experimental infection in mice (biodeme) - Experimental infection was performed in 4- or 5-week-old BALB/c female mice. A total of 144 mice were inoculated intradermally with 1×10^4 bloodstream trypomastigotes from infected mice for each strain. Parasitemia was evaluated every three days during the infection by microscopic examination of fresh blood samples obtained from the tail. Parasitemic levels were expressed as logarithms of the media of parasites in peripheral blood of six mice for each group. Mortality rate in relation to infection was evaluated from 7 to 48 days after infection. Morphology of trypomastigotes in peripheral blood was evaluated by the percentage of slender and broad forms, as seen in smears stained with the Giemsa method.

Histopathological analyses - These analyses were performed on 144 of the infected mice corresponding to each *T. cruzi* strain and on 48 uninfected control mice. Mice were killed by cervical dislocation and tissues were obtained each week for 7 weeks. Histological studies were carried out in infected and control animals from skeletal muscle, heart muscle, esophagus, colon, spleen, and liver. Samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and examined by light microscope.

Two serial sections cut from three different parts of each organ were examined. The total surface of the sections was at least 2 cm, and the lesions were recorded and classified according to their severity. The severity of the lesions was generally evaluated with respect to histological characteristics rather than to the number of lesions, which varied between the sections. Each type of lesion was assigned a severity index (scored from 1 to 3) according to its severity and potential pathological consequences.

Statistical analysis - For both MLEE and RAPD data, Jaccard's distance (Jaccard 1908) was used. Each RAPD and MLEE gel band was coded with a number, starting with 1 for the slowest band. The distance was estimated according to the formula:

$$D = 1 - [a/(a + b + c)]$$

where a is the number of bands that are common to the two compared genotypes, b is the number of bands present in the first genotype and absent in the second, and c is the number of bands absent in the first genotype and present in the second.

The UPGMA method (unweighted pair-group method with arithmetic averages) (Sneath & Sokal 1973) was used and for comparative evaluation of pathological profile of mice infected with human, vector and reservoir *T. cruzi* strains a non-parametric Fisher test was applied.

Data analysis of the biological parameters - In a first approach, the average values of all biological parameters were compared by Student's t-test between each of the different strains (human, vector and reservoir T. cruzi strains) genetically characterized by MLEE/RAPD analysis.

RESULTS

Genetic diversity - The phylogenetic picture obtained from 22 isoenzyme loci for the three Mexican strains and seven *T. cruzi* standard strains type into different DTUs are shown in Fig. 1. Parasite strains from human (patient with chronic Chagas disease) RyC-H; reservoir (*P. peromyscus*) RyC-R; and vector (*T. barberi*) RyC-V all belong to *T. cruzi* I, DTU 1 (DTU-1). Thus, *T. cruzi* parasites from human were characterized as HUM/ME/1997/MEX/ RyC-H (*TC* 1); from vector-like VCT/ME/1997/MEX/RyC-V (*TC* 1); and from reservoir-like RES/ME/1997/MEX/ RyC-R (*TC* 1).

Biological characterization - Genetic characteristics of *T. cruzi* parasites isolated from human, reservoir, and vector were analyzed in comparison with the pathological profile in in vitro and in vivo analysis. Several parameters were taken into account and included (a) growth kinetics of epimastigotes forms; (b) curves of parasitemia; (c) morphology of the parasite in the peripheral blood; (d) tissue tropism; (e) histopathological lesions (virulence); and (f) mortality rate of the infected animals.

The growth kinetics of the strains in LIT medium are shown in Fig. 2. By taking the average of six mice, parasitemic peaks were considered as "high" above 500 trypomastigotes and as "low" from 100 to 500 trypomastigotes. Thus, parasites from human and vector hosts showed slow multiplication in LIT medium and low parasitemia. The morphological study of peripheral blood trypomastigotes showed that the human and vector strains presented a predominance of broad forms (70%) by day 28 post-inoculation (pi) and a low percentage of slender forms, while parasites isolated from reservoir show a high multiplication rate with predominance of slender forms (60%) (Table I).

