

## AMASTIGOTES OF *TRYPANOSOMA CRUZI* SUSTAIN AN INFECTIVE CYCLE IN MAMMALIAN CELLS

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*Trypanosoma cruzi*, the causative agent of Chagas' disease which affects a large number of individuals in Central and South America, is transmitted to vertebrate hosts by blood-sucking insects. The infective forms of the parasite are the metacyclic trypomastigotes released with the feces of the insect. After invading the mammalian host, they penetrate cells, multiply as amastigotes, and transform again into trypomastigotes, which in turn are released into the bloodstream. The majority of acute human cases of Chagas' disease resolve in a few months but many individuals develop chronic manifestations of a systemic disease. Paradoxically, these individuals have very few parasites in circulation or in their tissues, but have severe heart and digestive tract lesions. It is generally assumed that the chronic infection is maintained by a few tryposmastigotes in the blood that escape the immune response and enter new cells, and that amastigotes are exclusively the replicative intracellular stage of the parasite (1, 2). Here we demonstrate that amastigotes of *T. cruzi* enter and multiply in cells in vitro and, when injected intraperitoneally, are as infective to mice as trypomastigotes.

### Materials and Methods

**Parasites.** Strain Y of *T. cruzi* was maintained in monolayers of LLC-MK<sub>2</sub> cells in DME containing 2% FCS medium (Gibco Laboratories, Grand Island, NY) at 37°C in a 5% CO<sub>2</sub> atmosphere (3). To obtain recently released trypomastigotes, LLC-MK<sub>2</sub> cells were infected with parasites and 4 or 5 d later, were washed to remove free parasites, and were reincubated in fresh medium. Trypomastigotes were collected 4 h later.

In vitro extracellularly developed amastigotes were obtained by incubation of recently released trypomastigotes in liver infusion tryptose medium (LIT)<sup>1</sup> (4) for 24, 48, or 72 h at 37°C in a 5% CO<sub>2</sub> atmosphere at a density of 10<sup>7</sup> parasites/ml. LIT medium was prepared by heating at 68°C for 1 h a solution containing 68 mM NaCl, 5.4 mM KCl, 56 mM Na<sub>2</sub>HPO<sub>4</sub>, 11 mM D-glucose, 3 g/liter of liver infusion (Difco Laboratories Inc., Detroit, MI) and 5 g/liter of Bacto Tryptose phosphate broth (Difco Laboratories, Inc.). After cooling, the pH of the medium was adjusted to 7.2 and 2% hemoglobin from SRBC (Colorado Serum Co., Denver, CO) was added. The LIT medium was sterilized by filtration and stored at 4°C. The parasites were sedimented by centrifugation (1,000 g for 5 min) and further purification

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<sup>1</sup> Abbreviation used in this paper: LIT, liver infusion tryptose medium.

was achieved by allowing contaminating motile trypomastigotes to accumulate in the supernatant during a subsequent incubation at 37°C for 2 h. These trypomastigotes were discarded. The amastigotes developed in LIT medium will be referred to as extracellularly derived amastigotes, and the number of hours of in vitro cultivation will be given in parentheses.

**Entry into Cells and Determination of Multiplication Rates.** PBMC were isolated from venous blood of a normal human donor by centrifugation in a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Piscataway, NJ). Monocytes were further purified by the removal of cells that did not adhere to the bottom of plastic plates (24-well, Falcon Laboratories, Oxnard, CA). As seen by transmission electron microscopy, the resulting culture consisted overwhelmingly of monocytes, with a few contaminating lymphocytes (not shown). The monocytes were then cultured in RPMI 1640 (Gibco Laboratories) containing 10% fresh human serum, penicillin and streptomycin (Gibco Laboratories). 6 d later the cultures, each containing  $\sim 2 \times 10^5$  cells, were exposed to a suspension of  $10^5$  parasites (amastigotes or trypomastigotes) per well. After 4 h of incubation (time zero) the medium was aspirated, and the wells were carefully washed with RPMI 1640 to remove free organisms.

