Biological Comparison between Three Clones of *Trypanosoma cruzi* and the Strain of Origin (Bolivia) with Reference to Clonal Evolution Studies

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After isolating three clones of Trypanasoma cruzi *(Bolivia), we first characterized them according to parasitaemia, pleomorphism and virulence, and then histopathologically. The study's interest lies on the hypothesis that clonal evolution of* T. cruzi *has a major impact on biologically relevant properties of this parasite. Data obtained from the studies of parasitaemia, pleomorphism and virulence showed no differences between the groups studied. As a final point, the histopathological study shows us a muscular tissue tropism both in clones and in their mother strain (Bolivia). In this paper, we conclude that Bolivia strain and clones isolated from it, pertaining to the same major clone share similar biological properties.*

Key words: *Trypanosoma cruzi* - histopathological study - clonal variability - biological characterization

The high biochemical heterogeneity of the causal agent of Chagas' disease, *Trypanasoma cruzi* , has been known for some time, it manifests a great diversity of medical and biological properties which in turn could be the origin of such clinical variability in the disease (Morel et al. 1980, Dvorak 1984, Tibayrenc et al. 1986, Tibayrenc & Ayala 1991).

Extensive genetic studies of populations show the multiclonal structure of natural populations of *T. cruzi,* (Tibayrenc et al. 1986, Tibayrenc & Ayala 1988), which might be the reason for such varied behaviour. The distinctive characteristics of clonal lines are potentially important to the knowledge of human disease, particularly when groups of clones are genetically heterogenous, as happens in the case of *T. cruzi.* Having obtained a serie of clones and compared them with the mother strain of *T. cruzi* (Bolivia) for parasitaemia, morphology, virulence and particularly histopathology (tissular tropism), we have tried to verify the hypothesis that the clones behave as independent genetic entities.

MATERIALS AND METHODS

Strain of T. cruzi - Bolivia strain was isolated from *Triatoma vitticeps* captured in Vittichi (Bolivia) in 1971. This strain has been maintained frozen in our laboratory. The strain belongs to "major clone" 20, according to Tibayrenc and Ayala's classification (1988), where natural populations of *T. cruzi* are subdivided into clones, of which a few are able to spread unchanged over large geographical areas and long periods of time, these are the "major clones", the authors express the hypothesis that the major clones play an important role in Chagas' diseases epidemiology and pathogenicity.

Cloning procedure - A drop of blood from a mouse's tail was put into 1ml of sterile saline solution, a microdrop of the homogenized solution was placed on a cover slip which was then inverted over a concave slide and examined under the microscope. The drop filled a microscopic field with objective of 40X.

When a single tripanosoma was seen, it was washed with a saline solution, inoculated in the medium of N.N.N. culture, and incubated at 26^{o}C. Observation began at tenth day postinoculation.

Experimental animals - Male mice *Mus musculus* of the Swiss strain Ico (OF1: IOPS Caw), maintained under the following conditions: photoperiod: 12 hr light/12 hr darkness, temperature; 21^{o}C ± 1^{o}C, humidity: 55 ± 10%, air renewal: 10 to 15 changes/hr.

Parasitaemia study - Beginning at 7th day postinoculation, a parasitaemia study was done at weekly intervals in thick film (Lumsden et al. 1973). 100 microscopic fields were observed with an objective of 100X, and the number of blood forms counted. This was done in sets of mice for each one of the clones obtained and also for the original strain during the acute phase of nine weeks.

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With the data obtained parasitaemia curves were done using arithmetic mean and standard deviation.

Virulence study - The virulence of each clone and of the original Bolivia strain was evaluated by determining the mortality of inoculated mice, following the experiments of Phillips (1960) and Andrade and Andrade (1968).

Histopatological study - Three sets of mice were sacrificed for each of the clones and the Bolivia strain at intervals of seven days during the acute infection phase starting at the 7th day of infection.

The following organs were extracted from each mouse: heart, brain, skeletal muscle (quadriceps), spleen, liver and colon. All the organs were placed in jars of saline formol 10%. The organs were observed macroscopically before being cut up and placed in paraffin. Subsequently, cuts of 5 µm were made in each organ and dyed with Hematoxilin-Eosin.

Once dyed, the cuts in each organ were examined microscopically for inflammatory lesions and/or pseudocysts, and graded on a 3-point scale:
less than 25% of affected tissue: +
between 25% and 50% of affected tissue: ++
more than 50% of affected tissue: +++
In the heart specimens a more detailed study was carried out with the different cardiac chambers being examined for inflammatory lesions and/or pseudocysts.

