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INFECTIVITY OF AMASTIGOTES OF TRYPANOSOMA CRUZI

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SUMMARY

The infectivity amastigotes of **Trypanosoma cruzi**, isolated from the supernatant of the J774G8 macrophage-like cell line infected with trypomastigotes to normal macrophages **in vitro** was tested. After a period of 1 h of **T. cruzi**-macrophage interaction about 2% of the mouse peritoneal macrophages had ingested amastigotes. In contrast 12% of the macrophages had ingested epimastigotes. Treatment of the amastigotes with trypsin did not interfere with their ingestion by macrophages. Once inside the macrophages the amastigotes divided and after some days transformed into trypomastigotes. When i.p. inoculated into mice the amastigotes were highly infective, inducing high levels of parasitaemia and tissue parasitism. As previously described for trypomastigotes, amastigotes were not lysed when incubated in the presence of fresh guinea-pig serum. Contrasting with what has been described for trypomastigotes, the resistance of amastigotes to complement-mediated lysis persisted after treatment with trypsin.

KEY WORDS: — Chagas'disease — Trypanosoma cruzi — Experimental infection in mice — Infectivity of amastigotes

INTRODUCTION

Three developmental stages exist in the life cycle of **Trypanosoma cruzi:** epimastigote, trypomastigote and amastigote. The first stage is the dividing form, found in the intestine of the invertebrate host and can be easily maintained **in vitro** in axenic culture. The trypomastigote stage is not able to divide. It can be found in the intestine of the invertebrate host and in the bloodstream of the vertebrate host. This form is able to infect most of the vertebrate cells, where it transforms into the amastigote stage. This amastigote is the only form of **T. cruzi** in the vertebrate under which multiplication occurs and is, therefore, responsible for the parasitological amplification of Chagas'disease.

It has been shown conclusively that epimastigote and trypomastigote forms of **T. cruzi** are ingested by macrophages. However, while epimastigotes are digested, trypomastigotes survive and divide within normal macrophages (NOGUEIRA & COHN [19]). Therefore, while epimastigotes are not infective, trypomastigotes are highely infective to the vertebrate host Few data exist on the infectivity of amastigotes to the vertebrate host due to the difficulties in obtaining parasites which can be safely identified as amastigotes and which are not altered by the drastic treatments with which they were obtained. Using amastigote-like forms from different sources, contradictory results have been reported (ABRAHAMSON et al.[1]; ARAÚJO et al[3]; CARVALHO et al.[6]; GUTTERIDGE et al.[10]; HUDSON et al.[11]; LANAR [13]; MAcCABE et al [14]; PAN [23]; RIBEIRO et al [24]; SEGURA et al.[25];

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UMEZAWA et al.[26]; VILLALTA & KIERSZENBAUM[27]. For some Authors amastigotes are infective while for others they are immediately digested by macrophages.

We described recently a procedure for obtaining amastigotes of **T. cruzi** (CARVALHO & SOUZA[7]) and showed, by freeze-fracture and determination of the surface charge (CARVALHO et al.[8]), that the forms obtained by us have surface properties which distinguish them from epimastigotes and trypomastigotes. In the present work we report the effect of complement on amastigotes of **T. cruzi** and their infectivity to mice and to mouse peritoneal macrophages maintained **in vitro.**

MATERIALS AND METHODS

Cell Interaction

The Y strain of **T. cruzi** was used. It had been maintained by weekly i.p. injections in mice. Trypomastigotes were obtained from the blood of mice 7 days post inoculation. Epimastigotes were cultivated for 4 days in Warren's medium (WARREN[28]). For isolation of amastigotes we used, as previously described, the macrophage-like cell line J774G8 (CARVALHO & SOUZA[7]). The cells were infected with bloodstream trypomastigotes and the cultures examined every day using an inverted microscope. When a large number of amastigotes were seen in the supernatant they were isolated by centrifugation in a Metrizamide gradient. The cells were processed for electron microscopy as described previously (CARVALHO & SOUZA[7]).

