In vitro cytocidal effect of novel lytic peptides on *Plasmodium falciparum* and *Trypanosoma cruzi*[1]

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ABSTRACT

Plasmodium falciparum and *Trypanosoma cruzi* were killed by two novel lytic peptides (SB-37 and Shiva-1) in vitro. Human erythrocytes infected with *P. falciparum,* and Vero cells infected with *T. cruzi,* were exposed to these peptides. The result, in both cases, was a significant decrease in the level of parasite infection. Furthermore, the peptides had a marked cytocidal effect on trypomastigote stages of *T. cruzi* in media, whereas host eukaryotic cells were unaffected by the treatments. In view of the worldwide prevalence of these protozoan diseases and the lack of completely suitable treatments, lytic peptides may provide new and unique chemotherapeutic agents for the treatment of these infections. —JAYNES, J. M.; BURTON, C. A.; BARR, S. B.; JEFFERS, G. W.; JULIAN, G. R.; WHITE, K. L.; ENRIGHT, F. M.; KLEI, T. R.; LAINE, R. A. In vitro cytocidal effect of novel lytic peptides on *Plasmodium falciparum* and *Trypanosoma cruzi. FASEB J.* 2: 2878–2883; 1988.

Key Words: lytic peptides • in vitro cytocidal effects • malaria • Chagas' disease

THE WORLD HEALTH ORGANIZATION (WHO) has identified malaria and Chagas' disease, which are caused by *Plasmodium sp.* and *Trypanosoma cruzi,* respectively, as posing significant health hazards for 2200 million people, or 46% of the world's population (1). In recent years, chemotherapy has been an important factor in reducing the mortality caused by malaria; however, in many countries, the prevalence of this disease is increasing at a rate of approximately 10 million new cases a year (2). This is due primarily to the development of drug resistance by *Plasmodium sp.* and to vector resistance to insecticides (3). Treatment of Chagas' disease has focused on the utilization of purine derivatives (e.g., allopurine), which disrupt normal nucleic acid metabolism (4). However, the deleterious effects of these drugs are not limited to the parasite, but are also toxic to host cells (5). Therefore, the established chemoetherapeutic treatments are less than completely effective or ideal for these diseases. Vector eradication programs, thus far, have also been unable to control these protozoan-caused diseases, and the development of usable vaccines is not imminent (6). With this recognizable persistent threat to a large proportion of the world's population, a search for novel chemotherapeutic agents based on nontraditional modes of action is important.

Many workers describe peptides or proteins capable of lysing organisms or cells (7–9). The pioneering work conducted by Hultmark et al. and Andreu et al. describes the humoral defense system used by *Hyalophora cecropia,* the giant silk moth, as a protective mechanism against bacterial infection (10–15). Specialized proteins in the insect's hemolymph after induction by either live or heat-killed bacteria are capable of membrane perturbation, which results in bacterial cell lysis. Among these proteins is a type known as the cecropins. The three principal cecropins, A, B, and D, are highly homologous (16), small, basic proteins that each contain a comparatively long hydrophobic region. Their primary mode of action appears to be one of membrane disruption and subsequent lysis owing to the target cell's loss of osmotic integrity (17). Perhaps these types of lytic proteins will also be found to play key roles in providing protection from disease in other organisms. Indeed, similar types of peptides have been isolated from amphibians by Gibson and co-workers (18) and by Giovannini and co-workers, and were designated PGS and $Gly^{10}Lys^{22}$-PGS (19), respectively. Somewhat later, the same peptides were described by Zasloff and called magainins (20).

Although the antibacterial effect of lytic peptides from insects has been well documented, there are no reports of their potential effectiveness against lower eukaryotic cells.

This paper describes the in vitro effects of two synthetic lytic peptides on the limitation of growth and multiplication of *P. falciparum* and *T. cruzi.* One peptide is a closely related derivative of cecropin B, SB-37, with minor changes made in the sequence by substitution of

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Met[11] with Val and addition of an NH_2-terminal, MetPro. These changes were made to plan for subsequent gene construction to produce a CNBr-cleavable repeat peptide. The other is a distinct peptide, Shiva-1, which was designed with significant differences in sequence homology (about 60% different) to test whether or not the lytic properties of cecropin-like peptides are highly sequence-dependent. However, the charge distribution and the amphipathic and hydrophobic properties of the natural molecule were conserved (**Fig. 1**).

