Effect of actinomycin D on *Trypanosoma cruzi*

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Summary. Viable metacyclic forms of *T. cruzi*, Y strain, treated with an adequate dose of actinomycin D (50 μg Act-D/ml/10^7 parasites/ml for 72 h at 28 °C) showed the following properties: 1) they lost their ability to replicate in culture medium, in blood and in tissues of normal mice and were no longer able to incorporate tritiated thymidine; 2) they could not penetrate into Vero cells and could not replicate inside normal macrophages; 3) they retained their immunogenicity and the ability to protect mice against a virulent infection; 4) they did not induce histological lesions as described in chronic experimental Chagas' disease.
Key words. Actinomycin D; *Trypanosoma cruzi;* vaccination.

It has been shown that the treatment of *T. cruzi* with actinomycin D (Act-D) inhibits its ability to multiply, in an apparently irreversible manner, even when the parasites are washed and transferred into a culture medium free of Act-D [1–3]. The aim of the work reported in this paper was to show that *T. cruzi*, treated with an adequate dose of Act-D (50 μg/10^7 parasites/ml), does indeed lose its ability to multiply, but retains its immunogenicity and the ability to protect mice against a virulent infection.

Material and methods

Animals. BALB/c adult female mice, 6 to 8 weeks old, were used in all experiments.

Parasites. Epimastigote forms of *T. cruzi*, Y strain, were obtained from culture in liver infusion tryptose (LIT medium) at 28 °C. The culture medium used was Yaeger's LIT medium as described by Camargo [4]. Metacyclic forms were obtained from culture in blood agar base + LIT medium (NNN-LIT medium) at 28 °C and purified on a DEAE-Cellulose column eluted with phosphate buffer pH 8.0, pI-0.181.

Treatment with Act-D. Viable parasites at a concentration of 10^7/ml in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) were treated with 50 μg Act-D at 28 °C for 72 h and then washed twice with RPMI 1640 containing 5% FCS.

Experiments. I) *Replication of Act-D-treated epimastigotes in vitro.* Epimastigote forms (1×10^7/ml) were treated with Act-D (50 μg/ml), incubated at 28 °C in tubes with LIT medium and transplanted weekly into new culture medium during 5 successive weeks. Normal parasites were similarly transplanted, as a control.

The ability of Act-D-treated and untreated parasites to replicate was determined weekly by counting the number of motile parasites and by measuring the amount of tritiated thymidine incorporated by the parasites. II) *Replication of Act-D-treated metacyclic parasites in vivo.* Three groups of mice were used in these experiments: 1) 5-day-old normal mice; 2) adult female normal mice; 3) adult female mice injected i.p. every other day with 10^6 metacyclic parasites previously treated with Act-D. Control animals only received normal parasites. Five animals of each group were sacrificed every 3 months for 12 months, and a search was made for parasites in their blood by direct examination, xenodiagnosis, repeated hemocultures in NNN-LIT medium, tissue histological examinations, and intraperitoneal inoculations of tissue homogenates into animals previously immunosuppressed. III) *Incorporation of tritiated thymidine by Act-D-treated parasites.* A suspension of 10^7 Act-D-treated epimastigotes was incubated at 37 °C for 24 h with 1 μCi/ml of tritiated thymidine (^{3}HTDR, sp. act. 6.7 Ci/mM, New England Nuclear Co., USA). The parasites were centrifuged and the sediment washed twice with 0.4 M per chloric acid and twice with 80% ethanol. The final sediments were suspended in 0.1 N NaOH and radioactivity measured in a liquid scintillation spectometer. IV) *Interiorization into Vero cells of T. cruzi treated with different doses of Act-D.* Suspensions containing 10^7/ml living metacyclic forms in RPMI medium were treated at 37 °C during 24 h with 1, 10, 25 and 50 μg Act-D/ml. Untreated and Act-D-treated suspensions with 10^5/ml of washed living parasites in RPMI, supplemented with 5% *FCS*, were used to infect confluent monolayers of Vero cells in Leighton tubes. The percentage of infected cells and the number of parasites per cell (index of interiorization) were determined in cultures after 72 h. The index of interiorization was obtained by calculating the percentage of Vero cells with parasites interiorized multiplied by the average number of parasites interiorized by each cell, according to Bianco et al. [5]. V) *Replication of Act-D-treated T. cruzi in macrophages.* A suspension containing 10^5/ml of living metacyclic forms in RPMI supplemented with 10% FCS was used to infect monolayers of mouse peritoneal macrophages in Leighton tubes. Peritoneal macrophages were collected and treated according to Cohn and Benson [6]. The percentage of parasitized cells and the number of parasites/cell were determined daily for 6 days. The index of endocytosis of parasites by macrophages was also obtained following Bianco et al. [5]. VI) *The protection induced by T. cruzi treated with Act-D.*