Macrophages were collected from the peritoneal cavities of uninfected Swiss mice after injection of 3 to 5 mls of Hank's balanced solution (HBS). Samples of a 0.6 ml suspension containing 2 x 10^6 mononuclear cells per ml were placed into Leighton tubes with flying cover.slips. After incubation for about 1 hr at 37°C the non-adherent cells were removed, the macrophage monolayers were washed twice with 199 medium and new 199 medium supplemented with 10% inactivated fetal calf serum was added and the cells were incubated for 24 hrs at 37°C. Parasites (amastigotes, epimastigotes) were suspended in 199 medium in order to achieve a ratio of 10 parasites per macrophage, and 0.5 ml of the suspensions was added to the macrophage cultures. Parasites were maintained in contact with the macrophages for periods varying from 1 to 24 h after which time the cells were rinsed with Ringer solution, fixed with Bouin's fixative and then stained with Giemsa. The percentage of infected macrophages was determined by randomly examining at least 200 cells in triplicates with high magnification under a Zeiss Universal Photomicroscope. The experiments were repeated at least 3 times. In some experiments, after 120 min. of interaction the cultures were washed twice with HBS to remove extracellular parasites, new 199 medium supplemented with 10% fetal calf serum was added and then incubated at 37°C for periods varying from 1 to 6 days At intervals of 24 hs the culture medium was changed. These cultures were also fixed with Bouin's fixative and stained with Giemsa.

Enzymatic Treatment

Amastigotes and trypomastigotes were washed twice with 199 medium and then incubated for 15 min. at 37°C in the presence of 500 μg/ml of trypsin (Sigma Chemical Company, type III) dissolved in 199 medium without serum, pH 7.2, or for 30 min. at 37°C in the presence of 0.2 U/ml of neuraminidase (Sigma, type X from **Clostridium perfringens**) in Tyrode's solution, pH 6.0. The effect of trypsin was stopped by addition of fetal calf serum. After enzyme treatment the cells were collected by centrifugation, washed twice in Tyrode's solution or 199 medium without serum and used for experiments of interaction with macrophages and complement-mediated lysis.

Lytic Assays

To measure lysis by complement, 100 μl of control or enzyme treated parasites were mixed with 100 μl of normal pig serum and incubated for 30 min. at 37°C. Aliquots were examined in a Neubauer Chamber before and after the incubation of the parasites in the presence of serum. The number of viable parasites, determined by using the trypan blue exclusion test, was expressed as the mean of two replicated tubes.

Infectivity to Mice

Groups of 12 mice were inoculated intraperitoneally with 10^5 isolated amastigotes or blood-

stream trypomastigotes. Parasitaemia was recorded every 48 hs. Six mice were, 10 days after inoculation, sacrificed and fragments from liver, heart and spleen were removed, fixed in 10% formaldehyde, and embebbed in paraffin. Five to 10 μm sections were obtained, stained with hematoxylin-eosin and observed under the microscope.

RESULTS

By using the methodology previously described for obtaining of amastigotes, very pure preparations were obtained some of which containing less than 0.1% of typical trypomastigotes (Fig. 1). In some experiments, for unknown reasons, the preparations obtained contained about 2% trypomastigotes. However, for the experiments described here only pure preparations of amastigotes were used. Examinations of thin sections of these preparations by transmission electron microscopy showed that they contained some forms apparently in transition to trypomastigotes (Figs. 4-5). These forms had a kinetoplast with a structure typical of amastigotes but located laterally in relation to the nucleus. They also possessed a flagellum slightly larger than that of amastigotes. By using the trypan blue exclusion test, we found that more than 95% of the cells were viable.

When incubated **in vitro** in the presence of normal mouse peritoneal macrophages amastigotes were ingested by the macrophages. However, the percentage of macrophages which ingested the amastigotes was much lower than that observed, under the same experimental conditions, for epimastigotes. Incubation of the amastigotes in the presence of trypsin before interaction did not increase their ingestion by the macrophages (Table I).