The reservoir strain which showed high parasitemia and predominance of slender forms, determined high mortality rates (31.25%) whereas this parameter was 5 to 10% for the human and vector strains (Fig. 3).

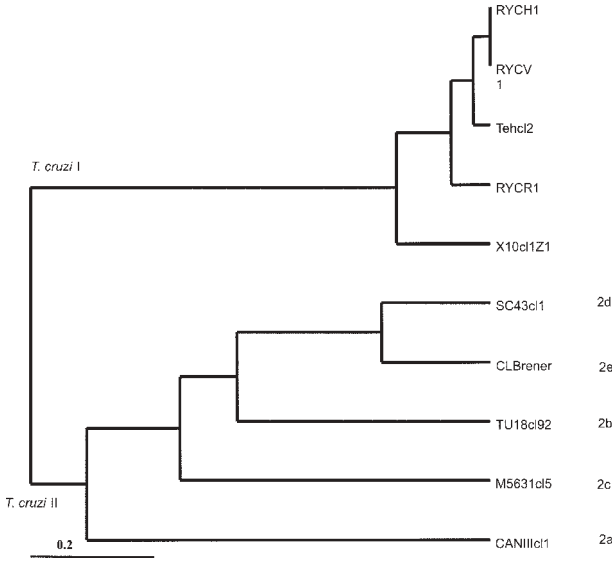

Fig. 1: neighbor-joining dendogram with the Unweighted Pair-Group Method with Arithmetic Average (Sneath & Sokal 1973) and the the scales with Jaccard distances (Jaccard 1908) based on the electrophoretic analysis of 20 isoenzymes showing the genetic relationships among three *Trypanosoma cruzi* strains from the same area (San Andrés Mimiahuapan, Molcaxac, Puebla): human strain (RYCH1), vector strain (*Triatoma barberi*) (RYCT1), and from reservoir (*Peromyscus peromyscus*) (RYCR1) from the same area (San Andrés Mimiahuapan, Molcaxac, Puebla) at the same time, compared with seven strains from different origins: Tehcl1 (vector, *Triatiamo* sp., Tehuantepec, Mexico) and X10 cl1Z1 (human, Brazil) typed as *T. cruzi* I, DTU1 and typed as *T. cruzi* II: DTU2a (human, Brazil) CANIIIcl1, DTU2b (vector, *T. infestans*, Bolivia) TU18cl2, DTU2c (reservoir *Didelphis marsupialis*, Brazil) M5631cl5, DTU2d (vector, *T. infestans*, Bolivia) SC43cl1 and DTU2e (vector, *T. infestans*, Brazil) CLBrener. Parasite strains from human (patient with chronic Chagas disease) RyC-H, reservoir (*P. peromyscus*) RyC-R, and vector (*T. barberi*), RyC-V, all belong to *T. cruzi* I, discrete typing Unit 1 (DTU-1).

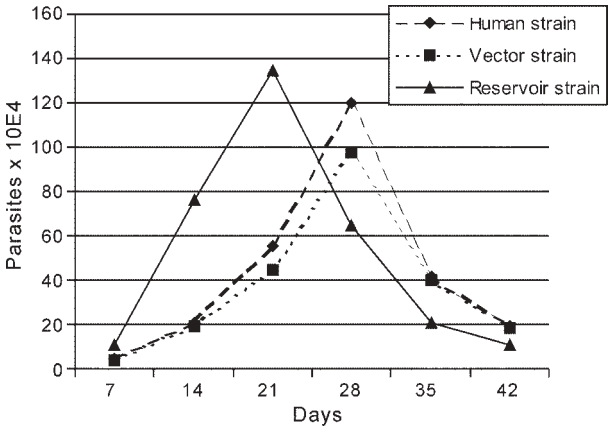

Fig. 2: growth curves of the three strains in LIT medium.

