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DIFFERENCE IN SUSCEPTIBILITY TO LYSIS BETWEEN CLONES OF THE Y STRAIN OF *TRYPANOSOMA CRUZI*

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Three clones isolated from the Y strain of Trypanosoma cruzi *– YP1, YP2 and YP3 – were adapted to* in vitro *cultivation in VERO cells. The recovery of the parasites from the Y strain and clone YP3 was similar after 24 hr of contact with cells (3.2% and 2.7%, respectively) and much lower than the recovery of clones YP1 and YP2 (56.7% and 60.0% of inoculum, respectively). After five days incubation, the ratio Trypomastigotes/Amastigotes released into the supernatants was about 90/10 for clone YP1, YP3 and Y strain, and 50/50 for clone YP2. After nine days, the ratio was 62/38 for clone YP1, 97/3 for clone YP3, 81/19 for Y strain and 50/50 for clone YP2.*

The susceptibility of tissue culture derived trypomastigotes (TCT) to lysis in the presence of chronic chagasic human sera and human complement was assessed using Complement Mediated Lysis reaction (CML). Trypomastigotes from clone YP2 were consistently less susceptible to CML (% lysis less than 20), than parasites from the other clones and Y strain. Parasites of clone YP3 had susceptibility to CML comparable to that of the Y strain (about 70%), while TCT of clone YP1 had intermediary susceptibility (40%).

Key words: *Trypanosoma cruzi* – clones – tissue culture – complement mediated lysis

There is ample evidence that *Trypanosoma cruzi* (Chagas, 1909), the causative agent of Chagas' disease, consists of a pool of parasite populations which circulate among man, vectors, sylvatic reservoirs and domestic animals (Miles, 1979). Heterogeneity in regard to morphology, virulence, pathogenicity and tissue tropism, has been found not only among several strains but also among different clones from a single strain (Engman et al., 1987). In addition to the usual heterogeneity found within strains isolated from different hosts, the prolonged maintenance and variability in handling of the parasites in laboratory may interfere with the original characteristics of the isolates. The study of cloned *T. cruzi* populations is needed to avoid misinterpretation of events which may pertain to the parasite alone.

It has been demonstrated that clones of *T. cruzi* present moderately stable differences in the growth kinetics (Crane & Dvorak, 1981; Engel et al., 1982; Gomes et al., 1991); total DNA content/organism (Dvorak et al., 1982), virulence and pathology to mice (Postan et al., 1983) as well as infectivity to vertebrate cells (Doyle et al., 1984); isoenzyme pattern (Romanha et al., 1981; Goldberg & Silva Pereira, 1981; Breniere et al., 1991); kDNA restriction mapping (Morel et al., 1980) and antigenic composition (Bongertz & Dvorak, 1983; Kirchhoff et al., 1984; Zingales et al., 1984; Plata et al., 1984; Lima & Villalta, 1989).

Marques de Araújo (1985) demonstrated that three clones derived from the Y strain obtained according to method described by Goldberg & Chiari (1980) were very distinct when inoculated into C_3H mice and evaluated for morphology of bloodstream trypomastigotes, infectivity, parasitemia levels and mortality.

Clones YP1, YP2 and YP3 and parental Y strain were adapted by us to growth in tissue

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culture and analyzed comparatively for interaction with VERO cells *in vitro,* presence of amastigotes and trypomastigotes in supernatants, and susceptibility of trypomastigotes emerged from tissue culture to lysis in the presence of chagasic human sera and complement.

MATERIALS AND METHODS

Parasite populations – The *T. cruzi* Y strain (Silva & Nussenzweig, 1953) and its clones YP1, YP2 and YP3 (Marques de Araújo, 1985) were studied.

Tissue culture – (1) Maintenance of cell line – VERO cells, originated from African Green monkey kidney obtained from Flow Laboratories were dispersed once a week with trypsin 0.25% in PBS (SIGMA CHEM. Co.) and subcultured at a ratio of 2 x 10^5 cells / 25 cm^2 tissue culture flasks (Falcon) in Minimum Essential Medium (MEM-SIGMA CHEM. Co.) supplemented with sodium bicarbonate (22 g/l) 200 mM glutamine, 100 IU/ml penicillin (P), 100 µg streptomycin (S) and 2% foetal bovine serum (FBS); *(2) Infection of tissue culture cells* – Parasites were cultured in liver infusion tryptose – LIT medium (Camargo, 1964) for 60 days at 28 °C starting from blood of mice infected with different isolates. Metacyclic trypomastigotes as well as non-infective epimastigotes were washed three times in PBS, resuspended in MEM and inoculated into tissue culture flasks containing a 24-48 hr old monolayer of cells at a ratio of 5 metacyclic trypomastigotes per cell. Subsequent weekly infection of new cells was initiated with tissue culture trypomastigotes (TCT) released into supernatants of infected cultures. After 24 hr at 37 °C parasites remaining in the supernatants were removed off, the medium replaced and the flasks incubated at 33 °C (Bertelli et al., 1977). Medium was replaced again after 4-5 days when cells were full of amastigotes ready to differentiate into trypomastigotes, and daily subsequently.