METHODS

Peptide synthesis

Cecropin B and the two lytic peptide analogs were synthesized on a Biosearch Sam Two peptide synthesizer using MBHA (4-methyl benzhydral amine) resin with a COOH-terminal amide. All reagents used for these biosyntheses were obtained from Biosearch in San Rafael, CA. After extraction and Sephadex column chromatography, the purity of the peptides was determined by HPLC on a Varian 5000 HPLC unit. A Waters μBondepak C18 column, 8 mm × 10 cm Radial-Pak cartridge, employing the Radial Compression Module-100, was used. HPLC profiles of these peptides indicated a purity of more than 95% (**Fig. 2**). To determine that the syntheses progressed to completion, amino-terminus sequence analysis was performed on all peptides with an Applied Biosystems 470-A gas phase protein sequencer. PTH-derivatized amino acids, generated from the sequencer, were analyzed in a Waters PicoTag system employing a C18 column, 3.8 mm × 15 cm (Waters Nova-Pak). All experiments used peptides that were purified in the manner described previously.

In vitro growth of *P. falciparum*

P. falciparum cultures were derived from Sierra-Leone 1/CDC isolate and maintained in Petri plates under 5% oxygen, 3% carbon dioxide, and 82% nitrogen (21). In vitro growth of the parasites was assessed by [^{3}H]hypoxanthine incorporation (22), and the cultures were enriched for ring stage parasites with the addition of 5% mannitol (23). Twenty-four hours after mannitol treatment, the culture was diluted to a level of 0.4% parasitemia and 3% hematocrit; media that contained 20% human serum and 50 μl/well aliquoted into microtiter plates. SB-37 and Shiva-1 were dissolved in media without serum, dilutions at twice the desired final concentration were prepared, and 50 μl/well were added to the malaria culture.

Radiolabeled hypoxanthine (50 μl/well), at 10 μCi/ml in media containing 10% serum, was added after 24 h of incubation. After an additional 24 h of growth, the parasites were harvested (Cell Harvester, Flow Labs, McLean, VA) and counted in a scintillation counter. Duplicate slides were prepared from unlabeled parasite cultures treated as above with SB-37 at 10, 50, and 100 μM. These preparations were Geimsa-stained and the level of parasitemia and percentage of the parasites in the various developmental stages were determined. To determine hemoglobin release from infected and uninfected red cells, an unsynchronized culture (at 1.5% parasitemia) was harvested by centrifugation at 250 × *g*