Observation by light microscopy of macrophage cultures fixed at various times after infection with amastigotes showed that the parasites were not digested, and dividing forms could be observed after 24 hs. (Fig. 2). After 72 hs. of infection the macrophages contained a large number of intracellular parasites, even trypomastigotes and intermediate forms (Figs. 3). After longer periods of interaction, trypomastigotes were released into the culture medium. Intracellular development of **T. cruzi** was observed in practically all macrophages which had ingested amastigotes.

Bloodstream trypomastigotes were seen in mice inoculated intraperitoneally with amastigotes. The parasitaemia was high (Fig. 7). The pattern of parasitaemic curve obtained with amastigotes was identical with that observed with bloodstream trypomastigotes, showing a peak at the 7th day and a decline thereafter. About 50% of the mice inoculated with 10^5 amastigotes died between day 13 and 15. Histopathological analysis of mice sacrificed 10 days after inoculation showed a marked parasitism of tissues (Fig. 6) and no apparent difference was observed in the grade of parasitism of mice inoculated with amastigotes or with bloodstream trypomastigotes. A large number of trypomastigotes was also observed in the peritoneal fluid of the mice.

Amastigotes were not lysed when incubated in the presence of normal, non inactivated guinea pig serum (Table II). We used bloodstream trypomastigotes and epimastigotes as controls. As described before, trypomastigotes were not while all epimastigotes were readly lysed. It had been shown previously that treatment of bloodstream trypomastigotes with tripsin renders them susceptible to complement-mediated lysis (KIPNIS et al.[12]). Our experiments confirmed these observations but showed that the same trypsin treatment did not interfere with the resistance of amastigotes to the lysis induced by guinea pig serum (Table II).

DISCUSSION

The infectivity of the amastigote form of **Trypanosoma cruzi** to the vertebrate host is still discussed. Some authors have reported that amastigotes are infective while for other they are not. It is possible that these discrepancies result (a) from the origin of the amastigote or amastigote-like forms used, and/or (b) from the methods used to obtain them.

Rounded forms, which have been considered amastigotes, have been obtained in axenic cultures under certain conditions (ENGEL et al. [9]; PAN [21,22,23]). When the Brazil strain is maintained in a special medium at 37°C, amastigotes are obtained which are able to infect human skin-muscle cells **in vitro** and mouse pe-

