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Isolation of the Intracellular Stage of *Trypanosoma cruzi* and Its Interaction with Mouse Macrophages In Vitro

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Interaction between *Trypanosoma cruzi* spheromastigotes (amastigotes) and mouse macrophages was studied. The spheromastigotes, isolated from the spleens of infected mice, were incorporated and digested by the macrophages. By use of horseradish peroxidase labeling of the macrophage lysosomes we showed that fusion of lysosomes with phagocytic vacuoles containing *T. cruzi* occurred. Parasites showing alterations indicative of digestion were seen inside the phagocytic vacuoles. Our results suggest that intracellular spheromastigotes of *T. cruzi*, isolated from the spleens of infected mice, are not able to induce a productive infection in mouse macrophages maintained in vitro.

Trypanosoma cruzi undergoes differentiation in the vertebrate host, assuming three developmental stages: trypomastigote, epimastigote, and spheromastigote (amastigote, micromastigote). In vivo and in vitro data show that trypomastigotes are nondividing forms responsible for the infection of cells whereas spheromastigotes are dividing forms which maintain and amplify the infection (3). In the vertebrate host epimastigotes are transitional forms which appear during the intracellular spheromastigote-trypomastigote transformation (8, 19).

Macrophages may play a role in host resistance to *T. cruzi* infection. However, *T. cruzi* can penetrate and multiply inside macrophages. Macrophage-*T. cruzi* interactions have been extensively studied (1, 8, 11, 14, 15, 18, 21, 22, 25, 27), using epimastigotes and trypomastigotes. Although both forms are incorporated by macrophages, epimastigotes are destroyed and trypomastigotes survive (22). In the present paper we describe data related to the interaction between *T. cruzi* spheromastigotes (amastigotes) and mouse macrophages.

MATERIALS AND METHODS

Parasite. The Y strain of *T. cruzi* was used. It was originally isolated from a human acute case of Chagas' disease (26). The parasite was maintained by weekly passages in mice. To obtain intracellular parasites, mice weighing 13 to 15 g were inoculated intraperitoneally with 1.5×10^5 bloodstream trypomastigotes. Spleens from mice on day 7 after infection were removed and rinsed in a 3.8% sodium citrate solution. The organ was cut into small fragments, suspended in Hanks solution, homogenized, filtered three times through gauze, and passed twice through glass wool to remove cell debris. The homogenate was centrifuged at $400 \times g$ for 5 min to separate blood and spleen cells from parasites. The $400 \times g$ supernatant was then centrifuged at $750 \times g$ for 10 min and left at 37°C for 30 min so that trypomastigotes could move into the supernatant. The pellet was suspended in either medium 199 or Eagle medium, layered on a Ficoll-sodium-diatrizoate solution (Bionetics Laboratory Products), and centrifuged at $1,000 \times g$ for 20 min. The amastigotes remained at the interface, were collected, washed three times with the above medium, layered on a two-step sucrose gradient (0.25 to 0.70 M), and centrifuged at $400 \times g$ for 5 min. The bottom layer, which contained the parasites, was removed and used in the experiments. Cell motility was observed by phase-contrast microscopy. Giemsa-stained preparations were used to detect any contamination of this fraction with trypomastigotes. The yield obtained by this method was about 5×10^6 parasites per spleen.

The viability of the isolated intracellular forms was controlled by using the trypan blue dye exclusion test and by measuring the oxygen uptake with a Clark-type oxygen electrode.

Macrophages. Peritoneal macrophages were collected from Swiss mice weighing 20 to 25 g. Animals were killed with ether, their peritoneal cavities were washed with 3 ml of either Hanks or Ringer solution, and the macrophages removed therefrom were plated on 125-mm^2 glass cover slips, which were introduced into Leighton tubes. The cells were allowed to adhere to the glass surface for 1 h at 37°C, after which the saline solution and the nonadherent cells were removed and culture medium (199 medium plus 5% fetal bovine serum) was added. After incubation at 37°C for 24 h, macrophage cultures were rinsed with Ringer solution and used for the phagocytosis experiments.

Macrophage-parasite interaction. Amastigotes obtained as indicated above were suspended in Ringer solution and added at a ratio of 10 parasites per macrophage on the slide culture. The number of macrophages in the preparation was estimated by counting 20 microscopic fields and varied from 200 to 400 cells

per mm^2. Parasites were in contact with macrophages for periods of ½ to 20 h. Cultures were then rinsed with Ringer solution, fixed with Bouin fixative, and stained with Giemsa. To check the viability of ingested parasites, some cultures were rinsed after 24 h of incubation to remove parasites which did not interact with the macrophages. Fresh medium was then added, and cultures were further incubated for 24 to 120 h with the medium being changed every 24 h.

The percentage of infected macrophages and the mean number of intracellular parasites per infected cell were determined by two independent observers examining randomly at least 200 cells with a Zeiss Universal Photomicroscope. In 200 infected macrophages we determined the percentage of parasites which showed signs of disintegration (disruption of either the kinetoplast or the nucleus and disorganization of the structure of the cytoplasm).