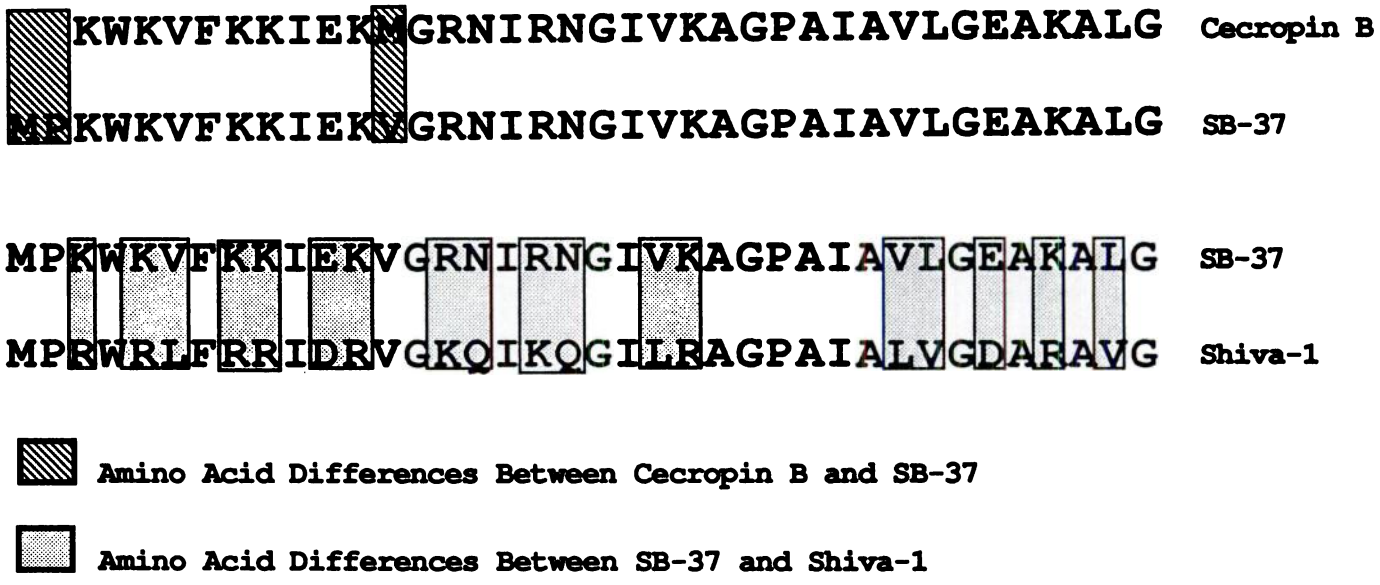

Figure 1. Sequence comparison of the natural cecropin B with the novel lytic peptides SB-37 and Shiva-1. The cecropin B derivative SB-37 was designed to have relatively minor changes from the native cecropin B peptide to facilitate future bioproduction and subsequent purification. Shiva-1, on the other hand, was designed with significant differences in sequence homology to test whether or not the lytic properties of cecropin-like peptides were highly sequence-dependent. The charge distribution, amphipathic, and hydrophobic properties of the natural molecule were conserved.

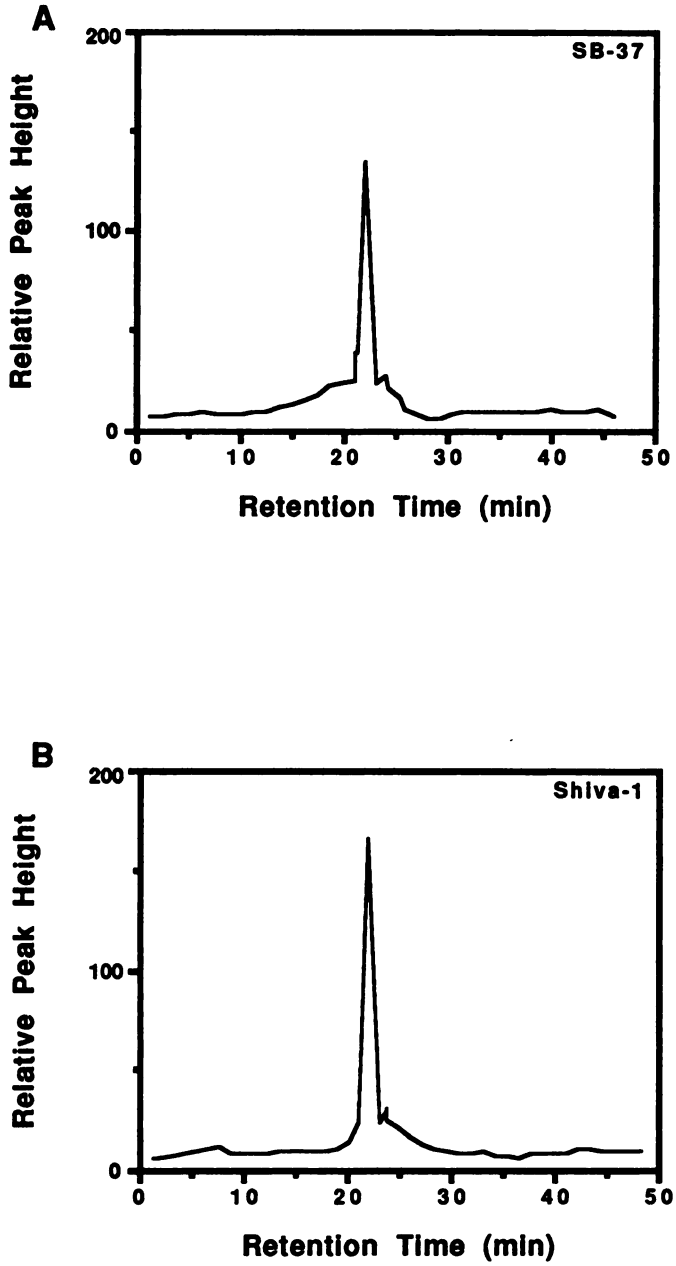

Figure 2. HPLC profiles of lytic peptides prepared via chemical syntheses SB-37 (*A*) and Shiva-1 (*B*). The peptides, purified in the manner described in the Methods section, were subjected to HPLC analysis on a linear gradient prepared from 0.01% TFA/H_2O (*A*) and 0.1% TFA/acetonitrile (*B*). The profiles indicate that the peptides used were more than 95% homogenous.

for 10 min and washed twice with sterile PBS. The final pellet was resuspended to 10% in PBS. The lytic peptides were diluted to 200, 100, and 50 μM with PBS and mixed with equal volumes of washed infected red cells and incubated for 30 min at 37°C. The mixtures were then centrifuged at 250 × *g* for 10 min, and the supernatants were removed and centrifuged at 1200 × *g* for 10 min. The optical density was determined with a Bausch & Lomb spectronic 2000 at 560 nm. Uninfected control red cells were analyzed in a similar manner.