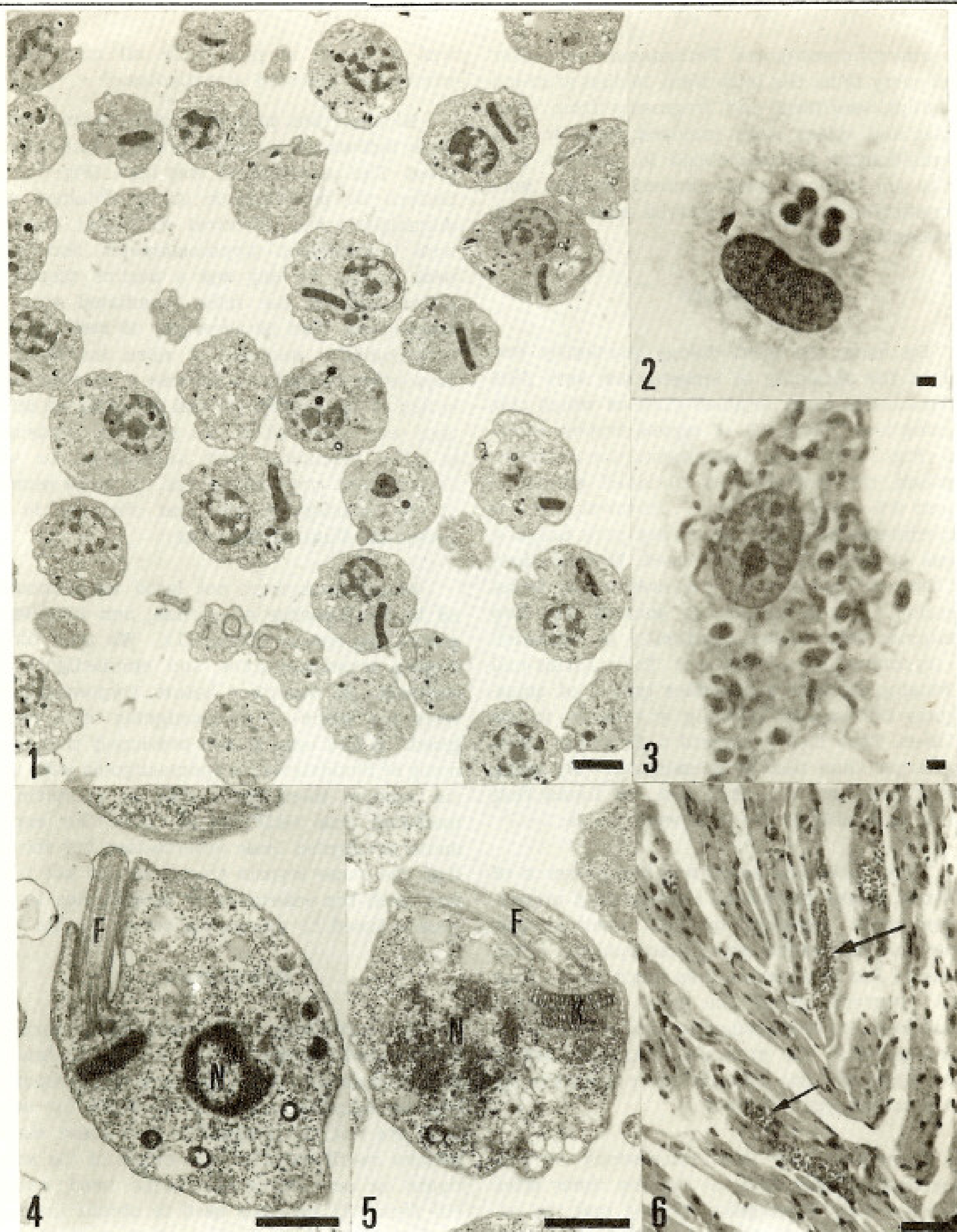

Fig. 1 — General aspect of the suspension of purified amastigotes as seen by transmisison electron microscopy X 7500. Bar = 1 μm

Fig. 2 — Photomicrograph of Giemsa-stained preparation showing dividing amastigotes after 24 h of **T. cruzi**-macrophage interaction. X 300. Bar = 10 μm.

Fig. 3 — Photomicrograph of Giemsa-stained preparation showing trypomastigotes, amastigotes and intermediate forms after 72 h of **T. cruzi**-macrophage interaction. X 300. Bar = 10 μm.

Fig. 4-5 — Detail of two forms which are in process of transformation into trypomastigotes, as seen by transmission electron microscopy. They have a kinetoplast (K) laterally located in relation to the nucleus (N), and a short flagellum) (F). X 13.000. Bar = 1 μm

Fig. 6 — Histological section of the heart of a mouse sachificed 10 day after inoculation of amastigotes. Infected cells can be seen (arrows) X 150. Bar = 50 μm

TABLE I

Ingestion of amastigote and epimastigote forms of **Trypanosoma cruzi** by mouse peritoneal macrophages **in vitro** (1)

Developmental form	Time of interaction (h)	Treatment	Percentage of infected macrophages
Amastigote	2	No	2
Amastigote	6	No	2
Amastigote	24	No	8
Amastigote	2	Trypsin	1.5
Amastigote	3	Trypsin	1
Epimastigote	2	No	12.5

(1) In all case the **T. cruzi**-macrophage ratio was 10:1. Results from one representative experiment.

