TRYPANOSOMA CRUZI: MECHANISM OF ENTRY AND INTRACELLULAR FATE IN MAMMALIAN CELLS*

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The factors involved in the resistance or susceptibility of mammalian hosts to infection with *Trypanosoma cruzi* remain obscure. A more detailed analysis of the interactions of the parasite and host cells would provide a better understanding of the pathogenesis of this infection. Several investigators have examined the mode of entry, intracellular development, and fate of *T. cruzi* in a variety of cells using both light and electron microscopic techniques, but the result of these studies are contradictory (1–11).

We report here our studies on the mode of entry and fate of both epimastigote and trypomastigote forms of *T. cruzi* at the light and electron microscope levels. The observations were mainly performed with mouse peritoneal macrophages, although parallel studies on several cultured cell types (HeLa, L cells, calf embryo fibroblasts) will also be described. Utilizing a technique to separate the cultured forms of epimastigotes and trypomastigotes (12), we were then able to study their different fates as well as the components of the host cell plasma membrane involved in the interiorization process. Some of these results have been previously published in abstract form (13).

Materials and Methods

Parasites. The Y strain of *T. cruzi* was obtained from Dr. S. C. Correa (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil). The PF strain was obtained from Dr. Z. Brenner (Fundação I. O. Cruz, Belo Horizonte, MG, Brazil) and the Tulahuen strain from Dr. B. Bloom (Albert Einstein School of Medicine, Bronx, N. Y.). The parasites were grown in Tobie's medium and passed weekly (14). Parasites were harvested from 8-day-old cultures, washed twice with phosphate-buffered saline (PBS)[1] (Dulbecco's, Grand Island Biological Co., Grand Island, N. Y.) at 750 *g* for 20 min, resuspended in minimal essential medium (MEM) and counted in a hemocytometer with a 40 × phase objective. The cultures of Y strain contained 85% epimastigotes and 15% trypomastigotes. The PF and Tulahuen strains contained 90 and 95% epimastigotes, respectively. Purified trypomastigotes were obtained as previously described (12) by lysing epimastigotes with fresh guinea pig serum (1:10 final dilution in PBS) and recovering the viable trypomastigotes on an albumin column (density 1.085 $g \cdot cm^{-3}$) after centrifugation at 10,000 *g* for 40 min. The parasites were diluted in 5 vol of PBS, washed twice, counted in a hemocytometer, and resuspended to the desired concentration in MEM.

Normal Macrophages. Mouse peritoneal macrophages were obtained from Swiss mice maintained at The Rockefeller University, as well as C3H/He and C57/BL strains obtained from The Jackson Laboratory, Bar Harbor, Maine. Cells were harvested according to the methods of Cohn

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[1] *Abbreviations used in this paper:* FCS, fetal calf serum; MEM, minimal essential medium; PBS, phosphate-buffered saline.

and Benson (15). They were cultivated on 13-mm round glass cover slips in MEM and 20% heat-inactivated (56°C, 30 min) fetal calf serum (FCS) or horse serum (Grand Island Biological Co.), 100 U/ml penicillin, and 100 μg/ml streptomycin for 24 h at 37°C before infection.

Thioglycollate-Induced Macrophages. Swiss Rockefeller mice were injected intraperitoneally with 1 ml of a 4.05% aqueous solution of Brewer thioglycollate medium (Difco Laboratories, Detroit, Mich.), and 4 days later their peritoneal cells were harvested and cultivated as described above.

Cell Lines. Continuous cell lines were maintained as monolayers on Falcon (Baltimore Biological Laboratories, Baltimore, Md.) tissue culture flasks (75 cm^3). HeLa cells were provided by Dr. S. Lazarowitz and L cells by Dr. S. Silverstein from The Rockefeller University. Calf embryo fibroblasts were obtained by successive trypsin digestion (37°C, 15 min) of embryonic thigh muscles, the cells incubated for 1 h at 37°C, and the nonadherent cell population discarded. After a second passage, the cultures consisted entirely of fibroblasts. All cells were kept in FCS-Eagle's MEM with antibiotics. Horse serum was used when indicated. The monolayers were cultivated for 48–72 h on glass cover slips for the infection experiments after removal from the flasks with purified trypsin (100 μg/ml in PBS).

Protease Treatment of Macrophages. Trypsin from bovine pancreas Type III No. T-8253 was obtained from Sigma Chemical Co., St. Louis, Mo. Macrophage monolayers were incubated for 30 min at 37°C with 500 μg/ml of trypsin in MEM and then washed three times with 500 μg/ml of ovomucoid trypsin inhibitor (Worthington Biochemical Corp., Freehold, N. J.) in MEM to end trypsin digestion. Chymotrypsin no. 06474112 (C. F. Boehringer and Sohne Gmblt, Manheim, Germany) treatment was performed as above and digestion terminated by washing the monolayers three times with MEM-50% FCS and again three times with MEM to remove the serum.

Phase Contrast and Electron Microscopy. Cover slip cultures were fixed with 1.25% glutaraldehyde in PBS (pH 7.2) for 5 min at room temperature, washed with distilled water, stained with Giemsa stain (Gradwohl Laboratories, Philadelphia, Pa.) for 15 min, and mounted on a glass slide for examination by phase contrast microscopy with a 100 × oil objective. The percentage of macrophages infected and the number of parasites per macrophage were obtained by counting a minimum of 200 macrophages. Macrophage numbers were determined by counting ten 22,700 μm^2 microscopic fields and expressed as number of macrophages per mm^2. Observations were also made on unstained specimens fixed in 2.5% glutaraldehyde and on live preparations observed under phase contrast on a warm stage. The developmental forms of *T. cruzi* could be easily recognized in the stained preparations under phase contrast. Typical epimastigotes have a wide body, a kinetoplast that is anterior to the round, central nucleus and possess a long, free flagellum. Transition forms present a more elongated nucleus, and the kinetoplast is slightly posterior to the nucleus. Trypomastigotes possess a much more slender body (1–2 μm width), a terminal or subterminal kinetoplast, an oval and elongated nucleus, and a flagellum that is attached along the body forming the undulating membrane. Amastigotes are round or oval (2.4–6.5 μm) with a round central nucleus and are aflagellate (16). Cells were processed for electron microscopy as previously described (17) and examined in a Siemens Elmiskop 1A.

Preparation of Sensitized Erythrocytes. Sheep erythrocytes (E) coated with anti-E IgG [E(IgG)] or IgM and complement [E(IgM)C] and the assay of ingestion were performed as described previously (18, 19).

Infection of Monolayers. Cell monolayers on cover slips were washed with MEM and covered with 0.05 ml of a suspension of parasites in MEM-20% FCS for HeLa, L cells, and calf embryo fibroblasts or in MEM alone when macrophages were used. The cells were then incubated for 90–180 min at 37°C. Parasite/cell ratios were 0.5/1 or 1/1 for macrophages and 5/1 for other cultured cells. With these multiplicities a sufficient number of cells were infected to allow accurate observation without appreciable cell death. At the end of the exposure period, cover slips were washed extensively to remove all extracellular parasites and either fixed for microscopic observation or replenished with complete medium and incubation continued at 37°C for the desired time.

Cytochalasin B. Cytochalasin B was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., batch no. PH/2668/77K. A 1 mg/ml stock solution was prepared in dimethyl sulfoxide and diluted to the desired concentration in MEM. Macrophage monolayers were preincubated for 2 h with 5 and 10 μg/ml cytochalasin B, then 0.05 ml of a suspension of parasites in the same solution was added to the cover slips and incubated for 1 h at 37°C. Cells were fixed and scored as described above.