Histopathological study - The study of the tissue lesions and parasite tropism indicated that the reservoir strain determined progressive skeletal muscle and myocardium lesions from the 14th day of infection with slight

TABLE I

Morphobiology of three strains of *Trypanosoma cruzi*

Days	Human strain (parasites × 10^4)			Vector strain (parasites × 10^4)			Reservoir strain (parasites × 10^4)		
	Broad forms (%)	Slender forms (%)	Total	Broad forms (%)	Slender forms (%)	Total	Broad forms (%)	Slender forms (%)	Total
7	3.53 (70.6)	1.47 (29.4)	5	2.96 (74.2)	1.03 (25.8)	3.99	5.88 (55)	4.79 (45)	10.67
14	15.32 (74)	5.37 (26)	20.7	14.22 (73)	5.25 (27)	19.47	42.03 (55)	34.38 (45)	76.41
21	40.39 (73)	14.94 (27)	55.3	33.26 (74)	11.69 (26)	44.9	70.02 (52)	64.63 (48)	134.65
28	78 (65)	41.99 (35)	120	67.38 (69)	30.27 (31)	97.65	34.04 (53)	30.36 (47)	64.40
35	29.58 (71)	12.08 (29)	41.7	27.78 (70)	11.90 (30)	39.68	12.16 (58)	8.80 (42)	20.96
42	13.52 (69)	6.08 (31)	19.6	12.13 (67)	5.96 (33)	18.09	5.92 (57)	4.47 (43)	10.39

Experimental infection from infected mice for each strain of *T. cruzi* from different sources. *T. cruzi* parasites from reservoir (*Peromyscus peromyscus*) RyC-R show highest percentage of slender forms associated with mortality.

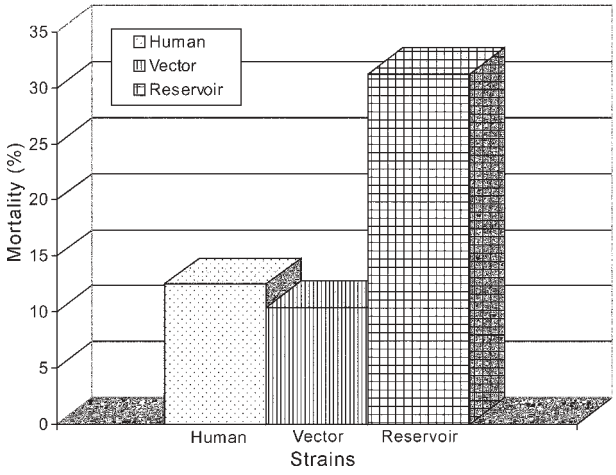

Fig. 3: mortality rate of the three strains in mice was evaluated from seven to 48 days after infection and was expressed as percentage of dead animals, varying from a low (5 to 10%) for human and vector strains (RyC-H and RyC-V), to a high level (31.25%) with the reservoir strain (RyC-R).

mononuclear infiltrates and low parasitism of myocytes. By the 21st day of infection, a progressive parasitism of myocardiocytes and destruction of myocells with dense inflammatory infiltration was seen, with focal myocyte necrosis and diffuse mononuclear cell infiltration. Tissue parasitism was prominent in skeletal muscles and progressive until 24 days of infection.

Histopathological lesions determined by human and vector *T. cruzi* strains from 14 to 30 days of infection showed the same evolution and histopathological aspects. In general, the parasitism and inflammatory infiltration were present at 14 and 20 days pi, varying from slight to moderate. Lesions were more prominent in cardiac muscles and intensified up to the 28th day of infection. Analysis of the severity of tissues lesions showed an intense inflammatory infiltrate, which was clearly more severe in BALB/c mice infected with parasites from reservoir RyC-R than human RyC-H and vector RyC-V, *T. cruzi* strains.

In summary, the biological profiles show a different pathogenic pattern among *T. cruzi* strains. The human and vector *T. cruzi* strains show low mortality and a slow increase of parasitemia with peaks of parasitemia between 26 and 28 days pi associated with a profile characteristic of the biodeme type III (Table II). Parasites isolated from the reservoir show parasitemic profiles of the biodeme type II with a mild multiplication of parasites and with peaks of parasitemia between 18 and 21 days pi and also show high mortality (Table II).

