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Autor(en): **Almeida Maria, Thaisa de / Alcantara, A. / Brener, Z.**

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Departments of Morphology and Parasitology, I.C.B., University of Minas Gerais, and Centro de Pesquisas René Rachou, Belo Horizonte, Brazil

Ultrastructural studies on the *in vitro* interaction of *Trypanosoma cruzi* bloodstream forms and mouse peritoneal macrophages¹

THAISA DE ALMEIDA MARIA, A. ALCANTARA, Z. BRENER

Summary

Macrophages are normally host cells for *T. cruzi* in the vertebrate host. Since the process of interiorization of this parasite into those cells is still controversial, we decided to investigate by electron microscopy the *in vitro* uptake of *T. cruzi* bloodstream forms by mouse peritoneal macrophages. After 15 min of interaction with macrophages, parasites are interiorized by a process of phagocytosis in which both parasite and host cell seem to play a specific role. Sequential steps of the uptake process and the simultaneous ultrastructural alterations of the host cell morphology have been studied. A dense amorphous substance interposed between the parasite and the macrophage surface was regularly observed. Although its origin, significance and nature remain unknown, we discuss the possibility that it might be released extracellularly by the macrophage in response to the parasite stimulation.

Key words: *Trypanosoma cruzi*; phagocytosis; peritoneal macrophages; electron microscopy.

Introduction

T. cruzi culture and bloodstream forms penetrate and develop in several types of mammalian cells, *in vitro* and *in vivo*. The mechanism by which these forms gain access to the intracellular medium, however, remains unclear and divergent conclusions have been presented by authors using different ex-

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perimental conditions (Dvorak and Hyde, 1973; Kongtong and Inoki, 1975; Tanowitz et al., 1975; Kress et al., 1975; Nogueira and Cohn, 1976; Kipnis et al., 1979). Ultrastructural evidence showing a second membrane surrounding the parasite in the host cell cytoplasm (Milder et al., 1973; Sanderson and Souza, 1979) suggests the process of phagocytosis as the mechanism responsible for uptake of the parasite. Active penetration, however, of bloodstream parasites has been also suggested (Kipnis et al., 1979).

This paper describes the early events of the *in vitro* interaction between *T. cruzi* bloodstream forms and mouse peritoneal macrophages in an attempt to characterize the mechanism by which these forms are interiorized into the macrophage.

Material and Methods

Parasites: Bloodstream trypomastigotes were collected from mice infected with *T. cruzi* Y strain (Silva and Nussenzweig, 1953) by bleeding the animal through the orbital sinus on the 7th day of infection. The blood was defibrinated with glass beads, centrifuged at 100 g for 8 min at 4° C and the supernatant centrifuged at 1000 g for 15 min at 4° C. The parasites were then suspended in tissue culture medium 199 (M 199) plus 20% fetal calf serum (FCS) and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin).

Macrophages: Peritoneal macrophages were obtained from DBA and outbred albino mice as described by Cohn and Benson (1965), suspended in medium (M 199–2% FCS, 10 units/ml of heparin and antibiotics) and centrifuged at 100 g for 5 min. The pellet was resuspended in the same medium without heparin to a concentration of 4×10^6 cells/ml. In order to obtain an adherent layer of macrophages, about 0.10 ml of this cell suspension was dropped on 8×16 mm glass cover-slips which were placed in glass or plastic Falcon Petri dishes and incubated at 37° C for 15 min in a 5% CO₂ atmosphere. To remove non-adherent cells the cover-slips were repeatedly washed with Hanks' solution and again incubated with 199 medium–20% FCS in the same conditions.