Electron microscopy. For ultrastructural studies, macrophages were plated on glass flasks, cultivated as described above, and incubated with the parasites. After interaction of parasites and phagocytic cells, these were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 3.5% sucrose (pH 7.2), rinsed in cacodylate buffer with 3.5% sucrose, and left in this buffer for 24 h at 4°C. The cells were then gently scraped off with a rubber policeman, postfixed with 1% OsO_4, dehydrated in acetone, and embedded in Epon. Thin sections were cut in an LKB Ultratome III ultramicrotome, stained with uranyl acetate and lead citrate, and observed in an AEI EM6-B or Jeol 100C-X electron microscope.

In some experiments the fusion of lysosomes with the phagosome was studied. Macrophages were incubated in the presence of 2 mg of horseradish peroxidase per ml (13) for 2 to 3 h at 37°C, after which cells were rinsed with Hanks solution and incubated in the presence of the parasites. After parasite-macrophage interaction, cells were fixed in glutaraldehyde as described above. For detection of the peroxidase activity, cells were incubated for 30 min at room temperature in a medium containing 3,3'-diaminobenzidine (0.5 mg/ml) in 0.05 M tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.6) and 0.01% H_2O_2 (9). After being washed with cacodylate, buffer cells were postfixed with OsO_4, dehydrated, and embedded in Epon.

RESULTS

By using the method described above, a relatively pure preparation of spheromastigotes was obtained (Fig. 3 and 4). *T. cruzi* infection of spleen macrophages provided cells containing parasites at different developmental stages. Although in some experiments we had pure preparations of spheromastigotes, in others we detected more than 5% trypomastigotes. Such preparations were discarded.

Observation of the spheromastigote fraction by phase-contrast microscopy indicated that the parasites were mobile although the flagellum was not always seen. By using the trypan blue dye exclusion test we found that more than 95% of cells were viable. Also, cells were metabolically active as indicated by oxygen consumption (data not shown).

Ultrastructural observations on the spheromastigote fraction showed it to be considerably pure, although fragments of other cells were occasionally seen (Fig. 4 and 5). Almost all parasites had a structure similar to that of cells found inside the spleen macrophages. In favorable sections, the presence of a short flagellum was seen. As described previously (6), the flagella of spheromastigotes did not show a paraxial structure. This aspect was used to distinguish spheromastigotes from epimastigotes and trypomastigotes.

As shown in Fig. 6 spheromastigotes were readily ingested by mouse macrophages. After 30 min of incubation, about 30% of the macrophages had parasites in their cytoplasm. However, the mean number of *T. cruzi* cells per macrophage was relatively low, remaining in the range of 1 to 2.4.

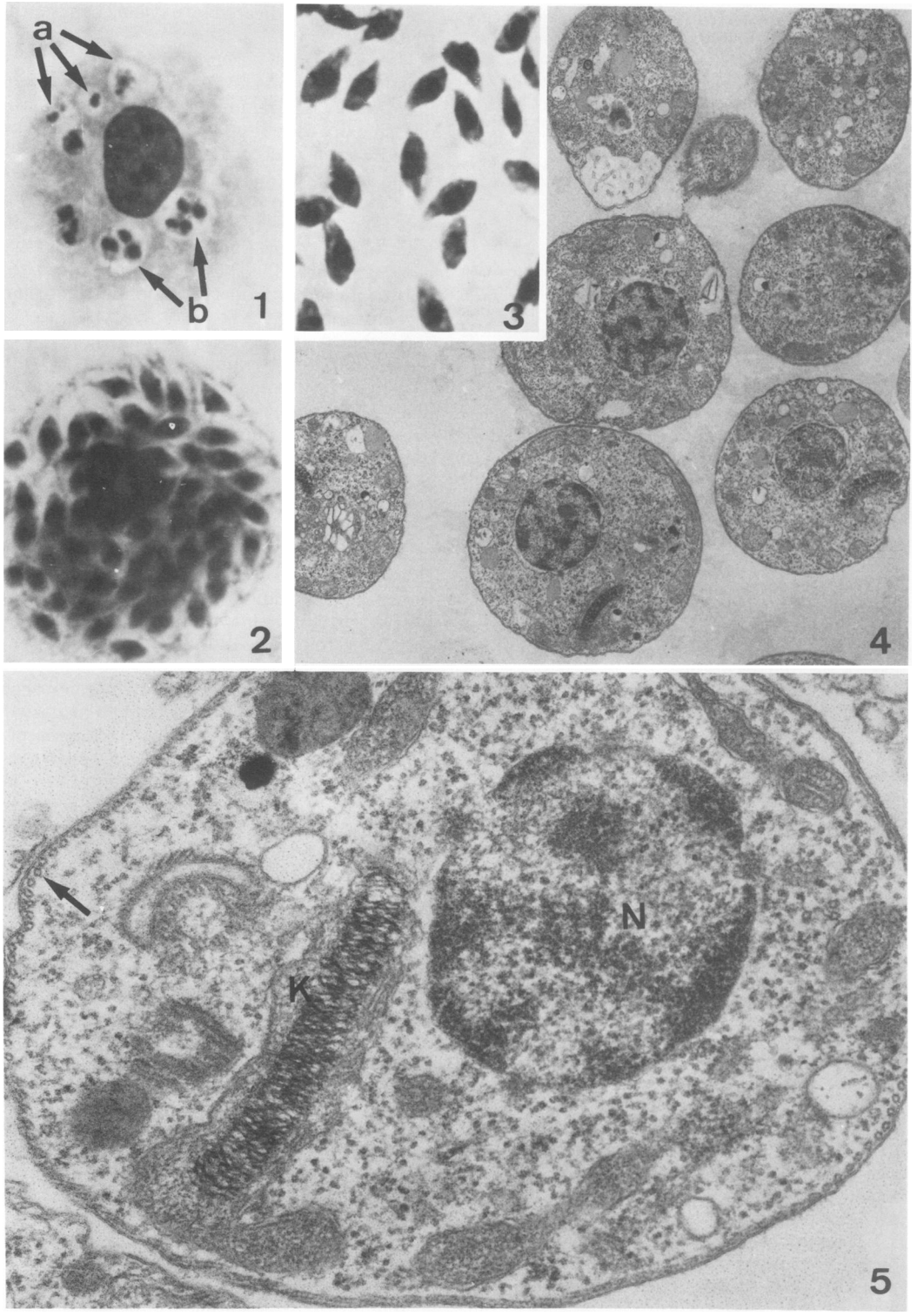
The process of digestion of internalized parasites started just after endocytosis. The number of parasites being digested increased with time (Fig. 7). After 48 h of *T. cruzi*-macrophage interaction, less than 5% of macrophages had viable parasites in their cytoplasm. These parasites were able to divide and differentiate later into trypomastigotes (Fig. 2).

