THE EXIT OF *TRYPANOSOMA CRUZI* FROM THE PHAGOSOME IS INHIBITED BY RAISING THE pH OF ACIDIC COMPARTMENTS

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Trypanosoma cruzi is the causative agent of American trypanosomiasis (Chagas' disease). Its host range is very broad, with more than 200 vertebrate species serving as animal reservoirs (1), and several species of reduviid insects as vectors (1, 2). *In vitro*, this protozoan can infect many cell types from different origins (3, 4).

The parasite's cycle in the vertebrate host begins when metacyclic trypomastigotes are released with the feces of the insect, reach a skin wound or a mucosa, and enter cells. The parasites are initially found in phagocytic vacuoles, which they subsequently leave to multiply free in the cytosol. Intracellularly, the flagellated trypomastigotes undergo profound morphological modifications, change their major surface glycoproteins (5–7), and transform into amastigotes. These are rounded forms that multiply and can transform again into trypomastigotes. Eventually, the infected cell breaks down and the parasites are released into the blood stream. Both the trypomastigote and amastigote forms infect mammalian cells (8).

Previous studies have demonstrated that lysosomes fuse with trypomastigote-containing vacuoles early after host cell infection (9–11), and that between 24 and 96 h later amastigotes are free in the cytosol (9, 10, 12–14). However, these studies have not clarified the time course and the escape mechanism. In this regard, one of us recently reported that *T. cruzi* secretes a hemolysin that is optimally active at low pH, and proposed that this agent might be involved in disruption of the phagosome membrane (15). Here we show supportive evidence for this hypothesis, demonstrating that the luminal pH of the vacuoles containing parasites is acidic, and that drugs that raise the pH of acidic compartments inhibit parasite escape.

Materials and Methods

Parasites and Mammalian Cells

The Y strain of *T. cruzi* was maintained in monolayers of LLC-MK_2 cells in DME con-

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taining 2% FCS (Gibco Laboratories, Grand Island, NY) at 37°C in a 5% CO_2 atmosphere. Trypomastigotes were collected from the supernatant of infected cultures (5). Amastigotes were derived from trypomastigotes by allowing them to differentiate for 24–36 h in liver infusion tryptose medium (LIT)[1] in the absence of host cells (8).

Madin-Darby canine kidney (MDCK) epithelial cells and 3T3 fibroblasts were obtained from American Type Culture Collection (ATCC, Rockville, MD). Human monocytes were isolated from the venous blood of normal donors as described (8). The mouse macrophage cell line J774-E (16) was a gift from Dr. Stephanie Diment, New York University, New York, NY.

Transmission Electron Microscopy

Cultures were fixed in situ with 2% glutaraldehyde, 0.1 M sucrose in 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C for 14 h. Cells were collected by scraping and pelleted by centrifugation. The pellets were osmicated (1% OsO_4 in 0.1 M cacodylate buffer), dehydrated in graded ethanol, and embedded in Epon 812. Thin sections were mounted on formvar-coated, carbon-stabilized grids. For immunodetection experiments, the samples were not osmicated and were mounted on noncoated nickel grids.

Localization of the Intracellular Parasites

Human monocytes, obtained as described (8), were plated on 35-mm diameter wells (Costar, Cambridge, MA) at 3×10^5 cells/well and 48 h later were exposed to trypomastigotes at a multiplicity of infection of 100 parasites per cell, in RPMI (Gibco Laboratories) containing 10% fresh human serum. After 1 h, the cultures were washed with medium to remove extracellular parasites and either fixed immediately or further incubated for two additional hours before fixation with glutaraldehyde in sodium cacodylate/sucrose buffer. Similarly, sparsely plated MDCK epithelial cells ($1.5 \times 10^4/cm^2$) were incubated with 100 trypomastigotes/cell (40×10^6 parasites/ml) in DME/10% FCS for 30 min. The cultures were washed with medium to remove extracellular parasites and the cells fixed or incubated in fresh medium for two additional hours before fixation. Cells were collected by scraping and processed for transmission electron microscopy. Sections of pellets from the different time points were examined and parasites inside intact vacuoles, membrane-disrupted vacuoles or free in the cytosol were quantitated. Approximately 200 parasites were counted for each experimental point.

