Trypanosoma cruzi: Metacyclogenesis *in Vitro* - I. Changes in the Properties of Metacyclic Trypomastigotes Maintained in the Laboratory by Different Methods

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In this work we have studied the modifications in the biological properties of Trypanosoma cruzi *when the parasite is maintained for a long time in axenic culture. The studies were done with a clone from an avirulent strain (Dm30L) and a non-cloned virulent strain (EP) of* T. cruzi. *Both parasites were maintained, for at least three years, by successive triatomine/mouse alternate passage (control condition), or by serial passage in axenic medium (culture condition), or only in the mouse (mouse condition). The comparison between parasites of culture and control condition showed that metacyclogenesis capacity was reduced in the former and that the resulting metacyclics displayed an attenuated virulence. In order to compare the virulence of metacyclics from the urine of the insect vector,* Rhodnius prolixus *were infected by artificial feeding with parasites of the control or culture condition. After three triatomine/triatomine passages, there was observed an almost identical biological behavior for these parasites, hence indicating that the maintenance of* T. cruzi *for a long time in axenic culture affects the differentiation capacity and the virulence of the parasite. Additionally, it was demonstrated that it is possible to maintain* T. cruzi *exclusively through passages in the invertebrate host.*

Key words: *Trypanosoma cruzi* - metacyclogenesis - Chagas' disease - *Rhodnius prolixus*

Trypanosoma cruzi alternates its morphology during its life cycle, showing stages with distinct biological potentials. In the laboratory, the parasite can be maintained in axenic media, in cell culture, by serial passages in the mammal host, and by alternating invertebrate/vertebrate host passages (Brener 1973). Long maintenance in axenic media modifies its growth pattern, the spontaneous meta-cyclogenesis rate, and the infectivity (Chiari et al. 1973, Chiari 1974a, b). There is, however, evidence that laboratory maintenance of *T. cruzi* causes little change in behavior in the vertebrate host (Magalhães et al. 1985).

The species *cruzi* consists of many subpopulations and clones, with differing genetic constitution (Morel et al. 1980, Gonçalves et al. 1984), DNA content, and biological behavior (Engel et al. 1982), so that the laboratory maintenance methods may favor the selection of certain lines (Deane et al. 1984, Marque de Araujo & Chiari 1988, Carneiro et al. 1990). Thus, changes of behavior of *T. cruzi* in the laboratory may be due to selection of certain subpopulations or clones from the different natural isolates of the parasite (Postan et al. 1983).

The proposal of this work was to study changes in the biological properties of the parasites according to the laboratory maintenance schedule. The results indicate that long-term maintenance of *T. cruzi* in culture leads to loss of the synchronization of morphogenetic events that occur in the vertebrate.

MATERIALS AND METHODS

Parasites and maintenance conditions - The Dm30 strain of *T. cruzi* was isolated in 1976 from the opossum *Didelphis marsupialis* and maintained in *Rhodnius prolixus* and mice, with vector/vertebrate passages at least three times yearly. *T. cruzi* Dm30L was cloned in 1986 by limiting dilution of metacyclics from the urine of *R. prolixus*, and inoculating one parasite into the buccal mucose of newborn mice, according to the method of Contreras et al. (1985a). Insect and mice were infected with parasites isolated from one mouse with patent parasitaemia. This clone was maintained under different experimental schedules, giving rise to distinct clonal populations. The clone Dm30L was thus maintained in

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Rhodnius and mice (control condition), in newborn mice (mouse condition), and in LIT medium (culture condition) (Evans 1978). The EP strain of *T. cruzi* was isolated from a fatal human case in 1967, and maintained by serial passages in mice until 1976, when it was transferred to *R. prolixus* and mice in alternation (control condition). The EP strain (culture condition) was obtained by hemoculture of EP-control in 1985, and was thereafter maintained in LIT medium with fortnightly passages.

Harvesting metacyclics - Metacyclic trypomastigotes were induced by the technique of Contreras et al. (1985a, b). Briefly, epimastigotes from the late exponentially growing phase in LIT medium were harvested by centrifugation at 8,500 xg and incubated for 2 hr in artificial triatomine urine (TAU, 190 mM NaCl, 17 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 8 mM phosphate buffer pH 6.8) in a density of 5 x 10^8 cell/ml. Thereafter, the parasites were incubated in TAU3AAG medium (TAU supplemented with 20 mM L-proline, 50 mM L-glutamate, 2 mM L-aspartate, 10 mM glucose) to a final concentration of 3 x 10^6 cell/ml in a final volume of 70 ml in Roux flasks (Goldenberg et al. 1987). They were incubated at rest at 27°C for 72 hr, then centrifuged at 10,000 xg, resuspended in TAU medium, treated for 30 min at 37°C with fresh guinea pig serum, and separated on DE52 cellulose (Sousa 1983).