Mice were vaccinated s.c. with 4 weekly doses of 5×10^7 motile Act-D-treated metacyclic forms. Forty days after the last immunizing dose all animals, and controls, were subcutaneously challenged with 10^4 untreated viable metacyclic Y strain parasites. Parasitemia, histological changes and percentage survival were registered at 30-day intervals for 12 months, when groups of 5 animals were sacrificed for parasitological and histological studies.

Statistics. The statistical analysis was carried out using the Student's t-test for independent small samples.

Results

Experiment I. The results of this experiment (fig. 1) showed: 1) Act-D-treated epimastigotes are no longer capable of replicating in culture medium; 2) the growth inhibition of these parasites is irreversible; it remains so until the death of the parasites, even when the parasites are twice washed and transplanted to fresh medium; 3) the Act-D-treated epimastigotes showed normal motility for at least two weeks. *Experiment II.* The results indicated (table 1) that: 1) none of the experimental animals (groups 1,2,3), injected with 10^6 viable metacyclic forms, showed the presence of parasites in their blood or tissues during the 12 months of observation; 2) replicating parasites could be regularly found in all control animals. *Experiment III.* The results (fig. 2) showed that metacyclic forms of *T. cruzi* treated with an adequate dose of Act-D are no longer able to incorporate tritiated thymidine into DNA. *Experiment IV.* The results showed (fig. 3) that: 1) treatment with 1 µg Act-D inhibited about 95% of parasite interiorization; 2) treatment with 10 µg of larger doses of Act-D totally inhibited this interiorization; 3) the small number of parasites treated with 1 µg Act-D/ml which were found inside Vero cells were identified as amastigotes. *Experiment V.* The results (fig. 4) showed that: 1) the replication index and the percentage of replication inside macrophages of Act-D-treated metacyclic parasites decreased to zero after 72 h and 96 h, respectively, whereas both phenomena increased continually in macrophages infected with untreated parasites; 2) Act-D-treated metacyclic forms no longer replicate inside macrophages, where they are easily destroyed. *Experiment VI.* The results (table 2) indicated that: 1) none of the experimental animals showed the presence of viable parasites in their blood or tissues up to 12 months after vaccination; 2) 100% of animals survived a challenge with 10^4 virulent parasites; 3) para-

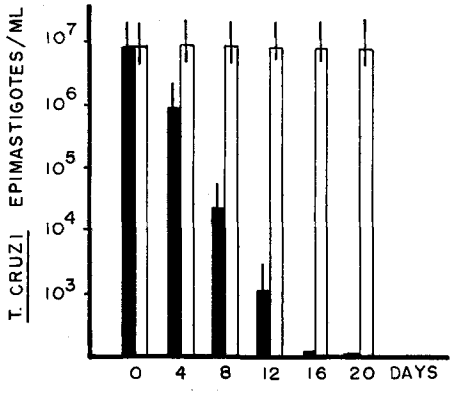

Figure 1. Act-D-treated epimastigotes (■) do not replicate in culture even when washed and transplanted to fresh medium. Parasites motility is usually maintained for three weeks. Untreated parasites (□) with normal replication.