Detection of DAMP in Acidic Compartments

a. By Immunofluorescence. NIH 3T3 fibroblasts or human monocytes were seeded in dishes containing 12-mm diameter cover slips. 24 h later, the cells were incubated with 100 trypomastigotes per cell in DME 10% FCS for 30 minutes. The monolayers were then washed to remove free parasites and reincubated in fresh medium containing 30 μM [3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropilamine] (DAMP; Oxford Biomedical Research, Oxford, MI) for 30 min at 37°C. In control experiments, cells were preincubated with 100 μM chloroquine or 10 mM ammonium chloride for 15 min before adding DAMP. Cells were fixed with 3% paraformaldehyde, washed with 50 mM ammonium chloride, and permeabilized with 0.1% Triton X-100 for 5 min at −10°C. The fixed cells were incubated with a mAb anti-dinitrophenol (DNP) (Oxford Biomedical Research), washed, incubated for 1 h at 25°C with anti mouse-IgG conjugated to fluorescein (Calbiochem-Behring Corp., La Jolla, CA), and viewed under a fluorescent microscope.

b. By Immunogold Labeling. NIH 3T3 fibroblasts (2.5×10^6 cells) cultured in 9-cm diameter dishes were incubated with 100 parasites/cell for 30 min, washed, and incubated with DAMP-containing medium (30 μM) for 30 min. Cells were fixed for 1 h at 25°C with 2% glutaradehyde in 0.1 M cacodylate buffer containing 0.1 M sucrose. The fixed cells were then washed once in buffer and incubated in 0.5 M ammonium chloride in PBS for 30 min at 25°C. Cells were scraped and pelleted, and the samples were processed for transmission electron microscopy as described. Thin sections mounted in uncoated grids were incubated with anti-DNP mAb for 16 h at 4°C. The grids were washed, incubated with 15 nm colloidal gold particles coated with *Staphylococcus aureus* protein A (Janssen Biotech N. V., Olen, Belgium),

stained with 5% aqueous uranyl acetate, and examined in a Phillips 300 transmission electron microscope.

Treatment with Weak Bases and Monensin

a. Effect on Parasite Intracellular Localization. J774-E mouse macrophages were seeded in 9-cm diameter dishes at 10^6 cells/dish, in αMEM (Mediatech, Washington, DC) 10% FCS, and incubated at 37°C in a 5% CO_2 atmosphere. After 24 h the cells were exposed to 50 amastigotes/cell for 40 min. One of the following drugs was then added: 10 or 100 μM chloroquine, 10 mM ammonium chloride, 20 mM methylamine, or 1 μM monensin (Sigma Chemical Co., St. Louis, MO), and the cultures further incubated for 30 min. The cells were washed to remove extracellular parasites and incubated in presence of the drugs for 1 additional hour. The cultures were fixed with 2% glutaraldehyde in cacodylate/sucrose buffer and processed for transmission electron microscopy.

b. Intracellular Development of Parasites after Removal of Drugs. J774-E cells were seeded at 10^4 cells/well in 48-well dishes (well diameter 11.3 mm) in αMEM 10% FCS and incubated at 37°C in 5% CO_2. After 24 h 5×10^4 amastigotes were added to each well and the cultures incubated for 40 min. The drugs were then added in the same concentrations above and the cells incubated for 30 min. Extracellular parasites were removed by washing with drug-containing medium and the infected cultures were incubated in presence of the drugs for one additional hour. The cultures were then washed twice and incubated with fresh medium for different periods of time. The number of intracellular parasites at each time point was determined by hybridization with a DNA probe specific for *T. cruzi* (8, 17).

c. Viability of Drug-treated Parasites. Amastigotes (5×10^6/ml) were incubated for 2 h at 37°C in DME containing 10% FCS and one of the following drugs: 10 μM chloroquine, 100 μM chloroquine, 10 mM ammonium chloride, 20 mM methylamine, 1 μM monensin. Control parasites were incubated in parallel with DME 10% FCS. The amastigotes were washed twice and added to wells (11.3 mm diameter) containing J774-E cells (10^4) at a multiplicity of infection of five parasites per cell. After 2 h at 37°C in a 5% CO_2 atmosphere the wells were washed to remove extracellular parasites and the cells were incubated in fresh medium for different time periods. The number of intracellular parasites at each time point was measured by hybridization with a DNA probe as described above.

The viability of the parasites was also assayed by their ability to secrete hemolysin in the presence of the pH-raising agents. Amastigotes (5×10^7) were incubated in 10 ml DME 10% FCS (pH 7.2) containing or not 100 μM chloroquine, 10 mM ammonium chloride, 20 mM methylamine, or 1 μM monensin, at 37°C in a 5% CO_2-containing atmosphere. After 2 h the parasites were centrifuged and resuspended in 0.5 ml of 10 mM acetate, 50 mM dextrose, and 100 mM NaCl, pH 5.5, containing the same drug concentrations above. The parasite suspensions were then mixed with 10^7 guinea pig erythrocytes (Colorado Serum Co., Denver, CO), incubated at 37°C for 3 h, centrifuged, and the hemoglobin released into the supernatant quantitated by reading the absorbance at 545 nm.

