Parasitol Res (2002) 88: 598–602
DOI 10.1007/s00436-002-0626-3

ORIGINAL PAPER

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Ultrastructural alterations and growth inhibition of *Trypanosoma cruzi* and *Leishmania major* induced by *Bothrops jararaca* venom

Received: 17 September 2001 / Accepted: 11 December 2001 / Published online: 23 March 2002

Abstract Snake venom can affect the growth of *Trypanosoma cruzi* and *Leishmania* spp. As new classes of therapeutic drugs against protozoan parasites could be derived from snake venom, alterations in the ultrastructure and growth of the epimastigotes, trypomastigotes and amastigotes of *T. cruzi*, as well as the promastigotes of *Leishmania major*, were analyzed after treatment with crude venom from *Bothrops jararaca*. Parasite growth (epimastigotes and promastigotes) of venom treated cultures showed a negative correlation between cell growth and venom concentration. No growth occurred at a dose of 100 μg/ml of venom, while 50% growth inhibition was obtained in the range 0.1–0.3 μg/ml. Ultrastructural observations of treated bloodstream trypomastigotes, intracellular amastigotes, as well as axenic cultures of epimastigotes and promastigotes, demonstrated mitochondrial swelling and kinetoplast disorganization. Our data show that *B. jararaca* venom effectively inhibited the growth of *T. cruzi* and *L. major* parasites. Growth inhibition was probably related to mitochondrial impairment.

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Introduction

Chagas' disease and leishmaniasis are severe diseases affecting about 16 million people in Central and South America (Desjeux 1992; Kirchhoff 1993). They are caused by infection with the intracellular protozoa flagellates *Trypanosoma cruzi* and *Leishmania* spp., respectively. Recent progress has been made in the chemotherapy of Chagas' disease (Vivas et al. 1997). However, new compounds are needed due to the side effects caused by the drugs currently in use. In the past few years, a new approach in the search for cheaper and less toxic chemotherapeutic agents has been the screening of natural compounds able to inhibit protozoan growth. It is known that snake venom, one of the most concentrated enzymes sources in nature, is a complex mixture of peptides and proteins whose composition varies from genera to genera, displaying a broad range of biological effects (Iwanaga and Suzuki 1979). Previous studies have shown that the crude venoms of *Cerastes cerastes*, *Vipera lebetina* and *Naja haje* inhibit the growth of *Trypanosoma cruzi* and *Leishmania donovani infantum* (Fernandez-Gomez et al. 1994). Recently, Tempone et al. (2001) have shown that the venom of *Bothrops moojeni* can inhibit the growth of different *Leishmania* species. Although the growth inhibition of parasites has been described, no reports exist characterizing the ultrastructural alterations caused by snake venom treatment to trypanosomatids.

In this study, we analyzed the inhibitory effect of crude extracts of *Bothrops jararaca* (a snake mostly found in South America) venom on the growth of the epimastigotes of *T. cruzi* and the promastigotes of *L. donovani*. The ultrastructural alterations induced by this venom were determined for the epimastigotes, trypomastigotes and amastigotes of *T. cruzi*, as well as for the promastigotes of *Leishmania major*.

Materials and methods

Snake venom

Lyophilized *B. jararaca* venom was purchased from Sigma or from the Instituto Butantan (São Paulo, Brazil). Venom stock solutions (1 mg/ml) were prepared in sterile distilled water and kept frozen at –20°C. Fresh or boiled venom solutions were diluted in the cell culture medium before use. Boiled snake venom was obtained by heating it for either 1 h at 90°C or 30 min at 70°C.

Parasites

The epimastigotes, amastigotes and trypomastigotes of *T. cruzi* (Y strain) were used. Epimastigote forms were maintained at 28°C by weekly transfers in LIT medium (Camargo 1964) supplemented with 10% fetal bovine serum (FBS). Five-day-old culture epimastigote forms (mid-log phase) were used in the experiments.

