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ACTIVITY OF LYTIC PEPTIDES AGAINST INTRACELLULAR *TRYPANOSOMA CRUZI* AMASTIGOTES IN VITRO AND PARASITEMIAS IN MICE

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ABSTRACT: Three cecropin-like lytic peptides (DC-1, DC-2, and DC-2R) were synthesized with virtually no sequence homology with the natural compound (cecropin B) while retaining the charge distribution, amphipathic, and hydrophobic properties of the natural compound. A fourth analog (α-Pi) without these later properties, but a similar molecular weight, was also synthesized as a nonlytic peptide control. The 3 lytic peptides were examined for their ability to kill *Trypanosoma cruzi* trypomastigotes in vitro, intracellular amastigotes in vitro, and their toxicity to a mammalian cell line. DC-2 at 5 μM and DC-1 and DC-2R at 10 μM were 100% effective in killing *T. cruzi* trypomastigotes in vitro, suggesting at least a 10-fold increase in lytic activity over previous tested lytic peptide analogues, SB-37 and Shiva-1. When *T. cruzi*-infected Vero cells were treated with a single or double exposure of low concentrations (2.5 μM) of DC-1, DC-2, and DC-2R there was a significant ($P < 0.05$) reduction in amastigote numbers/cell when compared to untreated and α-Pi-treated *T. cruzi*-infected cells. Vero cells alone treated with the lytic peptides showed no reduction in number or toxicity. One of the peptides (DC-1) was tested for its toxicity in AJ mice and its ability to reduce parasitemias in *T. cruzi*-infected AJ mice. No untoward effects were seen in AJ mice injected intravenously with 50 μg/mouse daily for 10 days. There was a significant ($P < 0.05$) reduction in parasitemia and mortality by day 14 postinoculation (from 100% to 0%) in *T. cruzi*-infected AJ mice given 25 μg of DC-1/mouse on days 2, 4, 6, 8, and 10 postinoculation.

Approximately 50,000 people die of Chagas' disease annually and it is estimated that 16–18 million people are chronically infected (Kirchhoff, 1993). Nifurtimox and benznidazole are the current recommended therapies for Chagas' disease (Viotti et al., 1994). However, both drugs have significant toxicity and do not effectively delay or prevent the development of clinical manifestations of disease (Marr and Docampo, 1986). In fact, toxicity is often so marked, some investigators propose not using these compounds (Marr and Docampo, 1986). Ketoconazole, gossypol, and allopurinol have been investigated in animals, in vitro, and in human patients, respectively, but, as yet, these studies show less than convincing results (McCabe et al., 1983; Gallerano et al., 1990; Rovai et al., 1990). There is a considerable need for the development of new novel approaches to chemotherapy for this disease.

Lytic peptides are small proteins of 23–39 amino acid sequences, which are thought to be components of the inducible defense system of some species of insects, molluscs, amphibians, and at least 1 mammal (Lee et al., 1989). Among these proteins is the type known collectively as cecropins. The principal cecropins (A, B, and D) are highly homologous small basic peptides the amino-terminal half of which contains a sequence that may form an amphipathic α-helix (Andreu et al., 1985). The carboxyl-terminal half comprises a hydrophobic tail. Various substitution analogs of a cecropin from the giant silk moth have a wide activity against a large number of bacteria and protozoa at very low concentrations (Hultmark et al., 1980; Jaynes et al., 1986, 1988, 1993). Although the mechanism of action is not completely understood, it is thought that cecropins localize in the cell membranes of cells to form pores resulting in disruption and lysis due to a loss of osmotic integrity similar to the effects of complement (Jaynes et al., 1988).

