PROTEIN STRUCTURE AND FOLDING:

Dermaseptins from *Phyllomedusa oreades* and *Phyllomedusa distincta* : ANTI-TRYPANOSOMA CRUZI ACTIVITY WITHOUT CYTOTOXICITY TO MAMMALIAN CELLS

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ANTI-*TRYPANOSOMA CRUZI* ACTIVITY WITHOUT CYTOTOXICITY TO MAMMALIAN CELLS*

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Amphibian skin secretions are known as a rich source of biologically active molecules, most of which are alkaloids, biogenic amines, and peptides. Dermaseptins are a class of antimicrobial peptides present in tree frogs of the *Phyllomedusa* genus. They are cationic molecules of 28–34 residues that permeabilize the membrane of Gram-positive and Gram-negative bacteria, yeasts, and filamentous fungi, showing little or no hemolytic activity. This work reports the isolation, molecular mass analysis, primary structure determination, biological activities, and potential therapeutic applications of an antimicrobial peptide found in the skin secretion of *Phyllomedusa oreades,* which is a newly described amphibian species endemic of the Brazilian savanna. DS 01 is a 29-residue-long peptide with a molecular mass of 2793.39 Da showing antibacterial properties against Gram-positive and Gram-negative bacteria in the range of 3–25 μM. Anti-protozoan activity was investigated using *T. cruzi* in its trypomatigote and epimastigote forms cultivated in both cell culture and blood media. Within 2 h after incubation with DS 01 at a final concentration of ~6 μM, no protozoan cells were detected. Two synthetic dermaseptins, described previously by our group and named dermadistinctins K and L (DD K and DD L), also had their anti-*Trypanosoma cruzi* activity investigated and demonstrated similar properties. Toxicity of DS 01 to mouse erythrocytes and white blood cells was evaluated by means of atomic force microscopy and flow cytometry. No morphological alterations were observed at a lytic concentration of DS 01, suggesting its therapeutic value especially as an anti-*T. cruzi* agent to prevent infections during blood transfusion.

The synthesis of gene-encoded antimicrobial peptides as an immune strategy is widely used in nature and has been conserved during evolution. Antimicrobial peptides have been produced by a number of Gram-positive and Gram-negative bacteria for billions of years to hinder the proliferation of other microorganisms that are closely related or confined within the same ecological niche (1–6). In vertebrates, these peptides are synthesized by lymphoid cells, granular epithelial cells of the skin, respiratory and urogenital tracts, and also by the gastrointestinal system (7–10). A defense strategy against invading microorganisms is well documented in amphibians and consists of the biosynthesis of a large number of small polycationic peptides integrated with the cell-mediated immune system.

Several antimicrobial peptides have been isolated from anuran species, and among the most studied are the ones from the dermaseptin family. Dermaseptins are molecules found in the skin secretion of tree frogs from the *Phyllomedusinae* subfamily. They consist of a characteristic polypeptide chain of 28–34 amino acids with 3–6 lysine residues and a very conserved tryptophan residue in the third position from the N terminus (10, 11). These peptides exert a lytic action on bacteria, protozoa, yeast, and filamentous fungi at micromolar concentrations. Unlike polylysines, the dermaseptins show little or no detectable hemolytic activity (11, 12).

The capacity of the dermaseptins to discriminate between mammalian and microbial cells has been known for more than a decade (13, 14), and it is mainly due to the amino acid composition of the peptides and to the differences encountered in the physical-chemical properties of membranes (13–20). Besides the phospholipid content, the cell lysis mediated by amphiphilic antimicrobial peptides such as the dermaseptins is attenuated by increasing amounts of membrane cholesterol (21, 22). The membrane of the *Trypanosoma cruzi* trypomastigote forms was demonstrated to be composed mostly of phosphatidylcholine and to a lesser extent phosphatidylethanolamine (23). There are no published data regarding the presence of cholesterol in *T. cruzi* bloodstream forms, although it has been shown to exist in high molar ratios in *Trypanosoma brucei* (24). Since *T. cruzi* cannot synthesize cholesterol, its source is restricted to the growth medium extraction (25), whereas in *T. brucei*, it is derived from host low density lipoprotein particles (26). The potential susceptibility of *Trypanosomatidae* to cationic amphiphilic peptides was stated by the discovery that apolipoprotein-A, which shares many features

