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THE INFLUENCE OF CYTOCHALASIN B ON THE INTERACTION OF T. CRUZI AND MOUSE PERITONEAL MACROPHAGES (*)

F. EBERT (1) and Helene Santos BARBOSA (2)

SUMMARY

Stimulated mouse peritoneal macrophages were cultured in M 199 containing 20% fetal bovine serum (FBS). Macrophage cultures were pre-incubated with Cytochalasin B in final concentrations of 1, 5 and 10 μg/ml (dissolved in DMSO 1 mg/ml) for two hours before the addition of **T. cruzi.** Strains of **T. cruzi** with significant differences in the proportion of trypomastigotes were added in the ratio 1:2 (peritoneal cell: parasite). The macrophage monolayers were fixed and stained at various time intervals. Both strains were found to attach to macrophages independent of the presence or absence of Cytochalasin B. The uptake of **T. cruzi**, however, was almost completely inhibited in macrophages treated with Cytochalasin B at concentrations of 5 μg/ml and 10 μg/ml. Cultures exposed to 1 μg Cytochalasin B/ml did not block the ingestion but the rate of infection of macrophages was significantly reduced. In comparison macrophages incubated in a M 199 + 20% FBS control culture, with DMSO, did not change the uptake of **T. cruzi.** No significant differences between the strains were found. Electron microscopic examinations of untreated macrophages showed the parasites either inside phagocytic vacuoles or attached to the membrane and surrounded by finger-like pseudopodia. The results suggest that epimastigotes and trypomastigotes from a culture of **T. cruzi** enter macrophages by a process of phagocytosis.

INTRODUCTION

Trypanosoma cruzi the agent of Chagas' disease is a parasite infecting a variety of mammalian cell types including those of the mononuclear phagocytic system. The interaction of **T. cruzi** (blood stream and culture forms) with tissue cultures and mouse peritoneal macrophages has been studied in several investigations (ALCANTARA & BRENER [3]; DVORAK & SCHMUNIS [6]; SOOKSRI & INOKI [15]; TANOWITZ et al. [16]). It seems that **T. cruzi** blood stream forms penetrate actively into both non-phagocytic and phagocytic cells. On the other hand there are conflicting reports on the mechanism by which culture forms of **T. cruzi** gain access to macrophages (ALEXANDER [4]; KIPNIS et al. [10]; NOGUEIRA & COHN [12]) treated with Cytochalasin B, a drug which inhibits phagocytosis by macrophages (ALLISON et al. [2]).

This paper reports the effect of Cytochalasin B on the uptake, by mouse peritoneal macrophages, of culture forms of **T. cruzi** strains, having different proportions of trypomastigotes.

MATERIALS AND METHODS

Parasites — The following **T. cruzi** strains were used. The Rato strain, isolated from a

(*) This study was supported by BMZ, Research Program — Fiocruz, Rio de Janeiro, BNI, Hamburg
(1) Dr. rer. nat. — Centro de Microscopia Eletrônica, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
Present address: Tropical Institute Hamburg, Dept. of Protozoology, Germany
(2) Pesquisadora Auxiliar — Centro de Microscopia Eletrônica, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

wild rat in Ribeirão Preto, SP., Brazil (RIBEIRO & BARRETTO [14]), was supplied to us by Dr. Barretto in 1978. The OPS-22 strain, isolated by mouse inoculation of feces from positive **Panstrongylus geniculatus** in Cojedes, Venezuela, 1977, was kindly provided by Prof. Mühlpfordt, Tropical Institute, Hamburg. Both strains were maintained in NNN-medium at 27°C at two weekly intervals and collected in the stationary phase. The Rato and OPS-22 strain contained 78% and 41% epimastigotes, respectively.

Macrophage culture — Albino mice were inoculated intraperitoneally with 2 ml of 3.7% Brain-Heartinfusion (Difco). Three days later macrophages were collected by peritoneal lavage with 2 ml of saline. The cells were centrifuged for 5 minutes at 1000g and suspended in medium 199 with 30 mM Hepes containing 20% fetal bovine serum (FBS), 200 μg/ml Streptomycin and 200 U/ml Penicillin. The concentration was adjusted to $2x10^6$ cells/1,5 ml. The cells were then distributed into 9,5 x 35 mm Leighton tubes with coverslips, incubated for four hours at 37°C and washed twice with M 199 to remove the non-adherent cells. The adherent cells were incubated overnight until used in experiments.