Bloodstream trypomastigote forms were obtained from infected CF1 Swiss mice. The animals were intraperitoneally inoculated with trypomastigotes and after 7 days blood was collected by cardiac puncture. The trypomastigotes were purified by centrifugation as described elsewhere (DaMatta et al. 2000).

Amastigotes were obtained by infecting Vero cell cultures with bloodstream trypomastigotes (Carvalho and De Souza 1983). Vero cells were cultivated in 25 cm^2 flasks with 199 medium supplemented with 10% FBS. After 4 days, most cells contained amastigotes.

Promastigotes of *L. major* (V1 strain) were maintained at 31°C by weekly transfers in Warren's medium (Warren 1960) supplemented with 10% FBS. Five-day-old culture promastigotes (mid-log phase) were used in the experiments.

Treatment of culture parasites

T. cruzi epimastigotes were seeded (10^6 cell/ml) into flasks (final volume 5 ml) containing medium supplemented with 0 (control), 0.1, 1, 10 or 100 μg/ml of fresh or boiled snake venom. There was no alteration in the pH of the medium after addition of the venom. Parasite growth was monitored, up to 7 days post-seeding, by the counting of formalin fixed parasites in a hemacytometer chamber daily. Parasite viability was evaluated by trypan blue exclusion.

A small percentage of the *T. cruzi* epimastigotes in axenic cultures usually differentiates into trypomastigotes (Camargo 1964). In order to determine if the differentiation rate changed after venom treatment, Giemsa-stained smears (Farias e Silva et al. 1996) of the treated epimastigote cultures were analyzed daily, up to 7 days post-seeding, and the percentage of epimastigote and trypomastigote forms was estimated.

L. major promastigotes were cultured in medium in the presence of 50 μg/ml venom. Parasite growth was monitored, up to 7 days post-seeding, by counting fixed parasites in a hemacytometer chamber daily. Parasite viability was evaluated by trypan blue exclusion.

Ultrastructural analysis

T. cruzi epimastigotes were cultured for 24 h in medium containing 50 μg/ml venom and processed for routine transmission electron microscopy. Some epimastigotes were alternatively incubated for 24 h in medium containing 50 μg/ml azide. Bloodstream trypomastigotes were washed with phosphate buffered saline (PBS), kept for 24 h in 199 medium supplemented with 10% FBS and 50 μg/ml venom, and then processed for transmission electron microscopy. *T. cruzi*-infected Vero cells cultured for 4 days were incubated in medium containing 50 μg/ml venom, cultured for a further 24 h and processed for transmission electron microscopy. *L. major* promastigotes were cultured for 24 h in medium containing 50 μg/ml venom and processed for transmission electron microscopy.

Transmission electron microscopy

Parasites were washed with PBS, fixed for 2 h with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Cells were washed in buffer, post-fixed for 1 h with 1% osmium tetroxide and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, dehydrated in graded acetone and embedded in epoxy resin. Ultra-thin sections