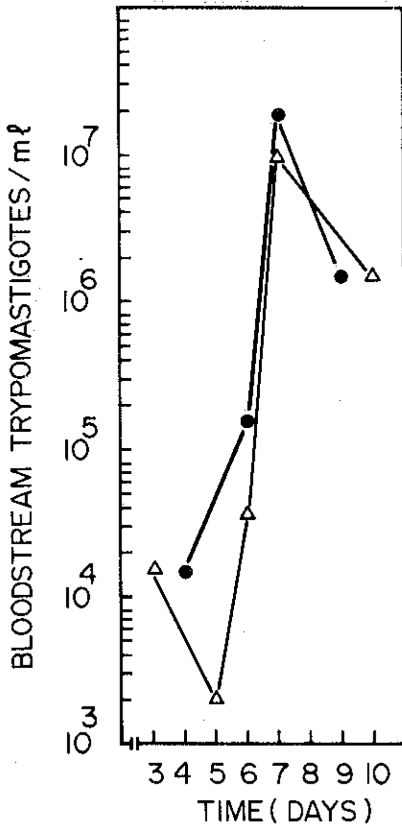

Fig. 7 — Parasitaemic curves in mice inoculated with 10^5 amastigotes (Δ) or 10^5 bloodstream trypomastigotes (●).

ritoneal macrophages **in vivo** (PAN [23]). When the Peru strain is cultivated in the presence of an estabilished cell line of **Triatoma infestans** embryo cells, rounded forms, which form clusters and which have been designated as "staphylomastigotes", appear. These forms divide outside the cells and are infective to mice, as seen by the appearance of bloodstream trypo-

TABLE II

Susceptibility of **T. cruzi** to lysis by complement (*)

Developmental form	Treatment	Percentage of Lysis
Amastigote	No	0
Amastigote	Trypsin	0
Epimastigote	No	100
Bloodstream trypomastigote	No	0
Bloodstream trypomastigote	Trypsin	98

(*) Data are from one representative experiment

mastigotes in the inoculated animals (LANAR [13]). It has also been shown that rounded forms isolated by centrifugation of axenic cultures, are infective to mice (RIBEIRO et al. [24]; SEGURA et al. [25]). Rounded forms found in clones of the CL strain were also infective (ANDRADE [2]).

In relation to the possible infectivity of amastigotes obtained from vertebrate cells it was observed that some rounded forms released by macrophages in culture are ingested by normal macrophages and can reproduce the intracellular cycle of the parasite (BEHEBEHANI [5]). When amastigotes are isolated from tissues of animals infected with **T. cruzi**, contradictory results have been reported. For instance, GUTTERIDGE et al. [10] showed that amastigotes, isolated from the muscle of heavly infected animals, are not infective for other animals. Others showed that amastigotes isolated from the spleen of mice were ingested by normal mouse peritoneal macrophages where they were digested (CARVALHO et al. [6]). However, it was also shown that amastigotes isolated from the liver and the spleen of mice are infective to mice (ABRAHAMSON et al. [1]; McCABE et al. [14]) and for macrophages in culture (McCABE et al. [14]; UMEZAWA et al. [26]).

Our observations show clearly that amastigotes isolated from the supernatant of the macrophage-like J774G8 cell line (CARVALHO & SOUZA [7]) and identified previously (CARVALHO et al. [6]) are infective to normal mouse macrophages **in vitro** as well as to mice. In the case of amastigotes it is interesting to point out that the percentages of macrophages which ingest amastigotes is much lower than that which is obtained when trypomastigote or epimastigote forms are used. This result is in contrast with previous observations (CAR-

VALHO et al.[6]; UMEZAWA et al.[26]), which show that amastigotes isolated from the spleen of infected mice were readly ingested by macrophages. Treatment of the amastigotes with trypsin did not increase their ingestion by macrophages. It has been shown that trypsinization increases the uptake of bloodstream trypomastigotes by macrophages (ARAÚJO JORGE & SOUZA[4]; KIPNIS et al.[12]; NOGUEIRA et al.[18]). It is possible that the procedures used for the obtention of the amastigotes from the spleen interfered with the surface components of the parasites rendering them susceptible to ingestion by the macrophages. Careful examination of macrophage cultures fixed at various periods of interaction with **T. cruzi** showed that parasites proliferated within most cells which had ingested amastigotes.