IgG Fraction of Rabbit Antiserum to Mouse Macrophages. This reagent was prepared as previously described (19, 20) and was kindly supplied by Dr. Frank Griffin (University of Alabama Medical Center, Birmingham, Ala.). 0.05 ml of the antiserum IgG fraction was added for 2 h at 37°C to 24-h-explanted macrophages. After washing, the cells were infected as described above.

Results

The observations were made on three different preparations: *(a)* mixed population, containing epimastigotes and trypomastigotes, from cultures in Tobie's medium; *(b)* purified trypomastigotes and transition forms, prepared as described previously (12); and *(c)* amastigotes harvested from infected cell cultures in 20% FCS. Morphologic features of the forms and details of their preparation are given in the Materials and Methods section above.

Mechanism of Uptake

MORPHOLOGIC OBSERVATIONS. Under phase-contrast microscopy the early interaction of the parasites with macrophages can be seen in Fig. 1. When a mixed population was used as the inoculum (Fig. 1 a), the epimastigotes were seen to attach to the surface of macrophages either by means of the flagellum or body of the parasite. However, in all instances, ingestion appeared to be initiated and proceed via the body of the parasite. The interiorized organism was often surrounded by a phase-lucent halo (Fig. 1 b). Trypomastigotes also attached to the macrophage membrane (Fig. 1 c) and were ingested in a similar fashion (Fig. 1 d). With other cultured cells, epimastigotes attached to the cell surface but failed to enter the cytoplasm. In contrast, trypomastigotes and transition forms entered HeLa, L cells, and calf embryo fibroblasts.

An electron microscopic study of these specimens showed that epimastigotes (in macrophages) and trypomastigotes entered all cells by a phagocytic mechanism. No evidence was found at any time of fusion of the parasite and host cell membranes or dissolution of the surface membrane at the points of entry. Figs. 2 a and b show, in specimens fixed 30 min after addition of parasites, early stages in the phagocytosis of an epimastigote and a transition form. Figs. 3 a and b show epimastigotes and a trypomastigote within phagocytic vacuoles; these preparations were fixed 1 h after exposure to the parasites. The surface membrane of the parasite is clearly identified and the phagocytic vacuole membrane is continuous about the circumference of the parasite. Fig. 3 b illustrates a trypomastigote within a vacuole juxtaposed to the nuclear membrane. The migration of vacuoles containing recently ingested trypomastigotes to the paranuclear region was a common occurrence.

MODIFICATIONS OF THE MACROPHAGE SURFACE MEMBRANE. We next employed reagents known to influence the expression of macrophage membrane receptors and examined their effect on the uptake of *T. cruzi*.

EFFECT OF PROTEASE TREATMENT ON *T. CRUZI* INGESTION. Trypsinization of macrophages destroys the plasma membrane complement receptor (19, 21) and prevents the attachment of glutaraldehyde-treated red blood cells (22), but has no effect on the macrophage Fc receptor. Treatment of normal or thioglycollate-activated macrophages with 500 μg/ml of trypsin completely abolishes the attachment and ingestion of both epimastigotes and trypomastigotes without impairing the ability of the macrophage to ingest E(IgG) (Tables I, II, and III).

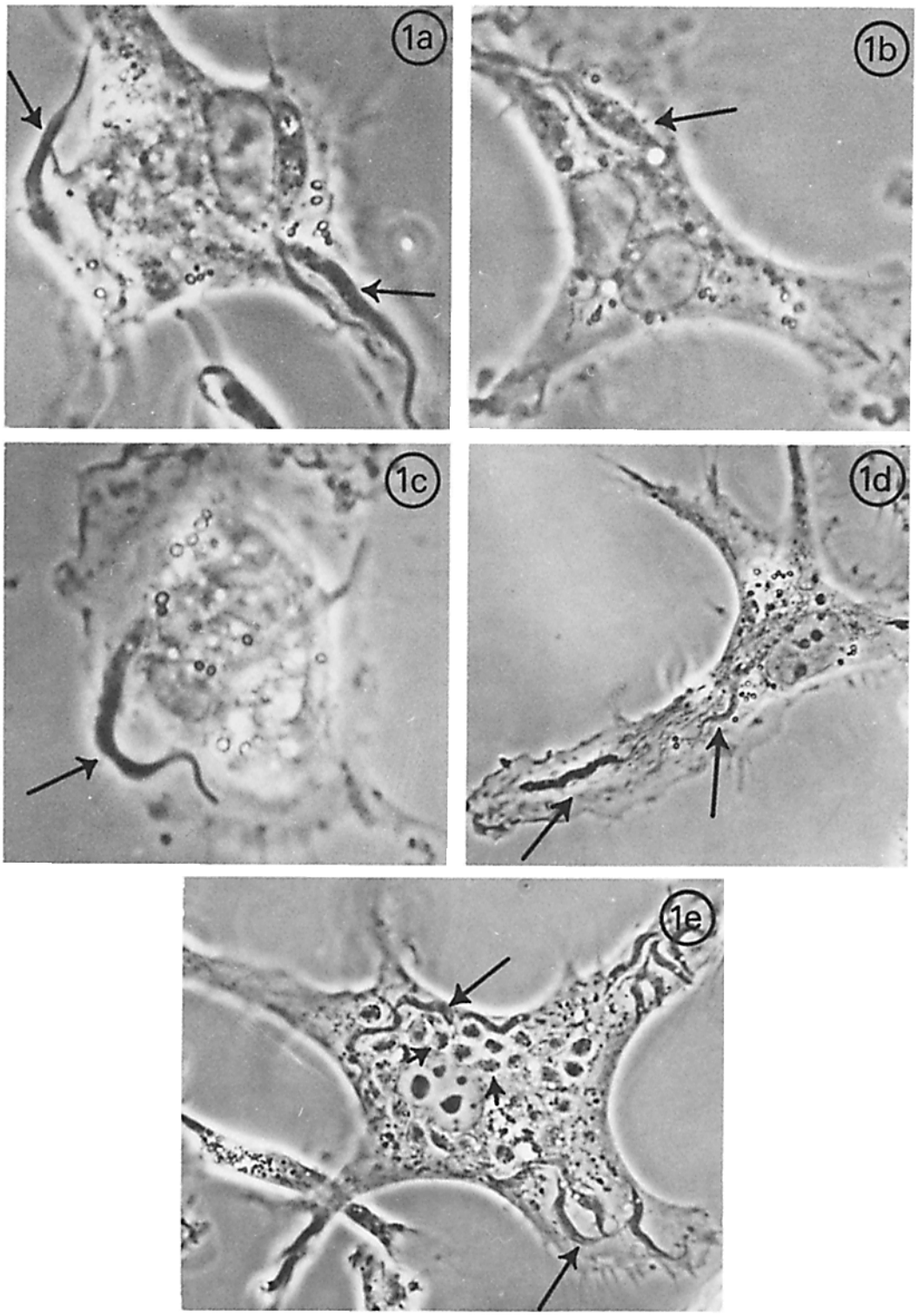

FIG. 1. Phase-contrast micrographs of the interaction of *T. cruzi* with mouse peritoneal macrophages in culture. (a) Attachment of epimastigotes (arrows) from a mixed population to the macrophage surface membrane. 30-min incubation. × 2,330. (b) Ingested epimastigote (arrow) within a phase-lucent vacuole. 60-min incubation. × 2,330. (c) Attachment of trypomastigote (arrow) from a purified inoculum to the macrophage surface membrane. 30-min incubation. × 2,560. (d) Ingested trypomastigotes (arrows) from a purified population. 60-min incubation. × 1,220. (e) Macrophage containing several amastigotes (short arrows) and fully differentiated trypomastigotes (long arrows) at 96 h after infection, in 20% horse serum. × 1,330.