Cytochalasin B (Serva, Germany) was dissolved in Dimethyl sulphoxide (DMSO) 1 mg/ml and diluted in M 199 + 20% FBS to final concentrations of 1,5 and 10 μg/ml.

The macrophage cultures were pre-treated with 1,5 and 10 μg Cytochalasin B for two hours. **T. cruzi** was then added at a ratio of **T. cruzi** to peritoneal cells of 2:1. Control cultures contained M 199 + 20% FBS and M 199 + 20% FBS and DMSO (1,5 and 10 μl/ml).

Preparation of cells for microscopy

a) **Light microscopy** — The coverslips were fixed in 2% OsO_4 in 0,1 M Cacodylate buffer, pH 7,2, for a few seconds and then stained in Giemsa solution; b) **Electron microscopy** — Macrophages were cultured in 100 ml flasks. Half an hour after addition of **T. cruzi** the cells were fixed by the method of HIRSCH & FEDORKO [9]. The process was done in situ. Then, the cells were gently scraped off with a rubber policeman. The procedure of embedding has been described previously (EBERT et al.[7]). Micrographs were taken with a Zeiss EM 10B.

Statistical Analysis

The median values of intracellular parasites were calculated by counting of 400 macrophages/time interval in each of the four experiments. The U-test of Wilcoxon, Mann and Whitney (SACHS [13]) was used for testing the significance between the groups. A p-level of 0,005 was chosen to indicate the statistical significance.

RESULTS

Culture forms of **T. cruzi** of both strains were found to attach to the membrane of the macrophages a few minutes after the infection independent of the presence or absence of Cytochalasin B (Figs. 1 and 2). There was no prefered position of contact of **T. cruzi** to the phagocytic cells, either the flagellum, the body or the posterior end of the parasite were seen in contact with the macrophages.

The engulfment of **T. cruzi**, however, was almost completely inhibited by the macrophage cultures treated with Cytochalasin B in the concentrations of 5 μg/ml and 10 μg/ml (Table I and II). Cultures exposed to 1 μg/ml of Cytochalasin B did no block the ingestion of **T. cruzi** to the extent seen in the higher concentrations but the rate of infection of macrophages was significantly reduced.

In comparison with macrophages incubated in M 199 + 20% FBS control cultures with DMSO did not change significantly the uptake of **T. cruzi.**

In all experiments it was found that the macrophage cultures treated with the higher concentrations of Cytochalasin B (5 μg/ml and 10 μg/ml) had a very low number of intracellular parasites, which were observed exclusively in macrophages localized on the periphery of the stained coverslips. Furthermore, in these cultures the macrophages retracted and became circular (Fig. 1). The effect of Cytochalasin B appeared to be completely reversible. Cells appeared normal within 20 — 30 minutes after washing with M 199 and replacement of growth medium. The phagocytosis was continued and parasites were seen to be intracellular.

The statistical analysis of the results with the two strains tested indicated that there was