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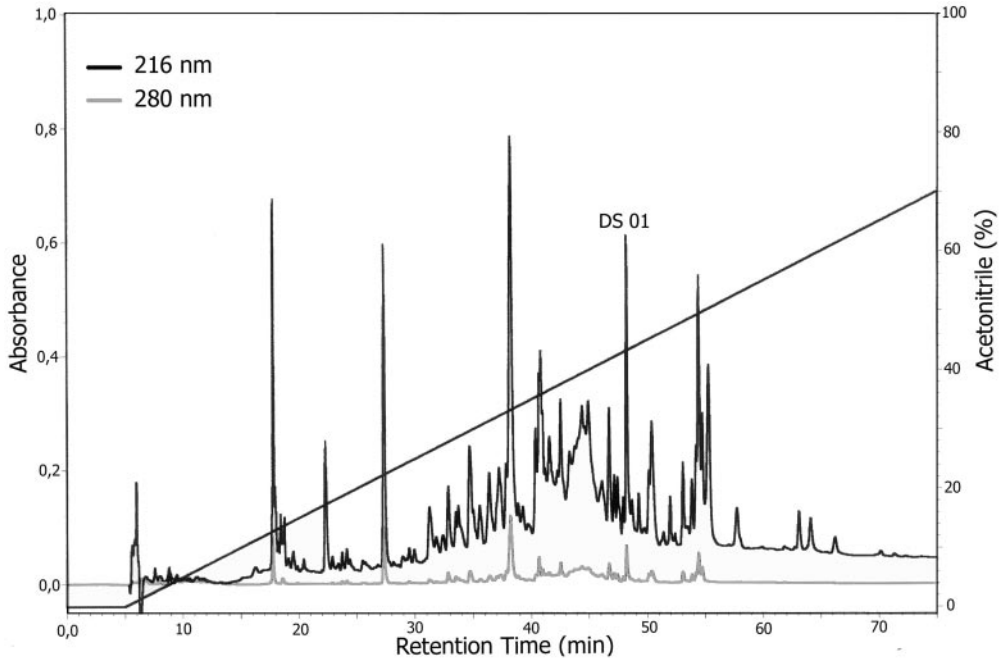

FIG. 1. **Crude skin secretion initial fractionation.** The skin secretion of *P. oreades* was loaded onto a C_{18} Vydac 218 TP 510 (10 × 250 mm) column and equilibrated with 0.1% trifluoroacetic acid. The peptide elution was performed using an acetonitrile with 0.1% trifluoroacetic acid solution with 2.5 ml/min flow rate. The absorbance was monitored simultaneously at two wavelengths (216 and 280 nm).