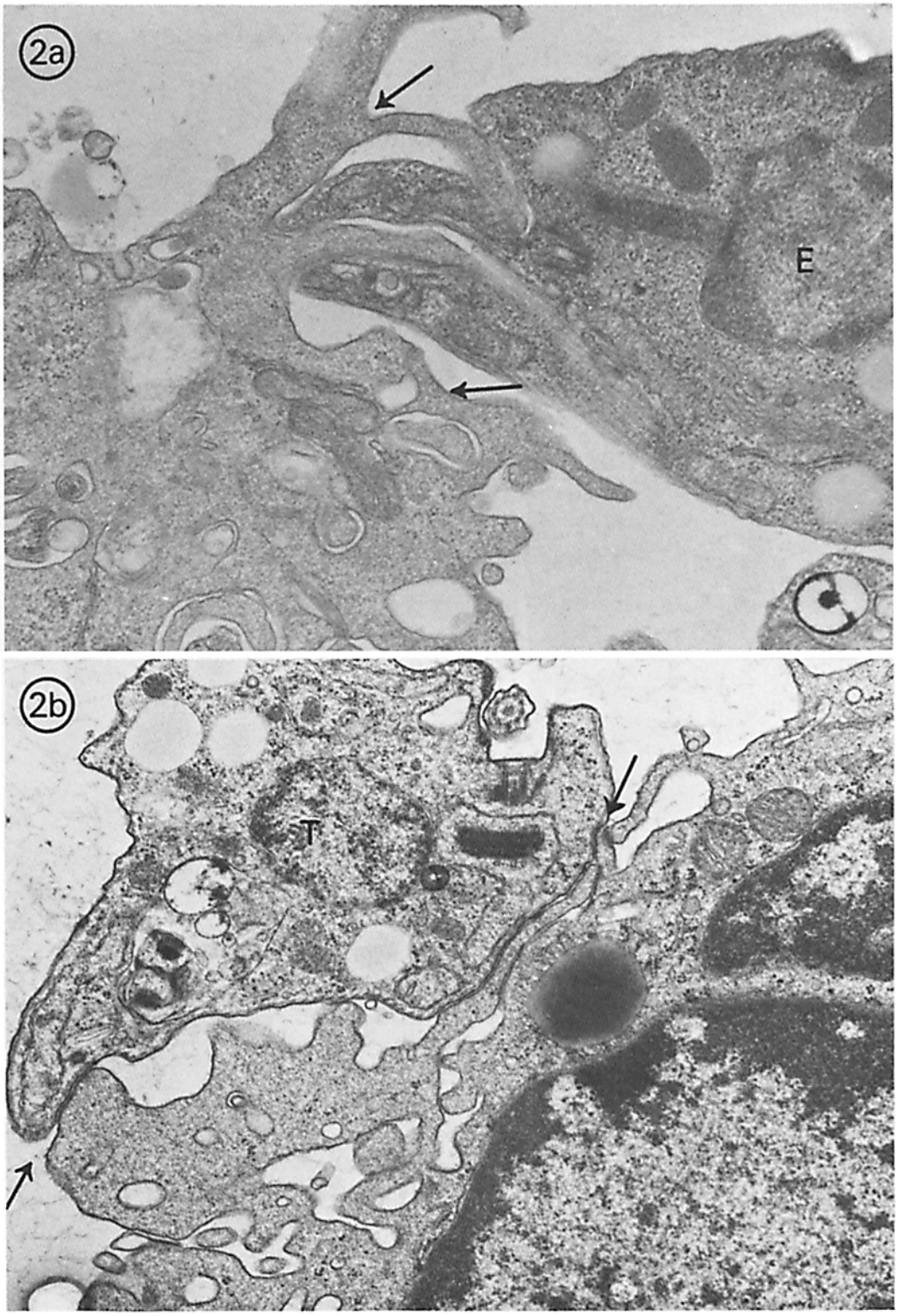

FIG. 2. Early stages in the phagocytosis of trypanosomes by mouse macrophages. 2 a shows the phagocytosis of an epimastigote (E) and 2 b of a transition form (T) from a purified trypomastigote population. Micropseudopods (arrows) are seen extending along the surface of the parasite. The membranes of both the parasite and macrophage appear unaltered. Fixed 30 min after the addition of parasites. (a) × 32,000 and (b) × 23,300.

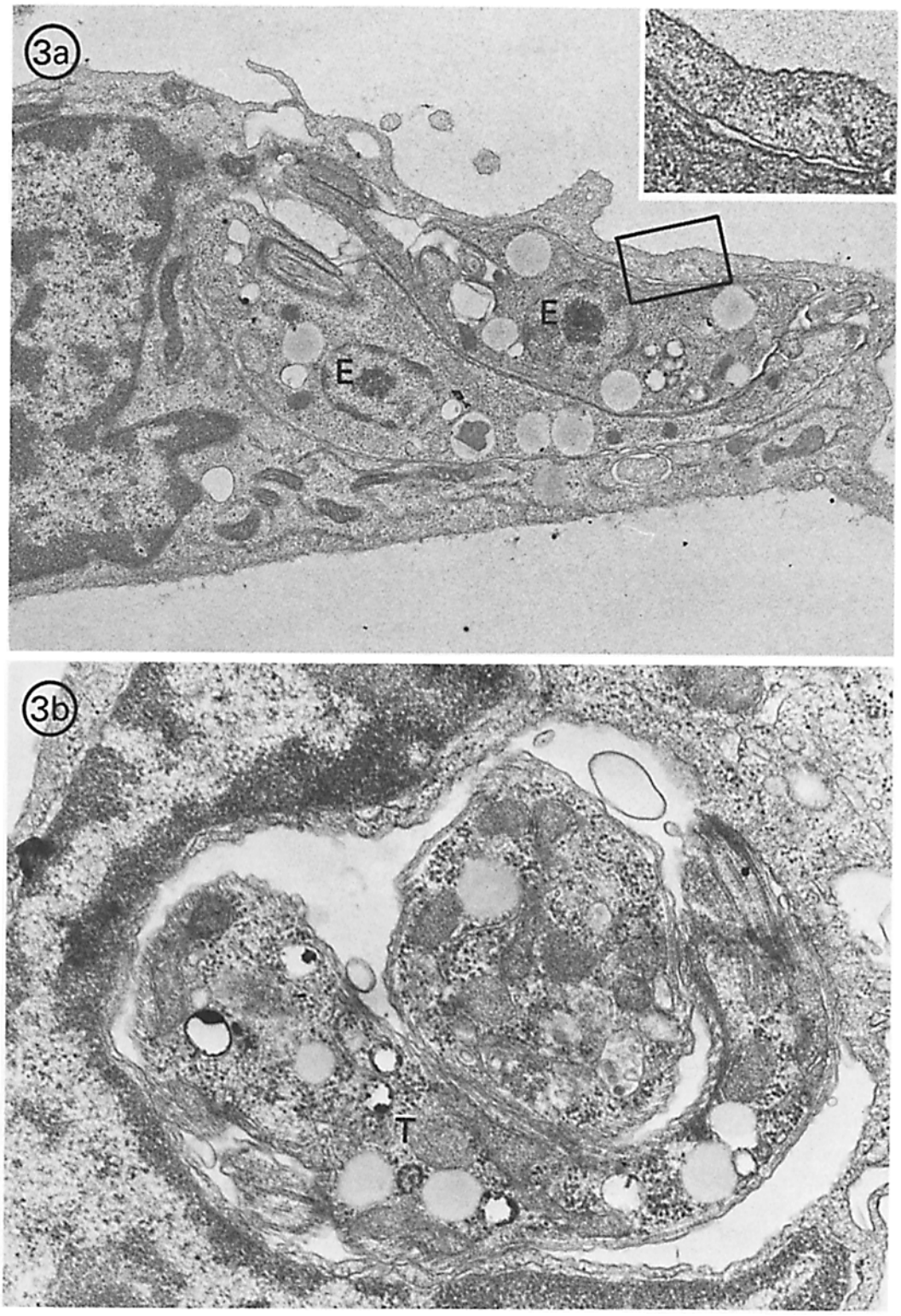

FIG. 3. *(a)* Epimastigotes (E) from a mixed inoculum in a macrophage, 60 min after the addition of parasites. The two parasites are seen within a single phagocytic vacuole. × 14,000. The insert shows the clearly defined membranes of the parasite and phagosome. × 35,400. (b) Trypomastigote (T) from a purified population within a macrophage, 60 min after the addition of parasites. The membrane of the phagocytic vacuole is clearly seen. × 24,000.