RESULTS

Blood study: examination of the parasitemia - In the Bolivia strain this becames measurable after the second week, reaching its maximum peak at 28 days with more than 100 parasites in 100 microscopic fields with 100X objective. This parasitaemia fell sharply becoming negative on day 56.

The three clones became positive at the 7th day and reached their maximum peak at day 21, Clone II being the one that reached the greatest parasitaemia with approximately 450 parasites in 100 microscopic fields. In Clone II parasitaemia fell sharply at day 35 becoming negative at day 42. With Clone III the same thing happened, becoming negative at day 49. Clone I fell more gradually, becoming undetectable at day 63 (Fig. 1).

Virulence study - Mortality commenced between 21 and 28 days postinoculation, and reached 40%-60% in every case at day 63 (Fig. 2).

Histopathological study - In the anatomopathological study of sections of the heart, brain, liver, spleen, skeletal muscle and colon of mice infected with *T. cruzi* (Bolivia) and clones I, II and III isolated from it, notable similarity in tissular tropism could be seen.

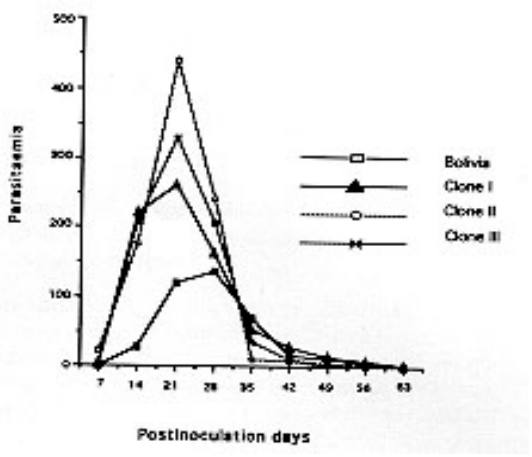

Fig. 1: parasitaemia evolution among *Trypanosoma cruzi* strain Bolivia, Clone I, Clone II and Clone III.

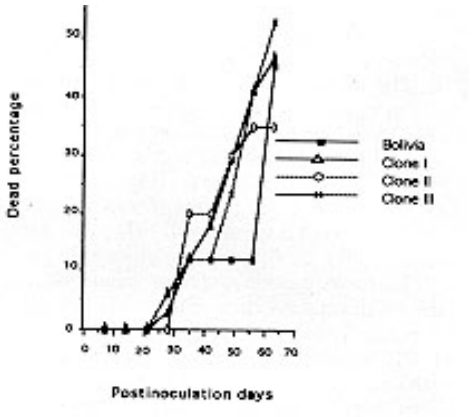

Fig. 2: percentage comparative of mortality among *Trypanosoma cruzi* strain Bolivia, Clone I, Clone II and Clone III.

In the Bolivia strain (Table I) inflammatory lesions appear principally in the liver, heart and muscle, the more important being in the latter two. Hearts and skeletal muscles disclose diffuse and meanly focal inflammatory infiltration with macrophages and in the last days lymphocytes and plasma cells. Pseudocysts were observed mainly in the heart (Fig. 3) and muscle. In this case, the presence of *T. cruzi* amastigotes was coincident with myocardial fiber degeneration and focal mononuclear infiltration. Isolated hyaline necrosis of non-parasitized myocardial fibers was also seen. Both, the inflammation and the presence of the parasite occurs meanly in the heart muscle, occupying in this case from 25% to 50% of the area and in the middle

TABLE I

Histopathological study of *Trypanosoma cruzi* (Bolivia strain)

Postinfection days		7	14	21	28	35	42	49	56	63
Brain	Inflammation	-	-	-	-	-	-	-	-	+
	Pseudocysts	-	-	-	-	-	-	-	-	-
Heart	Inflammation	-	-	++	++	++	++	+++	++	+
	Pseudocysts	-	-	++	++	+++	+++	++	++	+
Liver	Inflammation	+	+	+	+	+	+	+	+	+
	Pseudocysts	-	-	-	-	-	-	-	-	-
Spleen	Inflammation	-	-	-	-	-	-	-	-	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Colon	Inflammation	-	-	-	-	-	-	-	-	-
	Pseudocysts	-	+	-	-	-	-	-	-	-
Skeletal muscle	Inflammation	-	-	+	+	+	+	+	+	+++
	Pseudocysts	-	+	+	+	+	+	+	++	++