Results

Time Course of Escape of T. cruzi from the Phagosome

We have previously shown that the amastigote stage of *T. cruzi* can escape from the phagocytic vacuole of human monocytes (8). To determine the time course of exit from the vacuole and whether trypomastigotes or intermediate forms (those in the process of transformation into amastigotes) were also able to disrupt the membrane of the phagosome, we exposed human monocytes and epithelial MDCK cells to trypomastigotes, and fixed the cells at different times after infection. Cells were collected by scraping, pelleted by centrifugation, and processed for transmission electron microscopy. 1 h after infection, the majority of the parasites that had entered monocytes or MDCK cells were inside tight vacuoles (Fig. 1, *a* and *b*); ~25% were

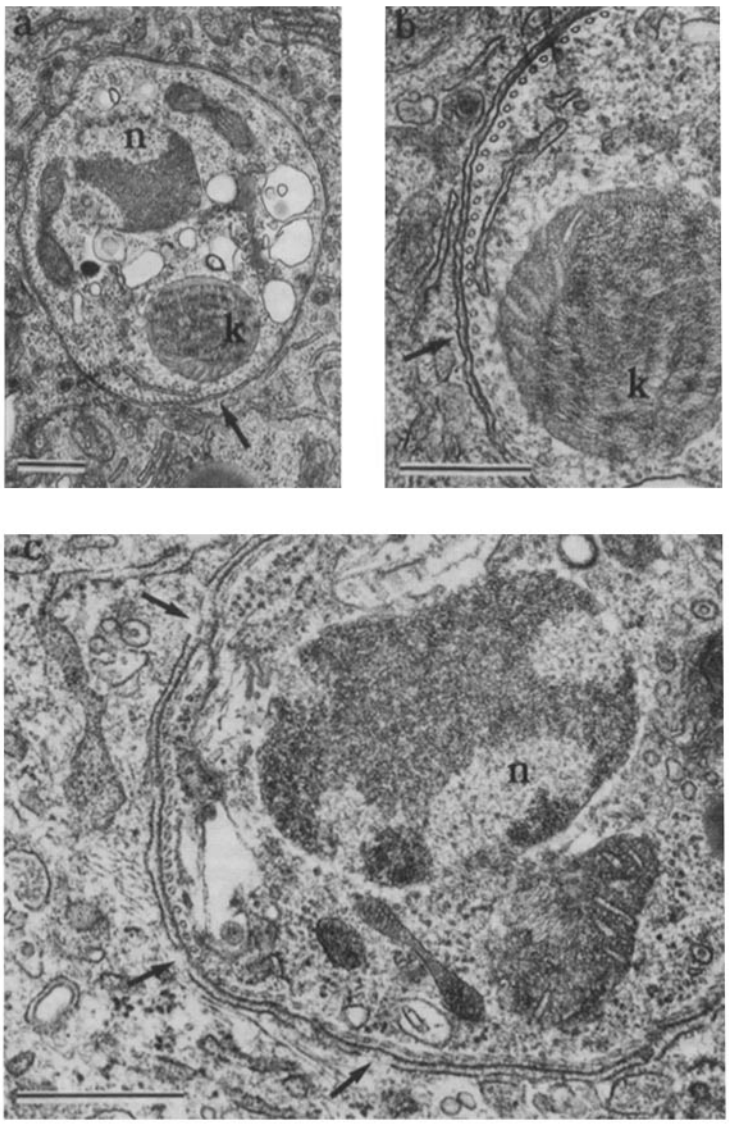

FIGURE 1. Recently interiorized parasites inside vacuoles. Infected human monocytes were processed for transmission electron microscopy as described in Materials and Methods. (*a*) Trypomastigote inside an intact vacuole, 1 h after infection. (*b*) Enlargement of a section of *a*. (*c*) Parasite inside a disrupted vacuole, 1 h after infection. Arrows point to the intact phagosome membrane in (*a*, *b*) and to discontinuities in (*c*). *k*, Kinetoplast; *n*, parasite nucleus. Bars, 0.5 μm.

inside apparently disrupted vacuoles (Fig. 1 *c*), and 10% were free in the cytosol (Fig. 2). After 2 h, the total percentage of parasites in disrupted vacuoles or free in the cytosol reached 70%. Among the free parasites we found trypomastigotes and intermediate forms (Figs. 2, *c* and *d*). These were identified with basis on kinetoplast morphology and presence of a long flagellum (Fig. 2 *c*).