TABLE I
Uptake of Mixed Populations of T. cruzi by Normal Macrophages

Pretreatment of macrophages	Percent of control*		
	Culture forms ingestion	E(IgG) ingestion	E(IgM)C attachment
Trypsin	2	98	22
Antimacrophage‡ IgG	44	10	Not done
Untreated (Control)	100 (28)	100 (87)	100 (78)

* Numbers in parenthesis represent actual values in control samples, expressed as percent of cells which ingested or attached the corresponding particles. 180-min incubation.
‡ IgG fraction of rabbit antiserum to mouse macrophages.

TABLE II
Uptake of Purified Trypomastigotes by Normal Macrophages

Pretreatment of macrophages	Percent of control*		
	Purified trypomastigotes ingestion	E(IgG) ingestion	E(IgM)C attachment
Trypsin	4	97	10
Chymotrypsin	18	79	113
Antimacrophage‡ IgG	51	9	Not done
Untreated (control)	100 (37)	100 (96)	100 (97)

* Numbers in parenthesis represent actual values in control samples, expressed as percent of cells which ingested or attached the corresponding particles. 180-min incubation.
‡ IgG fraction of rabbit antiserum to mouse macrophages.

However, chymotrypsin (500 μg/ml) treatment of normal and thioglycollate-activated macrophages, which we found not to affect C3 receptor-mediated binding or ingestion (Tables II and III), also abolished the binding and ingestion of epimastigotes and trypomastigotes. This rules out a requirement for the C3 receptor for parasite ingestion. Although protease treatment inhibited the ingestion of both epimastigotes and trypomastigotes, it is not known whether the same receptor is involved in the uptake of the two forms.

EFFECT OF IgG FRACTION OF RABBIT ANTISERUM TO MOUSE MACROPHAGES ON *T. CRUZI* INGESTION. The IgG fraction of an antiserum directed against the macrophage plasma membrane blocks ingestion mediated by the Fc receptor, but has no effect upon ingestion of formaldehyde-treated erythrocytes, zymosan, or latex particles (20). Macrophages pretreated with IgG antimacrophage antibodies were still capable of ingesting epimastigotes or trypomastigotes albeit to a degree less than that of untreated cells. Under these conditions, the uptake of IgG-coated erythrocytes is completely inhibited (Tables I and II). Since the purification of trypomastigotes required a serum-treatment step, the possibility existed that some adsorption of IgG had occurred, mediating uptake by the Fc receptor. To evaluate this, mixed populations of untreated parasites were studied in comparison with those incubated with fresh guinea-pig serum, as performed before the purification procedure. No significant difference in the ingestion of such serum-treated parasites by IgG antimacrophage-treated cells was

TABLE III
Uptake of Purified Trypomastigotes by Thioglycollate-Induced Macrophages

Pretreatment of macrophages	Percent of control*		
	Purified trypomastigotes ingestion	E(IgG) ingestion	E(IgM)C ingestion
Trypsin	7	87	5
Chymotrypsin	28	79	91
Untreated (control)	100 (23)	100 (93)	100 (32)

* Numbers in parenthesis represent actual values in control samples, expressed as percent of cells which ingested the corresponding particles. 90-min incubation.

found. Since the specificities of the antimacrophage antiserum are not known, it is possible that the inhibition is due either to steric interference or to the presence of antibodies directed against specific attachment sites for the parasite on the macrophage surface membrane.

EFFECT OF CYTOCHALASIN B ON THE INGESTION OF *T. CRUZI* BY MOUSE PERITONEAL MACROPHAGES. The antibiotic cytochalasin B has been shown (23, 24) to reversibly inhibit phagocytosis of various particles by mouse peritoneal macrophages. Cytochalasin B (5–10 μg/ml) blocked completely the ingestion of both epimastigotes and purified trypomastigotes by macrophages, but the attachment of the parasites to the macrophage plasma membrane was not altered. Parasite motility was unaffected by the drug concentrations used. Removal of cytochalasin B promptly allowed many of the attached parasites to be ingested and subsequent intracellular multiplication ensued, thus indicating that parasite viability was not altered by exposure to these drug concentrations.

Intracellular Fate

INTRACELLULAR GROWTH. Phase contrast observations on live cultures (warm stage) showed that when the mixed population of parasites were used, epimastigotes remained motile for minutes after the ingestion process had been completed. Later, parasite motility completely ceased, progressive disintegration of the epimastigotes ensued, and by 24 h no further morphologic details could be seen. At 24 h all the surviving parasites had the appearance of amastigotes. These amastigotes subsequently multiplied intracellularly in the presence of 20% FCS, whereas if 20% horse serum was employed, multiplication of amastigotes was followed by a much greater differentiation into trypomastigotes (Fig. 1 e).

A quantitative evaluation of these results with a mixed population inoculum is presented in the growth curve of Fig. 4 a. In the first 24 h after infection there was a sharp decline in the number of parasites/100 macrophages and in the number of macrophages containing morphologically intact parasites. This was correlated with the morphological disintegration of epimastigotes and the survival of a few amastigotes. With the ratios of parasite/cell used there was no significant loss in the cell number during the period of observation (Fig. 4 a; lower). However, a small amount of cell death and release of viable parasites

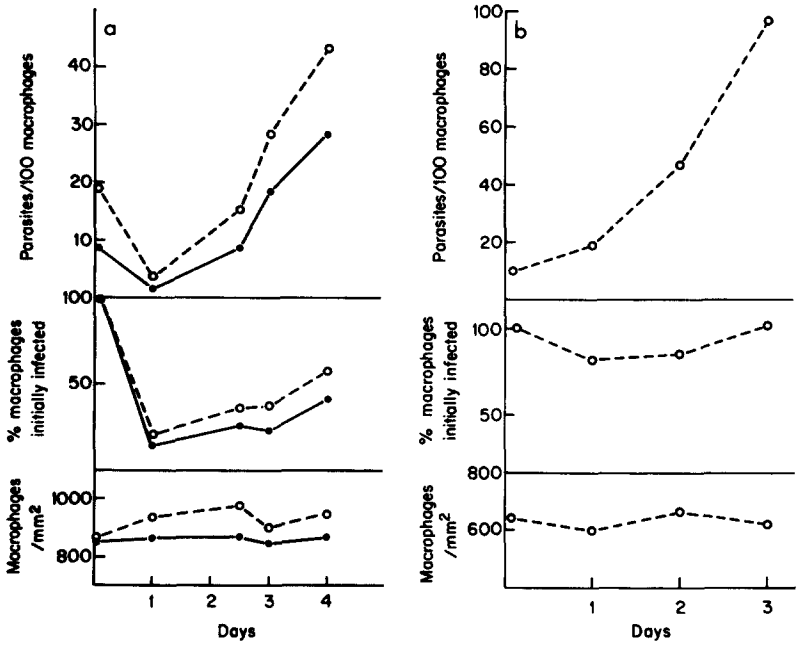

FIG. 4. The behavior of *T. cruzi* within unstimulated mouse peritoneal macrophages. (a) shows the fate of mixed populations and (b) of purified trypomastigotes. The percentage of macrophages initially infected expresses the number of cells containing one or more intact parasites at subsequent time points as compared to the number at the end of the infecting pulse (100%). Cells were exposed to parasites for 90 min in (a) or 180 min in (b) before removal of extracellular organisms. The continuous line represents a parasite/cell ratio of 1/2 and the dashed line 1/1.

was probably occurring, since the number of infected macrophages increases progressively (Fig. 4 a; middle). The number of parasites/100 macrophages also increased progressively with a doubling time of approximately 15 h.

