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EXTRACELLULAR "IN VITRO" EVOLUTION OF METACYCLIC TRYPOMASTIGOTES ISOLATED FROM TRYPANOSOMA CRUZI CULTURES

Eico KIMURA (1), Waltraut H. LAY (2) and J. F. FERNANDES (1)

SUMMARY

Metacyclic trypomastigotes of **Trypanosoma cruzi** are not a final stage of extracellular evolution. They were purified from cultures and were observed to develop into amastigotes, eventually multiply and subsequently transform into epimastigotes when maintained in LIT culture medium at 27°C. In certain strains or under certain conditions amastigotes also developed into short, broad trypomastigotes, which differed from the original metacyclic trypomastigotes. The raising of incubation temperature to 37°C accelerated the appearance of amastigotes in culture, but hindered further evolution.

INTRODUCTION

The transmission of **T. cruzi** to the vertebrate host through feces of hematophagous triatomids is currently attributed to the inoculation, along with the actively dividing epimastigotes, of infective metacyclic trypomastigotes which arise in the hind gut of the insect. The differentiation of epimastigotes into metacyclic trypomastigotes can also be observed in culture, under certain conditions (CAMARGO [1]; CASTELLANI et al. [2]), and the emerging forms are known to be able to infect a great variety of tissue culture cells, developing intracellularly. An extracellular evolution was seldom reported (MUNIZ & FREITAS [5]; PICK [11]). The purification of metacyclic trypomastigotes from **T. cruzi** cultures allowed us a long-term observation of their behaviour in an acellular medium and this furnished the proof that they are not a final stage of extracellular evolution. A sequential morphological transformation was consistently found, with peculiarities due to strain differences. The influence of temperature was also noted.

MATERIALS AND METHODS

Parasite strains — The following strains of **T. cruzi** were used: Y, MR, Brazil, Berenice, Rio, CL, SMJ, F. They were either isolated from human cases of Chagas' disease or from naturally infected bugs, having been maintained in the laboratory for years by regular blood passage in mice and/or in culture. Only the Y strain was used for quantitative data on the sequential evolution.

Culture procedures — There is no reliable method of regularly obtaining high proportions of metacyclic trypomastigotes in culture. However, reasonable quantities of these forms were often obtained after cultivation of **T. cruzi** on a medium similar to NNN, consisting of a solid phase composed of 30 g Bacto-Agar (Difco), 6 g NaCl, 900 ml H_2O and 100 ml human blood, overlaid with a thin layer of liver infusion tryptose medium (LIT) (CASTELLANI et al. [2]). Occasionally they were harvested from cultures in LIT medium alone. Purified metacyclic trypomastigotes were resuspended in LIT medium containing 10% fetal calf se-

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(1) Instituto de Química da Universidade de São Paulo, Caixa Postal 20780, São Paulo, Brasil.
(2) Instituto de Medicina Tropical de São Paulo (Depto. de Medicina Preventiva), Caixa Postal 2921, São Paulo, Brasil.

rum. All cultures were maintained at 27°C, unless otherwise stated.