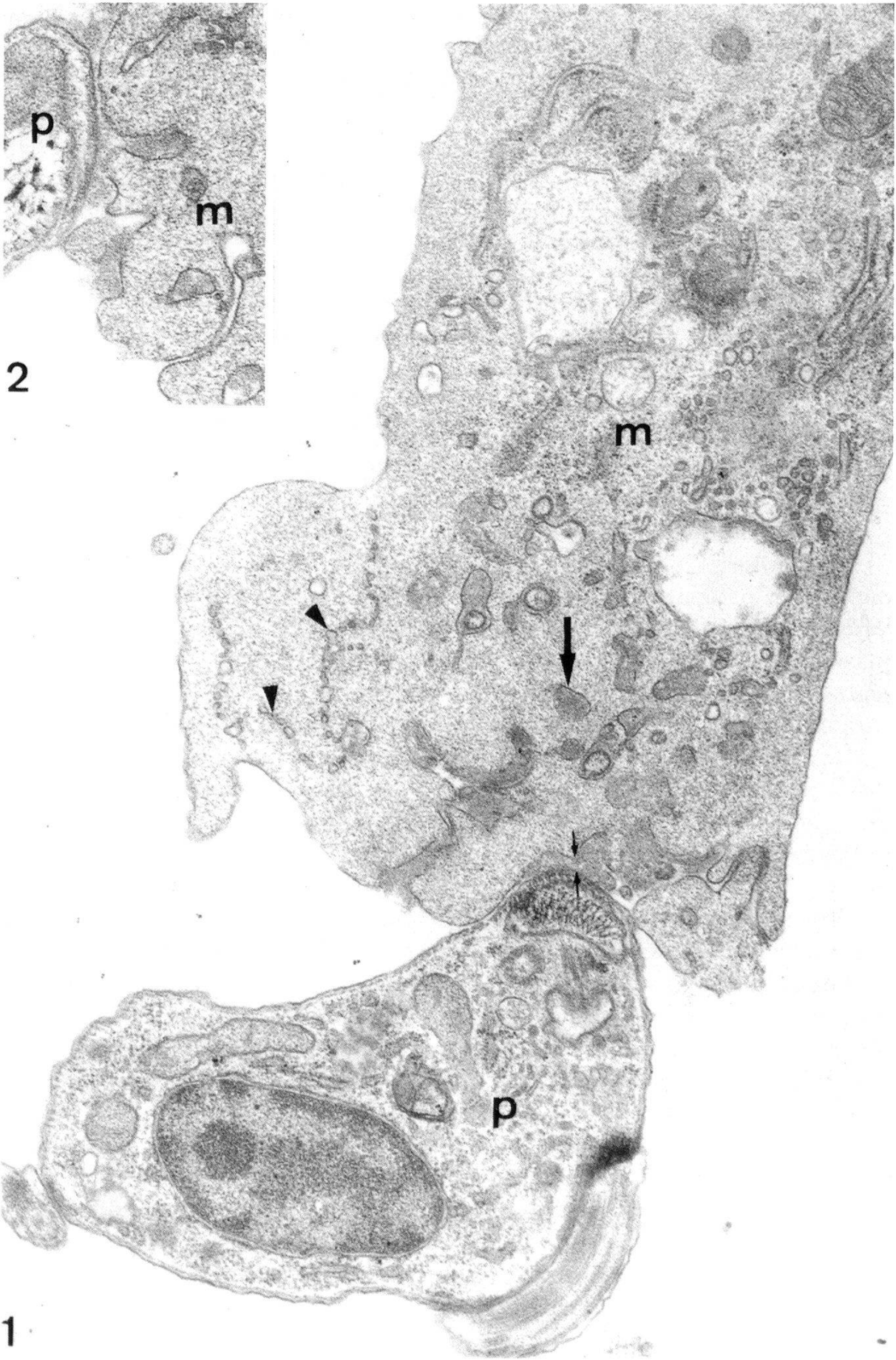
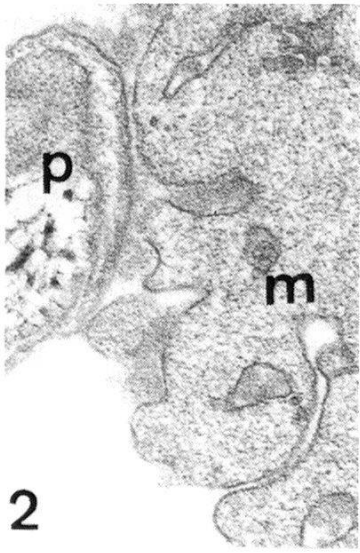
Macrophages infection and electron microscopic techniques: Macrophages on cover-slips, cultivated for 18–24 h, were incubated with the parasites for a period of 15 and 30 min at 37° C in 5% CO₂ atmosphere. The ratio of bloodstream parasites to macrophages used was about 20. The preparation was then rinsed in saline and prepared for electron microscopy (Hirsch and Fedorko, 1968). Ultra-thin sections were double stained with uranyl acetate and Reynolds' lead citrate and photographed in a Zeiss EM 10.