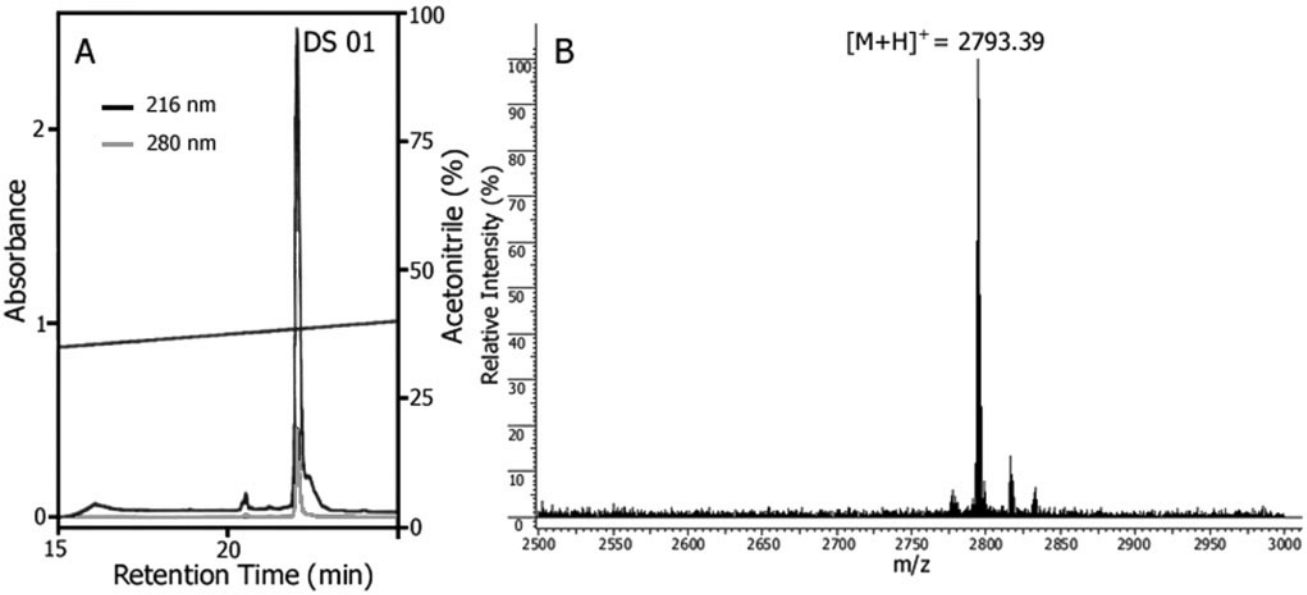

FIG. 2. **Further DS 01 purification.** *A*, DS 01 fraction after analytical RP-HPLC using a C_{18} Vydac 218 TP 54 (250 × 4.6 mm) column. Peptide fractions were monitored at 216 and 280 nm. *B*, MALDI-TOF/MS spectrum showing the molecular mass of DS 01. Sodium and potassium adducts were also detected in the experiment.

with antimicrobial peptides, has a trypanocidal effect on *T. brucei* (27).

Ultimately, there has been a growing interest in using dermaseptins as therapeutical agents in diseases caused by protozoa (28–30). Indeed, chemically modified dermaseptins have already been shown to effectively lyse the intra-erythrocytic form of *Plasmodium falciparum*, leaving the host cell unharmed (30). However, there is still a long way before dermaseptins can be considered as an actual and efficient therapy against protozoa infections since their interactions with mammalian blood cells are still poorly investigated.

The present work describes the isolation, molecular mass determination, and complete amino acid sequencing of a new dermaseptin, named DS 01,[1] isolated from the skin secretion of the recently identified species *Phyllomedusa oreades* (31). Due to the observed lytic action against a wide range of microorganisms, we also investigated the activity of DS 01 against *T. cruzi*. This protozoan pathogen is the causative agent of Chagas' disease, which affects 16–18 million people in Central and

[1] The abbreviations used are: DS 01, dermaseptin 01; DD K, dermadistinctin K; DD L, dermadistinctin L; HPLC, high pressure liquid chromatography; RP-HPLC, reverse-phase HPLC; MALDI-TOF/MS, matrix-assisted laser desorption/ionization-time of flight/mass spectrometry; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; AFM, atomic force microscopy.

