

Zeitschrift:	Acta Tropica
Herausgeber:	Schweizerisches Tropeninstitut (Basel)
Band:	42 (1985)
Heft:	1
Artikel:	"Trypanosoma cruzi" amastigotes : development in vitro and infectivity in vivo of the forms isolated from spleen and liver
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DOI:	https://doi.org/10.5169/seals-313449

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***Trypanosoma cruzi* amastigotes: development in vitro and infectivity in vivo of the forms isolated from spleen and liver**

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Summary

Trypanosoma cruzi amastigotes were isolated from liver and spleen of previously infected mice and purified in discontinuous gradients of Metrizamide and Percoll. The amastigotes were well preserved as judged by electron microscopy. The amastigotes were readily interiorized by macrophages and multiplied actively within these cells in vitro. However, their capacity of differentiation was hampered as estimated by the absence of trypomastigotes until day 6 of cultivation. The purified amastigotes were infective for mice but the onset of parasitemia was somewhat delayed and less intense when compared to mice infected with trypomastigotes.

Key words: amastigotes; *Trypanosoma cruzi*; macrophages; differentiation; amastigote infectivity for mice.

Introduction

Infection of a vertebrate host is essential to the maintenance of *Trypanosoma cruzi* complex life cycle. Amastigotes are the dividing forms of *T. cruzi* which are characteristically found in the vertebrate host. Inside the host cells' cytoplasm they undergo further differentiation to trypomastigotes which are nondividing forms (Brener, 1973 and 1980).

The infectivity, intracellular multiplication and further differentiation of trypomastigotes and epimastigotes have been extensively studied in vitro, mostly in macrophage cultures (Dvorak and Schmunis, 1972; Milder et al., 1973;

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Nogueira and Cohn, 1976; Milder et al., 1977; Kress et al., 1977; Alcantara and Brener, 1978; Milder and Kloetzel, 1980). In contrast, the few studies on the infectivity and differentiation of amastigotes have led often to conflicting conclusions. The inherent technical difficulties in obtaining purified viable amastigotes from their host cells may account for these discrepancies (Pan, 1978; Leon et al., 1979; Burger et al., 1982; Carvalho et al., 1981; Gutteridge et al., 1978; Villalta et al., 1980).

This study was undertaken to investigate the infectivity in vitro and in vivo of *T. cruzi* amastigote forms isolated from the spleen and liver of heavily infected mice.

Material and Methods

Parasites. Amastigotes of *T. cruzi* (Y strain) were isolated from the spleen and liver of mice infected with 1×10^6 bloodstream trypomastigotes and killed on the 7th day post-infection (PI). Isolation and purification of the amastigotes were done as previously described (Abrahamsohn et al., 1983). The technique consisted of centrifugations of the cells in a Metrizamide (Nyegaard, Oslo, Norway) gradient (10%/30%, 1000 g, 20 min) and in a Percoll (Pharmacia, Uppsala, Sweden) gradient (30%/60%, 800 g, 15 min). Bloodstream forms were collected on the 7th day of infection. The blood was defibrinated with glass beads and the parasites were separated by differential centrifugation (Milder and Kloetzel, 1980).

Macrophage cultures. Macrophages were harvested from the peritoneal cavity of Swiss out-bred mice stimulated three days before with 0.5 ml of sterile peptone (10% in water). Following centrifugation and washing in Hanks' Balanced Salt Solution (HBSS) the concentration of cells was adjusted to 1.5×10^6 /ml. Aliquots (0.1 ml) were dispensed on 18×18 mm glass coverslips and incubated in the same medium for 60 min at 37°C. The coverslips were thoroughly washed to remove non adherent cells and further incubated for 24 h in tissue culture medium 199 supplemented with 20% (v/v) Fetal Bovine Serum (TCM/FBS). The cultures were maintained in a humidified atmosphere containing 5% CO₂ in air (v/v).

Infection of the macrophage cultures with amastigotes. A suspension of purified amastigotes and of bloodstream forms in TCM/FBS 10% was added to the coverslips, in order to have a final parasite/macrophage ratio of 1:1, and incubation proceeded for further 2 h, at 35°C. The coverslips were then thoroughly washed with HBSS (37°C) to remove extracellular parasites, and reincubated for periods up to six days. Fresh medium was provided every two days. Coverslips were removed 2 h, 48 h, and 6 days after infection, washed in HBSS, fixed in Bouin's fixative and stained with May-Grunwald Giemsa stain. The percentage of infected macrophages and the number of amastigotes per infected macrophage were determined by counting 500 cells randomly in triplicates. The experiments were repeated four times. The cultures were examined daily and the supernatant every two days.