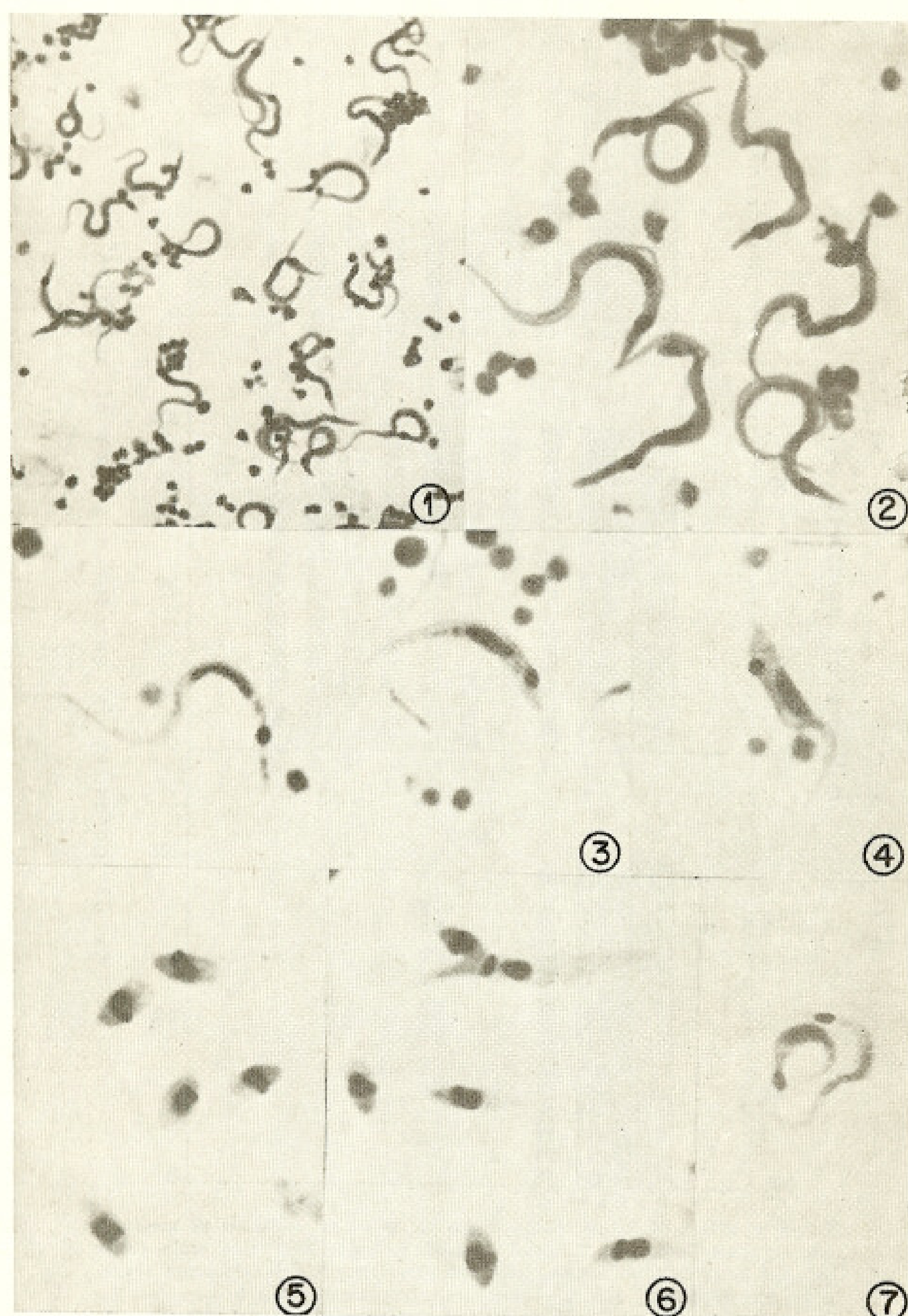
Purification of metacyclic trypomastigotes — The method employed took advantage of the fact that epimastigotes lyse when exposed to fresh serum of certain species (MUNIZ & BORRIELLO [4]; RUBIO [13]) in contrast to metacyclic trypomastigotes which survive under the same conditions. This lysis is dependent upon the alternate pathway of complement activation (NOGUEIRA et al. [8]). The use of whole blood instead of serum, and the sedimentation of the cells followed by resting periods, allowed the removal of dead and already affected epimastigotes, which are retained in the sediment while metacyclic trypomastigotes move into the superposed plasma. In brief, the method consisted of the following steps: rat blood was collected by cardiac puncture into ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetracetic acid (EGTA) and supplied with an excess of Mg^{++}: 1 ml of 0.2 M EGTA and 0.1 ml of 0.2 M $MgCl_2$ per 10 ml of blood. Aliquots of 4 ml of parasite culture containing 50% or more of metacyclic trypomastigotes were distributed into conical centrifuge tubes (17 x 120 mm). Two ml of the mentioned fresh normal rat blood were added to each tube. A mild centrifugation (400 g) was applied for 10 min in order to induce sedimentation, followed by 30 min of resting to allow motile parasites to reach the plasma. The supernatant was removed, examined, and usually the persistence of contaminating epimastigotes demanded a second step of the purification process. The plasma was then centrifuged at 1000 g for 10 min. The resulting sediment, after decantation of the supernatant, was vigorously shaken and resuspended in 1.5 ml of fresh normal mouse blood collected into 3.8% sodium citrate. Saline was added to each tube to double the volume, centrifugation followed for 5 min (400 g) and the tubes were left again for 30 min at room temperature. The plasma was carefully withdrawn and pooled. Estimations, when undertaken, showed 65 to 75% recovery of the metacyclic trypomastigotes originally present in the culture, and the purity of these populations was usually close to 100% (Figs. 1 and 2). This refers to the Y, MR and Brazil strains of **T. cruzi**. The epimastigotes of some other strains, however, such as Berenice and Rio, seemed to be less susceptible to complement lysis.