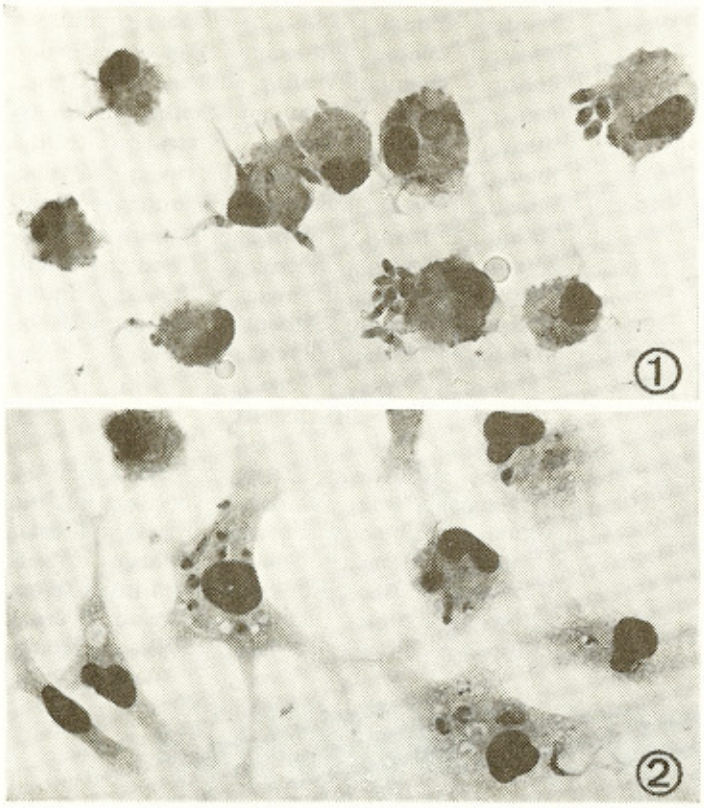

Fig. 1 — **T. cruzi** culture forms in Cytochalasin B-treated macrophages. (10 μg Cytochalasin B/ml, 48 hrs. after addition of **T. cruzi**). Many parasites are seen attached to macrophages (640 ×). Fig. 2 — **T. cruzi** culture forms in control macrophages. (10 μl DMSO/ml, 4 hrs. after addition of **T. cruzi**). Parasites can be seen within macrophages (640 ×)

T A B L E I

Median number of intracellular **T. cruzi** / 100 macrophages (strain rato, epimastigotes 78%). The minimum and maximum values are given in brackets

Medium	M 199	M 199 + Cytochalasin B			M 199 + DMSO		
Time	$\tilde{x}$ (x min. — x max.)	1 μg/ml	5 μg/ml	10 μg/ml	1 μg/ml	5 μg/ml	10 μg/ml
30'	30 (12 — 46)	18 (9 — 32)	1 (0 — 3)	0 (0 — 2)	30 (16 — 41)	19 (15 — 45)	24 (10 — 39)
1h	43 (27 — 55)	23 (11 — 37)	1 (0 — 3)	0 (0 — 2)	37 (27 — 56)	24 (13 — 50)	35 (17 — 43)
2h	55 (21 — 69)	30 (9 — 40)	1 (0 — 5)	0 (0 — 2)	53 (26 — 75)	39 (16 — 63)	45 (13 — 57)
4h	58 (21 — 69)	27 (14 — 53)	0 (0 — 1)	0 (0 — 2)	53 (27 — 62)	44 (21 — 63)	44 (26 — 65)
24h	53 (27 — 82)	24 (8 — 51)	0 (0 — 2)	0 (0 — 1)	26 (34 — 70)	45 (15 — 77)	54 (15 — 69)
48h	54 (10 — 80)	28 (8 — 60)	0 (0 — 2)	0 (0 — 1)	68 (19 — 84)	50 (22 — 77)	61 (19 — 83)

T A B L E II

Median number of intracellular T. cruzi / 100 macrophages (Strain OPS-22, epimastigotes 41%)
The minimum and maximum values are given in brackets

Medium	M 199	M 199 + Cytochalasin B 10 μg/ml	M 199 + DMSO 10 μg/ml
Time	$\tilde{x}$ (min. — max.)	$\tilde{x}$ (min. — max.)	$\tilde{x}$ (min. — max.)
30'	18 (11 — 30)	0 (0 — 3)	15 (7 — 29)
1h	34 (21 — 45)	0 (0 — 1)	25 (14 — 46)
2h	55 (28 — 65)	0 (0 — 6)	47 (23 — 66)
4h	58 (34 — 69)	0 (0 — 4)	52 (35 — 67)
24h	61 (49 — 77)	0 (0 — 4)	62 (48 — 75)
48h	63 (44 — 77)	0 (0 — 2)	69 (42 — 84)

no significant difference in their behaviour in treated and untreated macrophage cultures. Electron microscopic examination of untreated cultures 30 minutes after addition of **T. cruzi** OPS-22 showed the parasites either inside typical phagocytic vacuoles or attached to the membrane and surrounded by finger-like pseudopodia (Fig. 3). In contrast, macrophages treated with 10 μg/ml Cytochalasin B showed numerous membrane bounded spaces and the length of their pseudopodia appeared to be reduced. The parasites were seen only in contact with the membrane of the macrophages (Fig. 4).

DISCUSSION

The mechanism underlying the action of Cytochalasin B on cells is not yet clear but the antiphagocytic activity of this drug is well established in the peritoneal macrophages (ALLISON et al.[2]). The results presented in these experiments indicate that the uptake of **T. cruzi** culture forms by peritoneal macrophages occurs