Inoculation of amastigotes in liquid media. Amastigotes were incubated at 35°C in the following media (without other cells): TCM 199 plus 10% FBS. Eagle's Minimal Essential Medium plus 10% FBS. Dulbecco's Modified Eagle Medium plus 2% FBS. Incubation in Liver Infusion Tryptose (LIT) medium was at 28°C. The concentration of parasites was 5×10^6 /ml and the cultures were examined after 3 h and 20 h of incubation, in order to assess the transformed parasites.

Electron microscopy. Each preparation of amastigotes was fixed in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (1 h at room temperature) and post-fixed in 1% (W/v) OsO₄, dehydrated and embedded in Araldite.

Infectivity of amastigotes for mice. It was almost impossible to rule out a minimal (1% to 2%) contamination of the amastigote preparation with trypomastigotes. Therefore, we chose to use a very low infecting dose of amastigotes. Two hundred purified amastigotes were inoculated in-

traperitoneally into Swiss outbred male mice weighing 20 g. Mice were also inoculated with 10 bloodstream forms as a corresponding control for the eventual minimal contamination of the amastigotes with trypomastigotes.

Inoculation with 20 bloodstream forms was chosen as an additional control. Parasitemia was determined daily by the method of Brener (1962) from the 5th until the 11th day PI and twice weekly thereafter.

Results

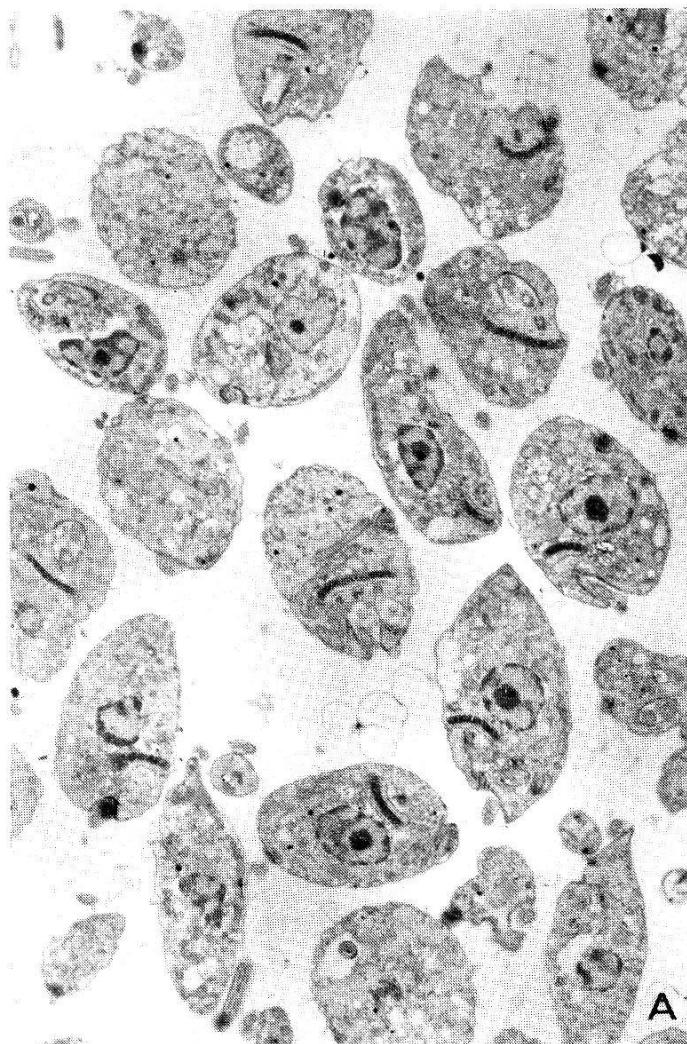
Ultrastructural observation of the purified parasites showed well preserved amastigotes, without contamination with trypomastigotes. Less than 1% transitional forms, probably in an intermediate stage between amastigotes and trypomastigotes were observed. These transitional forms were identified by the kinetoplast which already presented its DNA fibers in a typical trypomastigote's arrangement but not yet located at the posterior end of parasite's body. Occasionally membrane remnants of other cells could be seen (Fig. 1 A).