Results

After 15 min, *T. cruzi* bloodstream trypomastigotes were observed mostly outside macrophages, either close or adherent to the host cell surface. Preparations fixed after 30 min showed a high percentage of the parasites inside phago-

Fig. 1. *Attachment phase.* Macrophage (m) and parasite (p) are seen in direct contact. Morphological alterations are observed in the macrophage cytoplasm characterized by the appearance of dense irregular bodies (thin arrow), deep invaginations of the surface membrane, dense extracellular substance between parasite and macrophage (thick arrow) and row of vesicles (arrow head). $\times 20,000$.

Fig. 2. *Attachment phase.* Presence of dense extracellular substance inside invaginations at the surface of the macrophage and also interposed between this cell and the parasite (p). $\times 28,000$.



cytic vacuoles in the macrophage cytoplasm. Parasites and macrophages established contact on all macrophage surface areas and the flagellates could be found attached to the phagocytic cell by different parts of their body of flagellum.

The endocytosis process observed was divided into three sequential steps:

1. *Attachment phase*: ultrastructural modifications in the macrophage cytoplasm are observed near the area of parasite adhesion. These involved the appearance of membrane bound round or irregularly shaped dense bodies containing fine granular material (Figs. 1, 3). We also observed channels or invaginations lined by the plasma membrane around the area of parasite contact. The channels penetrated deeply into the cytoplasm and in many cases connected the dense granular bodies described above to the extracellular space (Fig. 1). At the end of this phase the macrophage surface displayed a fringed aspect because of the invaginations (Figs. 1, 2, 3). A dense granular material occurred between the parasite surface and the macrophage plasma membrane (Figs. 1, 2, 3, 4); in some cases a direct connection could be seen between this dense extracellular material and the material present inside the channels (Fig. 2). This material has a limited distribution, but it is always related to the presence of the parasite. Its thickness varies from 50 nm to 150 nm. Rows of small vesicles, with a diameter from 40 nm to 80 nm, appear in the macrophage cytoplasmic area near or in contact with the parasite (Figs. 1, 5, 7).
2. *Engulfment phase*: at this point, one or more pseudopodia start to form at the macrophage surface (Figs. 3, 6), eventually surrounding the parasite along with the dense extracellular substance so that both were ultimately enclosed inside the phagocytic vacuole (Fig. 7). The row of vesicles is still present and seems to be an essential part of the processes of membrane fragmentation and fusion leading to the formation of projections and consequent cytoplasmic movement during parasite engulfment. Another interesting aspect observed is the presence of small rounded portions structures that are morphologically identical to the parasite cytoplasm within the macrophage cytoplasm. These were surrounded by a dense granular substance. Different regions of the parasite could be simultaneously surrounded by several macrophage pseudopodia (Figs. 1, 3). Up to this point all events described above were observed mainly within the first 15 min of host-parasite interaction.
3. *Complete interiorization phase*: the next and final step was characterized by total engulfment of the parasite which was now inside the phagocytic vacuole (Figs. 8, 9). This phase was predominantly observed in the material fixed after 30 min. The space between the parasite surface membrane and the phagocytic vacuole membrane was generally filled with a fine amorphous granular substance, vesicles, irregular debris and the cytoplasmic portions limited by a unit membrane and surface coat (Fig. 8). In some cases the vacuole appeared enlarged and clear, with the parasite occupying only a small portion of the organelle.