We have previously demonstrated that 2 cecropin analogs are 100% effective (at concentrations of 100 μM) in killing *T. cruzi* trypomastigotes in vitro and reducing parasitosis in African green monkey kidney (Vero) cell culture (Jaynes et al., 1988). In this paper, we extend these studies by examining the in vitro activity of 3 new cecropin analogues against *T. cruzi*. The new analogues were designed to vary even more markedly in sequence homology from cecropin B and the previously tested peptides while still retaining the α-helix amphipathic tail (Jaynes et al., 1988). We have further extended previous studies by assessing the toxicity of 1 of the peptides to mice and its ability to reduce parasitemias in *T. cruzi*-infected mice.

MATERIALS AND METHODS

Lytic peptides were synthesized using a Milligen/Biosearch model 9050 automated peptide synthesizer, which employs Fmoc chemistry (Fields and Noble, 1990). All reagents were purchased from Milligen/Biosearch (San Rafael, California). Upon completion of the synthesis, the peptide was cleaved from the resin and exhaustively extracted with ethyl ether, dissolved, frozen in a solid CO_2/acetone bath, and lyophilized. Sephadex column chromatography was used to remove all remaining small organic molecules and to fractionate the peptide mixture according to size. The fractions were shell-frozen, lyophilized, and subjected to HPLC for initial analysis and preparative work. The samples were prepared in aqueous buffer containing 50% methanol and analyzed in a Varian 5000 HPLC System and a Waters μBondapak C18 column, 8-mm × 10-cm radial pak cartridge, which employed the radial compression module. The appropriate fractions were then subjected to mass spectroscopy analysis to confirm the identity of the full-length peptides. The samples were analyzed using a 252Cf plasma desorption mass spectrometer (PDMS) BIO ION 20 (Bio Ion Nordic AB, Uppsala, Sweden). The operating conditions for analyses were as follows: the acceleration voltage was 15 kV with 8 K channels being monitored for a duration of 1 million counts (about 10 min). The samples were applied to a nitrocellulose-coated target in a 50:50 water/ethanol solution, allowed to absorb for 10 min, and then loaded into the instrument.

The Tulahuen strain of *T. cruzi* was maintained in AJ mice (Jackson Laboratories, Bar Harbor, Maine) by syringe passage of blood (Trischmann et al., 1978). Trypomastigotes used to infect AJ mice were obtained from the blood of infected AJ mice with high parasitemias. The Tulahuen strain was also maintained in Vero cell culture. Trypomastigotes (also of the Tulahuen strain maintained in Vero cell culture) were used for in vitro and Vero cell infection experiments at a concentration of 5×10^6 organisms/ml in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, New York), supplemented with 10% heat-inactivated (56 C, 30 min) fetal bovine serum (FBS). Parasites were

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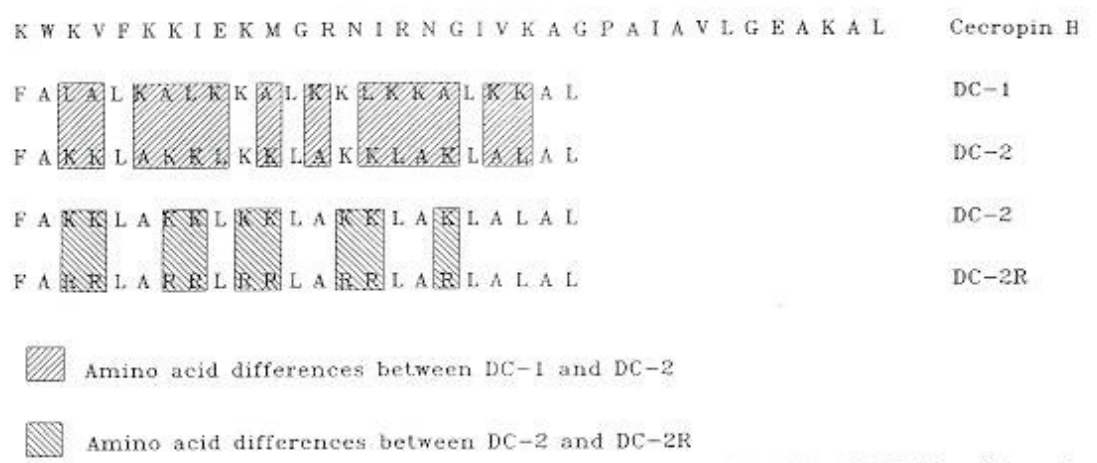