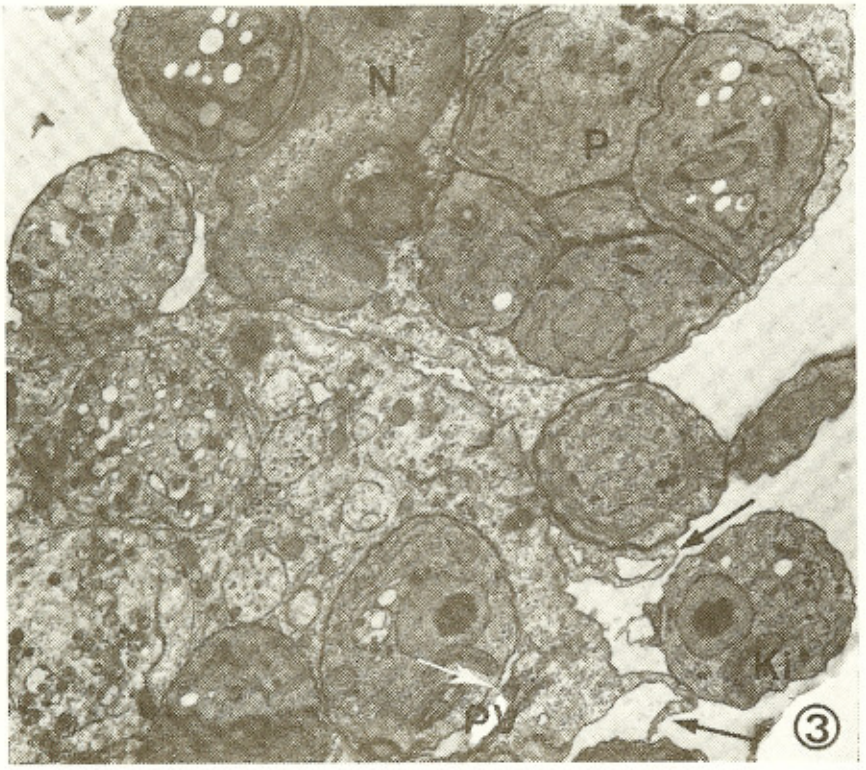

Fig. 3 — **T. cruzi** in control macrophages 30 min. after addition. Parasite is surrounded by finger-like pseudopodia and located in phagocytic vacuoles. (10.000 ×). N = nucleus; P = parasite; Mi = Mitochondria; Ki = Kinetoplast; PV = parasitic vacuole

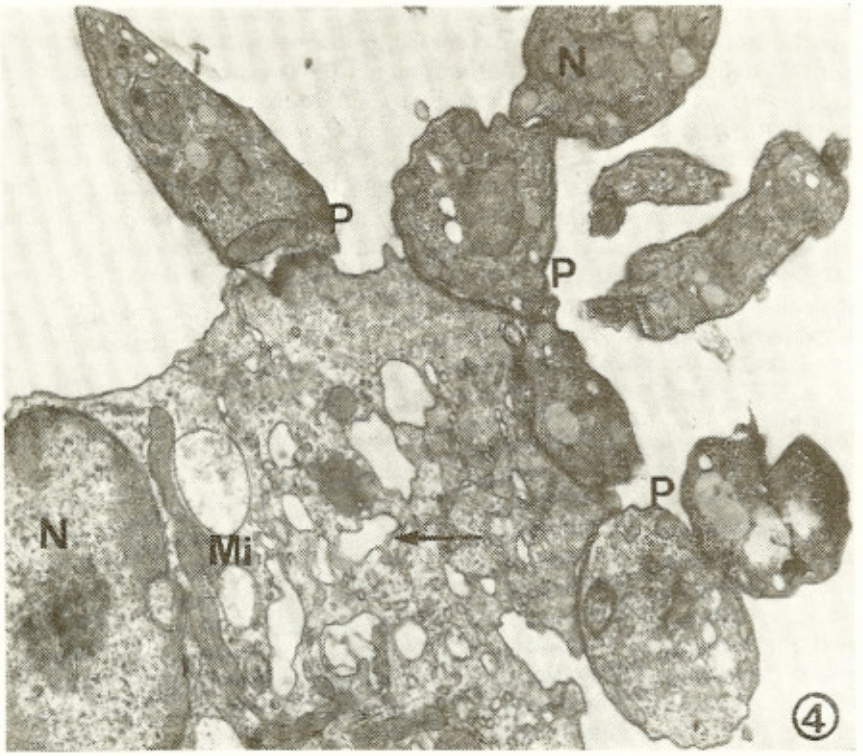

Fig. 4 — **T. cruzi** and macrophage treated with 10 μg/ml Cytochalasin B 30 min. after addition of the parasites (13.700 ×)

by a phagocytic process. This is true for both epimastigotes and trypomastigotes. The strain OPS-22 with the high proportion (59%) of trypomastigotes as well as the Rato strain which had only 22% trypomastigotes were both blocked by Cytochalasin B. On the other hand KIPNIS et al.[10] have demonstrated that the uptake of bloodstream trypomastigotes was not blocked by Cytochalasin B. Our results and those of other investigations mentioned above have shown that trypomastigotes from bloodstream gave different behaviour toward macrophages compared with trypomastigotes from culture. In agreement with our findings a complete inhibition of uptake of epimastigotes and trypomastigotes from culture has been described by KIPNIS et al.[10] as well as by NOGUEIRA & COHN[12] using concentrations of 5 and 10 μg/ml Cytochalasin B.