Cultivation of purified amastigotes in all the tested acellular media, for a period of 3 h yielded less than 5% trypomastigotes, while approximately 30% of the amastigotes became trypomastigotes after 20 h *in vitro*.

Macrophages readily phagocytosed amastigotes and after 2 h of contact 23.8% of the macrophages contained parasites (Fig. 2). The mean number of amastigotes per macrophage is better expressed by the index of infection. Most macrophages harboured only one parasite and the cultures presented an average index of infection of 1.5 (Fig. 2 and Fig. 1 B). Forty-eight hours after infection, the cultures presented the same percentage of infected macrophages, but the index of infection rose to 5.5 amastigotes per host cell (Fig. 2). The cytoplasm of a few infected cells was filled with parasites, some of which were dividing (Fig. 1 C and D). Unexpectedly, in all infected macrophages, the intracellular forms remained as typical amastigotes, well preserved, without any detectable sign of degeneration, inside of structurally well preserved macrophages for up to 6 days of culture (Fig. 1 E). In addition, during this period, we could never identify even a single cell presenting either trypomastigotes or transitional forms from amastigotes to trypomastigotes, nor were these forms ever found in the cultures' supernatant. There was no significant change of the number or proportion of infected macrophages in the culture during the period studied.

On the other hand in cultures of macrophages infected with blood trypomastigotes, 48 h after infection only two out of five hundred macrophages were parasitized. By 72–96 h, the number of infected cells did not change, but the parasites inside them were already completing their intracellular cycle and trypomastigotes were found in the cultures supernatant.

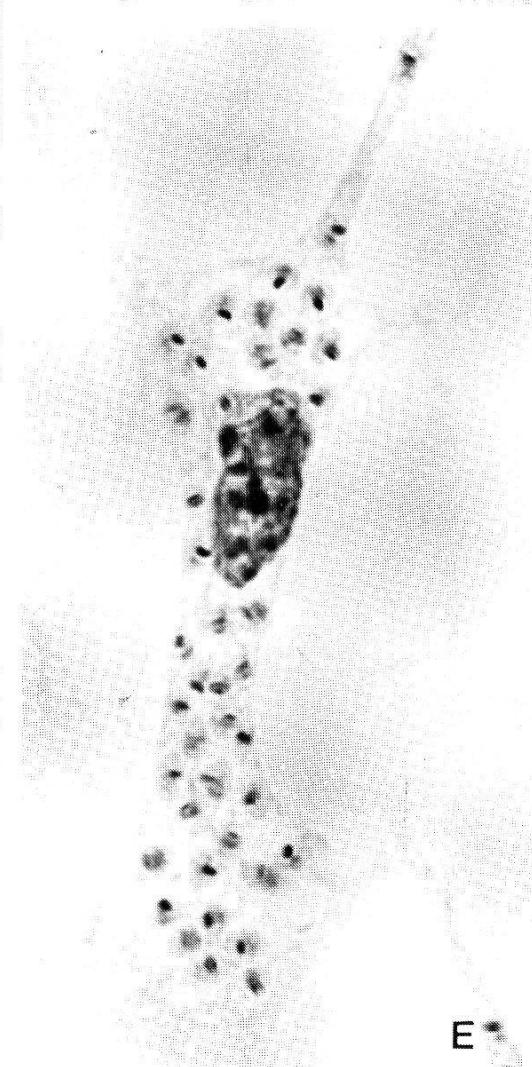
Fig. 3 shows the infectivity of purified amastigotes for mice. On day 18 PI, all animals inoculated with 200 amastigotes had patent parasitemia. The levels of parasitemia never reached more than 1×10^6 parasites/ml, declining gradu-