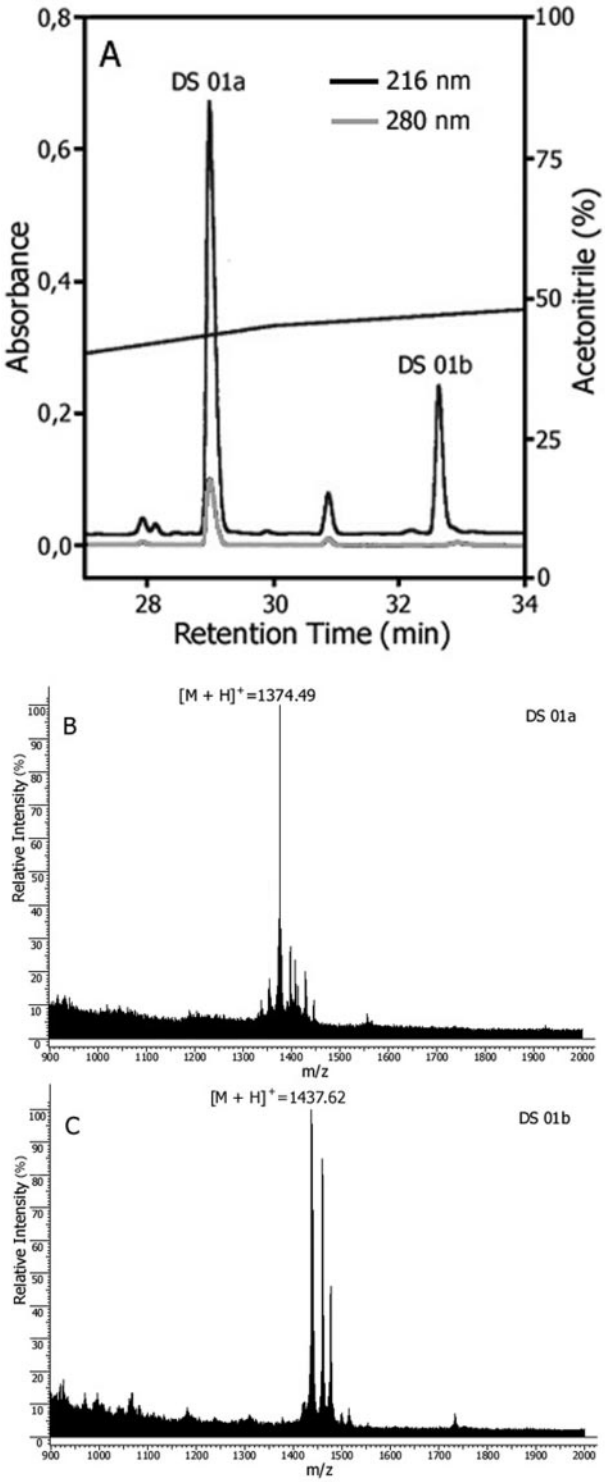

FIG. 3. **Analysis of the digested fractions.** *A*, DS 01 fraction after digestion with endoproteinase Glu-C yielding two fragments (*DS 01a* and *DS 01b*). The separation of the fragments was performed in a Vydac 218 TP 54 (250 × 4.6 mm) column with a three-step gradient (0–30% in 30 min, 30–40% in 20 min, and 40–50% in 10 min) of acetonitrile in 0.1% trifluoroacetic acid/water at a constant flow rate of 1.0 ml/min. *B*, MALDI-TOF/MS mass spectrum showing the molecular mass of the fragment DS 01a ($[M+H]^+$ = 1347.49 Da). *C*, mass spectrum of the fragment DS 01b ($[M+H]^+$ = 1437.62 Da).

South America (32). About 20,000 new cases are reported each year, all due to contaminated blood transfusions (33), thus creating a strong demand for effective trypanocidal agents that are non-toxic for blood cells and have little or no side effects.

The interaction of DS 01 with blood cells is investigated in a more comprehensive way than the conventional hemolysis assay. Possible cytotoxic effects on white blood cells and platelets were investigated by the use of flow cytometry, complemented by morphological observations under light microscope. Atomic force microscopy was also used to investigate any detectable morphological alterations of mice erythrocytes exposed to dermaseptins at high concentrations.

EXPERIMENTAL PROCEDURES

Amphibians—Frog skin secretion (crude extract) was obtained from adult specimens of *P. oreades*, a new specimen of the *Phyllomedusinae* subfamily captured in Serra da Mesa (Goiás), Central Brazil and described by Brandão (31). The Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis (IBAMA) license number was 097/96-DIFAS, and the process number was 0637/91A.C.