3T3 fibroblast cells were irradiated with 4,500 rad and plated onto 24-well plates ( $1-5 \times 10^5$  cells/well). Cells were incubated at 37°C in DME, 10% FCS for 24 h before adding the parasites. During infections, amastigotes were left in contact with the 3T3 cells for 14 h, then the cultures were washed and incubated with fresh medium at 37°C in a 5% CO<sub>2</sub> atmosphere from 1 to 6 d.

To permit intracellular development of the parasites, the washed cells were then incubated for varying periods of time in RPMI 1640, 10% fresh human serum at 37°C in a 5% CO<sub>2</sub> atmosphere. At different time points the cells were scraped off the wells and the whole contents (cells + medium) were centrifuged and processed for parasite DNA determinations. Pellets were resuspended in a lysis buffer: 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.4% SDS, containing 200 µg/ml of proteinase K (Bethesda Research Laboratories, Bethesda, MD). After incubation for 1 h at 65°C and 3 h at 37°C, lysates were extracted with phenol/chloroform and the DNA was blotted onto nitrocellulose discs. A *T. cruzi*-specific DNA probe (5) was labeled by the random priming method ( $1-4 \times 10^9$  dpm/µg) (6) and hybridized for 20 h at 42°C in 50% formamide, 5 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, and 0.2% cow's milk. Filters were washed twice in 2 × SSC, 0.5% SDS at room temperature, then in 0.5 × SSC, 0.5% SDS at 68°C, and finally in 0.2 × SSC, 0.5% SDS at 68°C. They were then air dried and counted in a scintillation counter. The amount of *T. cruzi* DNA was calculated from a standard curve made with known amounts of parasites. Standard curves obtained with equal numbers of trypomastigotes and amastigotes were identical.

To determine the proportion of infective parasites in different preparations, the medium and washes were combined, centrifuged and processed for *T. cruzi* DNA as described above. Percent infectivity was calculated by measuring the number of parasites associated (*A*) and not associated (*B*) with the cells by the formula:  $(A/A + B) \times 100$ . In the experiment represented in Fig. 3, 95% of extracellularly derived (24 h) amastigotes, 35% of intracellularly derived amastigotes, and 35% of trypomastigotes became associated with the monocytes.

**Intracellularly Derived Amastigotes.** Monocyte monolayers were first infected with extracellularly derived (24 h) amastigotes. After 45 h, the cells contained only replicating amastigotes bearing Ssp-4, a major stage-specific glycoprotein (3, 7). The cultured monocytes were scraped off the plastic culture plates and mechanically disrupted by passing them through a 25-gauge needle. The parasites were separated from the debris by low-speed centrifugation.

**Scanning Electron Microscopy.** Drops of 10 µl ( $10^7$  parasites/ml in PBS) were placed on 12-mm round glass coverslips precoated with 10 µg/ml poly-L-lysine (Miles-Yeda, Rehovet Israel). After 15 min at 4°C, the supernatant was aspirated and parasites were fixed with cold 2% glutaraldehyde, 0.1 M sucrose in 0.1 M sodium cacodylate buffer, pH 7.2. The coverslips were kept at 4°C in fixative until processed for scanning electron microscopy, at which time they were rinsed in buffer and processed as described (3).

**Transmission Electron Microscopy.** The parasites of the infected cells were pelleted and fixed for 2 h at 4°C with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose. After three 0.1 M cacodylate buffer rinses the pellets were osmicated in 2% OsO<sub>4</sub>

in the same buffer, dehydrated in ethyl alcohol, and embedded in Epon 812. Thin sections were picked up on formvar-coated, carbon-stabilized grids, stained with 1% aqueous uranyl acetate and 2% lead citrate, and examined in a Philips 300 transmission electron microscope.

**Escape from Endocytic Vacuoles.** Human monocytes were cultured as described for 6 d. The cells were exposed for 1 h to a suspension of extracellularly derived (24 h) amastigotes at ratios of 50 parasites per cell at 37°C in a 5% CO<sub>2</sub> atmosphere. Cultures were washed with RPMI to remove free organisms and reincubated for 1 h, 3 h, or 16 h. Then the infected cells were fixed with 2% glutaraldehyde, 0.1 M sucrose in 0.1 M sodium cacodylate buffer, scraped off the plastic, and processed for transmission electron microscopy as described.