Observation of cultures by light microscopy showed different stages of intracellular destruction of parasites (Fig. 1). By electron microscopy, images such as that shown in Fig. 8 were suggestive of phagocytosis. Inside the macrophage, parasites were seen in a vacuole clearly separated from the cytoplasm by a membrane (Fig. 9 and 10). Parasites showing alterations indicative of digestion were seen inside the phagocytic vacuoles. The subpellicular microtubules were structures more resistant to digestion (Fig. 13).

Macrophages incubated in the presence of horseradish peroxidase incorporated this protein by pinocytosis. Pinocytotic vesicles fuse with other cytoplasmic vesicles, forming larger structures. When parasites were incubated in the presence of such peroxidase-labeled macrophages, they were incorporated into phagocytic vacuoles. Using this system, we observed fusion of the macrophage lysosomes with vacuoles containing the parasites. An electrodense reaction product, indicative of peroxidase activity, was seen inside the vacuoles, surrounding the parasites (Fig. 12 and 13).

DISCUSSION

When trypanosomatids are ingested by macrophages, they are trapped inside a membrane-bound vacuole. Lysosomes can fuse with the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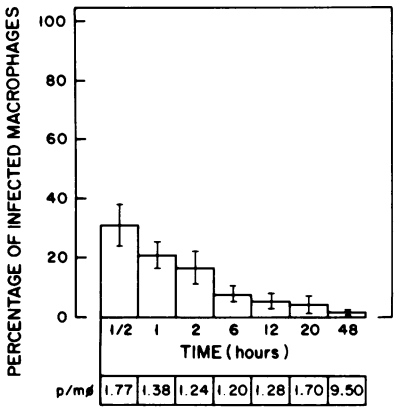


FIG. 6. *Penetration of spheromastigotes into macrophages. The percentage of infected macrophages and the mean number of intracellular parasites per infected macrophage (p/mφ) were determined after different periods of macrophage-parasite interaction. The vertical lines indicate standard errors.*

vacuole, liberating enzymes which may interact with surface structures of the parasites. For unknown reasons some developmental stages, as well as some strains of trypanosomatids, are resistant to digestion by lysosomal enzymes, whereas others are sensitive (2, 5, 21, 22). *T. cruzi* epimastigotes are susceptible, whereas trypomastigotes resist hydrolysis by lysosomal enzymes (22). Although lysosomes fuse with vacuoles (20), trypomastigotes apparently dissolve the membrane of the phagocytic vacuole by an unknown mechanism and, once in contact with the cytoplasm, complete their transformation to spheromastigotes, which are then able to divide.

In this study we isolated the dividing form of *T. cruzi* from spleen macrophages and studied their ability to enter and survive inside mouse macrophages maintained in vitro. Two methods have been described for the isolation of intracellular forms of *T. cruzi* from muscle (10) and from spleens (16) of experimentally infected mice. In both cases, enzymes such as deoxyribonuclease, collagenase, and trypsin were used. Since the use of such enzymes may interfere with the cell surface properties of parasites, we devised another procedure for the isolation of a relatively pure fraction of spheromastigotes. These forms seemed physiologically normal as judged by their motility, the trypan blue dye exclusion test of viability, oxygen consumption, and electron microscopy.