Determination of capacity of interaction of clones YP1, YP2 and YP3 with VERO cells – Cells were infected as described above with metacyclic trypomastigotes from acellular LIT medium. Tissue culture trypomastigotes (TCT) emergent from the first cycle in VERO cells (6-7 days), were counted and used to initiate new infection. After 24 hr at 37 °C, parasites remaining in the supernatants were recovered and counted in a hemocytometer. The mean of at least three counts of TCT recovered was recorded.

Determination of the proportion trypomastigotes/amastigote (T/A) released into supernatants of infected cells – Upon removal of non-penetrating TCT, medium was replaced and cells incubated at 33 °C. Extracellular parasites were removed daily from day 4 onwards and the ratio T/A in supernatants was evaluated on days 5 and 9 after infection by straightforward counts in a hemocytometer and results expressed as mean of at least three different counts.

Patients' sera – Human chagasic sera belonged to two groups: (1) nine sera from patients who had not been treated; (2) six sera from patients who had been submitted to specific chemotherapy but had not been cured. Both categories of human chagasic sera present lytic antibodies (LA) detectable by the CML reaction (Krettli et al., 1979; Krettli et al., 1982).

Complement mediated lysis (CML) – This assay was performed as described in the original article by Krettli et al. (1979) with small modifications. Tissue culture derived trypomastigotes (6 x 10^6/ml) were incubated with fresh human serum as source of complement, at 37 °C for 45 min and then had their number determined. Fifty microliters of the parasite suspension were added to 50 µl of the patient inactivad serum and the tubes were incubated at 37 °C, 30 min. Fifty microliters of the complement were added to tubes and the number of trypomastigotes determined in a haemocytometer; after incubation at 37 °C for 45 min the tubes were maintained in ice and the trypomastigotes again counted. The percentage of lysed parasites was calculated and results were considered positive when percentage of lysis was ≥ 20% and were expressed as mean of two to five experiments performed with each serum.

RESULTS

Recovery of TCT from different isolates after 24 hr of contact with VERO cells – After one intracellular cycle, the number of TCT given in the inoculum but remaining in supernatants of tissue cultures after 24 hr at 37 °C