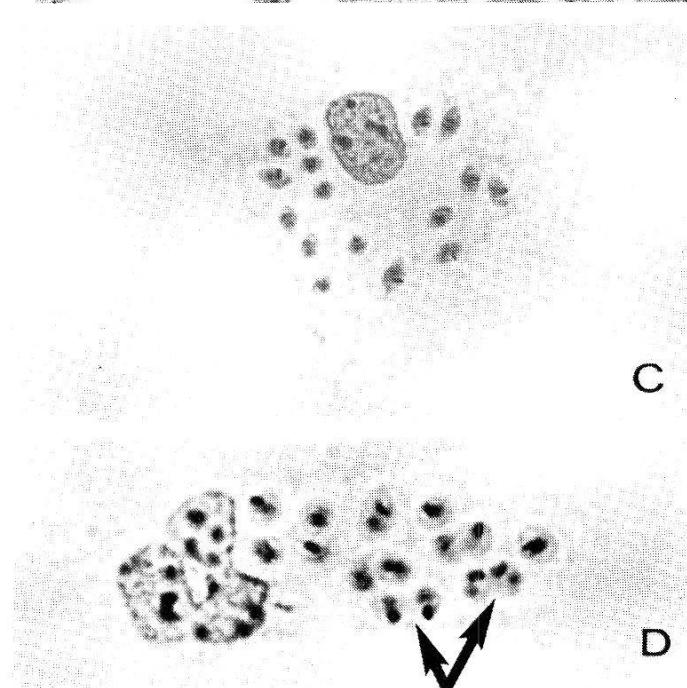
A



B



C



D



E

Fig. 1 A. Electron micrograph of purified *Trypanosoma cruzi* amastigotes. Parasites are well preserved. $\times 4500$.

Figs. 1 B to 1 E. Light micrographs of the interaction of macrophages and parasites.