Analysis of *T. cruzi* trypomastigotes

Trypomastigotes (total of 5 × 10^6), harvested from Vero cell culture, were incubated for 1 h at 37°C in MEM + 10% FBS with final concentrations of 100, 50, 25, and 10 μM of SB-37 and Shiva-1 were added. The number of parasites after treatment was determined by counting the motile organisms microscopically with a hemocytometer. For microscopic analysis, *T. cruzi* (1000 trypomastigotes/ml media) was incubated with 100 μM final concentration of Shiva-1 or medium for only 60 min at 37°C. The parasites were centrifuged and the supernatant was discarded. The parasite pellet was fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 15 min. The suspension was observed at 100% magnification with Nomarski differential interference optics.

Culture of *T. cruzi*-infected Vero cells

Vero cell monolayers were cultured for 24 h in eight chamber microscope slides with 100 cells/cm^2 in RPMI 1640 with 10% fetal bovine serum. The monolayers were infected with *T. cruzi* cell culture-derived trypomastigotes at a ratio of two parasites per Vero cell. The parasites were allowed to internalize within the Vero cells for 24 h. At this time, a set of slides was fixed and stained with Wright's stain and was designated as the 24-h control culture. The media from the remaining slides were removed and fresh media (control) or media containing SB-37 or Shiva-1 (100 μM each) were added to the cultures. These cultures were incubated for an additional 24 and 48 h, after which they were fixed, stained, and counted.

Cultures receiving a second exposure were treated by removing the media after the first 24 h of incubation, and by adding fresh media only or media containing 100 μM SB-37 and culturing for an additional 24 and 48 h. Numbers of parasites per infected cell were determined by counting the total number of intracellular parasites in no fewer than 200 infected cells and by dividing by the number of infected cells counted.

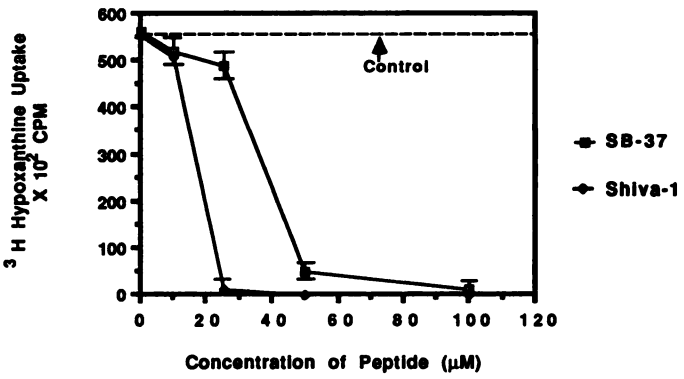

Figure 3. Effect of SB-37 on the in vitro growth of *P. falciparum*. A highly significant reduction ($P \geq 0.01$) was observed in the amount of [^{3}H]hypoxanthine incorporated by all treated cultures when compared with nontreated controls ($n = 3$).