DISCUSSION

Genetic and biological characteristics of the *T. cruzi* strains represent a tool for the understanding of different aspects of the epidemiology Chagas disease. Populations of *T. cruzi* are highly heterogeneous and by using genetic markers were distributed into two major phylogenetic lineages (Tibayrenc 1995, Souto et al. 1996), which were named as *T. cruzi* I and *T. cruzi* II (Anonymous 1999). Studies carried out in Southern Cone Countries of South America indicate that *T. cruzi* I is dominant in the sylvatic cycle and *T. cruzi* II is dominant in the domestic cycle of the parasite transmission (Zingales et al. 1998, Diosque et al. 2003). Very few data on the epidemiological distribution of the two *T. cruzi* major groups are available for the other countries of the Americas e.g. Mexico (Zavala Castro et al. 1992, Espinosa et al. 1998) and Central America (Higo et al. 2000). In this study, three Mexican *T. cruzi* parasites from human, vector and reservoir were genetically identified as belonging to *T. cruzi* I; moreover, high genetic identity was shown between them (Jaccard's distances < 0.20). This observation is in agreement with the data showing the predominance of *T. cruzi* I in stocks isolated from 8 of the 32 states of the Mexican Republic (Bosseno et al. 2002).

TABLE II

Characteristic of the isolates of human, vector and reservoir of *Trypanosoma cruzi* from the state of Puebla, Mexico

Name	RyC-H	RyC-V	RyC-R
Date	VII/98	IV-98	VIII/98
Biological source	Patient with chronic chagasic cardiomyopathy	*Triatoma barberi*	*Peromyscus peromyscus*
Geographical source	Molcaxac, Puebla, Mexico	Molcaxac, Puebla, Mexico	Molcaxac, Puebla, Mexico
Mortality rate in mice	12.5%	10.4%	31.25%
Peak of growth	28 days	28 days	21 days
Morphology	Broad forms (70%)	Broad forms (70%)	Slender forms (70%)
Tissue tropism	Skeletal muscle	Cardiac muscle	Cardiac muscle
Histopathogenicity	Cardiac muscle	Cardiac muscle	Cardiac muscle
Biodemes	III	III	II
Genetic characterization	Lineage1, DTU-1	Lineage 1, DTU-1	Lineage 1, DTU-1

Study of the various biological parameters in response to infection by strains of *T. cruzi* show clear differences in response to infection by strains from different sources. *T. cruzi* parasites from human (with chronic chagasic cardiomyopathy) and vector did not induce a significant inflammatory process in the heart and muscles. In contrast, *T. cruzi* parasites from reservoir induce an intense inflammatory process in the heart and skeletal muscles.

T. cruzi II has been shown to be heterogeneous and different genetic subdivisions have been described, named DTUs (Brisse et al. 1998). It was suggested that different DTUs correspond to distinct epidemiological pictures and are proposed as a reference framework for genetic variability and for biological characterization studies of *T. cruzi* strains (Barnabé et al. 2000). The geographical distribution of the six DTUs is present in the entire range of Chagas disease. On the other hand, DTU 2b has been mainly isolated in Brazil and Chile, while DTU 2d was mainly encountered in Chile and Bolivia (Barnabé et al. 2000). In this study, the three *T. cruzi* strains isolated in the same region from human, domestic and wild transmission cycles were characterized by multigene typing as belonging to DTU 1 and exhibited strong levels of linkage. *T. cruzi* I (DTU 1) has been considered a homogeneous group. The findings of the present study show clear biological differences between the three strains regarding the profile of parasitemia, tissue tropism, pathogenicity pattern, and mortality. In fact, *T. cruzi* parasites from human and vector (belonging to biodeme Type III) did not induce a significant inflammatory process in the heart and muscles. In contrast, *T. cruzi* parasites from the reservoir (belonging to biodeme Type II) induced an intense inflammatory process in the heart and skeletal muscles.