The possibility that the surviving amastigotes in these cultures were derived from the 15% trypomastigotes present in the mixed inoculum prompted us to study macrophage cultures infected with purified trypomastigotes. In contrast to the epimastigotes, the majority of the trypomastigotes remained morphologically intact and began to multiply intracellularly. Fig. 4 b illustrates the behavior of trypomastigotes within macrophages. No sharp decline in either the number of parasites/100 macrophages or the number of infected macrophages occurred. The number of parasites/100 macrophages increased with a doubling time of approximately 15 h, the same as that observed for the surviving parasites in the cultures exposed to mixed forms, discussed above. In the presence of 20% FCS little intracellular differentiation into trypomastigotes occurred, and amastigotes were released into the medium. These were subsequently phagocytized by macrophages, thereby propagating the infection. Behbehani (7) had observed a similar uptake of released amastigotes in vitro. As mentioned previously, use of 20% horse serum led to differentiation of amastigotes into trypomastigotes, and the latter were released upon cell rupture. The same growth curves were observed with peritoneal macrophages from C3H/He and C57/BL mice and with the Tulahuen and PF strains of *T. cruzi*.

FATE IN THIOGLYCOLLATE-ACTIVATED MACROPHAGES. Thioglycollate-induced macrophages, as well as macrophages obtained from animals systemically infected with bacillus Calmette-Guerin (BCG), display biochemical and functional properties different from their normal counterparts (15, 19, 25–29). Thioglycollate-activated macrophages ingested twice as many epimastigotes or trypomastigotes as normal resident cells did, but the subsequent intracellular fate and rate of growth of the surviving parasites was the same as in normal cells. Thioglycollate-activated macrophages, therefore, are not able to resist and contain intracellular infection by *T. cruzi* as has been described for BCG-activated macrophages (30–32).

DESTRUCTION OF EPIMASTIGOTES IN THE PHAGOCYTIC VACUOLE OF MACROPHAGES. Ultrastructural observation of cells infected with mixed populations, fixed at 6 h, showed nearly all intracellular parasites in various stages of degradation within phagocytic vacuoles (Fig. 5). If macrophages were prelabeled with Thorotrast according to the technique described by Jones and Hirsch (33), the marker was found inside the vacuoles, indicating delivery of the contents of secondary lysosomes into the epimastigote-containing vacuoles.

ESCAPE OF TRYPOMASTIGOTES AND TRANSITION FORMS FROM THE PHAGOCYTIC VACUOLE. Electron microscopic examination of cultures infected with purified trypomastigotes showed these forms initially inside phagocytic vacuoles (Fig. 3 b, 60 min). At later times, all the parasites were seen free in the cytoplasm (Fig. 6) and were often in close association with strips of rough endoplasmic reticulum. No evidence of the endocytic vacuole was observed. The parasite membrane was clearly defined as evidenced by the array of subpellicular microtubules (Fig. 7).

The mechanism by which the phagocytic vacuole membrane disappears is unknown. However, in rare instances, the parasite was seen inside a phagocytic vacuole with a modified vacuolar membrane having a thickness of approximately half the usual unit membrane, as early as 90 min after addition of the parasites (Fig. 8).

EPIMASTIGOTES AND TRYPOMASTIGOTES IN OTHER CULTURED CELLS. Epimastigotes were not taken up by HeLa, L cells, and calf embryo fibroblasts. In contrast, transition forms and trypomastigotes, as well as released amastigotes, were ingested by all cell types. The ingested trypomastigotes and transition forms were found inside phagocytic vacuoles at early times after infection, as is seen in Fig. 9 for an L cell, and later free in the cytoplasmic matrix, as shown in Fig. 10 for a calf embryo fibroblast.

Discussion

The experiments presented in this report indicate that the uptake of *Trypanosoma cruzi* by mammalian cells occurs by a phagocytic process. This is the case both for the ingestion of epimastigotes and trypomastigotes by macrophages and the entry of trypomastigotes and transition forms into "nonprofessional" phagocytes. The mechanism of entry of *T. cruzi* is thus similar to that of malarial merozoites into erythrocytes (34), of *Toxoplasma gondii* into macrophages and fibroblasts (33, 35), and of *Leishmania donovani* into macrophages (36).

The integrity of the macrophage plasma membrane was always preserved

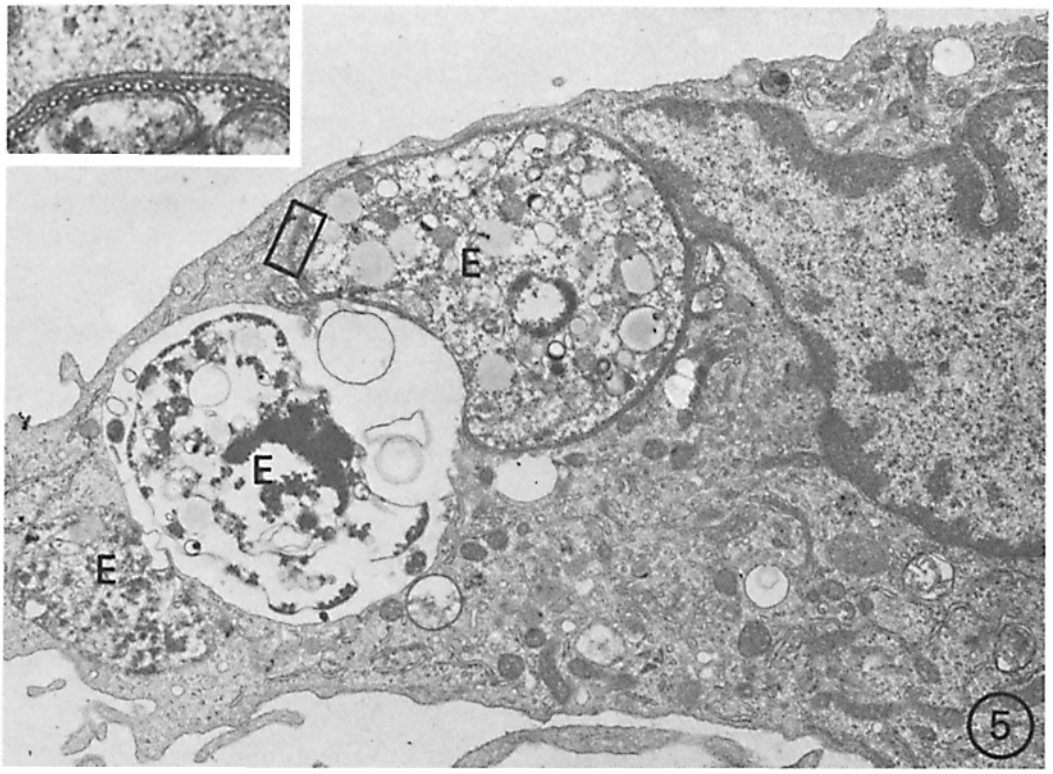

FIG. 5. Epimastigotes (E) within phagosomes in different stages of digestion. Fixed 6 h after the addition of parasites. × 14,000. Insert, high magnification of the phagocytic vacuole membrane. The membrane of the parasite is easily identified by the presence of the subpellicular microtubules. × 46,000.

during the ingestion process. In contrast, Sooksri and Inoki (11), studying the penetration of *T. cruzi* in HeLa cells, have interpreted their results as indicating an entry mechanism involving dissolution of the plasma membrane. However, their micrographs seem to show typical phagocytic figures with finger-like protrusions of the HeLa cell around trypomastigotes, and trypomastigotes completely surrounded by a phagocytic vacuole membrane.