+ < 25%; ++ between 25% and 50%; +++ > 50% of the surface affected

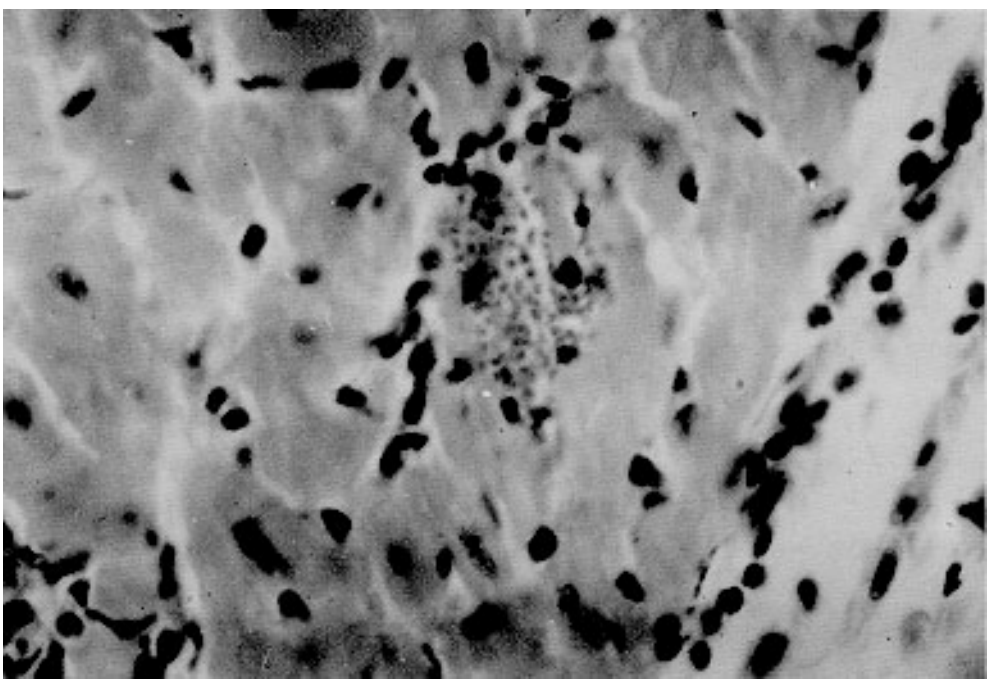

Fig. 3: amastigotes of *Trypanosoma cruzi* into myocardic fiber of mice infected with Bolivia strain. H/E X 400.

of the acute phase, more than 50%.

In Clone I (Table II), however, the inflammatory effect on the heart is very slight, and altogether absent in the liver; the predominant effect is muscular, with inflammatory infiltration in the brain and colon, and pseudocysts in the muscular layer of the latter organ. The heart pieces infected with Clone I showed the presence of the parasite only in the 49 and 56 days and in every case the foci occupied areas smaller than 25% of the total heart area.

TABLE II

Histopathological study of *Trypanosoma cruzi* (Clone I)

Postinfection days		7	14	21	28	35	42	49	56	63
Brain	Inflammation	-	-	-	-	+	+	+	+	+
	Pseudocysts	-	-	-	-	-	-	-	-	-
Heart	Inflammation	-	-	+	+	+	++	++	+	+
	Pseudocysts	-	-	-	-	-	-	+	+	-
Liver	Inflammation	-	-	-	-	-	-	-	-	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Spleen	Inflammation	-	-	-	-	-	-	-	-	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Colon	Inflammation	-	-	-	+	+	+	-	-	-
	Pseudocysts	-	-	-	+	++	+	-	-	-
Skeletal muscle	Inflammation	-	++	+	+	+++	++	++	+	+
	Pseudocysts	-	-	+	+	++	++	-	-	-

+ < 25%; ++ between 25% and 50%; +++ > 50% of the surface affected

Clone II (Table III) shows little alteration in the heart and slightly more in the muscle (Fig. 4), though never more than 25% of the affected area. Pieces infected with this Clone II showed also isolated hyaline necrosis of non-parasitized myocardial fibers. This clone had as a peculiarity an hepatic inflammation throughout the study.

Clone III (Table IV) shows cardiac and muscle alterations, in addition to cerebral inflammation which was evident in 78.6% of the mice studied.