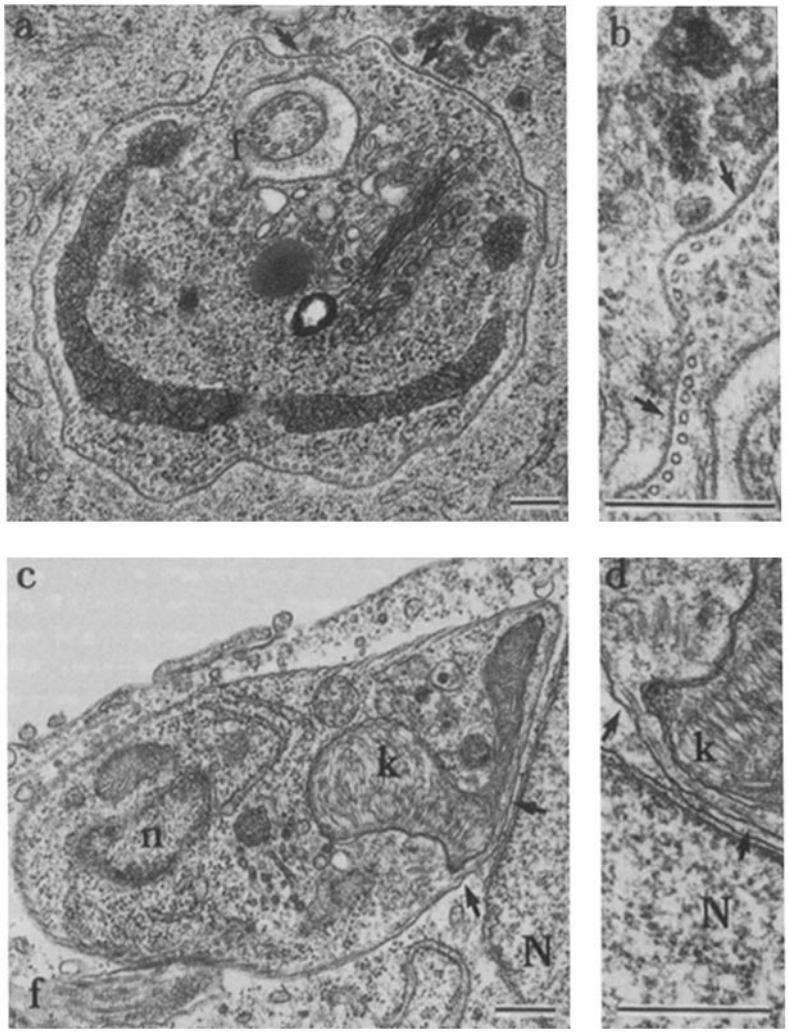

FIGURE 2. Parasites free in the cytosol. Infected cells were processed for transmission electron microscopy as described in Materials and Methods. (*a*, *b*) Parasite in a human monocyte 2 h after infection. (*c*, *d*) Trypomastigote or intermediate in an MDCK cell, 2 h after infection. (*b*) is an enlargement of a section of *a*, and *d* is an enlargement of a section of *c*. Arrows point to the parasite's plasmalemma, in direct contact with the host cell's cytoplasm. Note the parasite's subpellicular microtubule array, cross-sectioned in *a* and *b* and tangentially sectioned in *c* and *d*. *k*, Kinetoplast; *f*, flagellum; *n*, parasite nucleus; *N*, host cell nucleus. Bars, 0.3 μm.

T. cruzi-containing Phagosomes Are Acidic Compartments

To determine whether the vacuoles containing *T. cruzi* were acidified, we incubated recently infected cells (3T3 fibroblasts and human monocytes, exposed to parasites for 30 min) with the acidotropic compound DAMP (18, 19). The infected cells were processed in parallel for detection of DAMP by immunofluorescence or transmission electron microscopy and immunogold labeling. Results were the same with both

cell types, but they are documented only with 3T3 cells because the large amounts of DAMP accumulated in the extensive endocytic compartments of monocytes did not permit a sharp visualization of the parasites. Label was found in the phagosomes containing recently internalized parasites, as well as in other acidic compartments of the cell (Fig. 3 *a*). DAMP accumulation was prevented in cells preincubated with ammonium chloride (Fig. 3 *b*) or chloroquine (not shown), indicating that these treatments raised the pH of all acidic compartments, including those containing *T. cruzi*. Clear images of DAMP accumulation in the tight vacuoles surrounding parasites were observed by transmission electron microscopy using anti-DNP antibodies followed by protein A coupled to gold particles (Fig. 4, *a*, *b*, *c*). Parasites surrounded by DAMP label were often observed in the perinuclear region (Figs. 3 *a*, 4 *a*). Labeled endosomes or lysosomes were observed throughout the host cell, and adjacent to vacuoles containing parasites (Fig. 4, *a*, *c*).