**Infection of Mice with *T. cruzi* Parasites.** Groups of four BALB/c female mice (8–12 wk old) were injected intraperitoneally with 10<sup>4</sup>, 10<sup>3</sup>, or 10<sup>2</sup> extracellularly derived (24 h) amastigotes or with the same number of trypomastigotes. Mice were bled from the tail every day after infection and motile trypomastigotes were counted under the microscope.

## Results

In the initial experiments we compared the infectivity in vitro for cultured human monocytes of tissue culture–derived Y strain trypomastigotes and of extracellularly derived amastigotes. In the preparations of purified trypomastigotes and amastigotes used, the cross-contamination was <1%, as determined by scanning electron microscopy (Fig. 1). The extracellularly derived resembled the intracellularly derived amastigotes by scanning (Fig. 1, reference 3) and transmission electron microscopy (Fig. 2).

To measure the infectivity and the multiplication rates of the parasites inside cells, we used a DNA probe specific for *T. cruzi* (5). In several experiments, 85 to 95% of extracellularly derived (24 h) amastigotes, but only 10 to 35% of trypomastigotes, remained associated with the monocytes after four hours of incubation (Fig. 3). Identical results were obtained whether the culture medium contained fresh or heat-inactivated (56°C, 1 h) human serum, suggesting that complement factors are not involved. Following invasion, both amastigotes and trypomastigotes replicated as amastigotes with doubling times of about 10 hours. Trypomastigotes showed a prolonged lag between invasion and the start of DNA duplication, in contrast to amastigotes which commenced replication immediately (Fig. 3). Trypomastigotes exiting from infected cells were detected in all culture media after 4 to 5 d.

The very high infectivity of the extracellularly derived (24 h) amastigotes was further demonstrated by limiting dilution experiments. Monocyte monolayers were prepared in flat-bottomed 96-well plates (Falcon Labware). The amastigotes were suspended in RPMI 1640 medium at a concentration of 10 parasites/ml, and 200 µl was delivered to each well. The cultures were incubated for 5 d at 37°C and the wells were examined under the microscope for the presence of free trypomastigotes. These were found in 85% of the wells. This is very close to the expected result (86.5%), calculated from the Poisson distribution and assuming 100% infectivity. Taken together, these experiments eliminate the possibility that contamination of amastigotes with trypomastigotes accounted for the infection.

The ability of amastigotes to attach to monocytes in vitro did not diminish when they were kept in culture for an additional 48 h. 90% of such extracellularly derived (72 h) amastigotes became associated with the monocytes after 4 h of incubation, as determined with the DNA probe. After infection, the replication times of the “older” and “younger” amastigotes within the monocyte cytoplasm were the same, that is, 10 h (not shown). However, the number of parasites in the monocyte cultures at the