FIGURE 1. Sequence comparison of the natural cecropin B with the synthetic lytic peptides DC-1, DC-2, and DC-2R. The synthetic peptides were designed to have essentially no homology with the natural compound to test whether the lytic properties of cecropin-like peptides were highly sequence dependent. In fact, the lytic properties of the synthetic compounds were increased. In addition, DC-2 and DC-2R were designed to be considerably different from DC-1, but the properties of charge distribution, amphipathy, and hydrophobicity present in cecropin B were also conserved in DC-1, DC-2, and DC-2R.

counted and a viability check performed in a modified Neubauer hemocytometer.

To determine the activity of the peptides against trypomastigotes in vitro, trypomastigotes harvested from Vero cell cultures were incubated for 1 hr at 37 C in DMEM–10% FBS with final concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, and 0 μM of DC-1, DC-2, DC-2R (active lytic peptides), and α-Pi (control nonlytic peptide) were added (Jaynes et al., 1988). The number of motile viable parasites after treatment was counted in a modified Neubauer hemocytometer.

To determine the activity of the peptides against *T. cruzi*-infected Vero cells, Vero cell monolayers were prepared on tissue culture chamber slides (Lab Tek, no. 4804, Naperville, Illinois). Prior to infection, the cells in the 4 chambers on 1 slide were trypsinized, stained for viability using trypan blue, counted in a modified Neubauer hemacytometer, and averaged to obtain an estimate of the average cell count per chamber. The monolayers were washed 3 times with phosphate-buffered saline (PBS) at 37 C. Some monolayers were exposed to a suspension of trypomastigotes in DMEM–10% FBS (1 ml/chamber). The final concentration of trypomastigotes was calculated based on a ratio of 2:1 parasites to Vero cells. The parasites were allowed to internalize within the Vero cells for 24 hr. At this time, a set of *T. cruzi*-infected cultures was fixed and stained with Wright's stain and was designated as the 24-hr control culture. The media from the remaining slides was removed and fresh DMEM–10% FBS alone (control) or containing DC-1, DC-2, DC-2R, or α-Pi (2.5 μM each) were added to the cultures. After a further 24 hr of incubation at 37 C, a set of *T. cruzi*-infected cultures (untreated control and peptide-treated) was fixed and stained with Wright's stain. Media were removed from other cultures, and again, fresh DMEM–10% FBS alone (control) or containing DC-1, DC-2, DC-2R, or α-Pi (2.5 μM each) were added to the cultures. Cultures were incubated (37 C) for a further 24 hr after which they were fixed and stained with Wright's stain. Numbers of parasites/infected cell were determined by counting the total number of intracellular parasites in 200 infected cells/treatment and by dividing by the number of infected cells counted (Jaynes et al., 1988).

To determine the effect of the lytic peptides on the Vero cells themselves, we assessed the effects of low concentrations (2.5 μM) of each peptide on Vero cells alone. Monolayers of Vero cells were prepared on tissue culture chamber slides as above by plating out 10^5 Vero cells/ml (1 ml/chamber) in DMEM–10% FBS. After 24 hr, the cells in several chambers were trypsinized, stained for viability using trypan blue, counted in a modified Neubauer hemacytometer, and averaged to obtain a more accurate estimate of the average cell count/chamber. Other monolayers were washed 3 times with PBS at 37 C, then fresh DMEM–10% FBS alone (control) or containing DC-1, DC-2, DC-2R, or α-Pi (2.5 μM each) was added to the cultures. Cultures were incubated (37 C) for a further 24 hr after which several peptide-treated (representing single exposure of peptides) and untreated (control) cultures were trypsinized, stained for viability using trypan blue, and counted as above. Media were removed from other cultures, and again, fresh DMEM–10% FBS alone (control) or containing DC-1, DC-2, DC-2R, or α-Pi (2.5 μM each) was added to the cultures. Cultures were incubated (37 C) for a further 24 hr after which peptide-treated (representing double exposed cultures) and untreated (controls) were trypsinized, stained for viability using trypan blue, and counted as above.