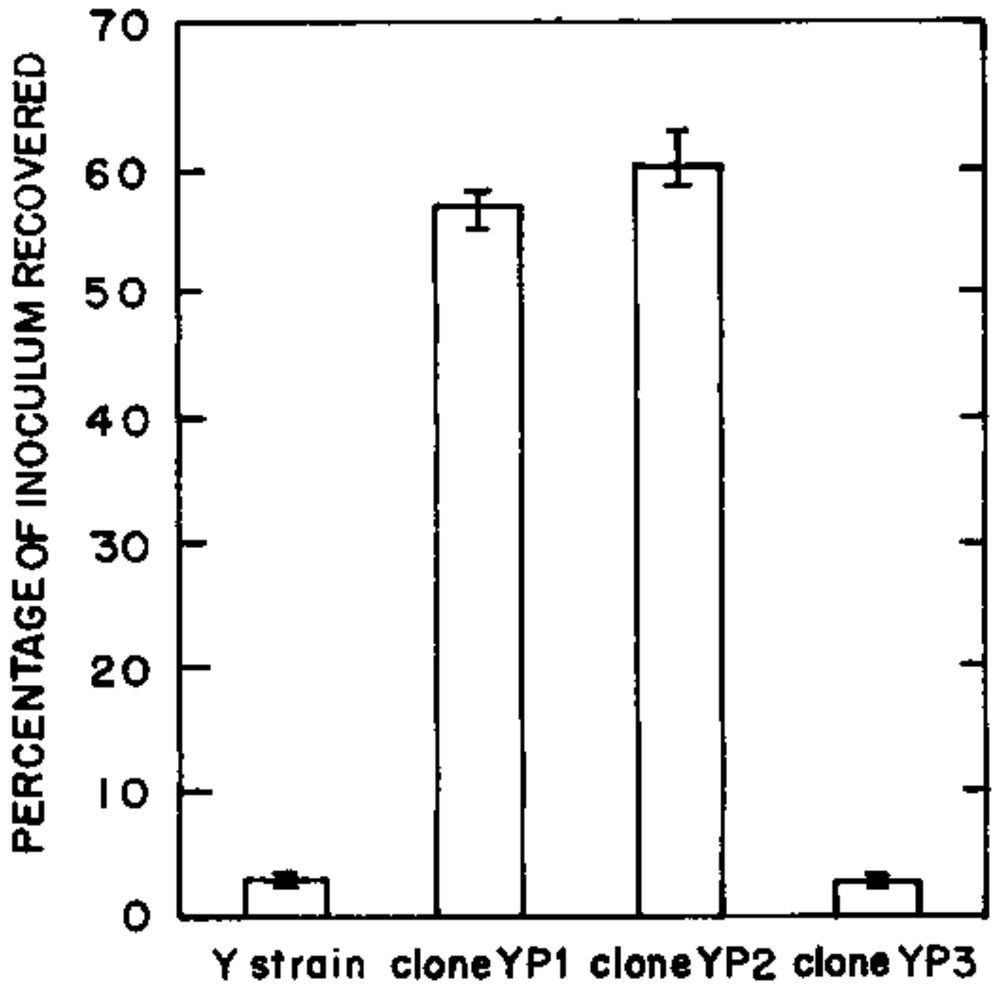
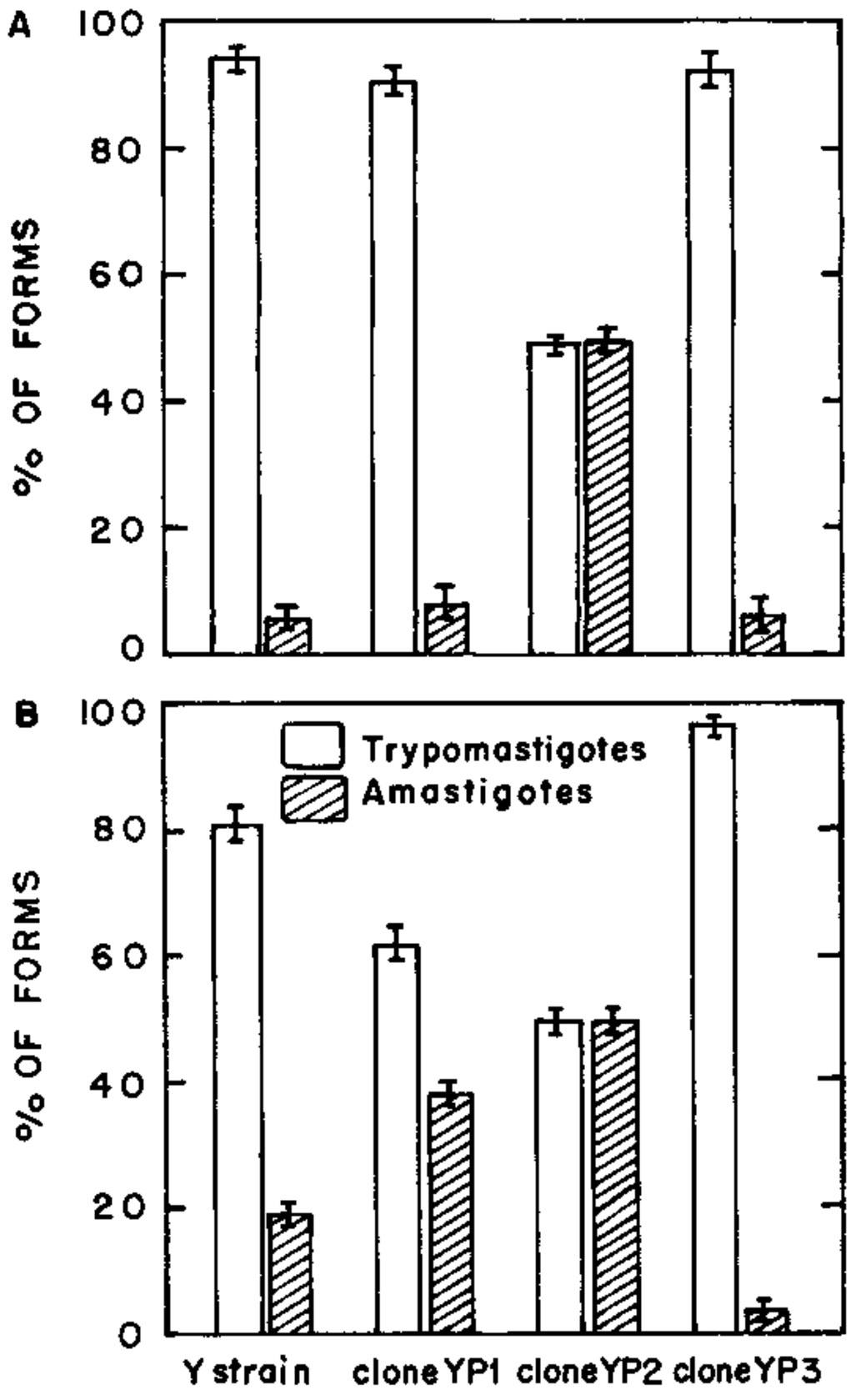