Present results show that the intracellular spheromastigotes isolated from the spleens of mice are digested by mouse macrophages. This conclusion is based on the following facts: (i) the proportion of macrophages containing parasites decreases with prolonged incubation; (ii) the proportion of disintegrated intracellular parasites increases with time. However, a small percentage of macrophage (less than 5%) still contained parasites after 48 h of infection, and these parasites were able to divide and differentiate (Fig. 2). Presumably, this could be due to the small percentage of trypomastigotes or intermediate stages or both which contaminated the preparation. Similar results are observed when acellular cultures of *T. cruzi*, consisting mainly of epimastigotes and a few trypomastigotes, are used in studies on the interaction with macrophages (22; unpublished data).

T. cruzi spheromastigotes usually are not in direct contact with the lysosomal enzymes since they are in the cytoplasm. The dissolution of the membrane of the phagocytic vacuole may represent the mechanism used by *T. cruzi* to escape from the action of lysosomal enzymes. Recent

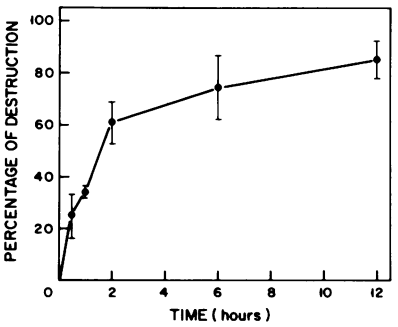


FIG. 7. *Intracellular digestion of the ingested parasites after different periods of macrophage-parasite interaction.*

FIG. 1. *Photomicrograph of Giemsa-stained preparation showing parasites inside the macrophage. Arrow a indicates parasites in degeneration, whereas arrow b indicates normal parasites. ×1,100.*

FIG. 2. *Photomicrograph showing a macrophage which was infected and in which the parasites divided. ×1,100.*

FIG. 3. *General aspect of the fraction containing spheromastigotes as seen by light microscopy of Giemsa-stained preparations. ×1,200.*

FIG. 4 and 5. *General aspect of the fraction containing spheromastigotes, as seen by electron microscopy. At higher magnification, the general structure of the parasites seems to be well preserved. The arrow indicates the subpellicular microtubules (Fig. 5). K, Kinetoplast; N, nucleus. Fig. 4, ×9,000; Fig. 5, ×44,800.*