Alternatively, metacyclics were obtained from the urine of *R. prolixus* infected with *T. cruzi* (Garcia et al. 1984).

Mammalian cell cultures - Vero and BHK-21 cell cultures (kindly supplied by Pfizer laboratories, the Universidad Simón Bolivar, and the Centro de Investigaciones Veterinarias) were routinely maintained at 35°C in MEM medium (GIBCO), supplemented with 10 mM HEPES at pH 7.0, 10% fetal bovine serum (Lab. Danibios Prod. Biol.), 2 mM L-glutamine, 0.02% Na_2CO_3, 500 U/ml penicilin and 500 µg/ml streptomycin. Cellular growth during infection was inhibited by reducing the fetal bovine serum to 2%. Unstimulated peritoneal macrophages harvested from 8 weeks old male NMRI mice were collected, sedimented, and adhered to 9 x 35 mm coverslips in Leighton tubes (Kierszenbaum et al. 1974). The macrophages were kept for 12-18 hr at 35°C in 5% CO_2. Only tubes showing at least 2/3 of the surface area of the coverslip covered with explated macrophages were used.

In vitro *cellular infection* - Monolayers of semiconfluent cells and macrophages, prepared as above, were inoculated with metacyclics, approximately 10 parasites/cell, and left in contact for 2-24 hr. The interaction experiments were performed at 35°C in a 5% CO_2 humidified atmosphere. The cultures were then washed with 0.15 M NaCl, fixed in Bouin and stained with Giemsa. The percentage of infected cells was evaluated in each coverslip from a total sample of 200 cells/slide, counting duplicates in each experiment. For determination of the kinetics of parasite active penetration in Vero or BHK-21 cell (kinetic of invasion), an hypotonic shock (2 ml of cold distilled water for 1 min) was applied at the end of the contact period and before fixation, resulting in the lysis of adherent parasites and allowing the quantification of interiorized parasites (Andrews & Colli 1982). The kinetics of infection (cellular colonization) was determined similarly, in tubes taken at the end of the contact time and incubated for an additional 72 hr. For the determination of passive penetration (phagocytosis) and determination of resistance to digestion by macrophages, Leighton tubes with infected macrophages, washed with maintenance medium at the end of the contact period, were incubated for additional 24 hr to determine invasion and 72 hr for infection, respectively (Contreras et al. 1988).

The experiments were repeated at least three times. The values obtained are accompanied by their standard deviations for each test.

Infection of mice - Groups of 9-10 female NMRI mice were inoculated with triatomine or induced metacyclics, blood trypomastigotes, or trypomastigotes from cell culture supernatant. Three days post-inoculation, blood was examined to determine parasitaemia (Brener 1962). Subpatent infections (45 days post- inoculation) were established by three separate xenodiagnoses with ten 4th-instar nymphs of *R. prolixus* at two day intervals, and the insects were examined 25, 45, and 60 days. Hemoculture was made to complement the xenodiagnosis. Mice with negative parasitaemia were reinoculated with metacyclics, blood trypomastigotes, or supernatants from cell cultures of the homologous clone, to determine infectivity, prepatency, and mortality.

Vector-vector maintenance - Groups of 60 3rd instar nymphs of *R. prolixus* were infected by artificial feeding with parasites, either with a 1/1 mixture of culture medium and heparinized mouse blood, or insect urine with the blood, following the protocols Dm30L-culture and Dm30L-control, respectively. The solutions contained 1 x 10^6 parasites/ml. The bugs were maintained to adulthood by feeding on non-infected mice. Infected urine was collected from 5th-instar nymphs and adults, and used to infect additional 3rd-instar stage nymphs. Triatomine metacyclic trypomastigotes obtained after three vector-vector passages were used in the test for infectivity to animals.