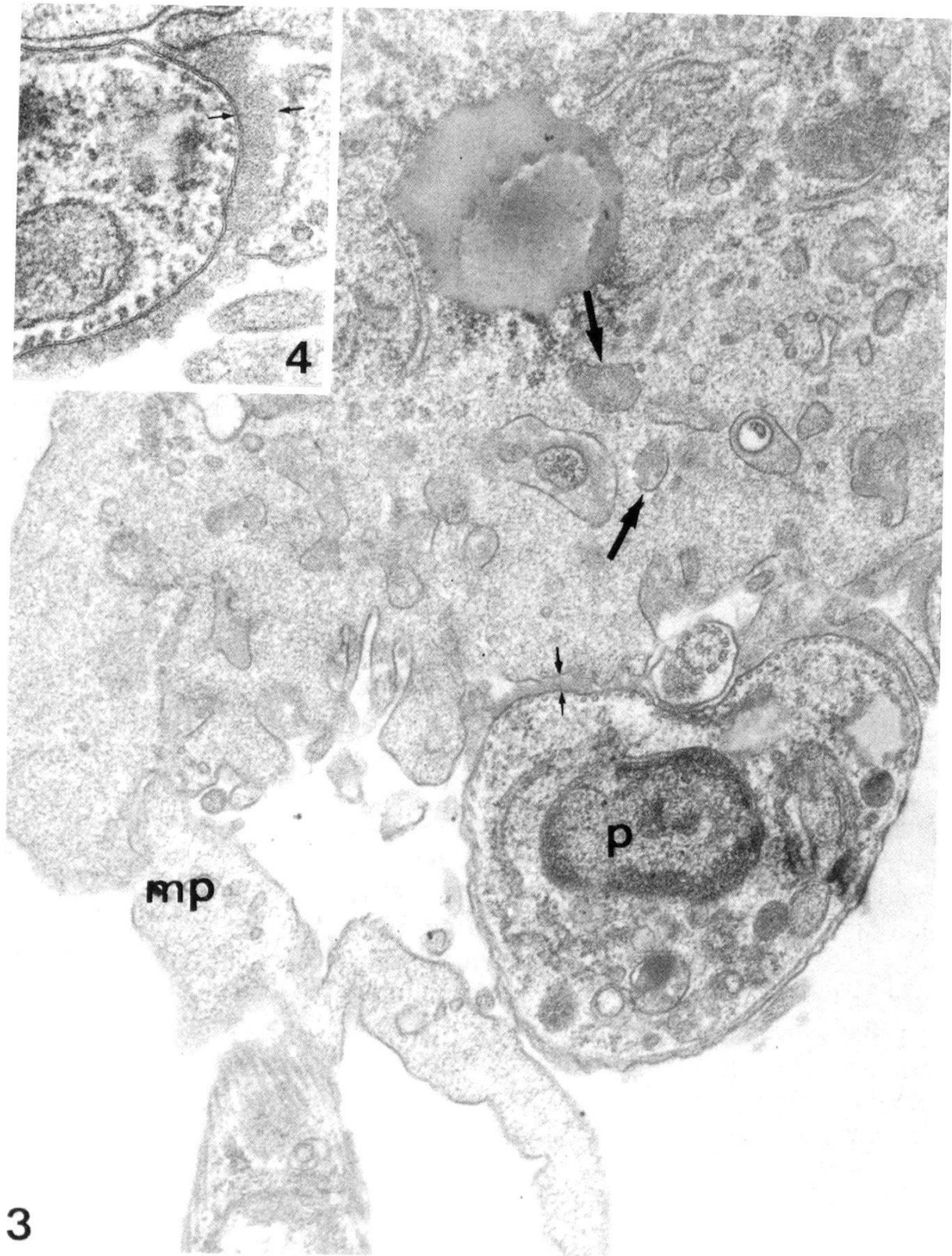


Fig. 3. *Engulfment phase*. Pseudopodium (mp) starts to form at the macrophage surface surrounding the parasite (p). Dense bodies (thin arrows), invaginations of the macrophage surface and dense extracellular substance (thick arrow) are still present near the area of interaction, $\times 28,000$.

Fig. 4. Detail of the dense extracellular substance between the macrophage and parasite surfaces (thick arrow), $\times 52,000$.

Discussion

The uptake of *T. cruzi* bloodstream trypomastigotes by mouse peritoneal macrophages through the classical process of endocytosis demonstrated here confirms most of the descriptions in the literature. This mechanism is considered the most common way of interiorization of several obligate intracellular protozoa such as malaria merozoites (Ladda et al., 1969); *Toxoplasma gondii* Jones et al., 1972); *Leishmania mexicana* (Alexander, 1975) and *Trypanosoma dionisii* (Baker and Liston, 1978).