Peptide Purification—Frog secretion was obtained by mild electric stimulation of the granular skin glands of *P. oreades* and freshly collected in distilled water as a soluble extract. The extract was filtered by gravity through filter paper, frozen, and lyophilized (Centrivap Concentrador, Labconco, Kansas City, MO). Peptide separation was performed by injecting 250 μl of the crude extract to a semipreparative Vydac 218 TP 510 (Hesperia, CA) reverse-phase chromatographic column, C_{18}, 10 μ (10 × 250 mm) in HPLC[1] system (Class LC-10VP from Shimadzu Corp., Kyoto City, Japan). Peptides were purified by using a linear gradient from 0 to 70% acetonitrile containing 0.1% trifluoroacetic acid for 75 min. RP-HPLC experiments were monitored in two different wavelengths (216 and 280 nm), and fractions were collected manually and subsequently lyophilized. The isolated fractions were submitted to a second chromatographic step using a Vydac 218 TP 54, C_{18}, 5 μ (0.46 × 25 cm) column with optimized gradients of acetonitrile in 0.1% trifluoroacetic acid over 40 min. Sample purity and mass analyses were made by MALDI-TOF/MS.

Enzymatic Digestion—The purified peptide was incubated with 0.5 μg/μl endoproteinase Glu-C (Roche Molecular Biochemicals) for 18 h at 25 °C. The buffer used was 25 mM ammonium carbonate at pH 7.8 (34, 35).

Molecular Mass Determination, N-terminal, and C-terminal Amino Acid Sequencing—The molecular masses of DS 01 and of the two fragments produced by endoproteinase Glu-C from digestion were determined by MALDI-TOF/MS (Voyager DE-STR, Applied Biosystems, Foster City, CA) using close external calibration under reflector mode. Approximately 20 nmol of lyophilized peptide was dissolved in Milli-Q water, mixed to a saturated solution of α-cyano-4-hydroxycinnaminic acid, spotted on a MALDI sample plate, and dried at room temperature. Amino acid sequencing was performed by the automated Edman degradation method on a PPSQ-23 protein peptide sequencer (from Shimadzu Corp.). C-terminal ladder sequencing was also performed on DS 01 using Perseptive Biosystems Sequazyme™ C-peptide kit and MALDI-TOF/MS.

Sequence Comparison—Dermaseptin DS 01 sequence alignment and similarity searches were performed using the FASTA 3 program on the Expasy Molecular Server (www.expasy.ch). Secondary structure prediction was performed using SOPMA, also at Expasy.

DS 01 and Dermadistinctins K (DD K) and L (DD L) Solid-phase Synthesis—Amidated DS 01 was synthesized on a Pioneer Synthesis System from Applied Biosystems. Fmoc amino acids and Fmoc-PAL-poly(ethyleneglycol)-polystyrene (FmocPAL-PEG-PS) resin were also purchased from Applied Biosystems. The resin leaves the amidated C-terminal after peptide extraction. The cleavage and deprotection procedures, after chemical synthesis, involve the addition of three scavengers, anisole, ethanedithiol, and thioanisole, in 100% trifluoroacetic acid (2:3:5:90, v/v). For 0.5 g of resin, 5 ml of cleavage mixture is needed, and reaction time lasted around 2 h. The resin was then washed with cold diisopropilic ether, causing peptide precipitation. 0.1% trifluoroacetic acid and 50% acetonitrile were then used to recover the crude synthetic peptide. To purify the peptide, the usage of a preparative C_{18} column (Vydac 218 TP 1022) on an HPLC system was required. Molecular mass and sample purity were checked by MALDI/TOF-MS.

DD K and DD L were synthesized using T-Boc amino acids as reported by Batista *et al.* (9). Purification of these two synthetic peptides was performed by RP-HPLC in a Vydac 218 TP 54 analytical column, and the purity was checked by MALDI-TOF/MS.