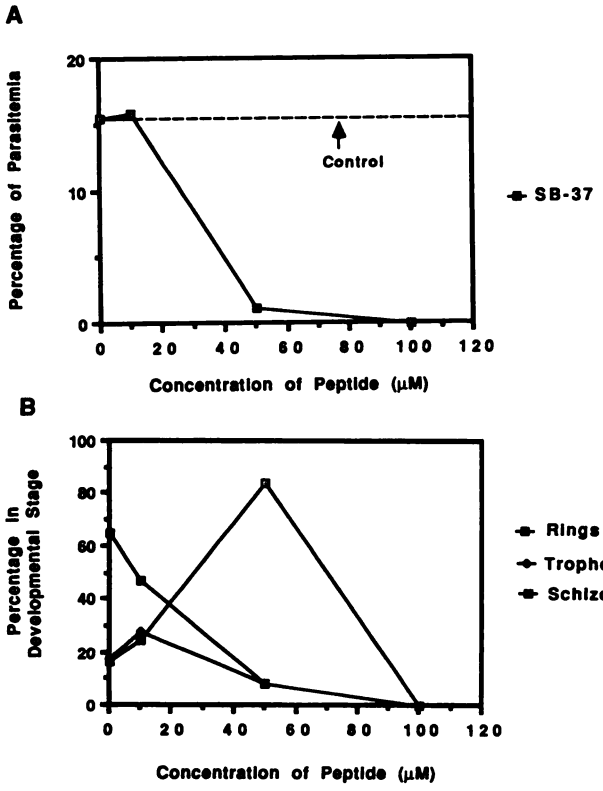

Figure 4. The effect of lytic peptides on the level of parasitemia and morphological stages of *P. falciparum* in human erythrocytes.

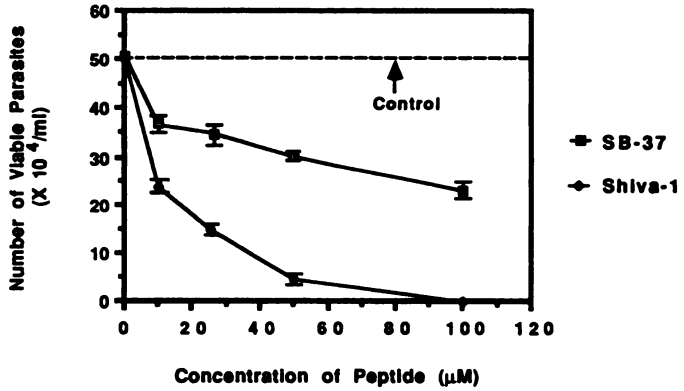

Figure 5. Viability of trypomastigotes after incubation with SB-37 and Shiva-1. A highly significant reduction ($P \geq 0.01$) was observed in the number of intact parasites in all samples treated with peptide when compared with nontreated controls ($n = 4$). A signficant reduction occurred at all concentrations of peptide tested.

RESULTS AND DISCUSSION

Human erythrocytes were infected with *P. falciparum* and exposed to various concentrations of SB-37 and Shiva-1, as described previously, and the uptake and incorporation of [^{3}H]hypoxanthine (22) were used as a direct measure of the in vitro growth of the parasites (before this, it had been established that there was no observable difference between the activity of cecropin B and SB-37, unpublished results). Uptake of [^{3}H]hypoxanthine was significantly diminished in the treated cultures when compared with untreated control cultures. The reductions observed were dose dependent for both of the lytic peptides. Shiva-1 was found to be approximately twice as effective (on a μM basis) as SB-37 in limiting the growth of the parasites (**Fig. 3**). The peptides caused no significant increase in lysis of either uninfected or infected erythrocytes (data not shown).