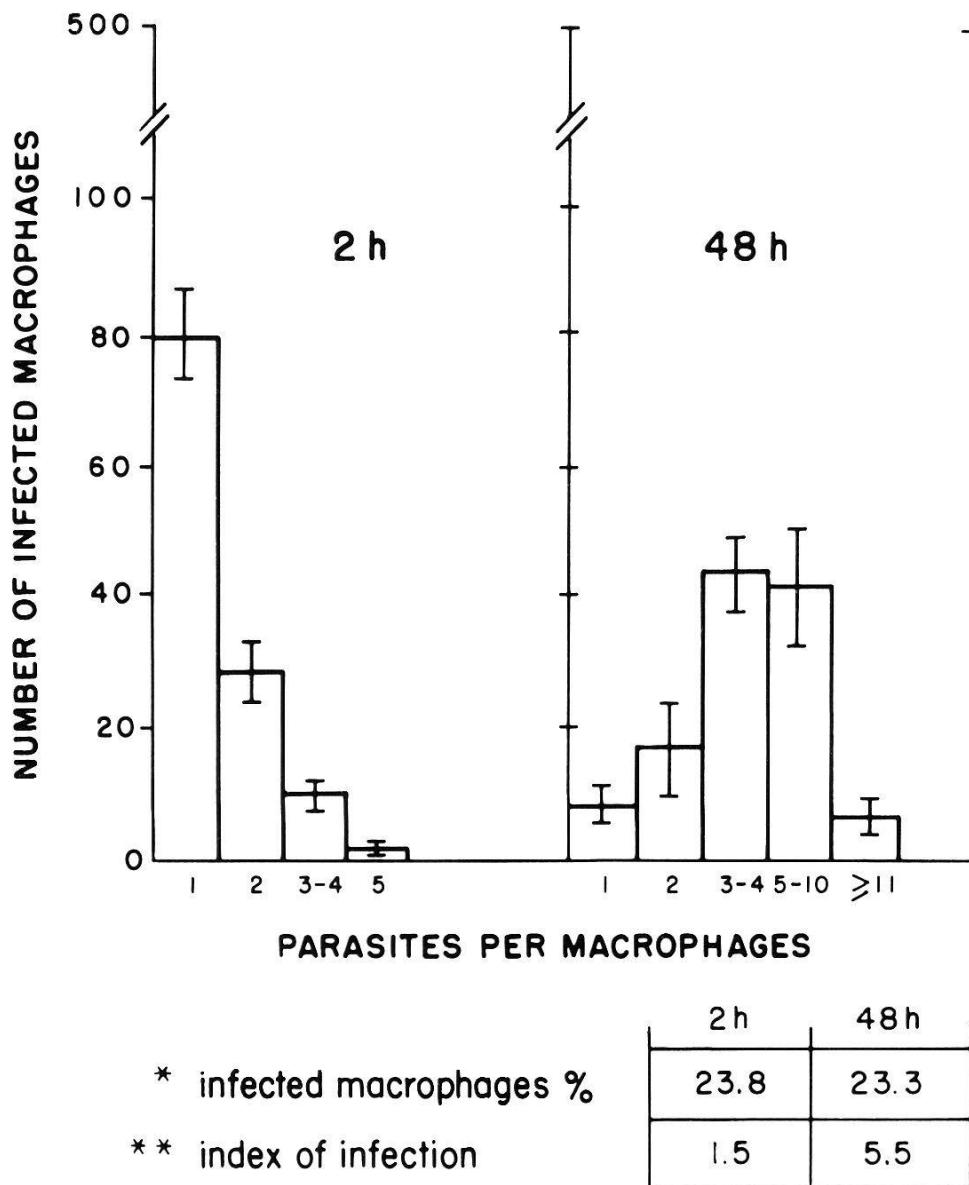


Fig. 2. Infection of macrophages by purified amastigotes. The vertical lines indicate standard errors.

* percentage of macrophages harbouring parasites at 2 h and 48 h

$$** = \frac{\text{total number of parasites}}{\text{total number of infected macrophages}}$$

ally after day 22 PI. Animals inoculated with trypomastigotes developed parasitemia earlier and at higher levels throughout the infection. The mortality rates (until day 45 PI) were 10% for the group injected with the amastigote suspension, 16% for mice injected with 10 trypomastigotes and 60% for mice injected with 200 trypomastigotes.

Fig. 1 B. 2 h post-infection – macrophages harbouring only one parasite. $\times 800$.

Figs. 1 C and 1 D. 48 h post-infection – macrophages containing numerous parasites, some of them in the process of division (arrows, Fig. 1 D). $\times 600$ and $\times 1320$.

Fig. 1 E. 6 days post-infection – at this time, parasites are still in amastigote forms. $\times 1320$.