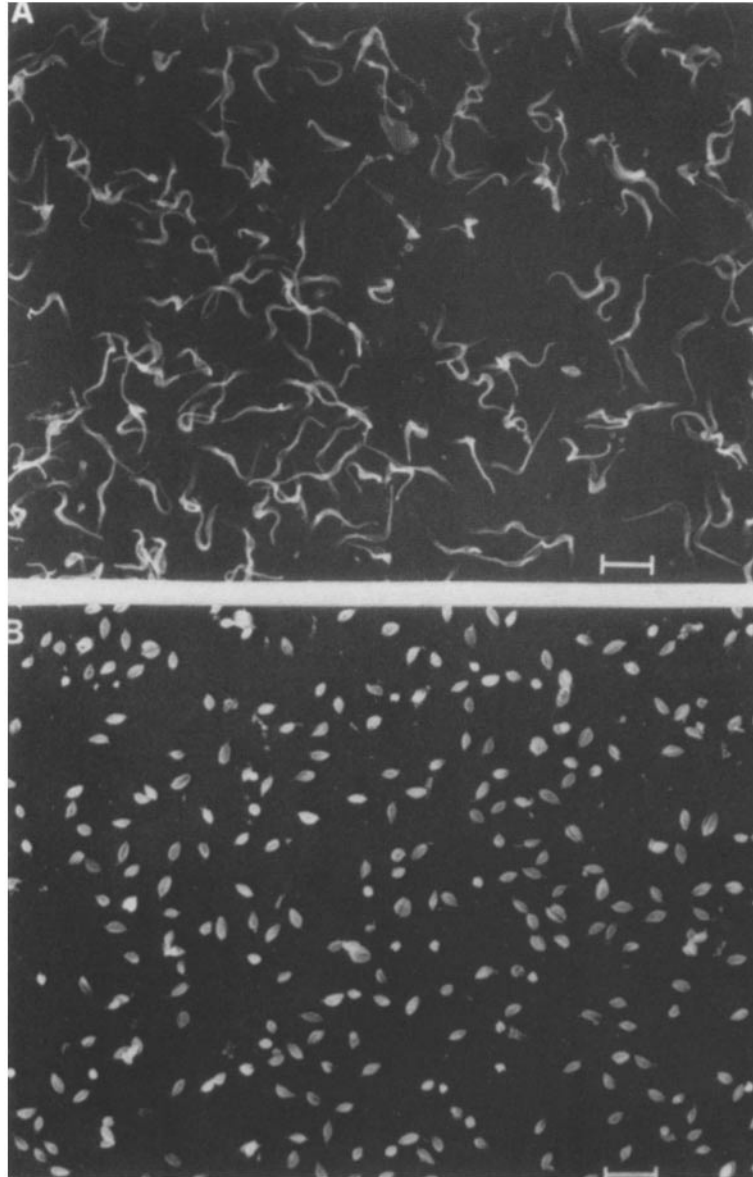


FIGURE 1. Scanning electron micrographs of *T. cruzi* parasites used for infections: (A) trypomastigotes, (B) extracellularly derived (24 h) amastigotes. Bars, 10  $\mu$ m.

end of 5 d was only 50% of that observed after infection of the cells with identical quantities of extracellularly derived (24 h) amastigotes. It is possible that half of the attached "older" parasites did not enter the monocytes or, more likely, that half of these parasites did not survive intracellularly. One difference between "younger" and "older" extracellularly derived parasites is the smaller amounts of the stage-specific glycoprotein Ssp-4 (7) in the latter, but whether this is the cause of the diminished infectivity is unknown.



**FIGURE 2.** Transmission electron microscopic appearance of extracellularly derived (24 h) amastigotes. The kinetoplasts have the typical bar morphology and the amastigote on the left has cristae in its mitochondrion. Bar, 1  $\mu\text{m}$ .

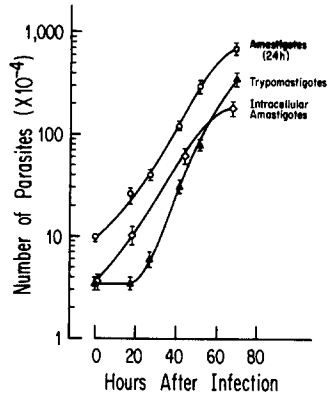


FIGURE 3. Infectivity and kinetics of multiplication of *T. cruzi* parasites in human monocytes. Values represent the mean amount of parasite DNA in four wells, and bars the standard deviations. Time zero is the time when the free parasites were removed, that is, after 4 h of incubation with the monocytes.

Because it could be argued that intracellularly and extracellularly derived amastigotes are different, we repeated the same experiments with a preparation of intracellularly derived amastigotes. These were suspended in medium and incubated with human monocyte monolayers for 4 h. 30% of them entered the cells and started to replicate without a lag (Fig. 3), with a doubling time of 10 h. After 4–5 d trypomastigotes were found in the culture medium.