RESULTS

Effect of the maintenance condition on T. cruzi *metacyclogenesis* - Table I shows the per-

centages and the yield of metacyclics after DE52 chromatography of parasites incubated for 72 hr in TAU3AAG medium. Phase contrast microscopy showed 66 ± 6% metacyclics for the clone maintained in the control condition, 75 ± 4% for the clone in mouse condition, and 27 ± 11% for the clone in culture condition. In the same differentiation medium the strain EP showed 48 ± 7% metacyclics for parasites from the control condition, and 22 ± 4% for the culture condition. However, the DE52-cellulose yield was 45 times lower for EP- culture (2.1 ± 0.9 x 10^6 metacyclics/ml) than for EP-control (90 ± 8 x 10^6 metacyclics/ml). Similar yields were obtained for Dm30L-culture when compared to Dm30L-control and Dm30L-mouse condition (Table I).

Effect of the maintenance condition on the virulence of TAU3AAG medium metacyclics - Table II compares the virulence of metacyclics of *T. cruzi* Dm30L and EP obtained from TAU3AAG medium maintained in the control condition, mouse condition and culture condition. Inoculation of 5 x 10^5 metacyclics/mouse of cloned parasite (Dm30L) obtained from the control condition infected all mice, nine of which showed parasites by blood examinations, and one by xenodiagnosis. The prepatent period was 28 ± 7 days; there was no mortality. Mouse condition metacyclics infected all mice tested, with a prepatent period of 24 ± 3 days, with 30% mortality (3/10), and a survival time of 56 ± 16 days. Dm30L-culture metacyclics infected 40% of mice (4/10), two infections being detected by blood examination and two by xenodiagnosis. Reinoculation of the animals with blood trypomastigotes from the mouse condition produced patent parasitemias in 100% of the mice in 29 ±3 days, but caused no mortality (not shown).

Metacyclics (3 x 10^5/mouse) of EP-control, infected 100% of the mice (10/10) with a prepatent period of nine days, 70% mortality (7/10), and survival of 30 ± 3 days (Table II). However the inoculation of metacyclics of the EP-culture produced a sub-patent infection, detectable only by xenodiagnosis 45 days post-inoculation, with no mortality.

Active penetration and intracellular multiplication of the Dm30L clone and EP strain - Table III compares percentages of BHK-21 cell culture invaded and infected by metacyclics of *T. cruzi* Dm30L of parasites maintained in the control condition, mouse condition, and culture condition. Metacyclics/cell ratio was 10:1 for contact periods between 2 and 24 hr. Comparing the percentage of cells infected and colonized during a 24 hr contact period, it can be seen that the clone maintained in culture was far less successful in invasion and infection than the parasites maintained in the vector or the vertebrate host. The Dm30L-culture clone, after additional 72 hr of incubation (96 hr total) had colonized no more than 1% of cells, and trypomastigotes were not seen in the supernatant. The clone maintained in the other two conditions infected aproximately 10% of the cells, and trypomastigotes were observed in the supernatant.

Table IV compares the percentage of Vero cells culture infected and colonized by metacyclics of the virulent strain *T. cruzi* EP. The percentage of Vero cells invaded by metacyclics of control condition increased from 53 ± 6 to 65 ± 5% between 2 and 24 hr of incubation, whereas the strain maintained in culture invaded 4 ± 2% during a 24 hr contact period. Furthermore, the percentage of cells infected by metacyclics of the control condition, increased from 40 ± 9 to 68 ± 4% between 2 and 24 hr of incubation and was less than 1% with metacyclics of culture condition. Trypomastigotes were seen in the supernatant of Vero cell cultures infected with the

TABLE I

Effect of maintenance conditions of *Trypanosoma cruzi* (Dm30L and EP) upon the percentage of metacyclics in TAU3AAG medium

Parasite	Maintenance	Pre-DE52		Post-DE52
		Percentage of live forms (means ± s.d.)		No. metacyclics (x 10^6/ml) (mean ± s.d.)
		epimastigotes	metacyclics	
Dm30L	control	34 ± 6	66 ± 6	67.0 ± 2.3
Dm30L	mouse	25 ± 3	75 ± 4	65.0 ± 8.1
Dm30L	culture	45 ± 8	27 ± 11	1.4 ± 0.3
EP	control	52 ± 5	48 ± 7	90.0 ± 8.0
EP	culture	61 ± 9	22 ± 4	2.1 ± 0.9

Note: culture conditions show immobile forms.