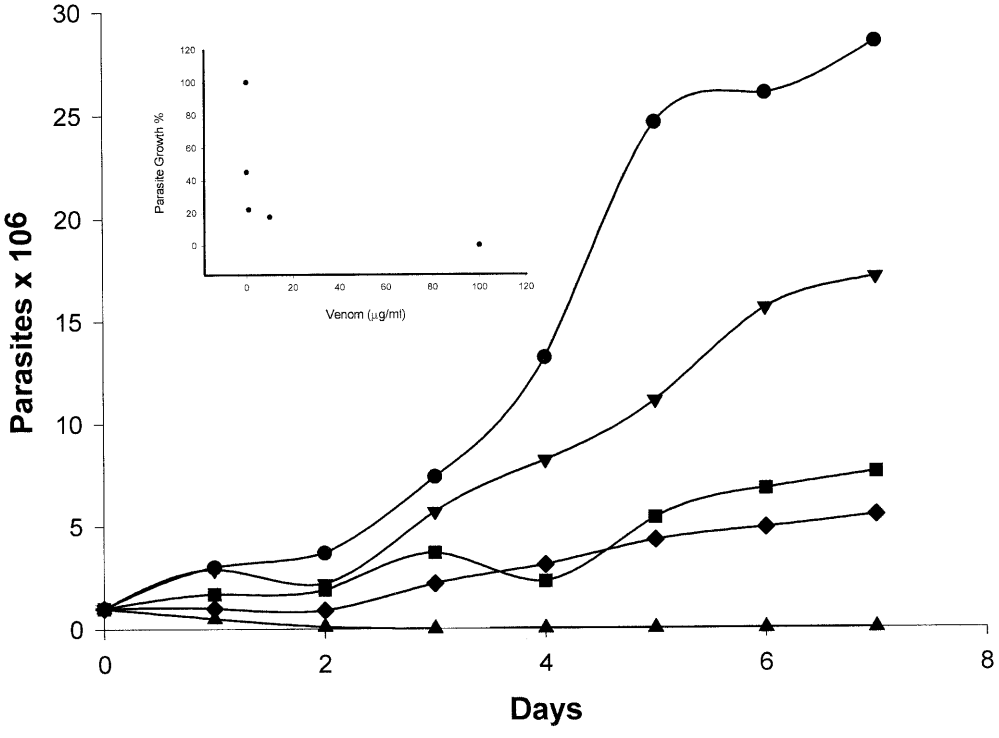

Fig. 1 Growth inhibition of epimastigotes of *Trypanosoma cruzi* by *Bothrops jararaca* venom. Cells were cultured for 7 days with increasing venom concentrations (*filled circle* 0; *filled triangle point down*0.1; *filled square* 1; *filled diamond* 10; *filled triangle point up* 100 μg/ml) and counted daily. The inhibitory $K_{0.5}$ (50% growth inhibition) was estimated by plotting the parasite numbers from day 5 as a percentage over venom concentration (*inset*)

were stained with uranyl acetate and lead citrate, and observed in a ZEISS EM900 transmission electron microscope.

Results and discussion

The culture epimastigote forms of *T. cruzi* were grown for 7 days in medium containing different concentrations of *B. jararaca* venom and the cell growth evaluated. While a venom concentration as low as 0.1 μg/ml partially inhibited the cell growth of the epimastigotes, no growth was detected when 100 μg/ml of venom was added to the culture medium (Fig. 1). The inhibitory $K_{0.5}$ (50% growth inhibition) at day 5 was in the range 0.1–0.3 μg/ml of venom (Fig. 1, inset). Comparing our results with those published by Fernandez-Gomez et al. (1994) using *C. cerastes* venom, it is evident that *B. jararaca* venom was about 10–100 times more effective in inhibiting *T. cruzi* and *L. major* multiplication.

No alteration in the differentiation rate could be observed after the addition of venom. Cultured parasites without motility were dead as demonstrated by their positive staining by trypan blue. Venom boiled for 1 h at 90°C had no effect on epimastigote growth, but boiling the venom for 30 min at 70°C resulted in the same inhibitory effect as observed with untreated crude extracts. This suggests that the venom component(s) involved in the inhibition of parasite growth was thermally quite stable. The treatment of *L. major* promastigotes with *B. jararaca* venom resulted in the same growth inhibitory effect observed with *T. cruzi* epimastigotes (not shown).