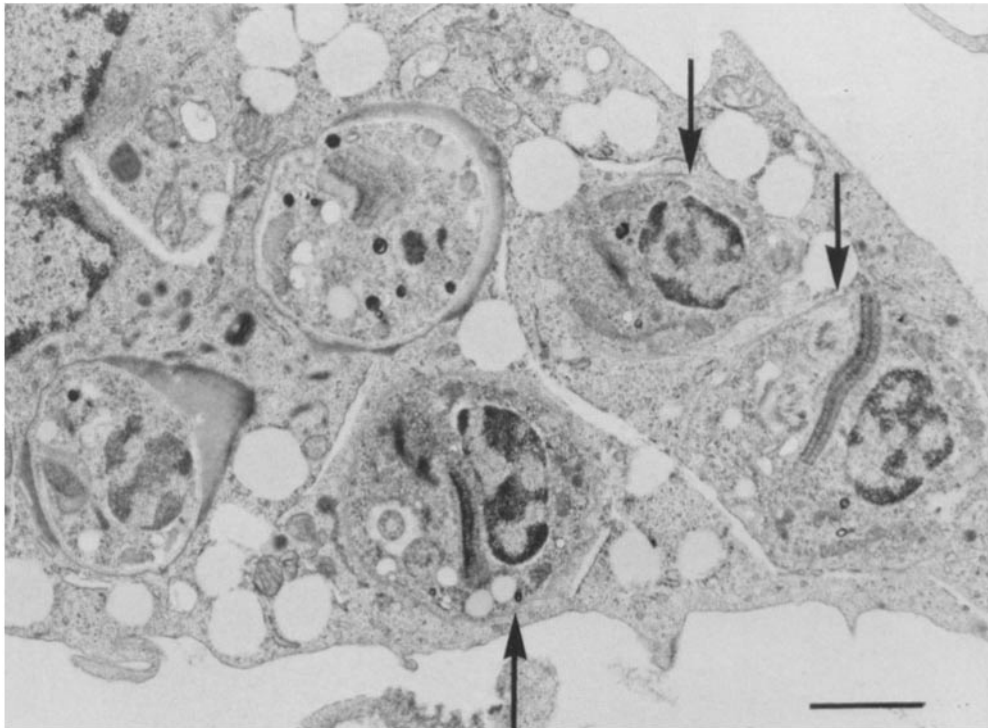


FIGURE 4. Human monocytes infected with extracellularly derived (24 h) amastigotes 1 h after the entry of the parasites, as seen by transmission electron microscopy. Some amastigotes are in phagocytic vacuoles and some are free in the cytoplasm (arrows). Lysosomes can be seen fused with the membrane of vacuoles containing parasites. Bar, 1  $\mu$ m.

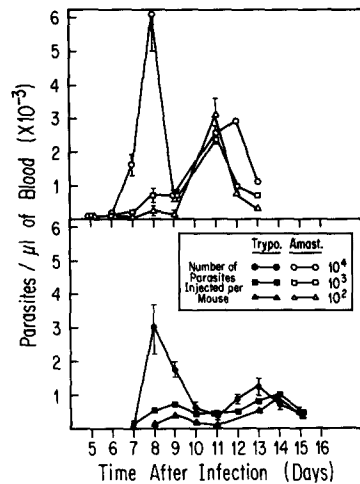


FIGURE 5. Parasitemia in infected mice. Groups of four mice were injected intraperitoneally with  $10^4$ ,  $10^3$ , or  $10^2$  amastigotes (*top panel*) or trypomastigotes (*bottom panel*). Parasitemia represents the mean number of motile trypomastigotes per microliter of blood in four animals, and bars the standard deviations. All mice died between 18 and 25 d after infection.

Amastigotes also infected nonprofessional phagocytic cells *in vitro*, albeit with a much lower efficiency. After 14 h of incubation, 20% of extracellularly derived (24 h) amastigotes entered 3T3 fibroblasts and these parasites replicated with a doubling time of  $\sim 12$  h (not shown).