Table 1. Infectivity of Act-D-treated *T. cruzi* metacyclic forms for mice

Intraperitoneal injection of 10^6 metacyclic forms of *T. cruzi*, Y strain	Parasites in blood or tissues during 12 months (Group 1) Five-day-old mice	(Group 2) Normal adult mice	(Group 3) Adult mice suppressed with cyclophosphamide
Treated with Act-D	(−)	(−)	(−)
Untreated	(+)	(+)	(+)

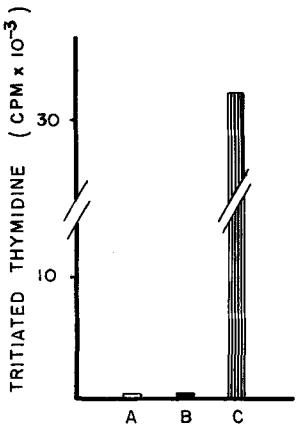

Figure 2. Epimastigotes forms of *T. cruzi* treated with Act-D (*A*) or killed by mertiolate (*B*) do not incorporate tritiated thymidine into their DNA. Control experiment with untreated parasites (*C*).

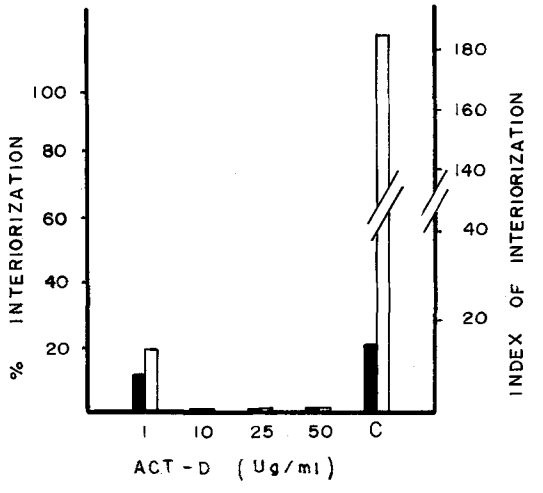

Figure 3. Percentage (■) and index of interiorization (□) into Vero cells of untreated (C) and Act-D-treated metacyclic forms of *T. cruzi* during 72 h. Treatment with 1 µg Act-D inhibited 95% of parasites interiorization, while treatment with 10 µg inhibited 100% of interiorization.

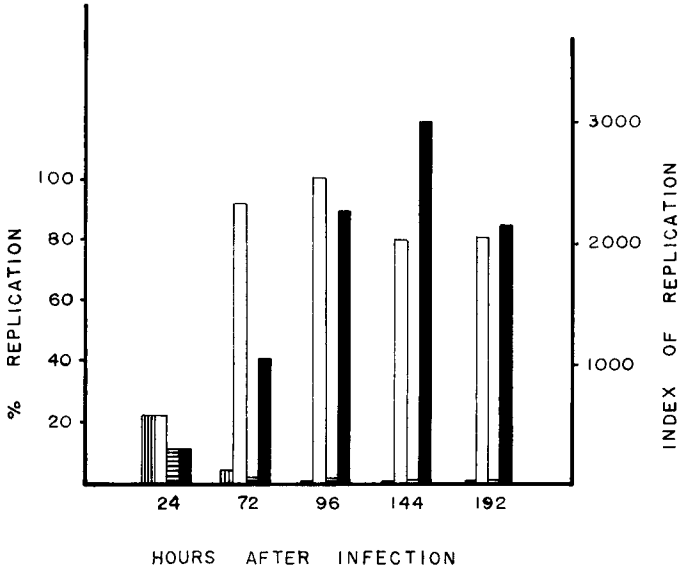

Figure 4. Percent replication (□ ▥) and replication index (▤ ■) into macrophages of untreated (□ ■) or Act-D-treated (▥ ▤) metacyclic forms of *T. cruzi*. The Act-D-treated parasites no longer replicate inside macrophages, where they are easily destroyed.