Methods of observation — Daily samples were withdrawn from the suspension of purified metacyclic trypomastigotes maintained in culture medium, transferred to a hemocytometer and observed and counted by phase-microscopy. Smears for photomicrography were prepared after addition of formaldehyde to the parasite suspension (2% final concentration), fixed in methyl alcohol and stained with Giemsa's solution.

RESULTS

Sequential evolution — Purified metacyclic trypomastigotes of the Y strain of **T. cruzi** resuspended in culture medium at a concentration of about 2×10^6 or 3×10^6 parasites per ml were maintained at 27°C. Daily observations revealed progressive transformations which consisted of gradual shortening, broadening and loss of motility of the original slender and actively moving forms (Fig. 3). They remained static although provided with a flagellum (Fig. 4). In a matter of days they attained the rounded shape of typical intracellular amastigotes, with rod-like kinetoplasts (Fig. 5). Pairs of amastigotes were observed, suggesting binary fission. They gradually transformed into short epimastigotes and later into characteristic epimastigotes (Fig. 6). Some amastigotes could still be observed on the 30th day of culture. Epimastigotes finally replaced all other forms as a consequence of continuous proliferation. Only very late, when the epimastigote culture was allowed to grow old, metacyclic trypomastigotes eventually reappeared.

Effect of temperature — The differentiation of metacyclic trypomastigotes of the Y strain of **T. cruzi** maintained at 27°C, 33°C, or 37°C is shown in Fig. 8. At 27°C amastigotes made their appearance already on the first day of culture, increased progressively in number up to a maximum around the 16th to 20th day, and then began to decline. Epimastigotes appeared around the 3rd day of culture, proliferated steadily and finally overwhelmed the culture. At 33°C differentiation into amastigotes was accelerated up to the 3rd day, but the total number of parasites declined thereafter, strongly suggesting a hindrance of differentiation into epimastigotes. The culture dwindled away and the most resistant



Figs. 1-7 — Sequential extracellular evolution of metacyclic trypomastigotes of **T. cruzi** in LIT medium (1-6: Y strain; 7: MR strain). 1. Population of metacyclic trypomastigotes after the purification process (the contaminating cells are platelets). 320X. 2. The same. 800X. 3. Metacyclic trypomastigotes, one of them showing beginning of shortening. 800X. 4. Transitional form between metacyclic trypomastigote and amastigote. 800X. 5. Amastigotes. 800X. 6. Amastigotes, short and long epimastigotes. 800 X. 7. Short, broad trypomastigote and slender metacyclic trypomastigote of the MR strain. 800X.