To determine if the lytic peptides had any toxicity in uninfected AJ mice, 6 AJ mice were given 50 μg (approximately 3 μg/g body weight) of DC-1/mouse/day, and 6 mice were injected with PBS (control group) for 10 consecutive days. Mice were weighed and observed daily for ill effects.

To test the efficacy of DC-1 in reducing parasitemia in AJ mice, 5–6-wk-old female mice were infected by inoculating intraperitoneally 10^5 blood-form trypomastigotes. The AJ mouse is an inbred strain that is very susceptible to the Tulahuen *T. cruzi* isolate producing 100% mortality by 14 days postinoculation (PI). *Trypanosoma cruzi*-infected mice were injected intravenously (i.v.) in the tail vein on days 2, 4, 6, 8, and 10 PI, with 25 μg of DC-1 in PBS (group 1, n = 10), α-Pi in PBS (group 2, n = 10), or PBS alone (group 3, n = 10). Mice were weighed daily and observed for ill effects to day 14 PI. At day 14 PI, mortality was also determined per group. Parasitemia was determined every third day starting on day 3 PI until day 12 PI. Tail vein blood samples (10 μl) from each infected mouse were mixed with 90 μl of 0.83% ammonium chloride to lyse red cells, and then parasites were counted in a modified Neubauer hemocytometer chamber (Postan et al., 1983).

RESULTS

By modifying the primary sequence of cecropin B, we have shown previously that the biological activity of the newly produced peptide may be maintained as long as α-helical amphipathy is retained even though sequence homology is lost (Jaynes et al., 1988). The peptides used in the following experiments (DC-1, DC-2, and DC-2R, and the control nonactive peptide, α-Pi) lacked sequence homology with cecropin B (Fig. 1), but

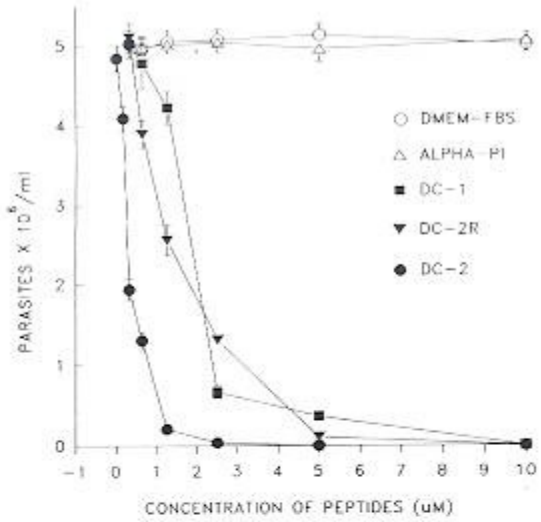

FIGURE 2. Viability of *Trypanosoma cruzi* trypomastigotes after incubation for 1 hr with 3 lytic peptides (DC-1, DC-2, and DC-2R), a nonlytic control peptide (α-Pi), and DMEM–10% FBS control (DMEM–FBS). A significant ($P < 0.01$) reduction from α-Pi and DMEM–FBS levels was observed in the numbers of viable parasites in samples treated with DC-2 and DC-2R, and with DC-1 at peptide concentrations beginning at 0.625 μM, and 1.25 μM, respectively.