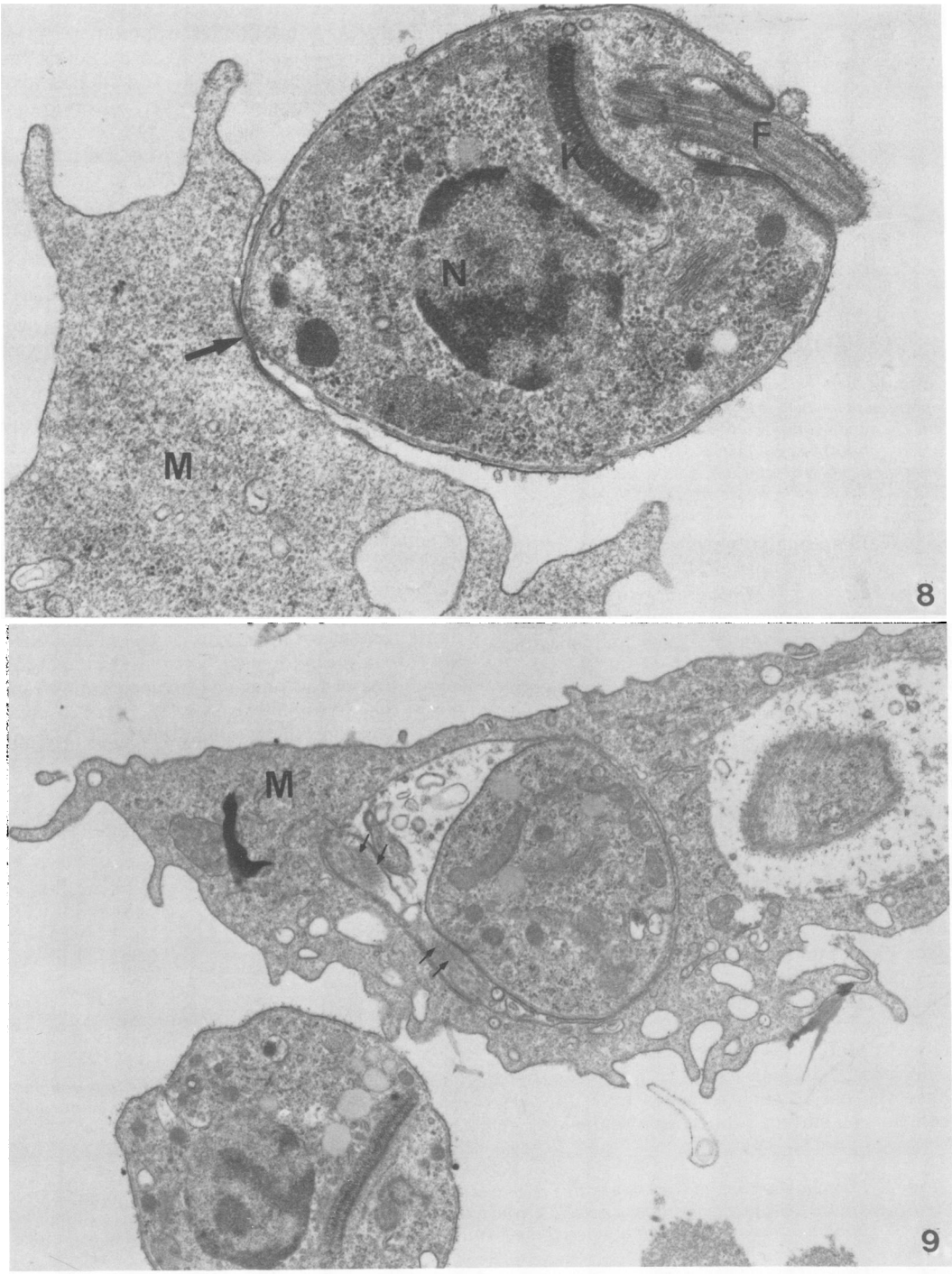


FIG. 8. *Attachment of the parasite to the plasma membrane of the macrophage (arrow). The parasite has a short flagellum. F, Flagellum; K, kinetoplast; M, macrophage; N, nucleus.* ×23,000.

FIG. 9. *Parasites are seen inside the macrophage. The arrows indicate the paraxial structure of the flagellum of one of the parasites, a structure which is not observed in spheromastigotes.* ×13,500.