Table 2. Immunization of mice with Act-D-treated *T. cruzi* metacyclic forms

Mice	Challenge with 10^4 viable metacyclic forms 40 days after vaccination	Parasites in blood	tissues	Histologic lesions
Vaccinated	(+)	(−)	(−)	(−)
Unvaccinated	(+)	(+)	(+)	(+)

sites capable of replication were regularly cultured from all control animals and presented the levels of parasitemia usually found between days 5 and 10 in normal animals infected with the Y strain; 4) all control animals died within 15–20 days after challenge; 5) no histological lesions similar to those described in chronic experimental Chagas' disease were found in vaccinated animals.

Discussion

Our experiments have regularly shown that the epimastigote forms of *T. cruzi,* Y strain, treated with an adequate dose of Act D (50 μg/ml/10^7 parasites/ml/28 °C/72 h), although they manifest their normal motility in vitro for about 15 days, irreversibly lose the ability to replicate in vitro (fig. 1), and the ability to incorporate tritiated thymidine into DNA (fig. 2). They also lose the ability to replicate in blood and tissues (table 1), to infect Vero cells (fig. 3) and to replicate inside macrophages (fig. 4). They retain, however, the capacity to induce a high degree of protection in animals against an infection with 10^4 parasites of a virulent strain of *T. cruzi* (table 2). Our results also showed repeatedly that when the dose of Act-D was not sufficient (below 1 mg/ml/10^7 parasites/ml), not all parasites lost their capacity to replicate (fig. 3). The small doses of Act-D used to block the replication of a high number of parasites possibly explain the results obtained by Fernandes et al.[2]. According to these authors, "in mice inoculated with high numbers of *T. cruzi* (1.5×10^7) cultivated for 3 days in the presence of 1 μg/ml Act-D, survival was 100%, and no parasites could be detected in blood or tissues. When challenged with virulent blood forms of the same strain, all mice survived while all controls died within 13–20 days. Parasitemia was much lower in the immunized than in the control mice, but the acute inflammation reaction was much more severe in the first group. This reaction frequently subsided, only a few discrete lesions being found in later examinations."

Two models have been proposed to explain the binding of Act-D to DNA: the first involves direct hydrogen-binding recognition between the guanine ring and the chromophore residue; the second involves interaction of the phenoxazone ring with DNA between the base-paired dinucleotide sequence dG-dC, while the peptide subunits lie in a narrow groove in the DNA helix and interact with deoxyguanosine residues on opposite chains, through specific hydrogen bonds[7]. Sobbel[8] proposed recently that Act-D binds to a premelted DNA conformation called beta-DNA found within the transcriptional complex. This acts to immobilize (or pin) the complex, preventing the elongation of growing RNA chains.

The in vitro toxicity of Act-D against the different forms of *T. cruzi* is very low; the parasites remain motile for several days at 28 °C in culture medium containing even 100 μg Act-D/ml/10^7 parasites[9].

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1 Fernandes, J. F., Halsman, M., and Castellani, O., Nature *207* (1965) 1004.
2 Fernandes, J. F., Castellani, O., and Okumura, M., Rev. Inst. Med. Trop. Saõ Paulo *8* (1966) 151.
3 Fernandes, J. F., Halsman, M., and Castellani, O., Exp. Parasit. *18* (1966) 203.
4 Camargo, E. P., Rev. Inst. Med. Trop. Saõ Paulo *6* (1964) 93.
5 Bianco, C., Griffin, F. J. Jr, and Silverstein, S. C., J. exp. Med. *141* (1975) 1278.
6 Cohn, Z. A., and Benson, B., J. exp. Med. *121* (1965) 153.
7 Sobbel, H. M., Prog. nucleic Acid Res. molec. Biol. *13* (1973) 153.
8 Sobbel, H. M., Proc. natl Acad. Sci. *82* (1985) 5328.
9 Brascher, H. M., Vargens, J., Queiroz Cruz, M., and Oliveira-Lima, A., Mem. Inst. Oswaldo Cruz *83* (1988) 152.