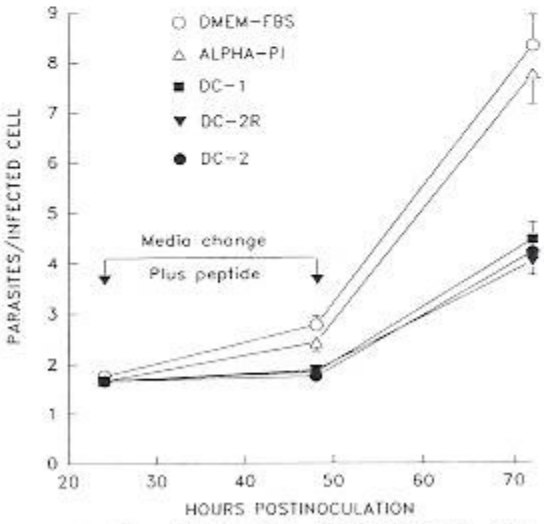

FIGURE 3. Effects of DC-1, DC-2, and DC-2R (2.5 μM each) on *Trypanosoma cruzi*-infected Vero cells with a double exposure of lytic peptide at 24 and 48 hr postinoculation. Parasitosis is expressed as the number of parasites per infected cell. A significant ($P < 0.01$) reduction in the number of parasites per infected cell was found when compared with infected control α-Pi- and DMEM–FBS-treated cultures at 24 and 48 hr posttreatment.

TABLE 1. Effects of peptides on Vero cell numbers after a single (A) or double (B) exposure (24 hr after the first exposure) of the peptides.*

Peptides (2.5 μM)	Vero cell numbers ($\times 10^5$)	
	A	B
DMEM–FBS	2.04 ± 0.21	3.24 ± 0.67
α-Pi	2.36 ± 0.25	5.02 ± 1.45
DC-1	2.14 ± 0.22	4.04 ± 1.47
DC-2R	2.02 ± 0.20	3.36 ± 0.69
DC-2	2.51 ± 0.28	3.16 ± 0.51

* No significant ($P > 0.01$) differences were noted in the numbers of Vero cells between each of the lytic peptides (DC-1, DC-2, and DC-2R) and the control peptide (α-Pi) or media alone (DMEM–FBS). Values represent the mean ± SD Vero cell numbers of 2 counts each from 2 replicates (4 measurements for each treatment).

the biological activity against *T. cruzi* of each of the new peptides was markedly enhanced (by approximately 100 times) over the activity of cecropin B as shown by the in vitro experiments.

All the lytic peptides (DC-1, DC-2, and DC-2R) killed Vero cell-derived trypomastigotes in a dose-responsive manner, and 100% killing was achieved at low molarities (5–10 μM). There was little difference between the 3 peptides (except that DC-2R was approximately 2-fold more effective than DC-1 and DC-2 on a μM basis) in killing trypomastigotes (Fig. 2). Significant reductions (using a 2-tailed Student's *t*-test; $P < 0.01$) in trypomastigote numbers (from control levels) occurred for DC-2 and DC-2R at peptide concentrations less than 0.625 μM and for DC-1 at a concentration of 1.25 μM. There was little doubt as to the difference between those parasites affected by the toxin and those not affected. Those unaffected were morphologically intact, motile, and were still capable of infecting Vero cell cultures (data not shown), whereas those affected appeared completely lysed as demonstrated previously (Jaynes et al., 1988).

In experiments to determine the activity of the peptides against *T. cruzi*-infected Vero cells, there was a significant ($P < 0.05$) reduction in the numbers of intracytoplasmic parasites/infected Vero cell for all the lytic peptides when treated with a single or double exposure when compared to treatment with the nonlytic peptide control, α-Pi (Fig. 3). The significance of the reduction was greater after double exposure compared to single exposure.

There was no significant ($P > 0.05$) reduction in the number of Vero cells when treated with peptides alone, showing little, if any, effect of the peptides at 2.5 μM on Vero cell cultures (Table I).

No ill effects or loss of body weight were observed in any of the 6 AJ mice given 50 μg of DC-1 for 10 days when compared with the age- and sex-matched PBS-treated control group (data not shown).