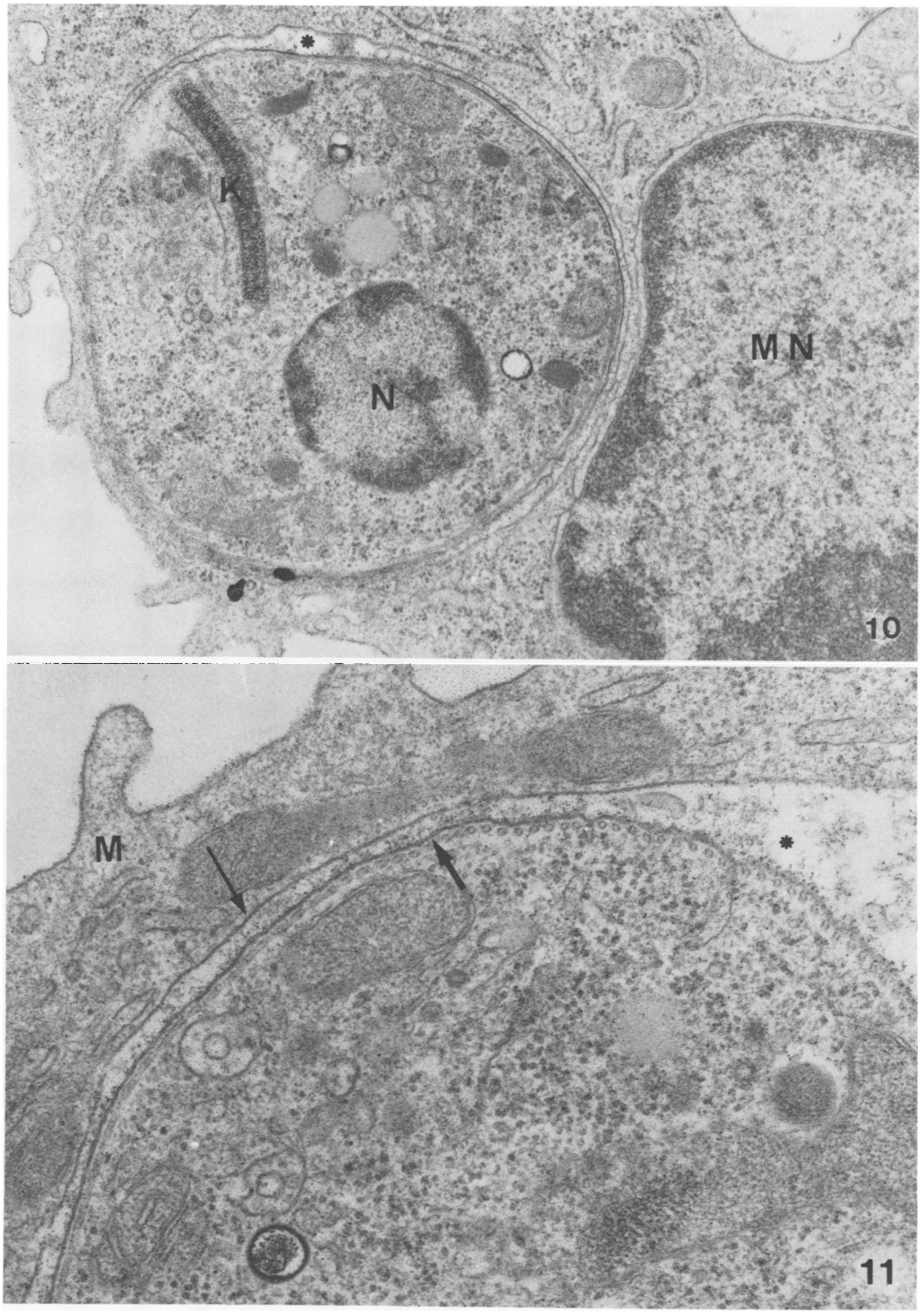
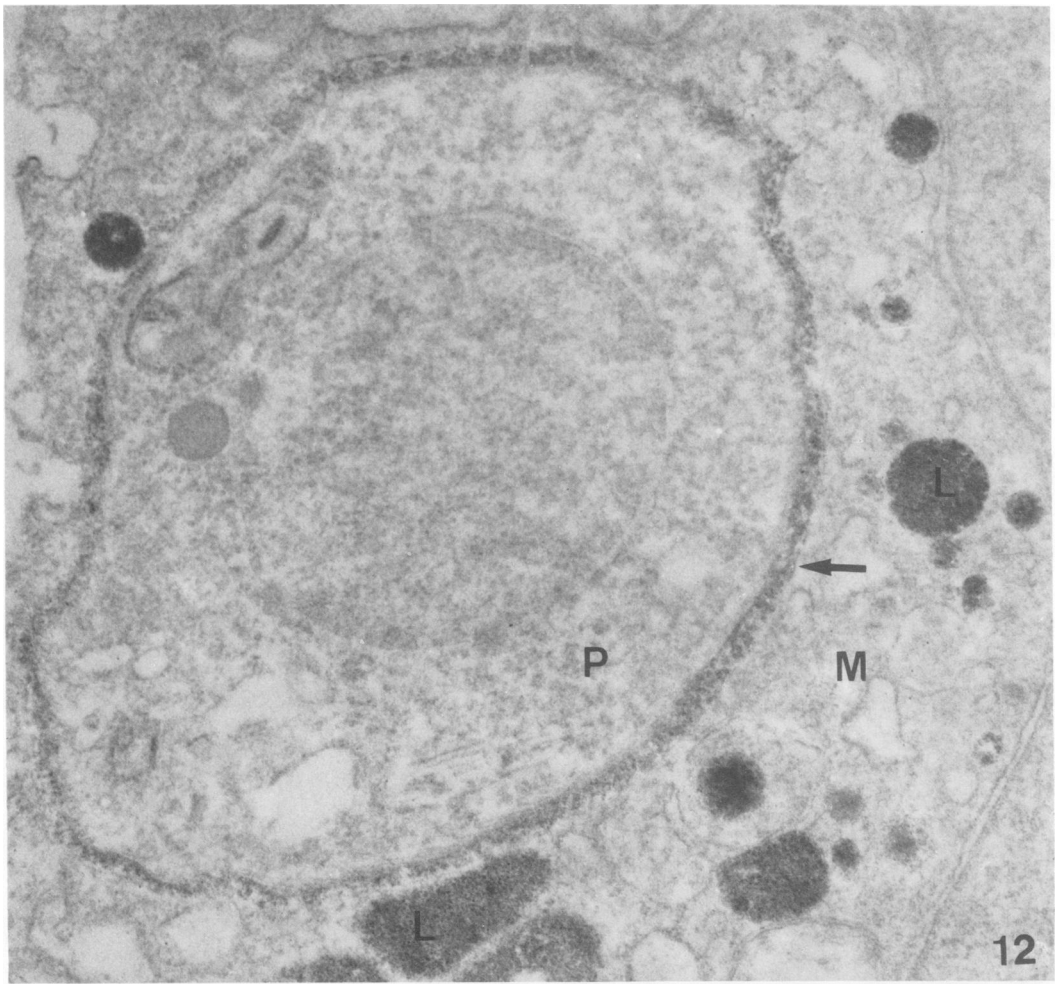