Fig. 1: percentage of tissue culture derived trypomastigotes (TCT) of Y strain and its clones YP1, YP2 and YP3 recovered after 24 hr of contact with VERO cells.

was determined. Fig. 1 shows that the recovery of TCT from parental Y strain and clone YP3 after 24 hr of contact with cells was similar and very low (3.2% and 2.7% of inoculum), while recovery of clones YP1 and YP2 were 56.7% and 60.0% of inocolum, respectively. Results represent the mean of three counts.

Proportion of trypomastigotes and amastigotes in supernatants of infected cultures – Fig. 2A, B show a steady stability in the release of parasites into supernatants of VERO cells infected with clone YP3 at day 5 and at day 9 of observation, when the vast majority of forms were trypomastigote (T/A = 97/3). Stability was also observed in supernatants of cultures infected with clone YP2 which, since day 5 of infection, showed a high proportion of amastigotes emerging from the first intracellular cycle (T/A = 50/50). However, supernatants of cultures infected with clone YP1 and parental Y strain showed an increase in the number of amastigotes released after nine days of infection: at day 5, the ratio T/A, for both populations, was 90/10 and at the day 9 of observation was 62/38 and 81/19 for clone YP1 and parental Y strain, respectively.

Complement mediated lysis – Susceptibility to lysis in the presence of individual human chagasic sera and complement was significantly different between TCT of clones YP1, YP2, YP3 and Y strain (Tables I, II). The mean lytic activity of chagasic patients' sera (untreated and treated, uncured) obtained with TCT of clone YP3 and parental Y strain was similar- both presented high susceptibility to lysis (% lysis about 70); clone YP2 was considered resistant to lysis (% lysis ≤ 20) and TCT of clone YP1 were between the other two (% lysis about 40).



amastigotes forms of Y strain and its clones YP1, YP2 and YP3 on day 5 (A) and day 9 (B) after infection of VERO cells.

DISCUSSION

The production of numerous, pure trypomastigote forms of *T. cruzi* is possible for strains and clones adapted to cultivation *in vitro* in tissue culture. The Y strain, used worldwide, is well adapted to growth in this condition, a great number of trypomastigotes being produced at 33 °C (Bertelli et al., 1977). However, in old cultures (more than seven days of infection), a number of amastigotes is seen together with trypomastigotes in supernatants of infected cultures (Fig. 2).

TABLE I

Average lytic activity of individual chagasic human sera (untreated patients) to TCT of Y strain and its clones YP1, YP2 and YP3

Sera	% CML TCT			
	Y Strain	YP1	YP2	YP3
CMCM	58.4 ± 30.8	40.5 ± 14.1	4.3 ± 5.7	70.8 ± 35.2
SCV	82.9 ± 5.2	70.7 ± 14.3	16.2 ± 11.2	68.3 ± 19.3
NMVB	82.6 ± 10.8	45.6 ± 8.9	8.7 ± 10.5	76.4 ± 23.6
VPB	88.4 ± 13.4	14.3 ± 7.6	26.7 ± 20.1	40.0 ± 15.7
VFS	77.6 ± 20.1	44.2 ± 5.6	28.0 ± 14.8	94.1 ± 6.7
MJMV	70.0 ± 5.1	10.0 ± 14.1	13.0 ± 6.2	73.0 ± 38.0
OJC	72.0 ± 20.3	56.5 ± 11.3	13.6 ± 14.0	80.7 ± 10.1
MZN	70.5 ± 13.6	33.5 ± 21.6	23.4 ± 16.4	80.0 ± 18.7
VPS	92.7 ± 3.9	92.8 ± 4.3	37.5 ± 20.2	98.3 ± 2.5
Mean	75.3 ± 9.5	43.6 ± 26.1	16.7 ± 8.6	75.8 ± 16.8
NHS[a]	9.8 ± 4.6	11.5 ± 2.0	0.0 ± 0.0	0.0 ± 1.0

a: NHS = normal human serum.