Drugs that Raise the Intraphagosomal pH Inhibit Escape

Next, we raised the pH of intracellular compartments by treatment with chloroquine, ammonium chloride, methylamine, or monensin, and studied the fate of the intracellular parasites. To obtain large numbers of cells infected with a homogeneous population of parasites, we infected J774-E mouse macrophages with extracellularly differentiated amastigotes. The cells were incubated with either 10 or 100 μM chloroquine, 10 mM ammonium chloride, 20 mM methylamine, or 1 μM monensin during and after parasite invasion, as detailed in Materials and Methods. After fixation, cells were processed for transmission electron microscopy, and sections were examined randomly. Intracellular amastigotes were scored as "in" or "out" of a vacuole (parasites inside an apparently broken vacuole were scored as "in"). 10 μM chloroquine, 100 μM chloroquine, 10 mM ammonium chloride, 20 mM methylamine, and 1 μM monensin caused 55, 91, 52, 72, and 75% reduction, respectively, in the number of parasites found free in the cytosol (Fig. 5). In similar experiments using human monocytes as host cells, inhibition rates of 95 and 65% were obtained by treatment with 100 μM chloroquine and 10 mM ammonium chloride, respectively (not shown).

Effect of the pH-raising Drugs on the Parasites

a. Reversibility of Their Effect on Exit from the Phagosome. Infected J774-E cells were washed to remove the drugs and incubated in fresh medium for different time periods. The parasite multiplication rates (Fig. 6) were normal and undistinguishable from the control after removal of all drugs, including 100 μM chloroquine, which caused the strongest inhibition of phagosome escape (91%). Since *T. cruzi* only multiplies when free in the cytosol, this result implies that after drug removal the parasites were able to escape from the phagosome.

b. Viability of Drug Pretreated Parasites. To test for possible drug toxicity, amastigotes were incubated for 2 h at 37°C with medium containing the above concentrations of chloroquine, ammonium chloride, methylamine, or monensin, or with plain medium. After washing, the parasites were allowed to infect J774-E macrophages and the infection and multiplication rates were measured (Fig. 7). All curves had similar slopes, indicating that the drug-treated parasites that entered cells were as viable as the nontreated controls.

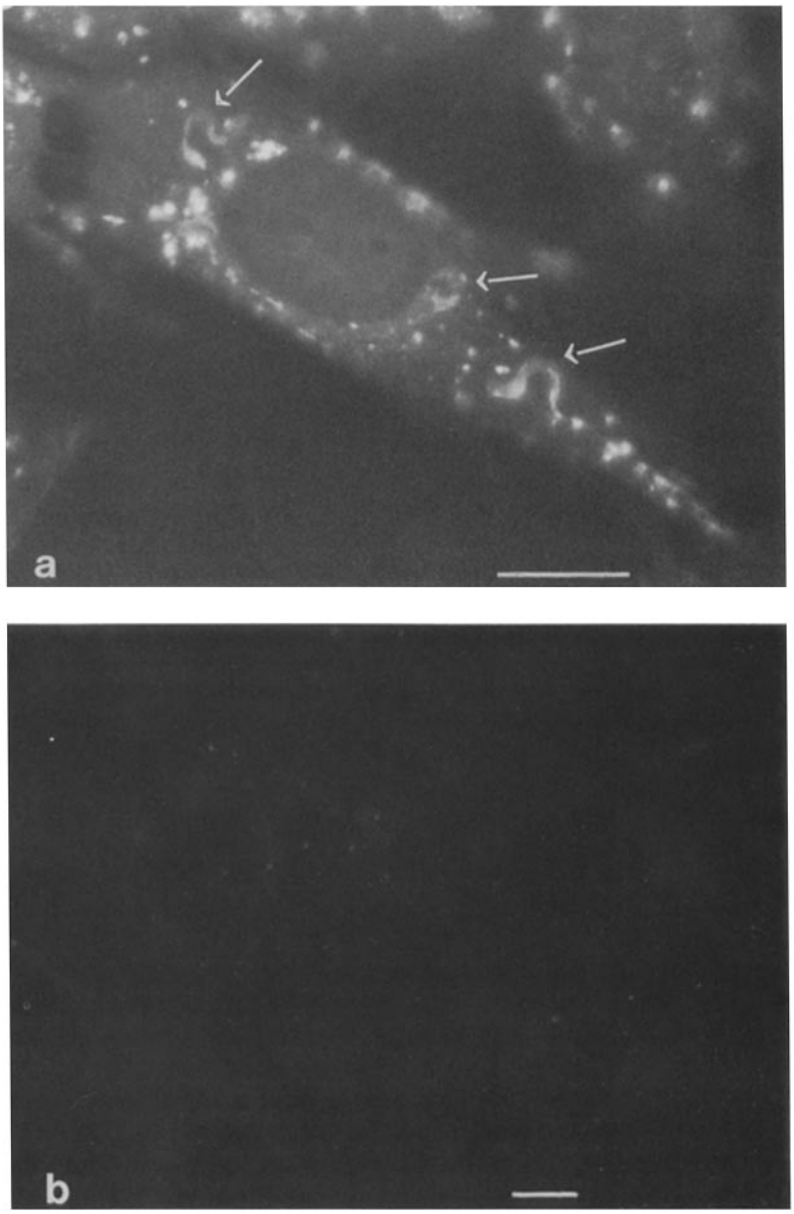

FIGURE 3. Immunofluorescence of DAMP accumulation in 3T3 fibroblasts recently infected with trypomastigotes. Cells were incubated with 30 μM DAMP for 30 min, fixed with formaldehyde, permeabilized with Triton X-100, and incubated with an anti-DNP mAb followed by anti-mouse IgG conjugated to fluorescein. Trypomastigote-containing vacuoles (*arrows*) can be visualized among other acidic compartments of the cell (*a*). Fluorescence was negative when the infected cells were incubated with 10 mM ammonium chloride for 15 minutes before the addition of DAMP (*b*). Bars, 10 μm.