According to Andrade and Magalhães (1997), although genetic studies are important to clarify the intraspecific heterogeneity of the parasite, only the study of the biological behavior could clarify the importance of different strains in the determination of clinicopathological manifestations of Chagas disease. Our results could be explained, in part, by the fact that many studies have been performed using *T. cruzi* strains isolated for more than 20 years and that genetic intrinsic characteristics and clonal selection could be influenced by handling in the laboratory. Although experimental evidence of the stability of strain behavior has been obtained by biochemical and biological characterization after parasites were subjected to different conditions of maintenance and cultivation, the main biological characteristics of the strains, such as infectivity and inoculum size, tended to decrease in association with maintenance and cultivation conditions (Magalhaes et al. 1985). Decrease in virulence has been observed when the culture forms were used or when the infection with low inoculum was employed. Additionally, the passage of *T. cruzi* parasites through the vector has a positive influence on the virulence (higher levels of parasitemia and predominance of slender forms in mice) (Magalhaes et al. 1996). In this work we employed *T. cruzi* parasites recently isolated from the same endemic area. This procedure would guarantee the reliability of the comparative studies.

Infection with *T. cruzi* is an enzootic disease extending from the US to South America (sylvatic cycle), that can lead to human infection when the insect vector (triatomine bugs) adapts to human dwelling (domestic cycles). This adaptation occurs in different manners and at different times (Prata 2001). The presence of different types of *T. cruzi* strains in endemic areas may bear influence on the biological characteristics as well as pathogenicity. Studies with cloned populations of natural strains have demonstrated either homogeneity or heterogeneity of several clones including differences in virulence and pathogenicity. Thus, in an epidemiological study, cloned populations cannot be taken as representative of the strains isolated from different geographical areas.

The expert committee in 1999 recommended that isolates typed as Biodeme Type III (Andrade 1974) are equivalent to *T. cruzi* I, and isolates typed as Biodeme Type II are equivalente to *T. cruzi* II (Anonymous 1999), our data

clearly show differences in the biological behavior of three strains classified as *T. cruzi* I, although *T. cruzi* I is considered a homogeneous group based on the analysis of genetic markers. Therefore, our *T. cruzi* I isolates were typed as Biodeme Type II and Type III.

In conclusion, our findings suggest that for Mexican isolates, characteristics such as pathogenicity and virulence of *T. cruzi* strains must be described by a combination of genetic and biological characteristics. Reliance on the genotype alone may confer significance of little biological relevance and could be determined by factors different from those identified in the genotypic characterization.

REFERENCES

Andrade SG 1974. Caracterização de cepas do *Trypanosoma cruzi* isoladas no Recôncavo Bahiano. *Rev Patol Trop 3*: 65-121.

Andrade SG, Magalhaes JB 1997. Biodemes and zymodemes of *Trypanosoma cruzi* strains: correlations with clinical data and experimental pathology. *Rev Soc Bras Med Trop 30*: 27-35.

Anonymous 1999. Recommendatiosn from a satellite meeting. *Mem Inst Oswaldo Cruz 94* (Suppl. 1): 429-432.

Barnabé C, Brisse S, Tibayrenc M 2000. Population structure and genetic typing of *Trypanosoma cruzi*, the agent of Chagas disease. A multilocus enzyme electrophoresis approach. *Parasitology 120*: 513-526.

Ben Abde-rrazak S, Guerrini F, Mathieu-Daudé F, Truc P, Neubauer K, Lewicka K, Barnabé C, Tibayrenc M 1993. Isoenzyme electrophoresis for parasite characterization. *Methods Mol Biol 21*: 361-382.

Bosseno MF, Barnabé C, Magallón-Gastélum E, Lozano-Kasten F, Ramsey J, Espinoza B, Breniére SF 2002. Predominance of *Trypanosoma cruzi* lineage I in Mexico. *J Clin Microbiol 40*: 627-632.

Brisse S, Barnabé C, Bañuls AL, Sidibé I, Noël S, Tibayrenc M 1998. A phylogenetic analysis of the *Trypanosoma cruzi* genome Project CL Brener reference strain by multilocus enzyme electrophoresis and random amplified polymorphic DNA fingerprinting. *Mol Biochem Parasitol 92*: 253-263.

Dias JC 1992. Epidemiology of Chagas disease. In S Wendel, Z Brener, ME Camargo, A Rassi (eds), *Chagas Disease (American Trypanosomiasis): Its Impact on Transfusion and Clinical Medicine*, International Society of Blood Transfusion, São Paulo, p. 49-80.