The time course of development of parasitemias in AJ mice infected with the Tulahuen strain of *T. cruzi* with no interventions is similar that depicted by mice treated with α-Pi and PBS-treated controls (Fig. 4). Typically, parasitemias are detectable by day 6 PI, then rise to a peak (approximately 10^7 parasites/ml of blood) by day 12 PI. Mice begin to die by day 12 PI and mortality reaches 100% by day 14 PI. *Trypanosoma cruzi*-infected mice treated with DC-1 still developed parasitemias that were significantly ($P < 0.05$) reduced at day 12 PI below parasitemias in mice treated with the α-Pi- and PBS-treated con-

trols alone (Fig. 4). Mice injected with DC-1 showed no ill effects or loss of weight as a result of the treatment. By day 14 PI, mortality was 100% in the α-Pi- and PBS-treated groups. Mortality was 0% in the DC-1-treated group by day 14 PI. By day 21 PI (the longest the animals were followed out), 2 of the 10 mice (20%) had died.

DISCUSSION

Lytic peptides fall into 1 of 4 classes based on the arrangement of amphipathic and high positive charge density within the molecule. Cecropins (35 amino acids in length) from the giant silk moth *Hyalophora cecropia* have a mostly hydrophobic C-terminal end and amphipathic N-terminal end (van Hofsten et al., 1985). Magainins (23 amino acids in length) from the African clawed frog *Xenopus laevis* are amphipathic the full length of the molecule (Zasloff, 1987). Melittins (26 amino acids in length) from the honeybee have an amphipathic C-terminal end while the N-terminal half is mainly hydrophobic (Haberman, 1972). Finally, sarcotoxins (slightly larger peptides than cecropins) are purified from the fleshfly *Sarcophaga peregrina* (Okada and Natori, 1985).

We have previously demonstrated that two analogs of cecropin B, SB-37 and Shiva-1, were 50% and 100% effective, respectively, in killing *T. cruzi* trypomastigotes in vitro when used at a concentration of 100 μM (Jaynes et al., 1988). These analogues varied only slightly from cecropin B in amino acid sequence homology, but the charge distribution, amphipathic, and hydrophobic properties of the natural molecule were conserved (Jaynes et al., 1988). In this paper, the analogues were designed to have essentially no sequence homology with cecropin B (Fig. 1) to test whether the lytic properties of cecropin-like peptides were in any way sequence dependent. Our data suggest that lytic properties are sequence independent. However, because the properties of charge distribution, amphipathy, and hydrophobicity present in cecropin B were also conserved in DC-1, DC-2 and DC-2R, but not in α-Pi (nonlytic control peptide), these properties would appear to be important in retaining the lytic property against *T. cruzi* of the analogues. In fact, the lytic properties of the analogs were increased as demonstrated in this paper by the ability of DC-2 at 5 μM, and DC-1 and DC-2R at 10 μM, to be 100% effective in killing trypomastigotes in vitro, about a 10–20-fold increase in activity over SB-37 and Shiva-1, respectively. A significant reduction in trypomastigote numbers from those treated with a control peptide occurred when DC-2 and DC-2R peptide concentrations of less than 0.625 μM, and DC-1 was 1.25 μM, were applied to trypomastigotes (Fig. 2). Furthermore, although these assays were performed over an hour, the majority of the parasites killed occurred within the first few minutes after peptide–parasite contact (results not shown). These results demonstrate that these 3 cecropins are extremely effective at low concentrations in killing *T. cruzi* trypomastigotes.

Why a certain percentage of trypomastigotes are not killed is not known. As a *T. cruzi* isolate has been shown to be made up of several clones with different characteristics (Postan et al., 1983), it is conceivable that those trypomastigotes surviving low concentrations of the lytic peptides are of a single clone of the Tulahuen *T. cruzi* isolate and do not represent a cross section of all the clones making up the isolate. Further studies are underway to elucidate if the peptides are clone specific in their killing within a *T. cruzi* isolate.