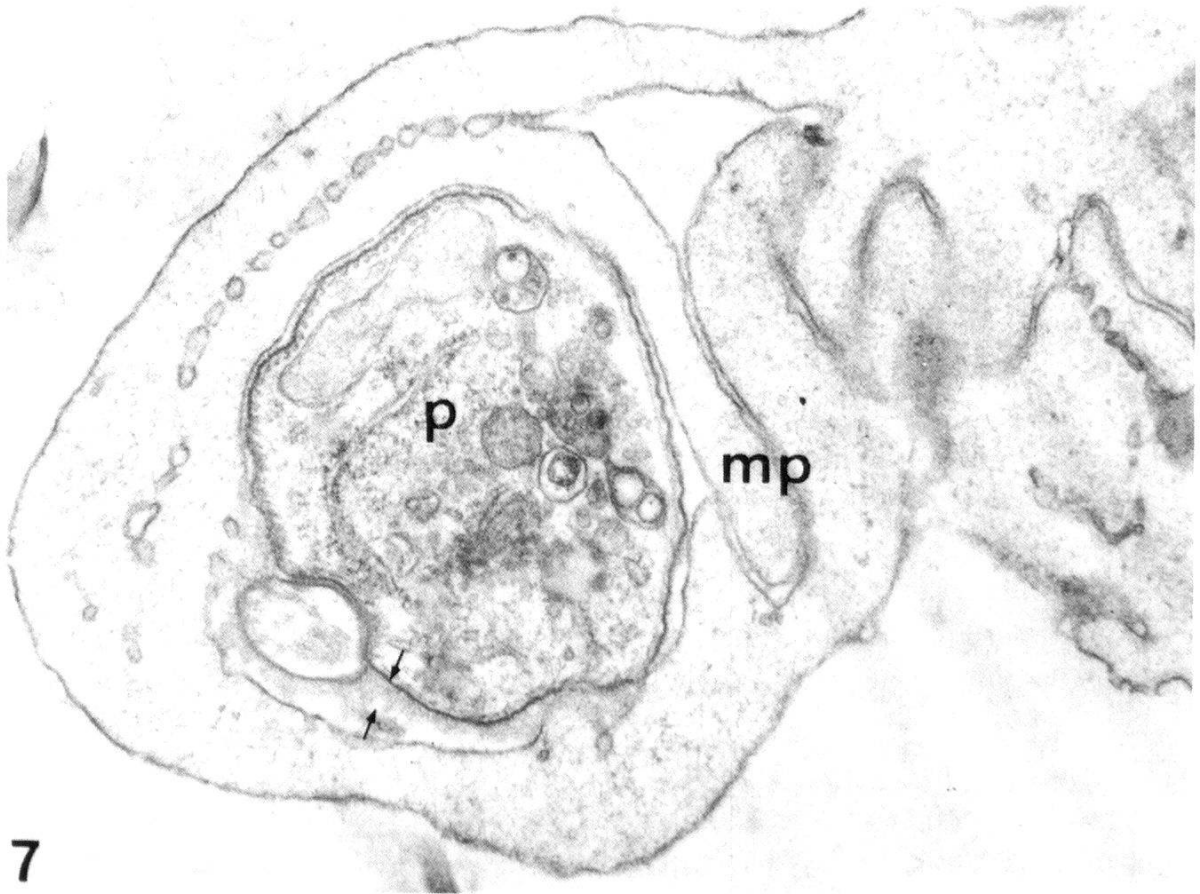
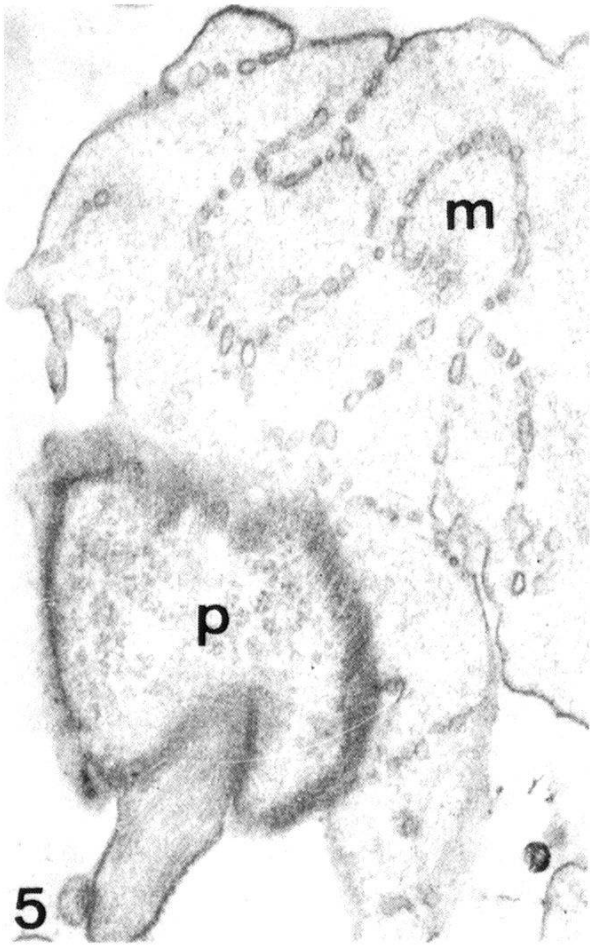
With respect to *T. cruzi*, observations on parasite entry are controversial. These different interpretations could be due to the inadequacy of the optic instruments utilized or to the presence of parasites inside cells that are not professional phagocytes (e.g. smooth or cardiac muscle cells and fibroblasts). The latter would lead authors to conclude that penetration of *T. cruzi* was an active phenomenon, not requiring host cell participation. Thus, some early workers observing the behaviour of *T. cruzi* in tissue culture by phase microscopy described the penetration of parasites after active movement and collision against the host cell (Kofoid et al., 1935; Meyer, 1942). Dvorak and Hyde (1973) using cinematography also reported active penetration of the host cell by the parasite.