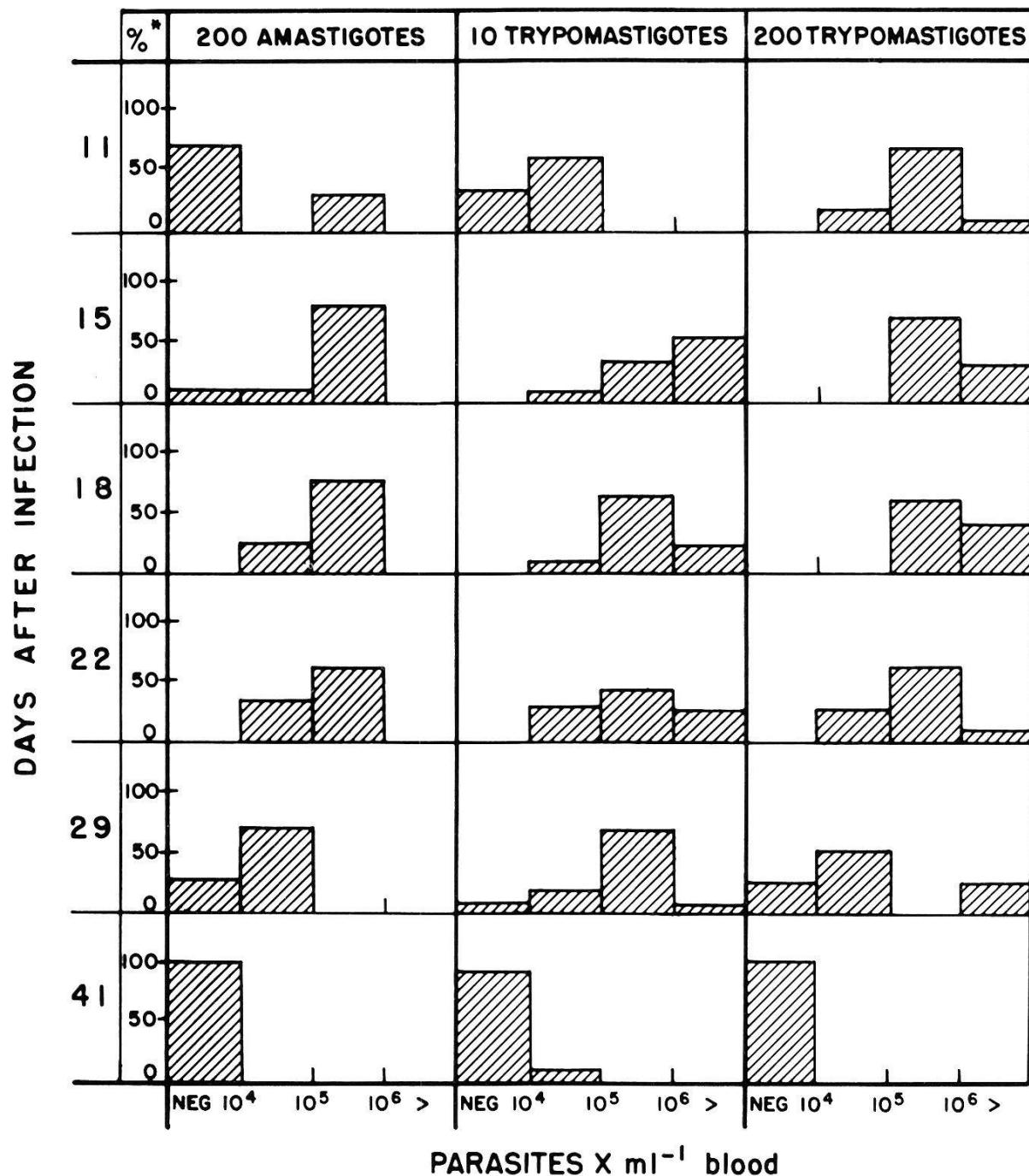


Fig. 3. Effect of inoculum size of purified amastigotes and bloodstream tryptomastigotes on the level of parasitemia.

* percentage of infected animals and parasitemia levels were plotted from day 11 until day 41 postinfection.

Discussion

Our results showed that intracellular amastigotes isolated from the spleen and liver were ingested by mouse macrophages in vitro and multiplied actively in their cytoplasm. It has been reported previously (Carvalho et al., 1981) that amastigotes are destroyed following ingestion by macrophages. The different

methods to isolate amastigotes might have led to different yields of viable organisms. Our preparations of isolated amastigotes showed rather pure and morphological well preserved forms.

It could be argued that the amastigotes observed inside the macrophages originated from ingested trypomastigotes either contaminating the amastigotes' preparation or alternatively derived from extracellular transformation of amastigotes to trypomastigotes. We think that this interpretation is unlikely for the following reasons: 1. the infectivity of trypomastigotes for macrophages is markedly lower than the observed for amastigotes (0.4% vs. 23.3% at 48 h of culture); 2. extracellular forms were never observed in the cultures infected with amastigotes; 3. the percentage of macrophages infected by amastigotes remained unaltered with time in culture, while there was an increase in the index of infection, suggesting that interiorization of parasites did not occur beyond the initial period of infection; 4. the low transformation rate of amastigotes to trypomastigotes (less than 5% in acellular medium) could hardly account for the infection rates observed.

Although capable of multiplication, differentiation of amastigotes to trypomastigotes was not observed in the cytoplasm of infected macrophages. Furthermore trypomastigotes were never detected in the tissue culture medium harvested from the macrophage cultures. The failure of the amastigotes to differentiate to trypomastigotes inside the macrophage could result from a minor damage during the process of amastigote isolation, although cell division, as a parameter of viability, was not impaired. Alternatively, early removal of amastigotes from their intracellular environment could hamper the natural programming of these cells to differentiate even after completion of several cycles of division.

Parasitemia and in vivo infectivity are rather crude parameters of viability. However, we had no reason to suppose that amastigotes would not be readily ingested (as observed in vitro), and thus expected anticipation of parasitemia by obviating the lag phase. Instead, a trend for lower levels of parasitemia and delayed appearance of circulating trypomastigotes was observed in the group infected with amastigotes when compared to the groups infected with trypomastigotes. This trend might reflect the delayed transformation to trypomastigotes observed in the in vitro conditions.

Acknowledgments

This work was supported by CNPq/PIDE V, FINEP, and UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. We thank Juan J. Lafaille for mouse infectivity control and Rosa M. Moraes for technical assistance.

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