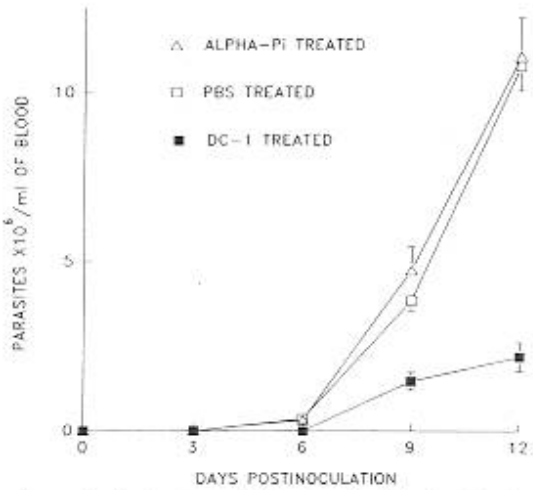

FIGURE 4. Parasitemias in AJ mice inoculated after i.v. injection with DC-1, α-Pi, or PBS (25 μg/mouse/injection) on days 2, 4, 6, 8, and 10 postinoculation with *Trypanosoma cruzi*. A significant ($P < 0.01$) reduction in parasitemia occurred on days 9 and 12 postinoculation in DC-1-treated mice when compared to either α-Pi-treated or PBS-treated mice.

We next treated *T. cruzi*-infected Vero cells with a double exposure of the peptides to determine the effects of the 3 peptides on the amastigote form of *T. cruzi* after internalization (Fig. 3). The marked reduction in numbers of amastigotes after 1 application of the peptide, but particularly after a double application, demonstrates that the peptide activity against the parasite is effective even when it is in an intracellular location. Furthermore, the fact that there was no significant reduction in the numbers of viable Vero cells from control levels after application of the peptides suggests that the peptides are nontoxic to the cells in spite of killing the organism located within (Table 1). How the lytic peptides destroy intracellular amastigote forms without damaging the host cells is unknown. It is thought that the peptide localizes in the host cell membrane by its hydrophobic portion. However, if it is to destroy the intracytoplasmic parasite, it needs to cross host cell membrane to exert its activity on the parasite. One explanation for the peptides' lack of toxicity to mammalian cells is that mammalian cells can repair the lesions induced by the peptides whereas the parasite cannot. We are currently conducting experiments to try to resolve these issues using radiolabeled lytic peptides.

As *T. cruzi* spreads throughout the body by the hematogenous route we determined that to have an effect on parasitemia, the peptide would need to reach the circulation. Because we felt that the peptide would have little chance of being absorbed into the circulation after dosage by another route, e.g., orally, subcutaneously, and intramuscularly, we chose to administer the peptide i.v. We first determined that DC-1 was nontoxic to mice when 50 μg/mouse was administered daily for 10 days. Because

we were unsure that this dosage regimen would be nontoxic in *T. cruzi*-infected mice, we used a reduced regimen of 25 μg of DC-1/mouse injected i.v. for 5 treatments. This regime significantly reduced parasitemia and mortality after 14 days PI (Fig. 4). We are currently assessing different methods of administration, dosage, and the effects of treatment on parasitemia and tissue parasitosis in both acutely and more chronically infected animals to improve the efficacy of these agents.

The mechanism of action of cecropins is not completely known. However, several modes of membrane interaction leading to cytolysis have been proposed. The amphipathic helix may localize on the membrane surface so that the presence of the helix in the head group region disorders the lipid bilayer (Dempsey, 1990). Another proposed mechanism suggests that the peptide oligomers form ion channels in the membrane, which results in osmotically induced lysis (Dempsey, 1990). Others suggest that the lytic peptide causes aggregation of native membrane proteins, which result in the formation of channels or pores (Hui et al., 1990). Finally, it has been suggested that the lytic peptide causes release of phospholipid, which results in osmotically induced lysis (Katsu et al., 1989). Irrespective of the mechanism, the results presented here suggest that some cecropin peptides may be effective agents against both trypomastigotes and intracellular amastigote forms of *T. cruzi*.

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