TABLE III

Histopathological study of *Trypanosoma cruzi* (Clone II)

Postinfection days		7	14	21	28	35	42	49	56	63
Brain	Inflammation	-	-	-	-	+	-	-	-	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Heart	Inflammation	-	+	+	+	+	+	+	-	-
	Pseudocysts	-	+	+	+	-	-	-	-	-
Liver	Inflammation	+	+	+	+	+	+	+	-	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Spleen	Inflammation	-	-	-	-	-	-	-	-	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Colon	Inflammation	-	-	-	-	-	-	-	-	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Skeletal muscle	Inflammation	-	-	+++	++	+	+	+	-	-
	Pseudocysts	-	-	+	+	+	+	+	-	-

+ < 25%; ++ between 25% and 50%; +++ > 50% of the surface affected

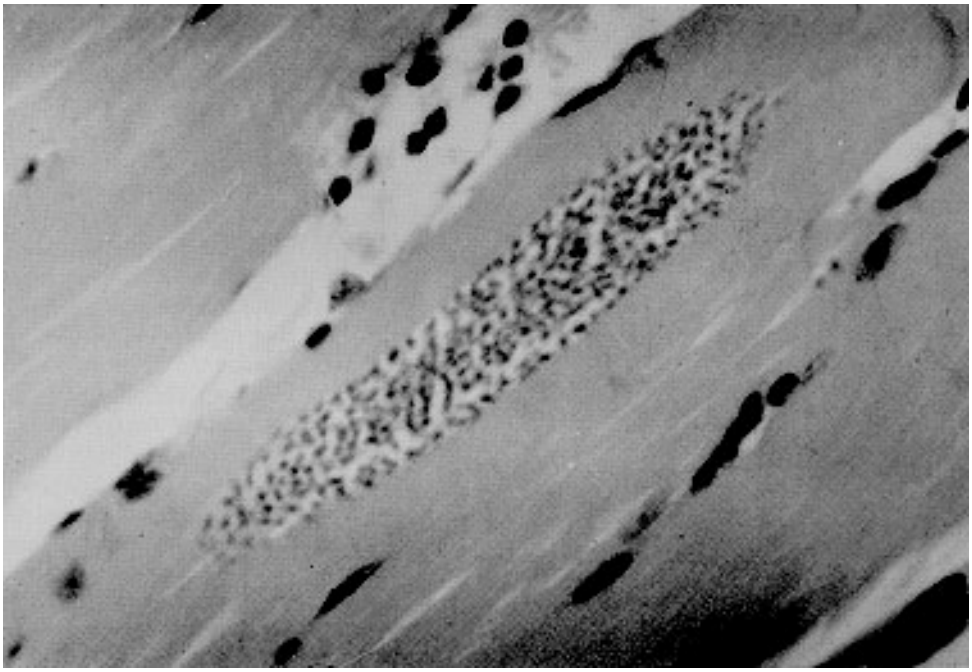

Fig. 4: inflammatiory infiltration and pseudocyst in skeletal mucle of mice infected with Clone II of *Trypanosoma cruzi*. H/E X 400.

TABLE IV

Histopathological study of *Trypanosoma cruzi* (Clone III)

Postinfection days		7	14	21	28	35	42	49	56	63
Brain	Inflammation	-	+	+	+	+	+	+	+	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Heart	Inflammation	-	-	+	++	++	+	+	+	-
	Pseudocysts	-	-	+	+	+	-	-	-	-
Liver	Inflammation	-	+	-	-	-	-	-	-	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Spleen	Inflammation	-	-	-	-	-	-	-	-	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Colon	Inflammation	-	-	-	-	-	-	-	-	-
	Pseudocysts	-	-	-	-	-	-	-	-	-
Skeletal muscle	Inflammation	-	+	++	+++	++	++	++	++	-
	Pseudocysts	-	+	++	++	+	+	-	-	-

+ < 25%; ++ between 25% and 50%; +++ > 50% of the surface affected

Due to the possible existence of non-specific lymphocytary proliferation we have to be very cautious with the interpretation of the inflammatory data (De Diego et al. 1991). Hearts and skeletal muscles disclose diffuse and focal inflammatory infiltration with macrophages, eosinophils, lymphocytes and plasma cells, never occupying areas bigger than 50% of the total tissue.

Both, in clones and in Bolivia strain, in heart lesions changes were most marked in the ventricle

and varied in intensity and extension from case to case. Infiltration with polymorphonuclear eosinophils was identified in several cases.