The presence of a protease-sensitive component on the macrophage plasma membrane, necessary for uptake of *T. cruzi*, suggests that such a membrane structure may also exist in other cell types, but this has not yet been studied. It is possible that these components determine the tropism of *T. cruzi* for different tissues. Previous studies have shown that the C3 receptor is sensitive to trypsin (19, 21). Extending these observations we have found that the macrophage C3 receptor is insensitive to chymotrypsin, while the receptors for *T. cruzi* are sensitive to both trypsin and chymotrypsin. This allowed us to rule out any involvement of the C3 receptor on the interiorization of *T. cruzi* under the experimental conditions used. Likewise, the receptor for immunoglobulin G does not seem to be involved under the present conditions. However, these receptors may play a role in the presence of specific antibody and complement, and this situation is being investigated.

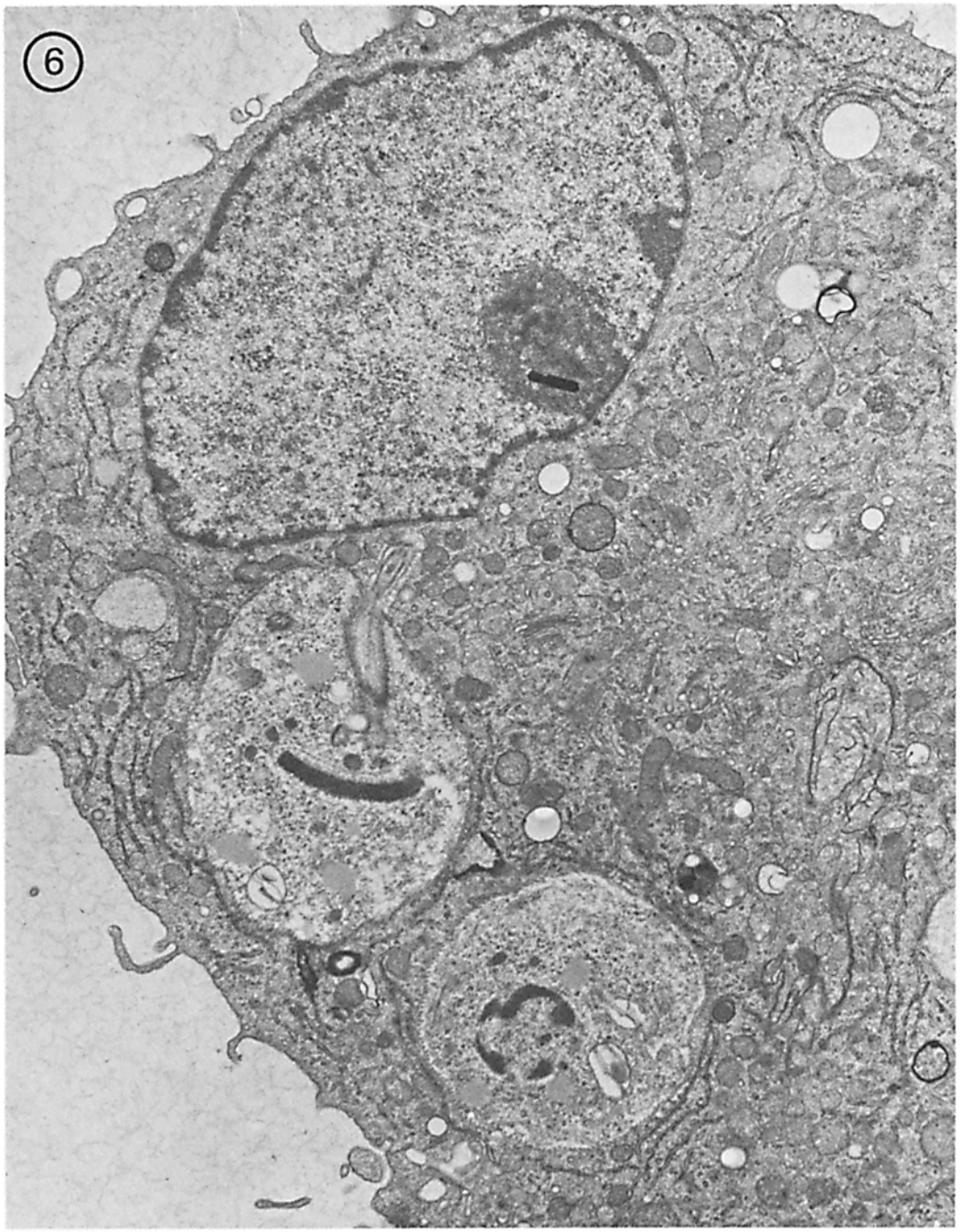

FIG. 6. Appearance of parasites at 48 h after macrophage infection. The organisms are free in the cytoplasmic matrix, and the phagosome membrane is absent. × 13,000.