The ability of the amastigotes to rapidly escape the phagocytic vacuoles was demonstrated by transmission electron microscopy. After 2 h of incubation with monocytes (1 h in the presence of parasites and one additional hour after the removal of those parasites free in the medium) a few extracellularly derived (24 h) amastigotes were seen free in the cytoplasm (Fig. 4) while most remained within the vacuoles. The number of free amastigotes increased with the time of incubation, and after 16 h, almost all of them were out of the vacuoles (not shown). Lysosomes fusing with the membranes of vacuoles containing parasites were frequently observed. In many instances the vacuoles contained an opaque material, most likely representing lysosomal products (Fig. 4).

To study the *in vivo* infectivity of the extracellularly derived (24 h) amastigotes, we inoculated groups of five A/J mice intraperitoneally with either  $10^2$ ,  $10^3$ , or  $10^4$  of them. Control animals were inoculated with the same number of tissue culture-derived trypomastigotes recently released from LLC-MK2 cells. All mice were infected and died 18–25 d afterwards, following two peaks of trypomastigote parasitemia on days 7 and 12 (Fig. 5). Significantly higher numbers of parasites were observed during the second peak of parasitemia in mice inoculated with amastigotes.

### Discussion

We showed here that both extracellularly derived (24 h) amastigotes and intracellularly derived amastigotes of *T. cruzi* entered human monocytes *in vitro*, divided with identical replication times (10 h), and developed into trypomastigotes. The extracellularly derived amastigotes were identical to the intracellularly derived amastigotes by several different criteria, that is, the morphology of the bar in their kinetoplasts as shown by transmission electron microscopy (Figs. 2 and 4), their morphology by scanning electron microscopy (Fig. 1), and the presence of an amastigote-specific surface glycoprotein (Ssp-4) on their cell membranes (7). Ssp-4 expression is maximal

in “young” amastigotes and is progressively shed during their development either intra- or extracellularly (7).

Greater than 90% of the extracellularly derived, but only 30% of the intracellularly derived amastigotes were infective for monocytes. Possible reasons for the lower infectivity of the intracellularly derived amastigotes are that they were isolated from cells by a relatively harsh procedure, and were contaminated by cellular materials, which could have affected the viability of the parasites.

Extracellularly derived (24 h) amastigotes also entered and developed within fibroblasts, cells which are not professional phagocytes. Although their infectivity was in this instance much lower, once they entered the cells the kinetics of intracellular multiplication and transformation into trypomastigotes was similar to that observed during their development in human cultured monocytes.

The development patterns of extracellularly derived or intracellularly derived amastigotes within monocytes were similar. Their replication times were identical (10 h), and there was no apparent lag period before the start of DNA replication, contrasting with the ~20-h lag seen when trypomastigotes invaded the same cells (Fig. 3). This lag was also reported by Dvorak and collaborators (8, 9) and, at least in part, it must represent the time necessary for the transformation of trypomastigotes into amastigotes. However, the cellular site where this transformation takes place is unknown. *T. cruzi* develops in the cytoplasm of host cells (10–12) and the parasite must destroy the membrane of the phagocytic vacuole to escape. As shown here, extracellularly derived (24 h) amastigotes have this ability, since they are found in the cytoplasm a few hours after entering cells. Perhaps the induction of the membranolytic agents, such as, for example, phospholipases, is stage specific and trypomastigotes first have to transform into amastigotes inside the vacuole before they can escape and start to replicate.

The developmental potential of amastigotes has been a matter of controversy. Villalta and Kierszenbaum (13) reported that amastigotes from continuous acellular cultures (in contrast to our recently transformed amastigotes) were destroyed in the phagocytic vacuoles of cultured human monocytes. Carvalho et al. (14) and Guttridge et al. (15) showed that amastigotes isolated from the spleen or muscle of mice were not infective to mouse peritoneal macrophages or infective when injected into animals. Umezawa et al. (16) reported that amastigotes entered mouse macrophages *in vitro* but did not differentiate into trypomastigotes. There are two possible explanations for these negative results. The survival of amastigotes in macrophages probably depends on the degree of activation of the phagocytes (17, 18), and/or the amastigotes used by these investigators might not have been viable intracellularly because they had developed too long *in vitro* or *in vivo*. As shown here, only “young” amastigotes are fully infective.