DISCUSSION

Trypanosoma cruzi, the etiological agent of Chagas' disease, shows great biochemical variability which enables a large variety of medical and biological properties to develop, depending on its geographic distribution. Many strains also develop variations over time (Morel et al. 1980, Dvorak 1984, Tibayrenc et al. 1986, Tibayrenc & Ayala 1991).

The general objective of this work has been to verify the hypothetical correlation between natural clones and their genetic variability.

Specifically, a comparative study has been done between three clones from Bolivia strain isolated from their natural vector; these clones have been compared among themselves and each of them with the mother strain, observing if they behave as independent genetic entities.

The high biochemical and medical heterogeneity of *T. cruzi* has already been shown (Dvorak 1984, Brenière et al. 1984, Tibayrenc et al. 1986, Tibayrenc & Ayala 1988, Tibayrenc et al. 1990), the principal question being whether the high clonal diversity might be responsible for all or part of this variability (Tibayrenc & Ayala 1987).

This genetic heterogeneity can be expressed phenotypically in different ways, such as in their different degrees of virulence. Differences in phenotypical variation can be detected in the antigenic composition of various strains of *T. cruzi.* When the clones are isolated from various strains and compared, they differ according to their antigenic composition, sequences of nucleotides of k-DNA, electrophoresis of isoenzymes, virulence, histotropism, and pathology.

In this work, a blood study was done, and in all cases the pattern of evolution of parasitaemia was similar.

No important variations were observed in virulence of any of the clones, or in the mother strain Bolivia, although recent studies in the cloning of *T. cruzi* show differences related mainly with the degree of virulence between those isolated from the mother strain (Andrade et al. 1985, Postan et al. 1987).

Blood pleomorphism of Bolivia strain has an absolute predominance of stout forms during the acute phase, which conforms to the characteristics of Andrade's group II (1974). Previous classifications by Brener and Chiari (1963) referring only to blood pleomorphism do not find these characteristics. However, research by Ribeiro et al. (1988) corroborates the predominance of stout forms in the Bolivia strain in infections in Swiss mice.

In our study, in Bolivia strain as in their isolated clones, as infection time passes, stout forms become a majority; this fact would make them fit within the second group of Brener and Chiari (1963), which gathers the strains with a predominance of stout forms and agrees with that described by Andrade (1974) for her group II.

In Clone III the last day the number of stout forms fall, with the slender forms increasing simultaneously, as described by Dvorak (1976) who finds that the morphogenesis of blood forms begins with a predominance of broad-stout, giving way to slender as happens in our study.

We can thus summarise that both Bolivia strain and clones show relatively slow multiplication with irregular peaks of parasitaemia, virulence with mean mortality into the acute phase and predominance of stout forms that fit within the framework of Andrade's group II (1974).

The histopathological study has demonstrated a clear tendency towards muscular tissue both in clones as in the Bolivia strain. However, we find some specific differences as follows: (a) clones II and III have a skeletal muscular tropism, (b) Bolivia strain is plainty cardiac, (c) clone I displays both muscular and cardiac alterations, with an important effect in the intestine. These preferences have been established as a function of the intensity of the presence of *T. cruzi* in each organ. Despite the strains' predilection for certain types of tissue, they can invade other tissues (Koberle 1968, Hoare 1972, Braun & Titto 1985).

Both the mother strain and the clones that fall within the framework of Andrade's Group II (1974) in their blood pleomorphism, exhibit the pattern of typically cardiac histopathological alterations, notwithstanding inflammatory lesions and pseudocysts that develop in other organs.

As a result we find that there are differences between the mother strain *T. cruzi* (Bolivia) and clones I, II, and III; this could be explained by considering that: (1) strain may have pluripotencial pathogenic effects; (2) changes in histopathogical patterns are considered to depend as much on the characteristics of the host as on the parasite (Postam et al. 1987); as tropism results from interaction between parasite and host membranes, an alteration of either of these characteristics could change the tropism.

To conclude, from a morphological, parasitological, anatomopathological and virulence study, there are great similarities between *T. cruzi* (Bolivia) and its clones I, II, and III. Indeed, it is worldwide known that when strains or isolates remain in a particular environment, some clones are selected. Thus it can be possible that our Bolivia strain could

be the result of a natural selection of its clones. In addition, Bolivia strain was checked by enzime profiles and these were similar to other included in one of the "major clones" studied by Tibayrenc and Ayala (1988), and very different from those of other major clones (unpublished data), being the underlining data that all stocks pertaining to a given major clone, independly of their geographical and host origin, could share common biological properties that could be radically different from the properties of other clones.

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