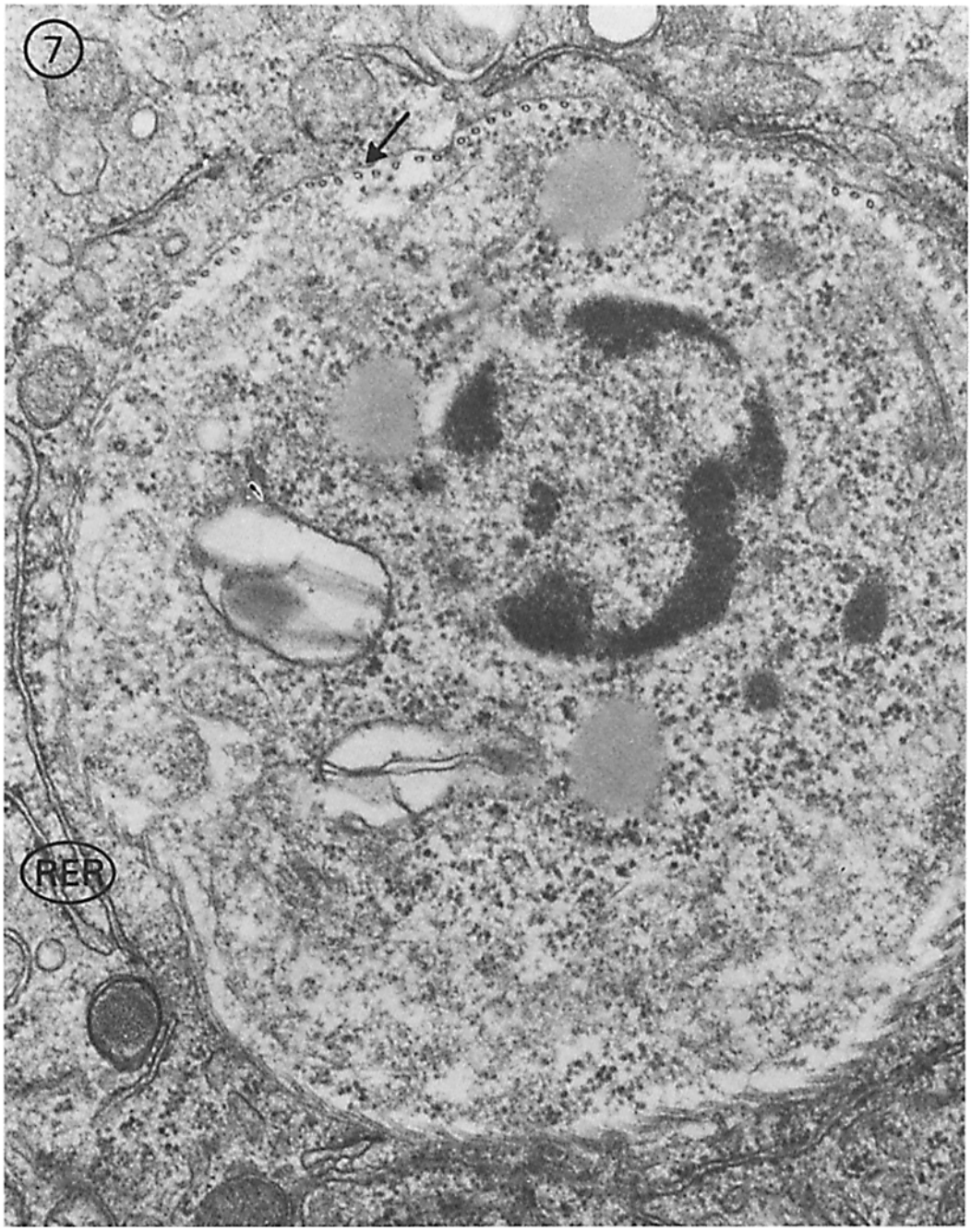

FIG. 7. High magnification of an intracellular parasite 48 h after macrophage infection, showing the typical unit-membrane structure of the parasite membrane (arrow), underlying subpellicular microtubules and absence of host membrane. Strips of rough endoplasmic reticulum (RER) are closely apposed to the parasite. × 46,000.

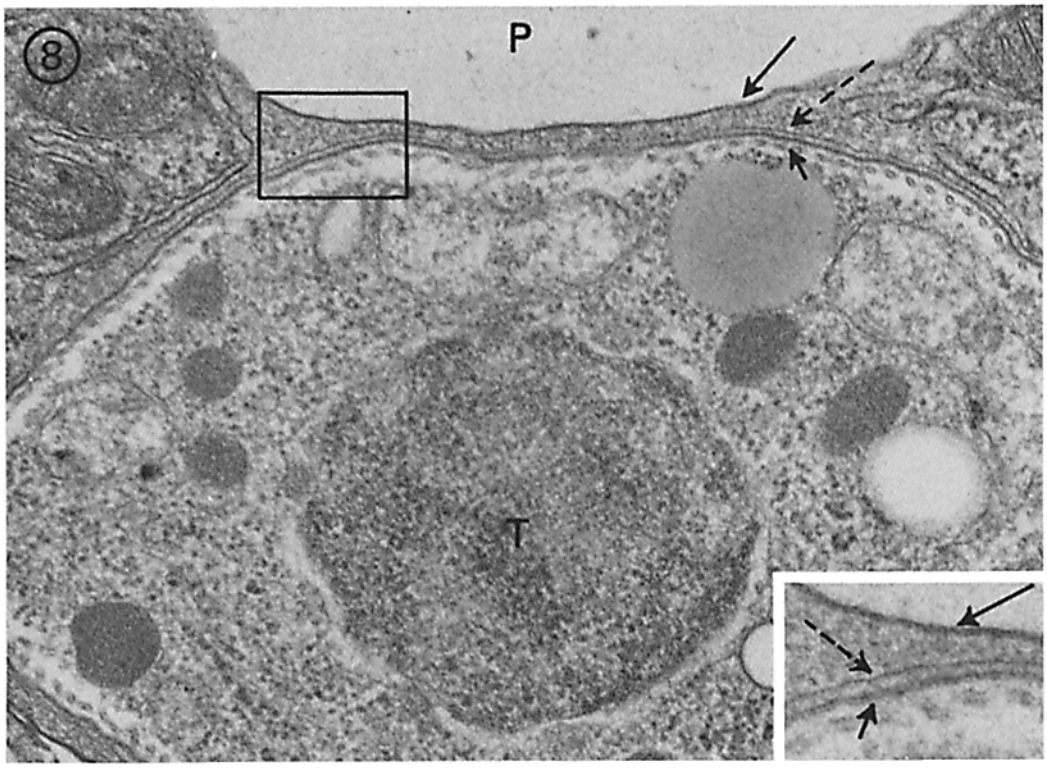

FIG. 8. Transverse section through an intracellular parasite (T), 90 min after macrophage infection with purified trypomastigotes. The parasite membrane (short arrow) has a typical unit membrane structure with underlying subpellicular microtubules. Another modified membrane is also seen around the parasite which is thinner and has only a single leaflet (dashed arrow). × 38,500. The insert illustrates this difference at higher magnification. The short arrow marks the membrane of the parasite and the long arrow an adjacent, typical phagosome (P) membrane. × 62,700.

A striking inhibition of the uptake of culture forms of *T. cruzi* by 5–10 μg/ml of cytochalasin B was also noted by Alexander (37). He argued that the failure of cytochalasin B to completely inhibit parasite uptake was consistent with active penetration of the macrophages by the parasites. In our hands, similar concentrations of cytochalasin B completely blocked the uptake of both epimastigotes and purified trypomastigotes by macrophages without affecting their attachment to the cells or their subsequent entry into cells after removal of cytochalasin B. These results are consistent with a classical endocytic mechanism for the interiorization of these organisms.

Epimastigotes were capable of attaching to, but not entering "nonprofessional phagocytic" cells, whereas trypomastigotes and transition forms entered all cell types studied. This could be explained by a difference in the cell surface receptors for epimastigotes and trypomastigotes or by the fact that the ingestion process requires a more active role for the parasites, a quality lacking in the epimastigotes.