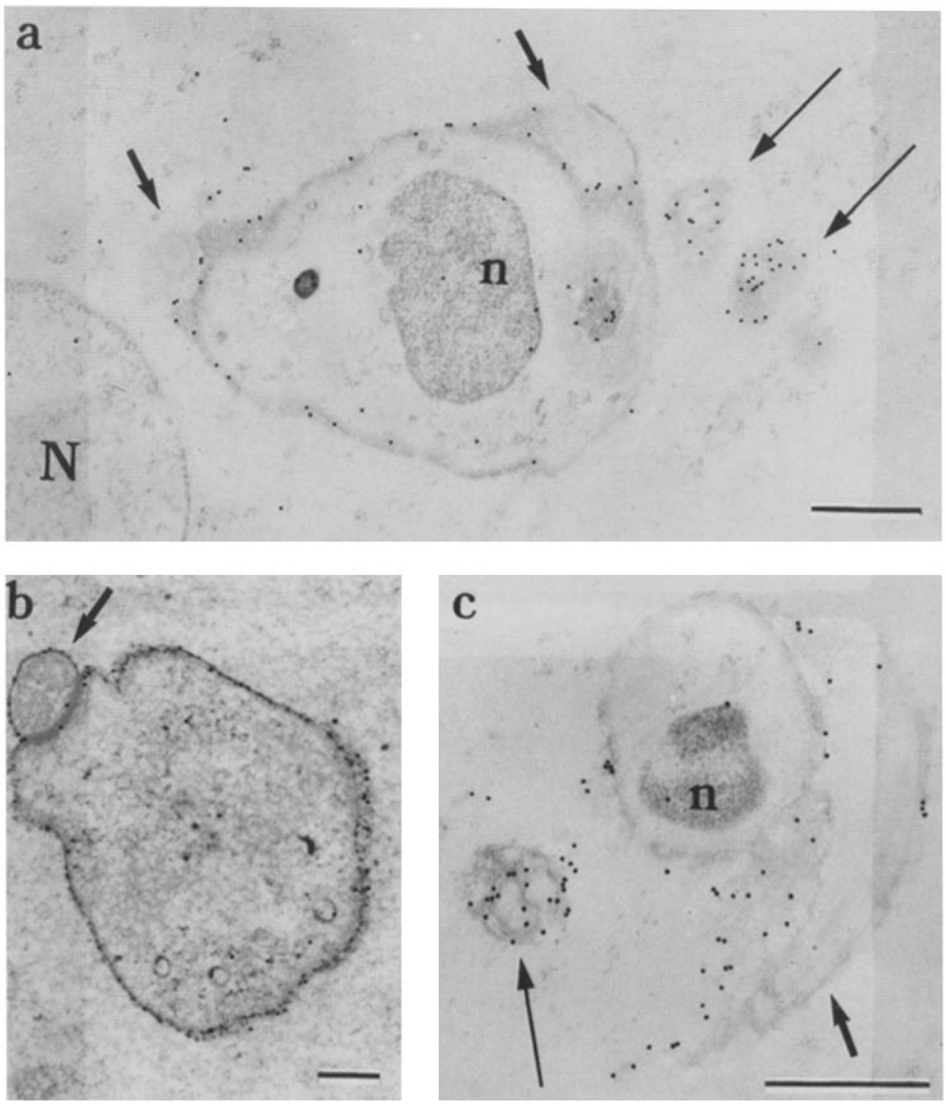

FIGURE 4. Immunogold labeling of DAMP in the tight vacuoles surrounding recently interiorized parasites. 3T3 fibroblasts infected with *T. cruzi* were incubated with 30 μM DAMP for 30 min before glutaraldehyde fixation. Sections were incubated with an anti-DNP mAb followed by 15 nm colloidal gold particles coated with protein A, and stained with uranyl acetate. Long arrows point to labeled host cell lysosomes/endosomes. Short arrows point to sections of parasites' flagella. *n*, Parasite nucleus; *N*, host cell nucleus. Bars, 0.5 μm.