FIG. 10 and 11. *Spheromastigotes are seen inside phagocytic vacuoles (*) clearly separated from the cytoplasm by a membrane. The thick and the thin arrows in Fig. 11 indicate the membrane of the parasite and the membrane of the phagocytic vacuole, respectively. K, Kinetoplast; M, macrophage; MN, macrophage nucleus; N, parasite nucleus. Fig. 10, ×37,000; Fig. 11, ×44,800.*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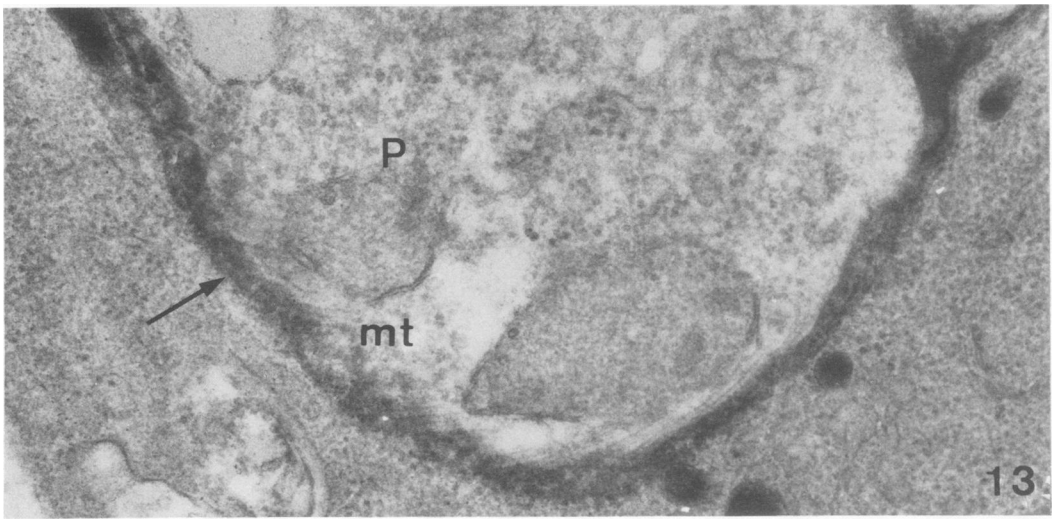
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studies indicate that trypomastigotes are not sensitive to lysosomal enzymes since lysosomes fuse with the membrane of the phagocytic vacuole (20). Other parasites, such as *Leishmania*, remain inside the vacuole, interact with lysosomal enzymes, but are able to divide (2, 5, 11). Both developmental stages of this parasite (promastigote and spheromastigote) infect and divide within vertebrate cells. In the case of *T. cruzi*, trypomastigotes are not able to divide and, therefore, to maintain and amplify the infection, they must transform into spheromastigotes. It is possible that changes occur at the cell surface during trypomastigote-spheromastigote transformation, rendering spheromastigotes sensitive to lysosomal enzymes. Dissolution of the membrane or vacuole may represent the basic mechanism developed by the parasite for its survival and maintenance of its parasitic condition.

By use of horseradish peroxidase labeling of lysosomes (13), we showed the fusion of lysosomes and phagocytic vacuoles, confirming previous data with vacuoles containing epimastigote (15, 22) and trypomastigote (20) forms of *T. cruzi*. In those studies, thorostrast was used to label lysosomes. Horseradish peroxidase has the advantage of being easily obtained from several commercial sources.

It has been reported (24) that amastigotes obtained in vitro in acellular cultures were able to enter and multiply in human skin-muscle cells. However, it is questionable whether parasites maintained in acellular cultures are similar to those inside vertebrate cells. In the cell-free F-6 medium, Pan (23) discerned the presence of at least 5% of promastigotes contaminating amastigotes. In our opinion, the promastigote stage is not found in the life cycle of *T. cruzi*. Therefore, it is necessary to clarify what Pan (23) considers a promastigote. Macrophages and human skin-muscle cells probably have different lysosomal contents and different abilities to destroy parasites. It has been reported that human skin cells are able to ingest promastigotes of *Leishmania braziliensis*. However, in this cell, fusion of lysosomes with the phagosome was not observed (4). It has been reported that amastigotes released from infected cells can infect new cells (22). However, the authors did not study this interaction in detail.

Our data suggest that spheromastigotes isolated from the spleens of infected mice are not able to induce a productive infection in mouse macrophages. Further studies are necessary to determine whether this is valid for spheromastigotes isolated by other methods, as well as for cells other than macrophages.