It was reported recently that no amastigote-trypomastigote transformation occurred in macrophages infected with amastigotes of **T. cruzi** (UMEZAWA et al.[26]). In our macrophage cultures, however, the intracellular cycle was completed and trypomastigotes were found in the supernatant of cultures maintained **in vitro** for 72 hours.

Our results show that amastigotes are highly infective when inoculated into mice where a large number of trypomastigotes in the bloodstream, as well as in the peritoneal fluid of the animals, can be seen. It is interesting to note that the pattern of the parasitaemic curve of mice inoculated with amastigotes is similar to that observed with trypomastigotes, supporting the view that this pattern is characteristic of the strain. The large number of cells containing parasites in the tissues of the mice infected with amastigotes is also an indication of the high infectivity of these forms. It was shown recently that amastigotes obtained from the fibrosarcoma line M_4 are infective for mice, producing low-grade infections in normal mice and acute fatal infection in mice previously irradiated (HUDSON et al.[11]).

It is well known that while trypomastigotes are resistant, epimastigotes are highly susceptible to complement-mediated lysis (MUNIZ & BORRIELO[16]; NOGUEIRA et al.[17]). In the case of trypomastigotes it was shown that also this form became susceptible to the complement mediated lysis when the parasites were treated with trypsin (KIPNIS et al.[12]). Our data confirm all these observations and extend them to the amastigotes, showing, however, that this form is highly resistant to complement-mediated lysis even after trypsin treament. This observation is in close agreement with previous results showing that rounded forms, obtained by incubation of trypomastigote or epimastigote forms of **T. cruzi** in axenic medium (ENGEL et al.[9]), are resistant to complement-mediated lysis.

All these data suggest that amastigotes are resistant to complement-mediated lysis and are able to infect vertebrate cells. It is possible that infection of vertebrate cells **in vivo** by amastigotes occurs mainly in the acute phase of Chagas' disease. Ultrastructural observations of heart tissue of mice heavily infected shows, occasionaly, the presence of amastigotes in the intercellular space (DE SOUZA, unpublished observations). Similar observations have been reported previously (BEHEBEHANI[5]). Studies carried out **in vitro** have shown that at the end of an intracellular cycle the amastigote-trypomastigote transformation is not synchronous so that when the cell has ruptured there is release of trypomastigotes, intermediate forms and also some amastigotes. Therefore it is possible that also these amastigotes may contribute to the spreading of the infection in the vertebrate host.

RESUMO

Infecciosidade da forma amastigota do
Trypanosoma cruzi

A infecciosidade da forma amastigota do **Trypanosoma cruzi**, isolada do sobrenadante de culturas da linhagem tumoral de macrófago J774G8 previamente infectada com formas tripomastigotas, para macrófagos normais mantidos **in vitro**, foi analisada. Apenas 2% dos macrófagos ingeriram amastigotas quando o período de interação parasito-célula era de 1 hora enquanto que para este mesmo período 12% dos macrófagos ingeriram formas epimastigotas. Tratamento prévio do amastigotas com tripsina não interferiu na sua ingestão por macrófagos. Uma vez no interior dos macrófagos os amastigotas se dividiam e posteriormente transformavam-se em tripomastigotas. A forma amastigota mostrou-se altamente infectiva

quando inoculada na cavidade peritoneal de camundongos, induzindo altos níveis parasitêmicos e parasitismo tecidual. Os amastigotas, não são lisados quando incubados na presença de soro fresco de cobaio à semelhança do que tem sido descrito para tripomastigotas. No entanto, ao contrário do que ocorre com tripomastigotas, os amastigotas não são lisados mesmo após prévia tripsinização.

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