Tanowitz et al. (1975) studying the invasion of macrophages and L-cells by culture forms of *T. cruzi* concluded that the entry of host cells by epimastigotes and trypomastigotes was not exclusively the result of parasite action but rather depended upon the host cell type used. According to these authors, macrophages would have a primary role in phagocytosing the parasite whereas L-cells play a less active role in the entry process, the parasite apparently actively penetrating the plasma membrane. Kress et al. (1975) confirmed the interiorization of *T. cruzi* culture forms in macrophages by phagocytosis, based on the presence of the parasite inside phagocytic vacuoles. Sooksri and Inoki (1972) reported that *T. cruzi* culture forms penetration into Hela cells was aided by flagellar movement and that, at the point of penetration, the Hela cell membrane breaks and disappears. Kongtong and Inoki (1975) described the penetration of trypomastigote and epimastigote culture forms into fibroblasts by scanning electron microscopy and confirmed the observations of Sooksri and Inoki (1972) in Hela cells. These authors admit, however, that epimastigotes probably enter by phagocytosis because of the occasional observation that some

Fig. 5. *Engulfment phase*. Detail of the drawing outlined by the row of vesicles on the peripheral region of the macrophage cytoplasm (m) close to the parasite (p), $\times 24,000$.

Fig. 6. *Engulfment phase*. Uptake of the parasite by macrophage pseudopodia, $\times 24,000$.

Fig. 7. *Engulfment phase*. Parasite (p) and dense extracellular substance (double arrow) surrounded by macrophage pseudopodia (mp), $\times 28,000$.



epimastigotes were trapped by elongated extensions and filaments of fibroblasts.

Other electron microscopic studies did not confirm the mechanism of active entry by *T. cruzi* into host cells. Nogueira and Cohn (1976) described the entry of acellular culture derived trypomastigotes by phagocytosis into macrophages, fibroblasts, Hela and L cells. Epimastigotes, however, although phagocytosed by macrophages, were not interiorized by L cells, Hela cells, 3T3 and bovine embryo fibroblasts. Trypomastigotes, therefore, showed a different entry behaviour from that by epimastigotes since they were able to enter macrophages as well as non-professional phagocytic cells by phagocytosis. Sanderson and Souza (1979) also described the interaction between *T. cruzi* tissue culture derived trypomastigotes and rat eosinophils, neutrophils or macrophages. Based upon the presence of the parasite inside phagosomes the authors confirmed the occurrence of phagocytosis as the mechanism of entry in these three cells.