Antimicrobial Activity—Six bacterial strains were used to investigate the DS 01 antimicrobial activity. *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25992 were purchased from American Type Culture Collection. *Acetobacter calcoaceticus* wild type, *E. coli* MR, and *S. aureus* MRSA (methy-

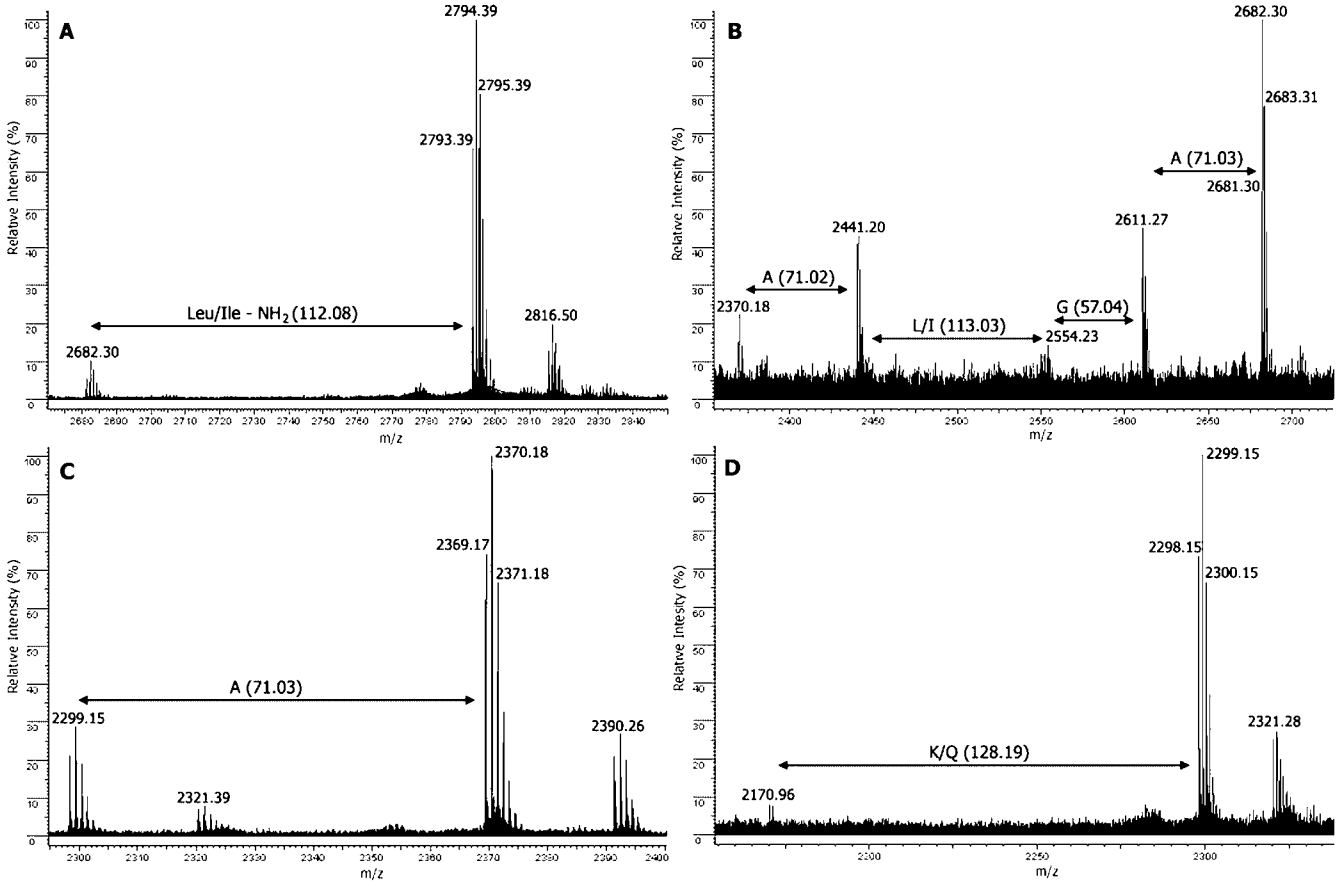

FIG. 4. **MALDI-TOF/MS spectra of the carboxypeptidase Y ladder sequencing of DS 01.** Mass intervals (indicated in *parentheses*) obtained from the difference of the most intense isotopic ions of each segment showing the last 7 C-terminal residues are shown.