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LITERATURE CITED

1. **Alcantara, A., and Z. Brener.** 1978. The *in vitro* interaction of *Trypanosoma cruzi* bloodstream forms and mouse peritoneal macrophages. Acta Trop. **35**:209–219.
2. **Alexander, J., and K. Vickerman.** 1975. Fusion of host cell secondary lysosomes with the parasitophorus vacuoles of *Leishmania mexicana* infected macrophages. J. Protozool. **22**:502–508.
3. **Brener, Z.** 1973. Biology of *Trypanosoma cruzi*. Annu. Rev. Microbiol. **27**:347–382.
4. **Chang, K. P.** 1978. Leishmania infection of human skin fibroblasts *in vitro*: absence of phagolysomsomal fusion after induced phagocytosis of promastigotes, and their intracellular transformation. Am. J. Trop. Med. Hyg. **27**:1084–1096.
5. **Chang, K. P., and D. M. Dwyer.** 1978. *Leishmania donovani* hamster macrophage interactions *in vitro*: cell entry, intracellular survival and multiplication. J. Exp. Med. **147**:515–530.
6. **De Souza, W., and T. Souto-Padron.** 1980. The paraxial structure of the flagellum of Trypanosomatidae. J. Parasitol. **66**:229–235.
7. **Dvorak, J. A.** 1976. New *in vitro* approach to quantitation of *Trypanosoma cruzi*-vertebrate cell interactions, p. 109–120. *In* American Trypanosomiasis Research. Pan American Health Organization.
8. **Dvorak, J. A., and G. A. Schmunis.** 1972. *Trypanosoma cruzi*: Interaction with mouse peritoneal macrophages. Exp. Parasitol. **32**:289–300.
9. **Grahan, R. C., and M. J. Karnovsky.** 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. **14**:291–302.
10. **Gutteridge, W. E., B. Cover, and M. Gaborak.** 1978. Isolation of blood and intracellular forms of *Trypanosoma cruzi* from rats and other rodents and preliminary studies of their metabolism. Parasitology **76**:159–176.
11. **Hoff, R.** 1975. Killing *in vitro* of *Trypanosoma cruzi* by macrophages from mice immunized with *T. cruzi* or BCG, and absence of cross-immunity on challenge *in vivo*. J. Exp. Med. **142**:299–311.
12. **Jones, T. C.** 1974. Macrophages and intracellular parasit-

FIG. 12 and 13. *Macrophages in which the lysosomes were labeled with horseradish peroxidase and then incubated in the presence of T. cruzi spheromastigotes. Reaction product is seen in the lysosomes and inside the phagocytic vacuoles which contain parasites (arrow). The microtubules show a normal aspect. L, Lysosome; M, macrophage; mt, microtubules; P, parasite. Fig. 12, ×37,000; Fig. 13, ×44,000.*

ism. RES J. Reticuloendothel. Soc. **15**:3439–3449.
13. **Kielian, M. C., and Z. A. Cohn.** 1980. Phagosome-lysosome fusion. Characterization of intracellular membrane fusion in mouse macrophages. J. Cell. Biol. **85**: 754–765.
14. **Kipnis, T. L., V. L. G. Calich, and W. Dias da Silva.** 1979. Active entry of bloodstream forms of *Trypanosoma cruzi* into macrophages. Parasitology **78**:89–98.
15. **Kress, Y., H. Tanowitz, B. Bloom, and M. Wittner.** 1977. *Trypanosoma cruzi:* Infection of normal and activated mouse macrophages. Exp. Parasitol. **41**:385–396.
16. **Leon, W., F. Villalta, T. Queiroz, and A. Szarfman.** 1979. Antibody-induced capping of the intracellular stage of *Trypanosoma cruzi.* Infect. Immun. **26**:1218–1220.
17. **Meirelles, M. N. L., T. C. De Araújo Jorge, and W. De Souza.** 1980. Interaction of epimastigote and trypomastigote forms of *Trypanosoma cruzi* with chicken macrophages *in vitro.* Parasitology **81**:373–381.
18. **Melo, R. C., and Z. Brener.** 1978. Tissue tropism of different *Trypanosoma cruzi* strains. J. Parasitol. **64**: 475–482.
19. **Meyer, H., and M. Y. De Oliveira.** 1948. Cultivation of *Trypanosoma cruzi* in tissue culture: a four year study. Parasitology **39**:91–94.
20. **Milder, R., and J. Kloetzel.** 1980. The development of *Trypanosoma cruzi* in macrophages *in vitro.* Interaction with lysosomes and host cell fate. Parasitology **80**: 139–145.
21. **Milder, R., J. Kloetzel, and M. P. Deane.** 1977. Observations on the interaction of peritoneal macrophages with *Trypanosoma cruzi.* II. Intracellular fate of bloodstream forms. Rev. Inst. Med. Trop. São Paulo **19**:313–322.
22. **Nogueira, N., and Z. Cohn.** 1976. *Trypanosoma cruzi:* mechanism of entry and intracellular fate in mammalian cells. J. Exp. Med. **143**:1402–1420.
23. **Pan, S. C.** 1978. *Trypanosoma cruzi:* intracellular stages grown in a cell-free medium at 37°C. Exp. Parasitol. **45**: 215–224.
24. **Pan, S. C.** 1978. *Trypanosoma cruzi: In vitro* interaction between cultured amastigotes and human skin-muscle cells. Exp. Parasitol. **45**:274–286.
25. **Sanderson, C. J., and W. De Souza.** 1979. A morphological study of the interaction between *Trypanosoma cruzi* and rat eosinophils, neutrophils and macrophages *in vitro.* J. Cell Sci. **37**:275–286.
26. **Silva, L. H. P., and V. Nussenzweig.** 1953. Sobre uma cepa do *Trypanosoma cruzi* altamente virulenta para o camundongo branco. Folia Clin. Biol. **20**:191–207.
27. **Williams, D. M., and J. S. Remington.** 1977. Effect of human monocytes and macrophages on *Trypanosoma cruzi.* Immunology **32**:19–23.