c. Hemolysin Production. *Trypanosoma cruzi* secretes a hemolysin active only at low pH (15). To verify if agents that raise the pH of acidic organelles interfered with production of this molecule, we incubated amastigotes with 100 μM chloroquine, 10 mM ammonium chloride, 20 mM methylamine, or 1 μM monensin under conditions similar to that described in previous experiments. The parasites were prein-

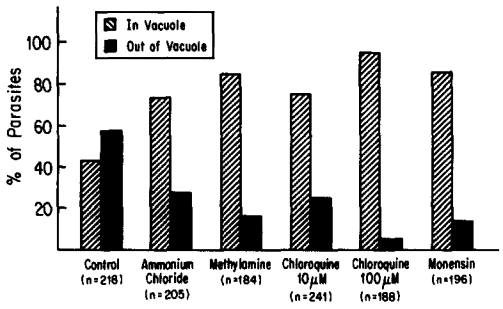

FIGURE 5. Effect of weak bases and monensin on the localization of intracellular parasites. J774-E mouse macrophages were incubated with amastigotes in medium with or without the drugs indicated. After a total period of 150 min, the cultures were washed, fixed, and processed for transmission electron microscopy. Intracellular amastigotes were scored as "in" or "out" of a vacuole. Values represent the percentage of parasites scored on randomly selected sections of each pellet. *n*, Total number of parasites examined.

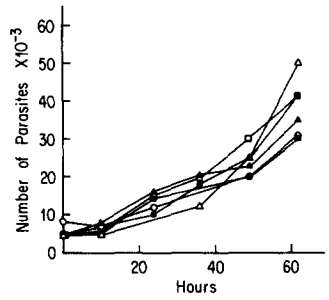

FIGURE 6. Kinetics of intracellular multiplication of amastigotes in J774-E mouse macrophages after removal of the pH-raising agents. After 2 h in the presence of the drugs the infected cultures were washed, incubated in fresh medium for different periods of time, and *T. cruzi* DNA determined by hybridization with a specific probe and used to calculate the total number of parasites at each point (8). (Δ) 10 μM chloroquine; (●) 100 μM choroquine; (□) 10 mM ammonium chloride; (▲) 20 mM methylamine; (■) 1 μM monensin; (○) control. The values represent the mean of triplicate determinations.

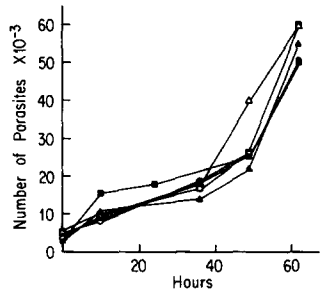

FIGURE 7. Kinetics of intracellular multiplication of amastigotes pretreated for 2 h with 100 μM chloroquine (●), 10 μM chloroquine (Δ), 10 mM ammonium chloride (□), 20 mM methylamine (▲) or 1 μM monesin (■), compared with amastigotes treated with normal medium (○). After washing, parasites were allowed to infect J774-E cells. The amount of *T. cruzi* DNA in each well was determined at different time points as described in the legend of Fig. 6.

cubated with the drugs at pH 7.0 for 2 h and then shifted to pH 5.5 in the continuous presence of the drugs. At this point guinea pig erythrocytes were added and the mixture was incubated for three additional hours at 37°C. As shown in Fig. 8, during this period comparable lysis of erythrocytes was caused by parasites in the presence or absence of the pH-raising agents. The values shown for hemoglobin release in the control samples correspond to lysis of ~40% of the erythrocytes. ATP depletion by addition of 5 mM sodium azide and 50 mM deoxyglucose inhibited production of the hemolysin by the parasites, as previously described (15).

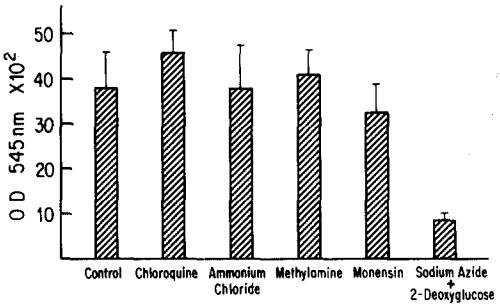

FIGURE 8. Hemolysin production by amastigotes in vitro. The parasites were incubated for 2 h in DMEM 10% FCS (control) or with DMEM 10% FCS containing the following drugs: 100 μM chloroquine, 10 mM ammonium chloride, 20 mM methylamine, 1 μM monensin, or 5 mM sodium azide + 50 mM 2-deoxyglucose. The amastigotes were centrifuged and reincubated in acetate buffer pH 5.5, in presence of the drugs at the same concentrations above and of guinea pig erythrocytes. After 3 h the hemoglobin released into the supernatant was determined by reading the absorbance at 545 nm. The values correspond to the mean of triplicate determinations, and the bars to the standard deviations.