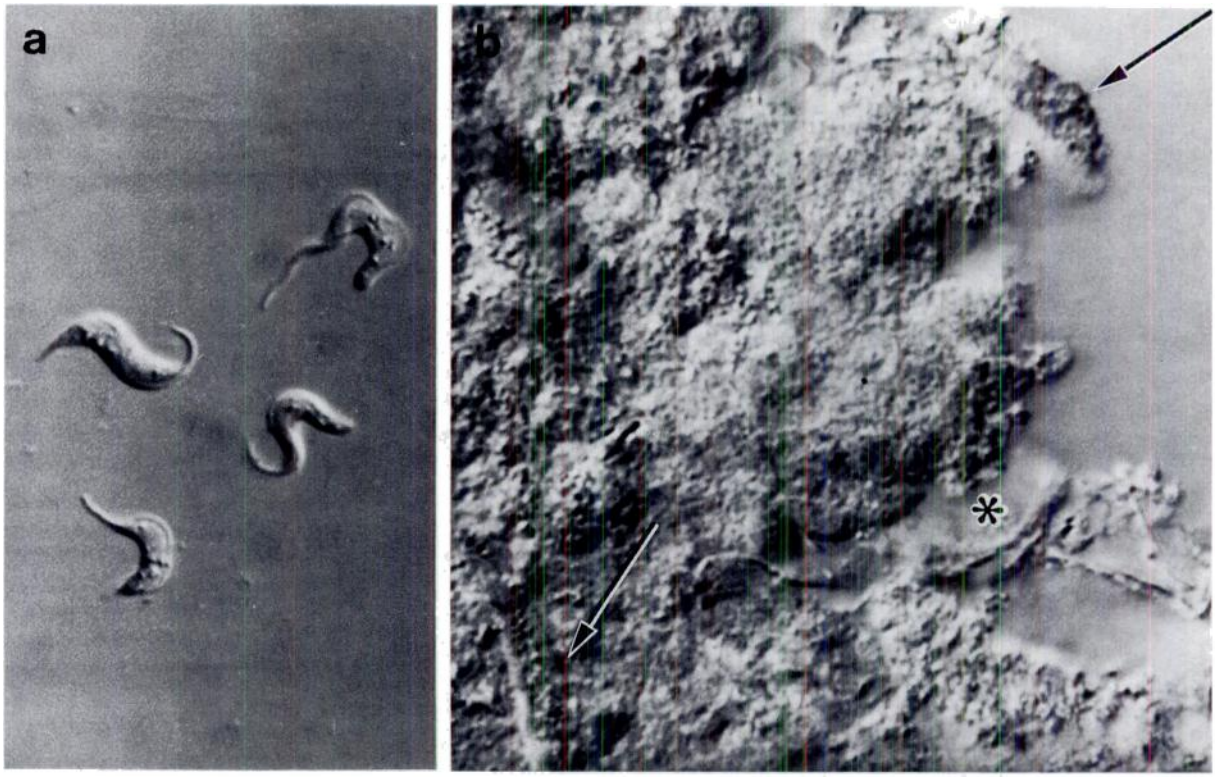

Figure 6. Microscopic analysis of the effects of *a*) media only and *b*) Shiva-1 (100 μM) on *T. cruzi* in vitro. The arrows indicate recently lysed trypomastigotes; the general shape of the parasite is still observable. The asterisk points out clumps of flagellar material.

An irrelevant 22 amino acid peptide, synthesized in the same manner as described above and at similar concentrations, was found to be inactive in reducing the uptake of [^{3}H]hypoxanthine by infected erythrocytes.

To ascertain the effect of SB-37 on the developmental stages of *P. falciparum*, infected human erythrocytes were cultured in the presence of three different concentrations of SB-37 (10, 50, and 100 μM, respectively). After 24 h of exposure, duplicate blood smears were microscopically examined. The percentage of parasitized cells and the distribution of the various developmental stages of the organism were determined. As the SB-37 concentration is increased, the level of host cell parasitemia is decreased; the most dramatic reduction occurs at the 50 μM concentration of lytic peptide (**Fig. 4*A***). Also, there is a concomitant increase in the number of the remaining parasites at the ring stage of development, which suggests that as the concentration of SB-37 is raised to 50 μM, there is an arrest in the life cycle of the organism at this developmental stage (Fig. 4*B*), or, alternatively, that the peptides are least effective against cells that contain this early developmental form. At 100 μM concentration of lytic peptide, there was no detectable parasitemia.

Similar effects of the lytic peptides were observed on the protozoan *T. cruzi*. Trypomastigotes, harvested from Vero cell culture, were exposed to various concentrations of SB-37 and Shiva-1 for 1 h at 37°C and were found to be killed in a dose-responsive manner.

However, under these conditions, Shiva-1 was approximately 10-fold more effective (on a μM basis) than SB-37 in destroying the trypomastigotes. The number of intact parasites was also dramatically reduced in treated samples when compared with untreated controls (**Fig. 5**), with many of the remaining parasites appearing lysed or damaged (**Fig. 6**). However, the intact trypomastigotes that remained in the treated samples were infectious when exposed to Vero cells, which established a reduced level of parasitemia (parasites within Vero cells; data not shown).

To determine the effect of SB-37 and Shiva-1 on *T. cruzi* after internalization of the parasite, *T. cruzi*-infected Vero cells were treated with a single exposure of the peptides. The numbers of parasites per infected cell were significantly decreased 24 h after exposure to the lytic peptides. However, 48 h after treatment, there was no significant difference between the number of parasites per infected cell in treated samples and untreated infected control cultures (**Fig. 7*A***). This result can be accounted for by the multiplication of surviving parasites. A second exposure of *T. cruzi*-infected Vero cells to the same concentration of SB-37 showed a more marked reduction in the numbers of parasites per infected cell after 24 and 48 h compared with cultures that received only a single treatment of SB-37 (Fig. 7*B*). There was no observable reduction in the numbers of control Vero cells that were treated with either of the peptides.