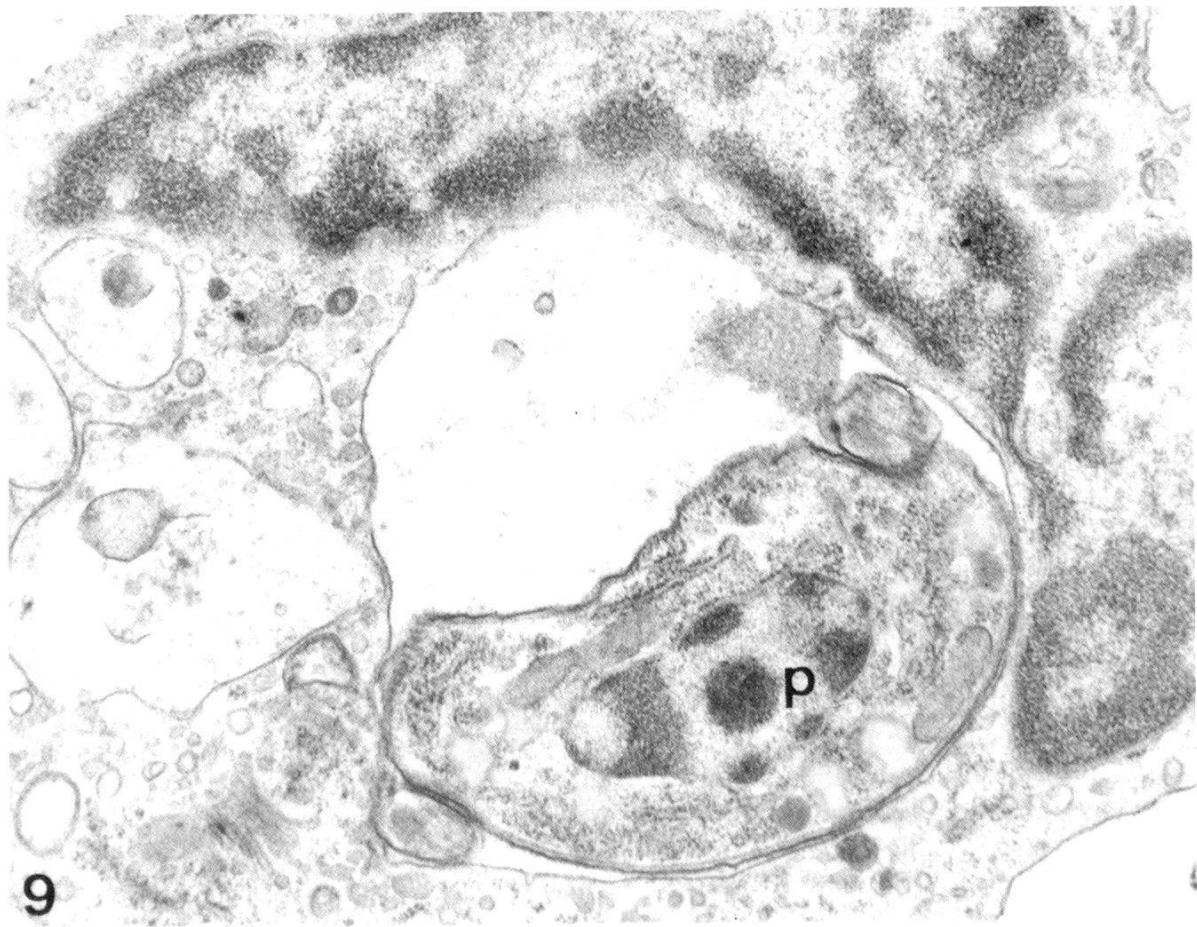
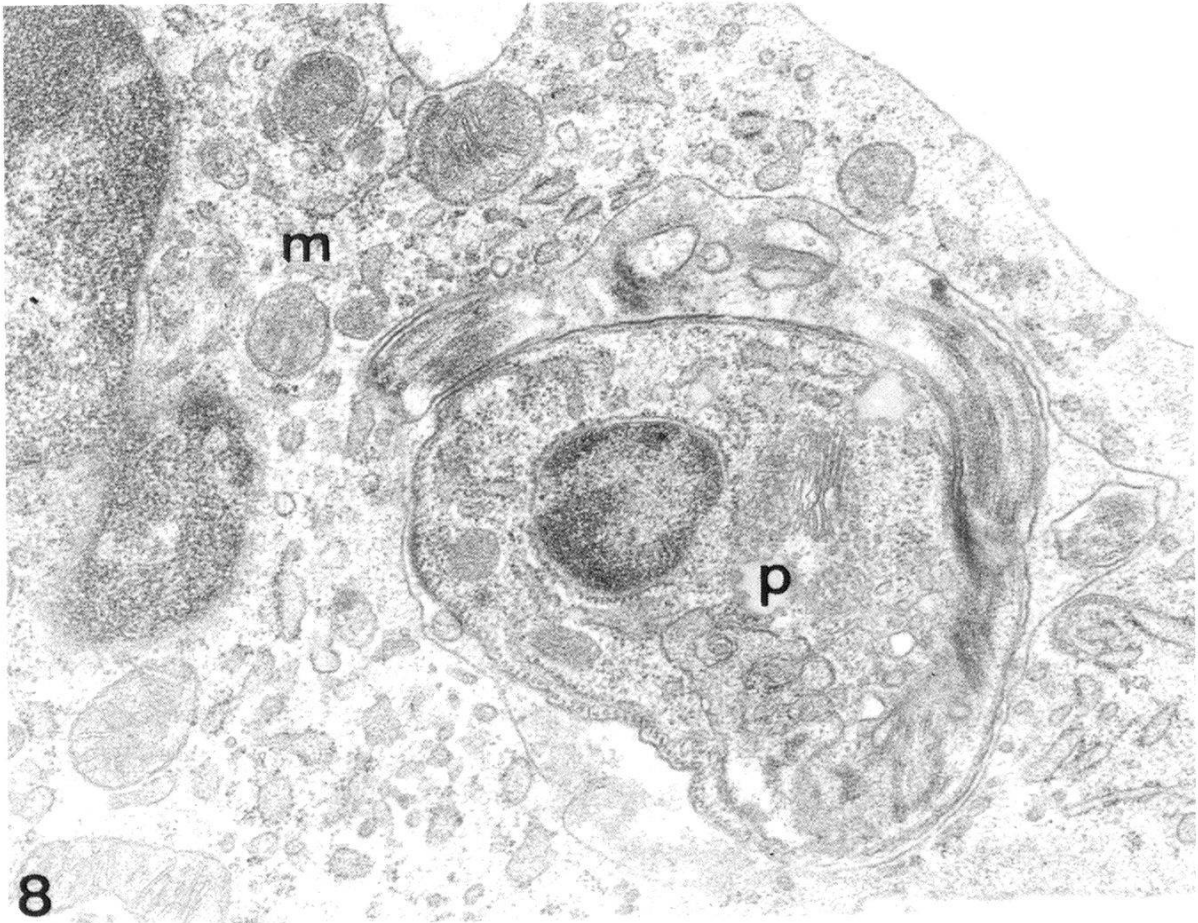
A few papers have been published dealing specifically with the mechanism of bloodstream trypomastigote penetration into macrophages. Milder et al. (1973) observing the interaction of peritoneal macrophages with *T. cruzi* bloodstream forms described the presence of a conspicuous membrane surrounding the whole parasite. This second membrane was considered an indication that the parasites were phagocytosed and that they did not penetrate actively. Their conclusion was contradicted by Kipnis et al. (1979) who infected macrophages with bloodstream forms in the presence of cytochalasin B (known to inhibit phagocytosis). The latter workers concluded that *T. cruzi* bloodstream forms penetrate actively.