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Class I and class II ribonuclease H activities in *Crithidia fasciculata* (Protozoa)

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Summary. The protozoan *Crithidia fasciculata* contains two different ribonuclease H activities. These enzymes display similar physical and biochemical characteristics to their homologues in higher eukaryotes, for instance calf thymus class I and class II ribonuclease H. Class I ribonuclease H of lower and higher eukaryotes can be activated by Mg^{2+}- and Mn^{2+}-ions. However, the presence of Mn^{2+}-ions is inhibitory for the Mg^{2+}-dependent class II ribonuclease H activity of *Crithidia fasciculata* and calf thymus. The protozoan class I-homologue enzyme appears to be serologically related to the class I ribonuclease H of calf thymus.
Key words. Ribonuclease H activity; kinetoplastida; *Crithidia fasciculata.*

Ribonucleases H are enzymes which specifically degrade the RNA moiety of RNA DNA-hybrids [1, 2]. Higher eukaryotes contain two enzymes with ribonuclease H specificity, a class I and a class II ribonuclease H [3–6]. The classification of these enzyme activities, in particular those of calf thymus (here ribonuclease H I and H IIb correspond to class I and II ribonuclease H, respectively), is based on their physical and biochemical properties as well as on serological analyses [3–8]. Although ribonucleases H of yeast are well characterized, an unambiguous attribution of the distinct activities to class I and class II ribonucleases H, based on the criteria mentioned above, is not possible [9–14]. Extracts of macronuclei of the ciliate *Tetrahymena pyriformis* contain three ribonuclease H activities with biochemical characteristics similar to the class II ribonuclease H of higher eukaryotes [15].
Here we show that extracts of the protozoan *Crithidia fasciculata* display two distinct ribonuclease H activities, which can be classified as class I and class II ribonuclease H activity, as judged by physical and biochemical parameters. Moreover, the enzyme preparation of *C. fasciculata* containing the presumptive class I ribonuclease H activity is specifically recognized by an antibody directed against calf thymus ribonuclease H I.

Materials and methods

Preparation of C. fasciculata crude extract. C. fasciculata clone 1 was grown in 3.7% (w/v) brain heart infusion broth supplemented with 20 mg/l hemin as described [16]. Cells were harvested by centrifugation, washed and lysed in 50 vols of TGED [50 mM Tris/HCl pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 25% (w/v) glycerol, 0.1 mM phenylmethylsulfonylfluorid] + 0.3 M $(NH_4)_2SO_4$ using a 'Stansted Cell Disrupter' [17]. The cell lysate was clarified by centrifugation and the sediment discarded. The crude extract contained 1.0 mg of protein/ml and 7.5 units/mg ribonuclease H activity (measured under Mg^+-conditions).
Separation of two ribonuclease H activities in C. fasciculata. C. fasciculata crude extract was absorbed to DEAE-cellulose, equilibrated in TGED and washed extensively. The DEAE-cellulose-binding ribonuclease H activity was eluted with TGED + 200 mM KCl. The DEAE-cellulose eluate contained one third and the flow-through two thirds of the remaining ribonuclease H activity. The yield of enzyme activity amounted to 77% compared with the crude extract. The DEAE-cellulose-binding and unbound ribonuclease H activities (1.0 mg of protein/ml each) displayed specific activities of 5.0 units/mg and 1.5 units/mg measured under Mg^{2+}-conditions, respectively. The ribonuclease H activity not bound to DEAE-cellulose bound to CM-Sephadex, and eluted from this column at around 0.2 M KCl.
Assay for ribonuclease H activity. Ribonuclease H activity determinations were carried out exactly as described elsewhere [1, 3, 5, 6]. The assay mixture contained, in a final volume of 500 µl, 30 mM Tris/HCl pH 7.8, 50 mM $(NH_4)_2SO_4$, 0.02% 2-Mercaptoethanol, 20 µl (^{3}H)RNA DNA-hybrid (2000 cpm, corresponding to 60 pMol of ribonucleotides), and either 10 mM $MgCl_2$ or 2 mM