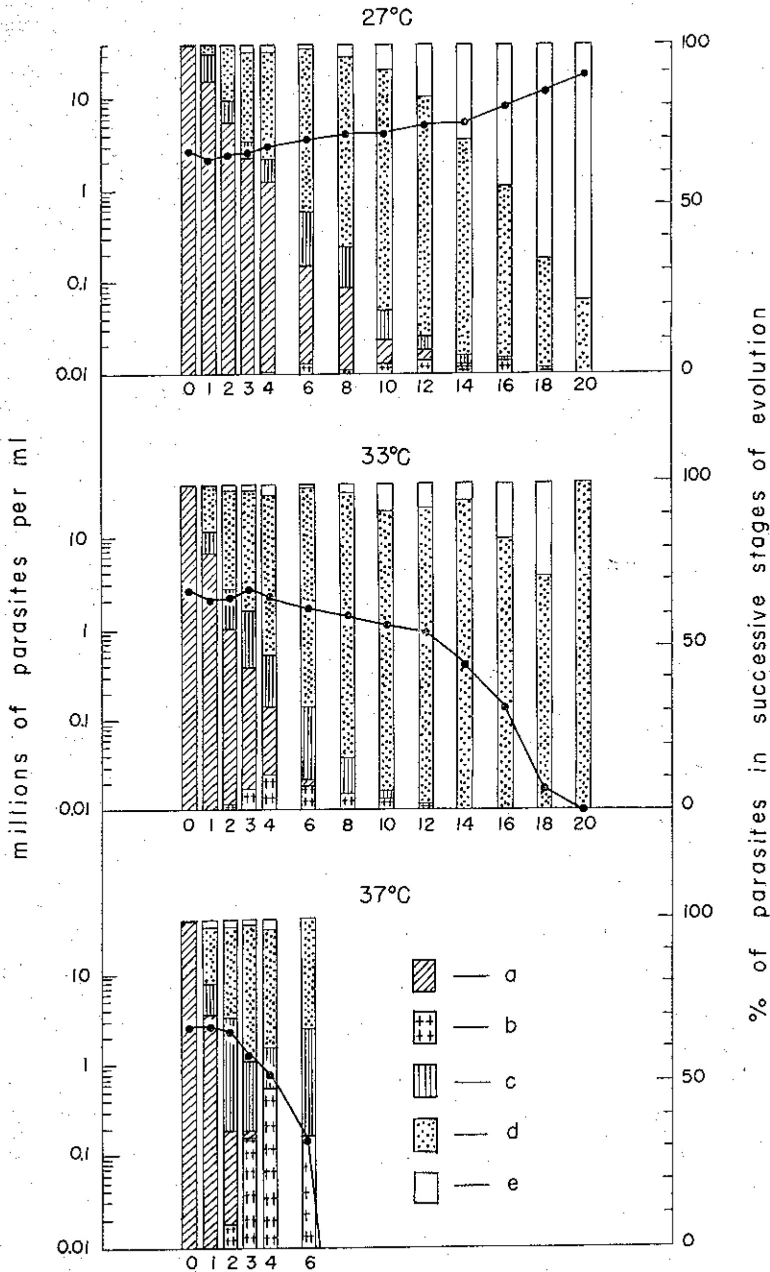


Fig. 8 — Effect of temperature on the extracellular evolution of metacyclic trypomastigotes of **T. cruzi** in LIT medium. Left side scale (lines through bars) = total concentration of parasites. Right side scale (bars) = relative frequency of: a — metacyclic trypomastigotes; b — metacyclic trypomastigotes showing prominent signs of degeneration; c — transitional forms between metacyclic trypomastigotes and amastigotes; d — amastigotes; e — epimastigotes.

amastigote form disappeared completely within 20 days of culture. At 37°C a drastic shift toward early differentiation of metacyclic trypomastigotes into amastigotes characterized the first day of culture, but the subsequent disappearance of parasites up to the 8th

day proved the deleterious effect of higher temperature on further development. The number of metacyclic trypomastigotes showing prominent signs of degeneration was also considerably higher at 37°C.

Strain differences — The sequential evolution described above for the Y strain of **T. cruzi** was observed in all examined strains, but the speed of transformation from one to another form sometimes differed considerably according to the strain. Metacyclic trypomastigotes from the Brazil strain, for instance, reorganized into amastigotes only very late, around the 5th day of culture, and these soon developed into small, slowly growing epimastigotes.