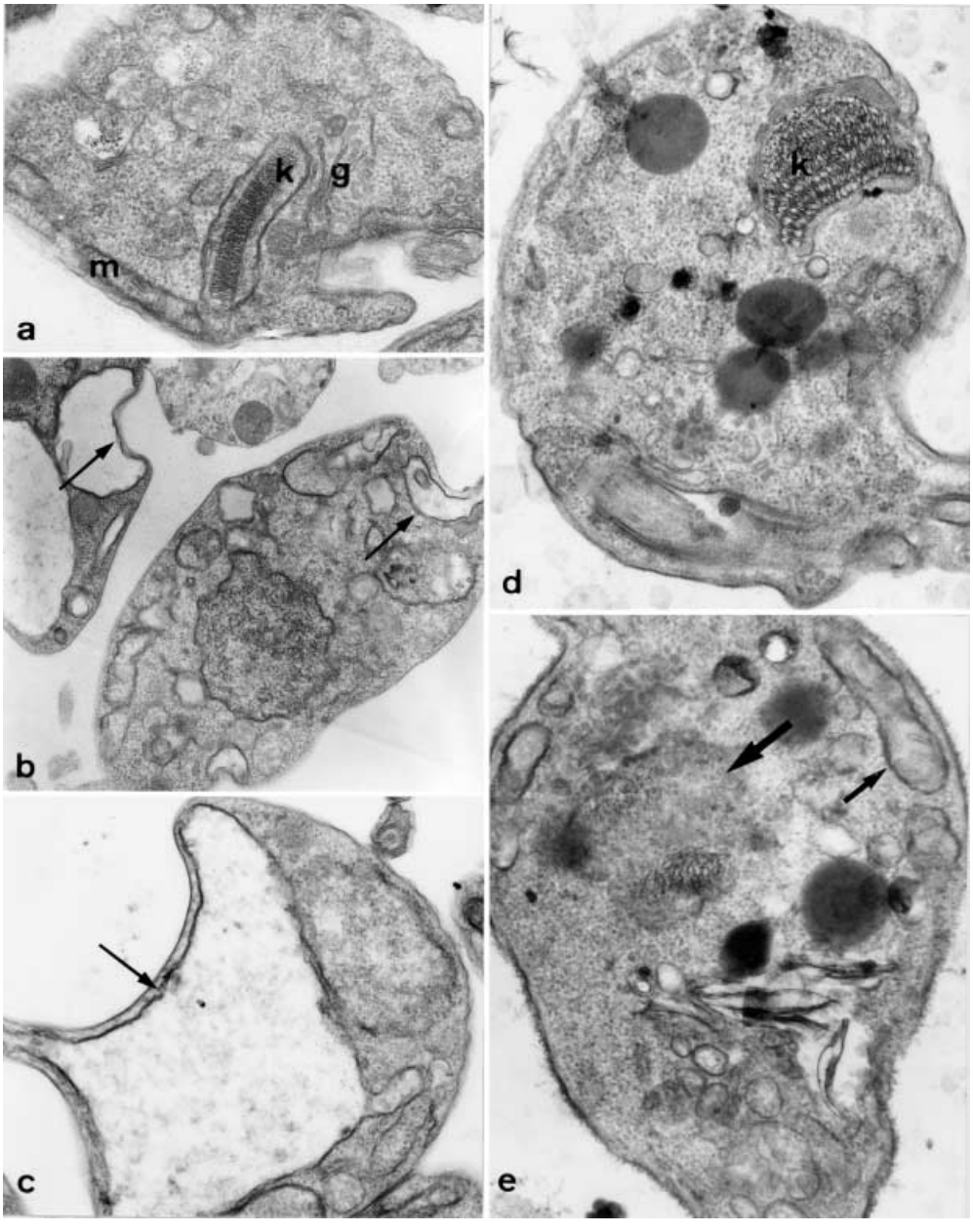

Fig. 2a–e Ultrastructural examination of the epimastigote and trypomastigote forms of *Trypanosoma cruzi*. **a** General view of an untreated epimastigote, showing kinetoplast (*k*), mitochondrion (*m*) and Golgi apparatus (*g*). **b** Epimastigote form treated with snake venom (50 μg/ml) for 24 h. Note mitochondrial swelling (*arrow*). **c** Epimastigote form treated with sodium azide (50 μg/ml) for 24 h. Note intense swelling of the mitochondrion (*arrow*). **d** Untreated trypomastigote form, showing the characteristic kinetoplast (*k*). **e** Trypomastigote form treated with snake venom (50 μg/ml) for 24 h. Note disorganization of the kinetoplast (*arrow*) and swelling of the mitochondrion (*small arrow*). Note normal appearance of biological membranes in both treated forms