Discussion

The main findings described here are that *T. cruzi* escapes from acidic phagosomes in a short period after cell invasion, and that its ability to gain access to cytosol of the host cell is blocked by agents that raise the pH of acidic compartments.

The results with DAMP labeling demonstrate that *T. cruzi*-containing phagosomes are acidic compartments (Figs. 3 and 4). Previous studies had shown lysosomal fusion with *T. cruzi*-containing phagosomes (8–10), but the possibility remained that the parasites might block the acidification process, as in the case of *Toxoplasma gondii* (20) and *Legionella pneumophila* (21). We found that the membranes of the phagosome and of the parasite are tightly apposed (Fig. 1, *a*, *b*, and Fig. 4). Comparisons between the intensity of label inside the parasite-containing phagosomes and in compartments for which the luminal pH is known (such as lysosomes and endosomes) were therefore not possible, due to the small volume available for DAMP accumulation.

Recently interiorized parasites were frequently observed in the perinuclear region (Figs. 3 and 4). In spite of their large size, *T. cruzi*-containing phagosomes seem to follow the same pathway as secondary lysosomes, which are known to accumulate around the cell center, probably driven by a microtubule-based motor of the dynein type (22, 23). It remains to be determined if the luminal pH of these phagosomes decreases as they approach the perinuclear region, similarly to what has been shown to occur along the endocytic pathway (24, 25).

Additional findings relate to the stage of the parasite which is capable of escaping from the phagosome, and to the time course of escape. Earlier ultrastructural studies had shown that soon after invasion *T. cruzi* trypomastigotes were surrounded by membranes, and that 24 h later amastigotes were free in the cytosol (9, 10, 12–14). In a previous report (8) we showed that amastigotes, after entering human monocytes, escaped from the phagosome and multiplied in the cytosol. This observation raised the question of whether trypomastigotes first had to transform into amastigotes before escaping. In agreement with a recent report (11), our present observations demonstrate that trypomastigotes can leave the phagosome before complete transformation into amastigotes, and that this occurs within the first 2 h after invasion. The fate of the phagosome membrane during the escape process is unknown; apparent discontinuities were frequently observed by transmission electron microscopy (Fig.

1 *c*). Perhaps the fragments reseal into small vesicles, which can in fact be observed in the vicinity of parasites free in the cytoplasm (Fig. 2 *c*).

A significantly smaller number of parasites escaped into the cytosol when infected cells were treated with the weak bases chloroquine, ammonium chloride, or methylamine, and also with the carboxylic ionophore monensin (Fig. 5). At concentrations similar to those that we have used, these agents raise the endocytic pH of 3T3 mouse fibroblasts and macrophages from ~5 to 6.2 (26, 27). The effect of all drugs was reversible, as evidenced by normal intracellular development of the parasites after shifting the infected cells into normal medium (Fig. 6). Furthermore, pretreatment with the drugs did not affect infectivity (Fig. 7), nor did it interfere with secretion of the acid-active hemolysin of *T. cruzi* (Fig. 8), indicating that the drugs were not toxic for the parasites. The simplest interpretation for these findings is that an acidic environment in the phagosome is necessary to activate the escape mechanism. However, the parasites probably also contain vacuolar compartments with a low luminal pH. Therefore, the observed phenomena might also be explained by rapidly reversible effects of the drugs on the endocytic/exocytic compartments of *T. cruzi*, provided that weak bases and monensin function identically in mammalian cells and in this protozoan parasite.

One of us reported recently that *T. cruzi* secretes a hemolysin that is optimally active at pH 5.5 (15). Epimastigotes, the insect stages that are destroyed inside phagosomes (13) induce significantly less acid pH-dependent lysis to target cells in vitro when compared with trypomastigotes and amastigotes (15). Taken together, the results presented here are compatible with the hypothesis that the acidic environment of the phagosome is required for activity of the *T. cruzi* lysin and its disruption of the vacuolar membrane. Work in progress will hopefully elucidate the mechanism of action of this lytic molecule and allow definitive conclusions about its involvement on phagosome escape.

Summary

The protozoan parasite *Trypanosoma cruzi* can infect many distinct mammalian cell types. The parasites enter cells through the formation of phagocytic vacuoles, but later are found free in the cytosol, where they multiply as amastigotes. Using transmission electron microscopy we found that within 2 h after infection 70% of the parasites, including examples of both mammalian forms (trypomastigotes and amastigotes), were inside partially disrupted vacuoles or free in the cytosol. We demonstrated that the pH of vacuoles containing recently interiorized parasites is acidic, through immunocytochemical localization of the acidotropic compound DAMP (18) in their interior. Increasing the vacuolar pH with chloroquine, ammonium chloride, methylamine, or monensin significantly inhibited the escape of the parasites into the cytosol. These results are compatible with the hypothesis that an acid-active hemolysin of *T. cruzi* (15) might be involved in the escape mechanism.

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