Other studies using amastigotes purified from different sources showed that they entered and developed in cells *in vitro* and in infected mice (11, 19–25). However, parasite development *in vitro* was estimated only by light microscopy, and the proportion of infective parasites in the population or their rates of multiplication were not evaluated.

Our findings settle the controversy as to whether or not amastigotes themselves can infect mammalian cells, since they showed directly by two independent experimental approaches that 90% or more of extracellularly derived (24 h) amastigotes



infected monocytes. Furthermore, these amastigotes were as infective to mice as trypomastigotes were; the prepatent periods and the number of trypomastigotes in the blood during the first peak of parasitemia were quite similar. In fact, the number of trypomastigotes in the blood during the second peak of parasitemia was significantly higher in mice inoculated with amastigotes. This difference can be explained if, in the animals infected with trypomastigotes, the immune response to trypomastigote-specific antigens (3, 26, 27) developed earlier than in mice infected with amastigotes. This is likely because the immune system of the latter animals came in contact with trypomastigotes only after the first intracellular replication cycle. These emerging trypomastigotes would then be less likely to be destroyed by immune mechanisms and the parasitemias would reach higher levels than in mice initially infected with trypomastigotes.

On the basis of these observations, the possibility of an alternative developmental cycle for *T. cruzi* within the mammalian host with the participation of infective amastigotes, should be entertained. In vivo, the infective amastigotes could emerge from cells containing either amastigotes or mixtures of trypomastigotes and amastigotes or, alternatively, originate from the extracellular transformation of trypomastigotes into amastigotes. Blood stage trypomastigotes can transform into amastigotes whether or not they enter cells (3) and, during the acute stage of the infection, amastigotes and intermediate forms have been found in large numbers in the circulation of mice (3) and also in the extracellular space between heart muscle cells (23). Extracellular amastigotes may also be present during the chronic stage of Chagas' disease. However, at this stage parasites in the blood are very scarce and the nonmotile amastigotes would be virtually impossible to detect by light microscopy. Amastigotes are also found in the intermediate host, but little is known about their developmental potential (2).

The present observations are of relevance to the biology, immunoprophylaxis, and pathology of *T. cruzi* infections in humans. Since host cells can be invaded by both trypomastigotes and amastigotes, which bear distinct surface antigens, different stages of *T. cruzi* most likely are able to use different mechanisms for attachment and penetration into cells. This represents an important survival advantage and could in part explain the remarkably wide range of definitive hosts of this parasite, which infects over 100 mammalian species from several orders, and develops in vivo in macrophages, as well as in muscle, epithelial, and nervous tissue cells (1, 2). The realization that different *T. cruzi* stages may use different strategies for entering cells of the vertebrate host complicates the development of vaccines, and also raises questions regarding the relative importance of the two infective stages in perpetuating the infection leading to the chronic phase of Chagas' disease.

### Summary

The two main stages of development of the protozoan parasite *Trypanosoma cruzi* found in the vertebrate host are the trypomastigote and the amastigote. It has been generally assumed that only trypomastigotes are capable of entering cells and that amastigotes are the intracellular replicative form of the parasite. We show here that after incubation for 4 h with human monocytes in vitro 90% or more of extracellularly derived (24 h) amastigotes of *T. cruzi* are taken up by the cells. Within 2 h they escape the phagocytic vacuole and enter the cytoplasm, where they divide and

after 4–5 d transform into trypomastigotes. Trypomastigotes also invade cultured human monocytes. However, they show a lag of several hours between invasion and the start of DNA duplication, while amastigotes commence replication without an apparent lag. Amastigotes also infect cultured fibroblasts, albeit with lower efficiency. When injected intraperitoneally into mice, amastigotes are as infective as trypomastigotes. Based on these results, and on prior findings that amastigotes are found free in the circulation of mice during the acute stage of the disease (3), it seems likely that the cellular uptake of amastigotes can initiate an alternative subcycle within the life cycle of this parasite in the mammalian host. Also, because trypomastigotes and amastigotes have diverse surface antigens, they may use different strategies to invade host cells.

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