The observation of untreated epimastigote forms of *T. cruzi* by transmission electron microscopy showed all the typical ultrastructural characteristics of the trypanosomatids. A main feature was the single elongated mitochondrion running along the cell body, containing a large condensation of mitochondrial DNA, the kinetoplast. A single Golgi complex could also be observed at the anterior end of the parasites, close to the flagellar pocket (Fig. 2a). Epimastigotes treated with crude snake venom showed ultrastructural alterations, consisting mainly of a large mitochondrial swelling (Fig. 2b). Epimastigotes treated with azide also displayed an intense swelling of the mitochondrion (Fig. 2c).

Untreated bloodstream trypomastigotes presented a small flagellar pocket, an anterior flagellum, electron-dense granules and a characteristic basket-shaped kinetoplast (Fig. 2d). After venom treatment, swelling of the mitochondrion and alteration of the kinetoplast morphology could be detected (Fig. 2e).

Untreated Vero cells infected with *T. cruzi* displayed amastigotes with normal nuclei and kinetoplasts (Fig. 3a). After 24 h of venom treatment, the amastigotes displayed disruption (Fig. 3b) and disappearance (Fig. 3c) of the mitochondrion, as well as disorganization of the kinetoplast (Figs. 3b, c). Furthermore, the morphology of the Vero cells was preserved after venom treatment (Fig. 3c). This suggests that components of the venom may diffuse through biological membranes and are cytolytic only for the intracellular parasites.

Untreated promastigotes of *L. major* showed typical nuclei and kinetoplasts (Fig. 3d). After 24 h of venom treatment, extensive swelling (Figs. 3e, f) and vacuolization (Fig. 3f) of the mitochondrion could be observed. The nuclear morphology was maintained (Fig. 3f). It is interesting to note that with the same trypanosomatids used in the present work, the mitochondrion was the first organelle to suffer morphological alteration after treatment with drugs which interfere with the biosynthesis of ergosterol, leading to parasite