An apparent peculiarity of evolution was observed with the MR strain: their metacyclic trypomastigotes transformed into amastigotes and then evolved either into epimastigotes, as described, or into short, broad trypomastigotes, which appeared in the culture around the 6th day. Figure 7 shows the difference in morphology between the original slender metacyclic trypomastigote, with sub-terminal kinetoplast, and the short, broad trypomastigote, with terminal kinetoplast. The motility of both trypomastigotes was also distinct: sinuous and forward-driven in the former, and sluggish in the latter. The short, broad trypomastigotes apparently transformed again into amastigotes, sometimes seen holding together in large clumps, and finally developed into epimastigotes.

This peculiar way of evolution was thought to be characteristic of the MR strain of **T. cruzi,** but subsequently proved not to be so because, on a single occasion, an old culture of the Y strain, observed 2 weeks after a "wave" of metacyclic trypomastigotes had occurred, showed a small but unequivocal proportion of short, broad trypomastigotes, besides amastigotes.

DISCUSSION

The present observations leave no doubt about the fact that metacyclic trypomastigotes of **T. cruzi** are not exclusively dependent on intracellular proliferation for further evolution. The theoretical possibility of the existence of sub-populations with distinct properties, one disposed toward intracellular proliferation, the other one capable of evolving extracellularly, has no morphologic support and could not be proved by selective enrichment: metacyclic trypomastigotes which had completed the extracellular evolution toward epimastigotes gave rise again to metacyclic trypomastigotes capable of infecting cell cultures.

The preference of metacyclic trypomastigotes for one or the other kind of evolution probably depends on several extrinsic factors and their interactions. The influence of temperature seems to be important: whereas intracellular parasitism was shown to increase in cell culture experiments the closer one reaches the vertebrate host's temperature (NEVA et al. [6]; TREJOS et al. [14]; RIBEIRO et al. [12]), extracellular evolution proved to be most prominent at 27°C, the temperature corresponding to optimum "in vitro" culture conditions of the parasite form which lives in the invertebrate host.

The described extracellular pathway could also be observed in cultures of **T. cruzi** which had not been submitted to complement lysis during the purification process of metacyclic trypomastigotes, although the clear sequential evolution is then difficult to follow. Amastigotes have been reported sporadically in long-term established **T. cruzi** cultures, either isolated or agglutinated into large masses (CHAGAS [3]; NOBLE [7]). The cultivation of large numbers of leishmaniform organisms in cell-free media described by PAN [9,10], starting with cultures of **T. cruzi** maintained routinely in a modified blood agar (NNN) medium, could also be the consequence of the triggering of extracellular evolution of metacyclic trypomastigotes, followed by some unknown factor of hindrance to further evolution. Data from published reports on the morphology of organisms thought to be released from cell cultures infected with metacyclic trypomastigotes should also be reconsidered under the present knowledge of the possibility of extracellular evolution.

In our view, the peculiar behaviour of the MR strain, giving rise in culture to two distinct types of trypomastigotes, should not be considered characteristic of the strain. We are inclined to believe that hitherto unknown progressive biochemical changes in the medium or the influence of previous conditions of ma-

intainance of the strain could eventually, be responsible for the appearance or not of the short, broad trypomastigotes in culture, which greatly resemble the so called "bloodstream type" of trypomastigotes described and photographed by PAN [10] in cultures of the Brazil strain. The morphological as well as biological differences observed between the two distinct types of culture trypomastigotes of the MR strain will be the subject of a subsequent paper.

RESUMO

Evolução extracelular in vitro **de tripomastigotas metaciclícos, isoladas de cultura de** T. cruzi

Tripomastigotas metacíclicos de **Trypanosoma cruzi** não constituem forma final na evolução extracelular do parasita. Após purificação dessas formas a partir de culturas verificou-se que, quando mantidas em meio LIT a 27°C, elas são capazes de se transformar em amastigotas, eventualmente multiplicar e depois evoluir para epimastigotas. Em algumas cepas, ou em certas condições de cultivo, os amastigotas extracelulares evoluiram dando origem a tripomastigotas curtos e largos, diferentes dos tripomastigotas metacíclicos originais. A elevação da temperatura de cultivo até 37°C acelerou o aparecimento de amastigotas na cultura, mas prejudicou sua evolução posterior.

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