This report has focused on the in vitro effect of novel lytic peptides on two pathogenic protozoa. Surprisingly, Shiva-1, the peptide that is the most divergent from

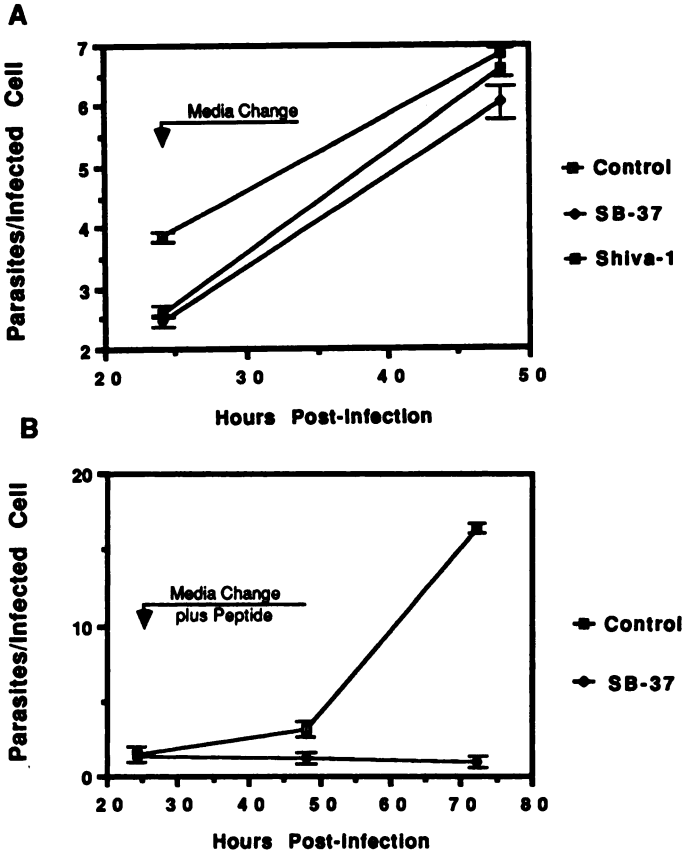

Figure 7. Effects of SB-37 and Shiva-1 (100 μM each) on *T. cruzi*-infected Vero cells with single exposure (*A*) and double exposure of lytic peptides (*B*). Parasitemia is expressed as the numbers of parasites per infected cell. A highly significant reduction ($P \geq 0.01$) in the numbers of parasites per infected cell was found when control nontreated infected cultures ($n = 5$) were compared with 24-h peptide cultures treated with peptide ($n = 7$) (*A*). However, no significant differences were noted in the numbers of intracellular parasites after 48 h in cultures that received a single exposure to the lytic peptides. When infected monolayers received a second exposure (*B*) to SB-37 or fresh media at 24 and 48 h, a highly significant reduction ($P \geq 0.01$) in the number of intracellular parasites was observed in the cells treated with SB-37 ($n = 14$) compared with infected control cultures ($n = 14$) at all time periods.

the sequence of the parent molecule, is also the most biologically active against these parasites (Shiva-1 is also more active on bacteria than either cecropin B or SB-37; to be reported elsewhere). However, the charge distribution, amphipathic, and hydrophobic properties of the natural cecropin B lytic peptide were conserved in Shiva-1, and thus it would seem that these physical characteristics are the parameters to be judiciously controlled in the design of new lytic peptide analogs.

At present, the exact mechanism of action of these peptides is unknown. The data suggest, however, that alterations of the eukaryotic host cell membrane that are caused by these parasites may increase the tendency of infected cells to undergo peptide-induced lysis. Additional studies, using different synthetic peptide analogs, are currently under way and should provide information on how the lytic effects are exerted.

Because of the global significance of protozoan diseases, it is of the utmost importance to develop and evaluate novel chemotherapeutic agents for antiprotozoan activity.

Our report illustrates the effectiveness of synthetic lytic peptides in limiting the level of infection by *P. falciparum* and *T. cruzi* in vitro. Further experimentation is under way to determine if the use of such lytic peptides can be extended to the clinical treatment of these and other recalcitrant diseases (J. M. Jaynes, G. W. Jeffers, G. R. Julian, K. L. White, and F. M. Enright, manuscript submitted). FJ

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