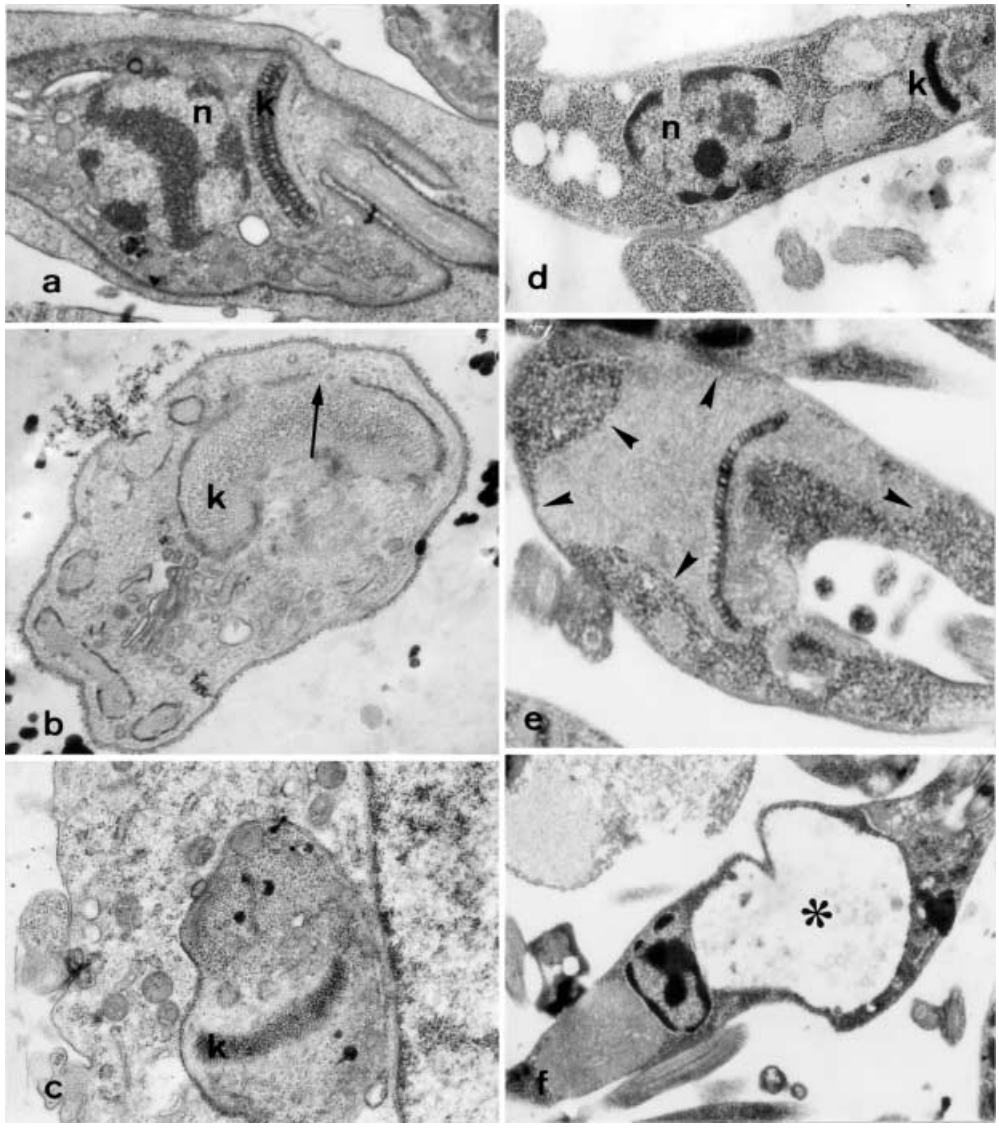

Fig. 3 Ultrastructural examination of Vero cells infected with amastigotes of *Trypanosoma cruzi* (**a–c**), or axenic promastigotes of *Leishmania major* (**d–f**). **a** Untreated infected Vero cells. Note the normal appearance of kinetoplast (*k*) and nucleus (*n*) in the parasite. **b**, **c** *T. cruzi* infected Vero cells treated with snake venom (50 μg/ml) for 24 h. Note the disorganization of the kinetoplast (*k*), as well as fragmentation (*arrow* in **b**) and disappearance (**c**) of the mitochondrial membrane in the parasites. Note the normal appearance of the plasma membranes in all treated parasite forms. **d** Untreated *L. major* promastigote, showing normal kinetoplast (*k*) and nucleus (*n*). **e**, **f** Promastigotes treated with snake venom (50 μg/ml) for 24 h. Note high swelling of the mitochondrion around the kinetoplast (*arrowheads* in **e**) as well as vacuolization of the mitochondrion (*asterisk* in **f**)

death (Vannier-Santos et al. 1995; Vivas et al. 1997). A possible explanation for the observed mitochondrial swelling may be that *B. jararaca* venom inhibited the parasite's respiratory chain, thus lowering ATP levels, leading to mitochondrial swelling and finally to parasite death. This hypothesis is further support by the observation of mitochondrial alteration after azide treatment.

Further studies are in progress to isolate and identify the biological compound(s) that inhibited growth and caused the ultrastructural alterations in these parasites. *B. jararaca* venom may be an alternative source of biological compounds against pathogenic trypanosomatids, and work on it could lead to the discovery of new, alternative chemotherapeutic treatments.

Acknowledgements We would like to thank Rosemary Cardoso Maciel for technical assistantship and Andrèa Carvalho César for reading the manuscript. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Rio de Janeiro (FAPERJ), Fundação Estadual do Norte Fluminense (FENORTE), Financiadora de Estudos e Projetos (FINEP) and Programa de Núcleos de Excelência (PRONEX). The experiments performed in this work comply with current Brazilian laws.

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