TABLE II

Comparison of the virulence of metacyclics of *Trypanosoma cruzi* (Dm30L and EP) from TAU3AAG medium. Effect of maintenance conditions of the parasites

Parasite	Condition	No. of metacyclics mouse ($x10^5$)	Virulence in terms of			
			Infectivity (%)	Prepatency (mean±s.d.) (days)	Mortality (%)	Survival (mean±s.d.) (days)
Dm30L	Control	5	100	28 ± 7	0	>70
Dm30L	Mouse	5	100	24 ± 3	30	56 ± 16
Dm30L	Culture	5	40	28 ± 0	0	>100
EP	Control	3	100	9 ± 0	70	30 ± 3
EP	Culture	3	100	<45	0	>100

TABLE III

Percentage of BHK-21 cells invaded and infected by metacyclics of *Trypanosoma cruzi* Dm30L from TAU3AAG medium. Comparison between parasites maintained in the control condition, in mouse condition and in culture condition

Contact time (hr)	Percentage of cells (mean ± s.d.)					
	Invaded			Infected		
	control	mouse	culture	control	mouse	culture
2	24 ± 2	20 ± 6	9 ± 5	4 ± 3	6 ± 3	9 ± 4
6	39 ± 16	30 ± 4	23 ± 6	14 ± 3	10 ± 1	5 ± 1
18	41 ± 4	40 ± 10	21 ± 1	11 ± 7	11 ± 3	3 ± 2
24	47 ± 7	45 ± 7	24 ± 7	10 ± 7	10 ± 4	1 ± 1

Note: metacyclics/cell ratio was 10:1 for each contact time.

TABLE IV

Percentage of Vero cells invaded and infected by metacyclics of *Trypanosoma cruzi* EP from TAU3AAG medium. Comparison between parasites maintained in the control condition and in culture condition

Contact time (hr)	Percentage of cells (mean ± s.d.)			
	Invaded		Infected	
	control	culture	control	culture
2	53 ± 6	7 ± 3	40 ± 9	<1
6	57 ± 6	15 ± 6	45 ± 11	2 ± 1
12	53 ± 9	9 ± 3	66 ± 9	4 ± 2
24	65 ± 5	4 ± 2	68 ± 4	<1

Note: metacyclics/cell ratio was 10:1 for each contact time.

strain maintained in the natural cycle (control) after 90 hr of incubation, while the supernatant of EP-culture parasites showed only large epimastigote forms even after five days of incubation.

Endocytosis by professional phagocytes of the Dm30L clone and EP strain and its resistance to digestion - Table V shows the percentage of mouse peritoneal macrophages invaded and infected by metacyclics of *T. cruzi* Dm30L maintained in the control condition, mouse condition and culture condition. Comparison of the percentage of macrophages invaded, for control condition parasites, showed that approximately 50% of the macrophages engulfed metacyclics from 2 to 24 hr of incubation, while de percentage in the mouse condition increased from 30% to 59% between 2 and 24 hr of incubation. In contrast, for the culture condition, the percentage of macrophages that engulfed metacyclics was significantly lower (about 15%) than the control condition. The resistante to macrophage digestion, as judged by the percentage of amastigote nests, was about 19% for control condition, increased linearly with the incubation time from 6 ± 1% to 28 ± 9% for mouse condition, and was less than 1% for the culture condition.

Table VI shows the percentage of mouse peritoneal macrophages invaded and infected by metacyclics of *T. cruzi* EP-control and culture condition. The results show that the percentage of macrophages engulfing metacyclics of the control condition does not vary significantly, 44 ± 12% to 34 ± 9%, between 2 to 18 hr of contrast, for the culture condition, the percentage of macrophages invaded was significantly lower (19 ± 5 to 12 ± 7%). The percentage of amastigotes nests after an additional 72 hr of incubation is approximately 30% for the control condition, while only 2 ± 4% of macrophages were colonized by metacyclics of the culture condition after 90 hr of incubation.

TABLE V

Percentage of macrophages invaded and infected by metacyclics of *Trypanosoma cruzi* Dm30L from TAU3AAG medium. Comparison between parasites maintained in the control condition, in mouse condition, and in culture condition

Contact time (hr)	Percentage of macrophages (mean ± s.d.)					
	Invaded			Infected		
	control	mouse	culture	control	mouse	culture
2	48 ± 8	30 ± 3	17 ± 7	16 ± 5	6 ± 1	2 ± 2
6	56 ± 2	37 ± 6	15 ± 3	19 ± 6	9 ± 3	4 ± 4
18	44 ± 6	49 ± 4	10 ± 8	19 ± 1	14 ± 5	5 ± 2
24	47 ± 8	59 ± 3	13 ± 5	13 ± 2	28 ± 9	1 ± 1

Note: metacyclics/macrophage ratio was 10:1 for each contact time.