Trypomastigotes and transition forms are able to survive the interaction with

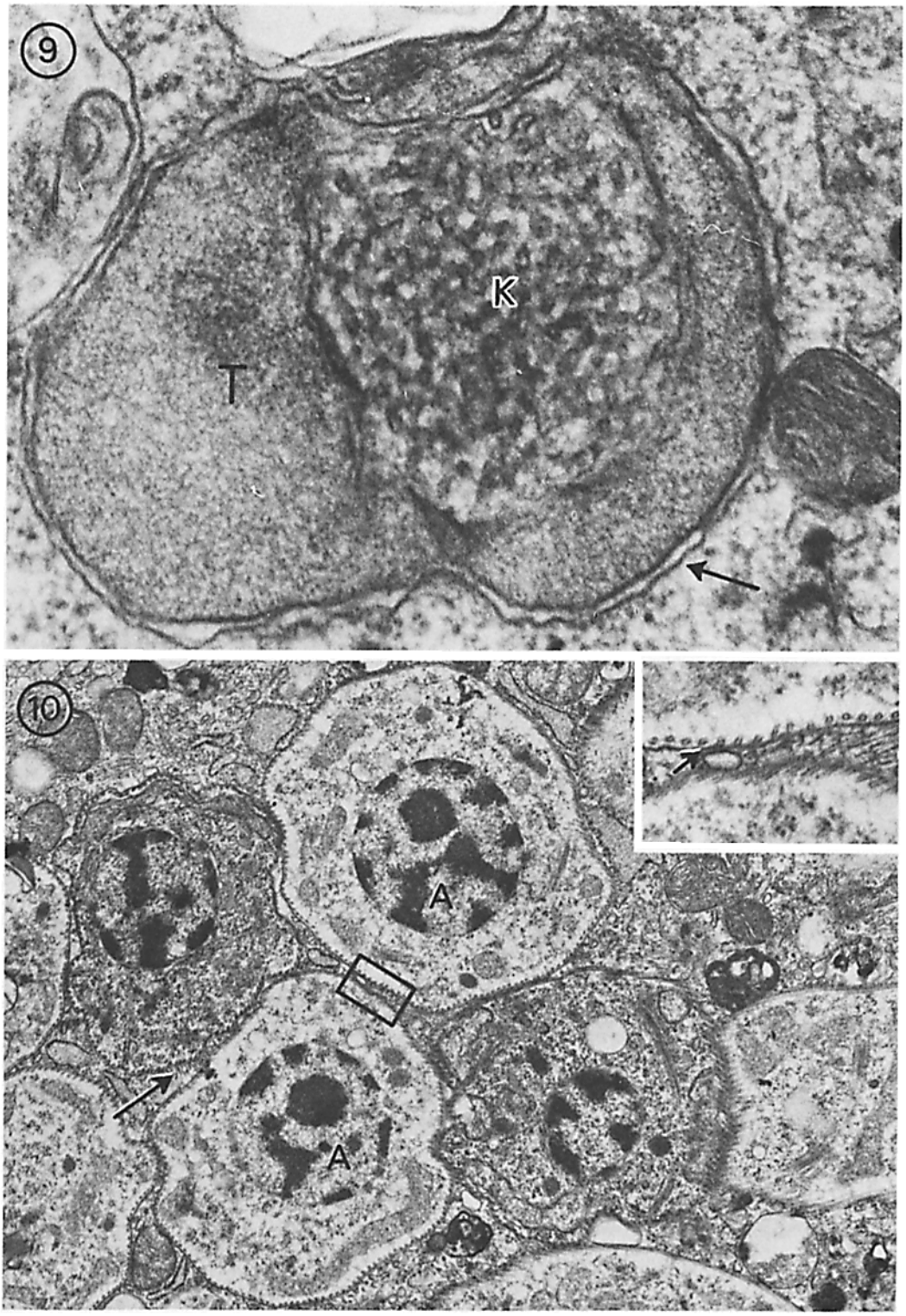

FIG. 9. A section through the kinetoplast (K) of a trypomastigote (T) within an L cell fixed 3 h after addition of the parasites. At this time, the phagosome membrane (arrow) clearly surrounds the parasite. × 55,000.

FIG. 10. Parasites in a calf embryo fibroblast fixed 60 h after ingestion. Amastigotes (A) are seen free in the cytoplasm. × 18,400. Insert, high magnification of the parasite membrane (short arrow) and absence of the phagosome. Microfilaments are seen (long arrow) between the developing organisms. × 45,000.

macrophages and other cell types, escape from the phagocytic vacuole, and then multiply in the cytoplasmic matrix. This is in contrast with *Toxoplasma, Leishmania,* and malarial trophozoites, which multiply within phagocytic vacuoles. A similar process of escape from the phagocytic vacuole has been described for a number of animal viruses (38, 39). In the case of vaccinia virus it has been found (38) that opsonization of the virus particle with specific antibody prevents the escape from the phagocytic vacuole, and the virus is then killed and digested by lysosomal enzymes. Perhaps specific antibody on the surface of trypomastigotes might similarly interfere with escape from the phagocytic vacuole, and this possibility is being investigated.

The mechanism by which the trypomastigote interacts with the host cell vacuolar membrane promoting its disappearance is not yet clear. Possible mechanisms might represent lysis of the phagosome membrane by a parasite-released factor or some more direct membrane-membrane interaction. It is not clear if the process is one of complete dissolution of the membrane or a more limited reaction in which disrupted membrane is ultimately disposed of by the cell. We tend to favor the first possibility in view of the finding of modified membranes as is shown in Fig. 8 and on the fact that no membrane remnants were seen in the course of this study. In addition, we do not know if the phagosomes containing trypomastigotes or transition forms fuse with lysosomes before the membrane alteration.

The situation with epimastigotes is quite different and after fusion with lysosomes has occurred, complete digestion of the parasites ensues. This result is in keeping with the microscopic observations of Pizzi et al. (40, 41) in which only the trypomastigotes were able to survive an in vivo interaction with macrophages. However, others have reached different conclusions (8, 42). In this regard, it is important to realize that transition forms are also able to survive lysis by normal fresh serum (12) and therefore behave functionally as trypomastigotes in terms of uptake, fate, and sensitivity to lysis. This fact may explain some of the discrepancies in the literature on the role of epimastigotes in infection. The fate of amastigotes, trypomastigotes, and transition forms may be different in immune and normal macrophages, as suggested by the in vitro results of Hoff (32) for mixed population of parasites and from the in vivo studies of Scorza (43). In thioglycollate-activated macrophages, however, no alteration in their fate was observed.

A previous report on the interaction of culture forms and blood form trypomastigotes in hamster macrophages (44) has reached opposite conclusions. Epimastigotes were thought to be free in the cytoplasm, whereas trypomastigotes were contained within a vacuole. The finding of trypomastigotes within a phagocytic vacuole at 1 h after infection is consistent with our results; however, at later times we would expect them to be free in the cytoplasm. The finding of epimastigotes free in the cytoplasm may be the result of poor membrane preservation during the processing of the specimens.

The availability of a purified population of trypomastigotes allowed us to establish the mechanism of uptake and the fate of the two culture forms of *T. cruzi*. The use of mixed populations obtained from cultures is not appropriate for in vitro work, since the majority of the population is composed of epimastigotes

that are unable to survive in the macrophage and are unable to penetrate other cell types. This makes it difficult to quantitatively assess uptake and intracellular survival of the parasites at early times. Epimastigotes are probably noninfectious in vivo for other reasons as well, since they can activate the alternate pathway of complement in the absence of specific immunoglobulin (12) and are lysed in normal serum. The observed uptake by macrophages and other cultured cells and intracellular development of released amastigotes imply that these forms are also infective and may play a role in the pathogenic process in vivo.

Summary

The mode of entry and intracellular fate of epimastigotes and trypomastigotes of *Trypanosoma cruzi* in cultured cells was studied. Electron microscopic observations indicated the uptake by phagocytosis of both forms into mouse peritoneal macrophages and of trypomastigotes and transition forms into other cultured cell types. In each instance the organisms were initially surrounded by a plasma membrane-derived phagosome. Trypsin and chymotrypsin treatment of the macrophages completely abolished attachment and ingestion of both forms, indicating that protease-sensitive structures on the macrophage plasma membrane mediate ingestion. The macrophage Fc or C3b receptors were not essential for uptake of *T. cruzi* in the conditions used. Cytochalasin B inhibited ingestion but not the attachment of both forms by macrophages.

Epimastigotes were not taken up by HeLa, L cells, and calf embryo fibroblasts. In macrophages, epimastigotes were killed and digested within phagolysosomes. In contrast, trypomastigotes and transition forms escaped from the phagocytic vacuole and then multiplied in the cytoplasmic matrix. Amastigotes released from infected cells exhibited properties similar to those of trypomastigotes and were able to enter all cell types studied and multiply intracellularly.

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