TABLE II

Average lytic activity of individual chagasic human sera (treated uncured patients) to TCT of Y strain and its clones YP1, YP2 and YP3

Sera	% CML TCT			
	Y Strain	YP1	YP2	YP3
SFG	82.6 ± 14.7	68.5 ± 24.0	10.8 ± 8.3	52.9 ± 8.4
RAA	90.7 ± 8.7	62.1 ± 14.1	1.6 ± 2.2	96.6 ± 3.5
JMA	48.8 ± 11.3	16.0 ± 6.6	2.9 ± 4.1	42.4 ± 13.1
AAS	71.5 ± 25.3	49.2 ± 22.5	14.8 ± 8.5	89.1 ± 11.1
PMS	63.3 ± 9.7	26.3 ± 16.0	0.0 ± 0.0	39.5 ± 23.2
ESC	71.0 ± 5.2	18.2 ± 7.9	26.7 ± 13.9	83.9 ± 19.8
Mean	71.3 ± 14.7	40.0 ± 23.0	9.5 ± 10.2	67.4 ± 25.4
NHS[a]	9.8 ± 4.6	11.5 ± 2.0	0.0 ± 0.0	0.0 ± 0.0

a: NHS = normal human serum.

The growth characteristics in tissue culture of clones YP1, YP2 and YP3 showed that the contact of YP3 subpopulation with VERO cells was similar to that of parental Y strain. Very few trypomastigotes from YP3 could be recovered after 24 hr in presence of VERO cells at 37 °C (2.7% of inoculum) suggesting that most parasites must have entered the cells and predominance of trypomastigotes was seen in supernatants of infected cultures mantained at 33 °C. Some parallelism is observed between results obtained *in vitro* and *in vivo:* Marques de Araújo (1985) reported that parental Y strain and its clone YP3 were more virulent to C_3H mice (high parasitemia, 100% mortality) than clones YP2 and YP1 (45.5% and 0% mortality, respectively). On day 5 and 9 of infection of cells with clone YP3, very few amastigotes could be seen in the supernatants. The obtaining of pure trypomastigotes is helpful in many studies where other forms of parasite are undesirable.

Susceptibility to lysis in the presence of chronic chagasic human sera was also evaluated for TCT of the three clones. Trypomastigotes (TCT) from Y strain were largely used by Galvão (1990) in a CML reaction which detects protective antibodies present at the chronic stage of active subpatent Chagas' disease and promote lysis of the trypomastigote form in presence of human complement. Susceptibility to lysis was different among the clones studied. Trypomastigotes of clone YP3

and parental Y strain were equally susceptible to CML in the presence of patients' sera presenting high lytic antibodies titer (70%). Assuming that parasites derived from a clone should be homogeneous, no explanation is available for the remaining unlysed 30% trypomastigotes.

The findings regarding capacity of interaction with VERO cells, ratio amastigotes/trypomastigotes recovered in supernatants of infected cultures and susceptibility to CML for clone YP3 when compared with the Y strain, suggest that this parasite subpopulation could predominate in the strain. However, Macedo et al. (1992), using non radioactive hybridization DNA probes, demonstrated that DNA fingerprinting is distinct for the three clones; clone YP1 presented a profile similar to the parental *T. cruzi* population, suggesting its predominance in the Y strain. Clone YP3 would be the less represented since its DNA profile is the most distinct and clone YP2 is almost identical to YP1. After hybridization with the same probe (Jeffreys et al., 1985 a, b) it was not possible to detect predominance of one clone over the other along different timing *in vitro* up to 25 days of cultivation of Y strain (Macedo et al., 1992). It may be that the clones studied do not represent all the subpopulations incorporated in Y strain.

The possibility that the resistance to lysis presented by TCT of clone YP2 could be an event associated with mutation on DNA should be considered; TCT of clone YP1 were less susceptible to CML than TCT of Y strain and those of clone YP2, whose DNA profile is very similar to YP1, were resistant.

The data presented in this paper suggest that variability obtained by different authors who have used the Y strain in CML reactions may be explained by the presence of at least two very distinct populations with regard to this character (YP2 resistant and YP3 susceptible) in this strain. Studies are underway using a wider range of sera from infected individuals to determine if resistance to lysis is inherent to clone YP2 itself or to the source of serum.

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