Diosque P, Barnabé C, Padilla AM, Marco JD, Cardozo RM, Cimino RO, Nasser JR, Tibayrenc M, Basombrio MA 2003. Multilocus enzyme electrophoresis analysis of *Trypanosoma cruzi* isolates from a geographically restricted endemic area for Chagas' disease in Argentina. *Int J Parasitol 33*: 997-1003.

Espinoza B, Vera-Cruz JM, Gonzalez H, Ortega E, Hernandez R 1998. Genotype and virulence correlation within Mexican stocks of *Trypanosoma cruzi* isolated from patients. *Acta Trop 70*: 63-72.

Higo H, Yanagi T, Matta V, Agatsuma T, Cruz-Reyes A, Uyema N, Monroy C, Kanbara H, Tada I 2000. Genetic structure of *Trypanosoma cruzi* in America continents: special emphasis on sexual reproduction in Central America. *Parasitology 121*: 403-408.

Jaccard P 1908. Nouvelles recherches sur la distribution florale. *Bull Soc Vaudoise Sci Nat 44*: 223-270.

Luquetti AO, Miles MA, Rassi A, De Rezende JM, De Souza A, Povoa M, Rodriguez L 1986. *Trypanosoma cruzi*; zymodeme asosociated with acute and chronic Chagas' disease in central Brazil. *Trans R Soc Trop Med Hyg 80*: 462-470.

Magalhaes JB, Andrade SG, Sherlock I 1996. *Trypanosoma cruzi* strains: behavior after passage into authoctonous or foreign species of traitomine (biological and biochemical patterns). *Rev Inst Med Trop São Paulo 38*: 23-28.

Magalhaes JB, Pontes AL, Andrade SG 1985. Behavior of the Y and Peruvian strains of *Trypanosoma cruzi* in mice, after passage through various media. *Mem Inst Oswaldo Cruz 80*: 41-50.

Moncayo A 1997. Progress towards the elimination of transmission of Chagas disease in Latin America. *World Health Stat Q 50*: 195-198.

Prata A 2001. Clinical and epidemiological aspects of Chagas disease. *Lancet Infect Dis 1*: 92-100.

Revollo S, Oury B, Laurent JP, Barnabé C, Quesney V, Carriere V, Noel S, Tibayrenc M 1998. *Trypanosoma cruzi*: impact of clonal evolution of the parasite on its biological and medical properties. *Exp Parasitol 89*: 30-39.

Sneath HA and Sokal RR 1973. *Numerical Taxonomy. The Principles and Practice of Numerical Classification*, WH Freeman & Co., San Francisco, 573 pp.

Souto RP, Fernandes O, Macedo AM, Campbell DA, Zingales B 1996. DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi*. *Mol Biochem Parasitol 83*: 141-152.

Tibayrenc M 1995. Population genetics of parasitic protozoa and other microorganisms. *Adv Parasitol 36*: 47-115.

Tibayrenc M, Le Ray D 1984. General classification of the isoenzyme strains of *Trypanosoma (Schizotrypanum) cruzi* and comparison *T. (S.) c. marinkellei* and *T. (Herpetosoma) rangeli*. *Ann Soc Belge Méd Trop 64*: 239-248.

Tibayrenc M, Neubauer K, Bernabé C, Guerrini F, Skarecky D, Ayala FJ 1993. Genetic characterization of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. *Proc Natl Acad Sci USA 90*: 1335-1339.

Tibayrenc M, Ward P, Moya A, Ayala F 1986. Natural populations of *Trypanosoma cruzi*, the agent of Chagas disease, have a complex multiclonal structure. *Proc Natl Acad Sci USA 83*: 115-119.

Zavala-Castro JE, Velasco-Castrejón O, Hernández R 1992. Molecular characterization of Mexican stocks of *Trypanosoma cruzi* using total DNA. *Am J Trop Med Hyg 47*: 201-209.

Zingales B, Souto RP, Mangia RH, Lisboa CV, Campbell DA, Coura JR, Jansen A, Fernandes O. 1998. American Trypanosomiasis in Brazil based on dimorphism of rRNA and mini-exon sequences. *Int J Parasitol 28*: 105-112.