TABLE VI

Percentage of macrophages invaded and infected by metacyclics of *Trypanosoma cruzi* EP from TAU3AAG medium. Comparison between parasites maintained in control condition and in culture condition

Contact time (hr)	Percentage of macrophages (mean ± s.d.)			
	Invaded		Infected	
	control	culture	control	culture
2	44 ±12	19 ± 5	26 ± 8	9 ± 4
4	40 ± 8	19 ± 3	26 ± 12	9 ± 6
8	36 ± 10	16 ± 9	30 ± 7	6 ± 4
18	34 ± 9	12 ± 7	31 ± 9	2 ± 4

Note: metacyclics/macrophage ratio was 10:1 for each contact time.

DISCUSSION

The present study confirms that different schedules of maintenance in the laboratory can modify the biological properties of epimastigotes and metacyclics of *T. cruzi*, in agreement with results from other groups (Bice & Zeledon 1970, Chiari et al. 1974a, b, Deane et al. 1984). The fact that the infectivity of metacyclics of a cloned parasite (Dm30L) has been modified demonstrate that such changes are inherent in the metacyclics *per se* and are not due to a population selection. Conversely, the changes observed with the EP strain might be due to population selection.

The experiments comparing the effect of the maintenance condition on *T. cruzi* metacyclogenesis in TAU3AAG, and yield of metacyclics of a clone (Dm30L), and a strain (EP), showed that maintenance of the parasites for a long time in culture resulted in a two fold reduction of their differentiation capacity, an increase of the epimastigote mortality in TAU3AAG medium, and a decrease in the yield of metacyclics post-DE52.

Experiments comparing the virulence of *T. cruzi* to mice showed that metacyclics from the control condition of strain EP were more virulent than those of the control condition of clone Dm30L. Metacyclics obtained from parasites maintained exclusively in culture medium are less infective, they produce subpatent parasitemias, they are less lethal, and they do not protect against subsequent reinoculations with homologous trypomastigotes. These observations are compatible with results obtained by others, who have demonstrated that the antigenic make up of metacyclics differs from that of trypomastigotes harvested from the supernatant of cell cultures, blood trypomastigotes, and metacyclics from the urine of the vector (Kanbara & Nakabayashi 1985, Yoshida et al. 1986, da Silva et al. 1989). Recent results from our laboratory have shown that the expressed antigens of the Dm30L clone vary according to different schedules of maintenance in the laboratory (Araque et al. in preparation).

The experiments dealing with the study of invasion, infection and colonization of cultured cells by metacyclics from the avirulent clone Dm30L or the virulent strain EP, indicate that the maintenance of the parasites in culture affects the metacyclics by reducing the following properties: (a) their invasive capacity, (b) their endocytosis by macrophages, and (c) their capacity to colonize tissue culture cells and macrophages. This may explain the fact that the metacyclics from culture condition were less virulent for mice, and the observation that mouse condition metacyclics increased their virulence for mice.

A detailed analysis of cell cultures infected with metacyclics originated from parasites maintained for long periods of time in culture medium showed absence of trypomastigotes in the supernatant. This might explain the fact that even a very large inoculum of these metacyclics did not infect mice. Furthermore, these parasites displayed loss of the amastigote intracellular multiplicative capacity, loss of the ability to transform from amastigote to trypomastigote, loss of resistance to macrophage digestion, and the presence of large epimastigotes. Hence, long-term maintenance of *T. cruzi* in culture might lead to loss of the synchronization of morphogenetic events that would occur in the vertebrate host.

In order to investigate whether it is possible to maintain *T. cruzi* exclusively by passages through its invertebrate host and to evaluate the infectivity of the culture condition by successive passages through the vector, we compared the virulence of clone Dm30L of the control condition with parasites of the culture condition, after three successive vector passages. Our results

demonstrated that *T. cruzi* can be maintained only in the vertebrate host and that the virulence of metacyclics of the control condition is not affected. Additionally, our results confirm those of Villalta and Kierszenbaum (1987), who found that metacyclics from insect urine are more virulent than those from axenic culture. However, the suggestion that lectins of the vector might influence the infectivity of this stage is not supported by the observation that the clone maintained in the culture condition did not recover virulence after three passages in the vector.

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