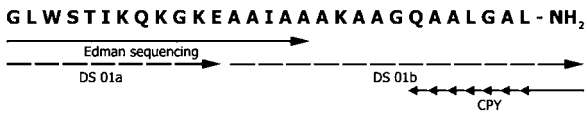

FIG. 5. **The complete amino acid sequence of DS 01.** The *long arrow* indicates automatic Edman sequencing of the intact DS 01. *Fragmented arrows* indicate endoproteinase Glu-C peptides sequenced by Edman degradation. *Small arrows* pointing to the *left* represent carboxypeptidase Y (*CPY*) ladder sequencing using MALDI-TOF/MS.

cilin/oxacylin resistant) were isolated from patients of the Laboratório SABIN de Análises Clínicas/Brasília-DF, Brasília, Brazil. Microorganisms were grown in stationary culture at 37 °C and then transferred to the Mueller-Hinton liquid medium (National Committee for Clinical Laboratory Standards (NCCLS)-approved standard M100-S9) in which the tests were performed (36).

The bacterial liquid growth inhibition assay was performed as described by Bulet *et al.* (37). Synthetic DS 01 was dissolved and diluted 8-fold in Mueller-Hinton broth. The highest peptide concentration used for the assay was 64 μg/ml. The initial inoculum was $\sim 1.8 \times 10^7$ colony forming units/ml. The final volume was 200 μl, 50 μl of the peptide test in broth, 150 μl of the inoculum in Mueller-Hinton. The experiment was carried on in stationary culture at 37 °C, and the spectrophotometric readings were performed 12 h after incubation. The minimal inhibitory concentration, as introduced by Park *et al.* (38), was measured from optical density (A_{595}) and is the result of three independent measurements.

Conventional antibiotics had their minimum inhibitory concentrations determined against the six experimental bacterial strains by automated biochemical analysis (Vitek, bioMériuex Inc.). Initial inoculum was adjusted to match 0.5 in the McFarland scale. The Mueller-Hinton broth was the chosen medium.

Trypanocidal Activity—Bloodstream forms of *T. cruzi* (Y strain) were harvested from BALB/c mice 7–10 days after the infection with 10^5 parasites. The infected blood was diluted to a final concentration of 5×10^5 parasites/ml with phosphate-buffered saline. The biological tests were performed as described previously (39). The plates were incubated at 37 °C followed by counting the parasites under a light microscope. Disappearance or immobility of parasites was judged to indicate drug action. The experiment was done in triplicate.

Assays for Hemolytic Activity, Erythrocyte, and Leukocyte Count—Investigation of the hemolytic activity of synthetic DS 01 was done as reported by Bignami (40). The peptide was diluted serially and incubated with human red blood cells at 37 °C for 30 min. After a $900 \times g$ centrifugation for 10 min, the absorbance of the supernatant phase was measured by spectrophotometry (Microplate Reader 3550, Bio-Rad) at 567 nm. Maximum hemolysis was determined by adding distilled water to a red blood cell sample.

Leukocyte counts were done as described by Ben-Ezra *et al.* (41) and Hove *et al.* (42). Aliquots of human fresh blood were collected, preserved in heparin as anticoagulant, and incubated with synthetic DS 01 at 37 °C during 4 h. The final peptide concentration in solution was 128 μg/ml. Total leukocytes and platelets counts were done in 200 μl of blood using Cell-Dyn 3500 SC/SL flow cytometry automated hematology analyzer (Abbott Laboratories, Abbott Park, IL). The data obtained are the result of two independent measurements. Aliquots of blood subjected to incubation with synthetic DS 01 were collected and stained by routine Wright's eosin methylene blue. Morphological monitoring of white blood cells was done by light microscopy.