The electron microscopic studies support the conclusion that uptake of **T. cruzi** occurs by phagocytosis. In untreated macrophages **T. cruzi** were seen surrounded by typical finger-like projections of the membrane during the ingestion process as described in other electron microscopic investigations with **T. cruzi** (MILDER et al.[11], NOGUEIRA & COHN[12]) and **Leishmania** (AKIYAMA & MCQUILLEN[1], EBERT et al.[7]).

In contrast, ALEXANDER[4] reports intracellular **T. cruzi** in macrophages, treated with similar concentrations of Cytochalasin B. He suggested that epimastigotes seem to be able to invade macrophages. Further evidences of a penetration of epimastigotes into macrophages and HeLa cells were described by DVORAK & SCHMUNIS[6] and SOOKSRI & INOKI[15], respectively.

It is difficult to explain why macrophages treated with 5 μg and 10 μg/ml Cytochalasin B were infected only on the periphery of the coverslips. In addition to these observations it was found that macrophages were not so well rounded at the edge of the coverslips as compared to the middle. It is possible that the cells at the periphery have preserved a reduced ability of phagocytosis and consequently were infected with **T. cruzi.**

During the initial phase of interaction of **T. cruzi** with peritoneal macrophages the parasites were seen to attach to the membrane of the macrophages either by means of the flagellum, the body or the posterior end corresponding to the findings of NOGUEIRA & COHN [12]. By direct observations, however, DVORAK & SCHMUNIS found that epimastigotes entered into macrophages with the flagellar end whereas trypomastigotes entered with the posterior end first. Similar "conflicting" results during the initial phase of studying host-parasite interaction are described for **Leishmania** (see ALEXANDER [4], EBERT et al. [8]).

It has been noted that **T. cruzi** as well as **Leishmania** show the ability to enter a cell actively depending on the cell type used. In nonphagocytic cells it could be shown that **T. cruzi** and **Leishmania brasiliensis** appear to penetrate these cells actively (CHANG [5], TANOWITZ et al. [16]). However, it is not understood why studies on **T. cruzi** macrophage interactions give such controversial results and consequently several interpretations. If these discrepancies are based on virulence, different strains should be examined in further experiments.

RESUMO

A influência de cytochalasina B na interação de T. cruzi e macrófagos peritoneais de camundongos

Macrófagos peritoneais de camundongos foram cultivados em Meio 199 contendo 20% de soro fetal bovino (SFB). Culturas de macrófagos foram pré-incubadas com Cytochalasina B nas concentrações finais de 1, 5 e 10 μg/ml (dissolvida em 1 mg/ml DMSO) 2 horas antes da adição de **T. cruzi.** Cepas de **T. cruzi** com diferenças significantes na proporção de tripomastigotas foram adicionadas na relação 1:2 (célula peritoneal: parasita). As culturas de macrófagos foram fixadas e coradas em vários intervalos de tempo. Constatou-se que ambas as cepas aderiram aos macrófagos independente da presença ou ausência da Cytochalasina B. O englobamento de **T. cruzi,** porém, foi quase completamente inibido em macrófagos tratados com Cytochalasina B nas concentrações de 5 μg/ml e 10 μg/ml. Culturas expostas a 1 μg/ml Cytochalasina B/ml não bloquearam a ingestão, mas o grau da infecção dos macrófagos foi significantemente reduzido. Em comparação aos macrófagos incubados em M 199 + 20% SFB os controles com DMSO não alteraram o englobamento de **T. cruzi.** Exames em microscópio eletrônico de macrófagos não tratados mostraram os parasitas dentro de vacúolos fagocíticos, ou aderidos à membranas, e cercados por pseudópodos em forma de dedo. Os resultados indicam que epimastigotas e tripomastigotas de culturas de **T. cruzi,** entram em macrófagos através do processo de fagocitose.

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