Our in vitro results suggest that *T. cruzi* bloodstream forms normally enter macrophages via the mechanism of phagocytosis. After 30 min of contact, parasites were interiorized by the classical process of endocytosis. Parasite phagocytosis was characterized by attachment, ingestion and eventual lodgement inside a phagocytic vacuole.

The constant presence of a dense amorphous substance between the parasite and macrophage surfaces during the initial stages of the interaction is an interesting finding. Although its origin and significance are not clear, this material seems to originate from the host cell. No morphologic evidence was found suggesting that it might be secreted by the parasite. The possibility that this substance was an artifact is not likely because it was observed only in regions of parasite macrophage contact. Moreover, the presence of this amorphous substance was morphologically similar and sometimes contiguous with

Fig. 8. *Complete interiorization phase.* Parasite (p) inside phagocytic vacuole in the macrophage cytoplasm (m). Note phagosome filled with dense material, $\times 18,000$.

Fig. 9. *Complete interiorization phase.* Parasite (p) inside phagocytic vacuole. Phagosome enlarged and with scarce dense material, $\times 20,800$.



material observed inside vacuoles and invaginations areas of interaction with the parasite. This morphological evidence supports the hypothesis that the substance might be released by the macrophage to the extracellular medium after stimulation by parasite contact. In fact, macrophages may release products of low molecular weight such as prostaglandins and cyclic nucleotides or much higher molecular weight substances such as components of the complement system and a variety of hydrolytic enzymes (Davies and Bonney, 1979). The capacity of macrophages cultivated *in vitro* to release biologically important substances in response to environmental factors has also been established (Unanue, 1976).

A classical mechanism of secretion where secretory granules fuse with the plasma membrane and discharge their contents extracellularly by exocytosis was not observed in our material, although in some cases evidence of fusion of vacuoles containing amorphous substances with deep invaginations or channels at the periphery of the macrophage cytoplasm was seen.

Another constant feature observed during the uptake of bloodstream forms by macrophages was the appearance of rows of small vesicles in the macrophage cytoplasm. The question remains whether this occurrence is a general characteristic of macrophage phagocytosis or is characteristic of this specific interaction.

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