Atomic Force Microscopy—Mouse erythrocytes were freshly prepared as described by Aboudy *et al.* (43). Aliquots of 3 ml of freshly prepared erythrocytes were mixed with an anticoagulant (EDTA) and washed with isotonic phosphate-buffered saline, pH 7.4 (phosphate-buffered saline), until the color of the supernatant turned clear. The washed erythrocytes were then diluted to a final volume of 20 ml with the same buffer. Peptide samples diluted in phosphate-buffered saline (12.4 μg/ml) were incubated at 37 °C for 30 min with the cell suspension in a total volume of 190 μl. Another erythrocyte sample was treated for 5 min with 0.05% Triton X-100 as positive control to indicate topographic alterations. Control samples were made in the absence of both peptide and Triton X-100.

An aliquot (4 μl) of each sample was manually spread onto a polished microscope glass slide to create a blood film, which was rapidly fixed by

TABLE 1
Amino acid sequence and similarity analysis

Amino acid sequence of DS 01, its alignment with Dermaseptin V and Adenoregulin precursor showing 59 and 76% of identity, respectively. Bold letters represent identical residues, and underlined letters represent conservative substitutions. The sequences of DD K and DD L, both isolated from *P. distincta* are also shown for comparison purposes.

Peptide	Sequence
	1 5 10 15 20 25 30
DS 01	**G L W S T I K Q K G K E A A I A A A K A A G Q A A L G A L-NH$_2$**
DS V	**G L W S** K **I K** T A **G K S** V **A** K **A A A K A A V** K **A** V T N **A** V
ADR Precursor	**G L W S** K **I K** E V **G K E A A** K **A A A K A A G** K **A A L G A** V
	50 60 70
DD L	A L W K T L L K N V G K A A G K A A L N A V T D M V N Q
DD K	G L W S K I K A A G K E A A K A A A K A A G K A A L N A V S E A V

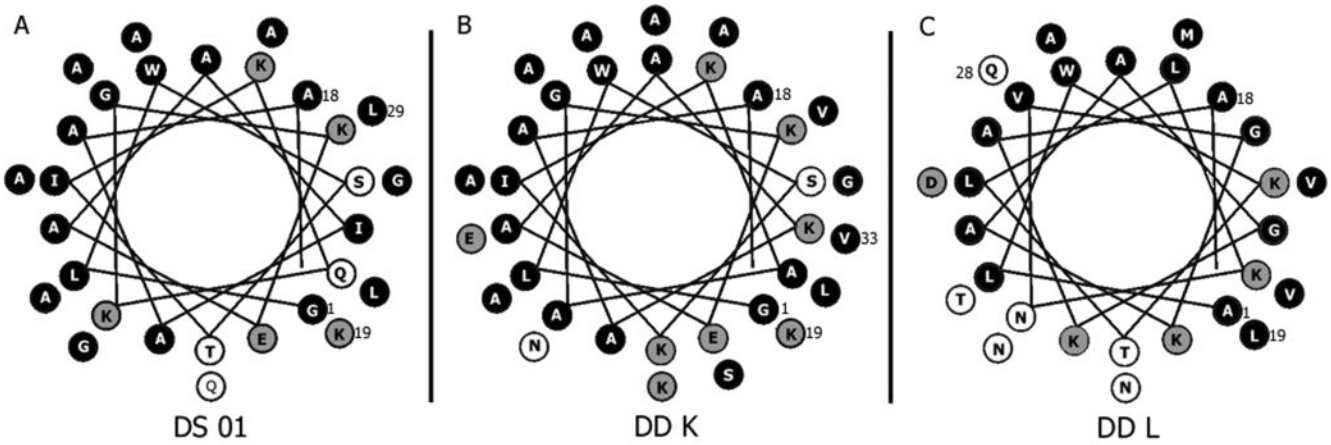

FIG. 6. **Helix-Wheel plots.** *A*, peptide DS 01 showing the amphiphilic character of the molecule. *B*, synthetic peptide DD K. *C*, synthetic peptide DD L. The residues shown in *black*, *gray*, and *white* have hydrophobic, charged and polar side chains, respectively. The first and last residues of each turn have the corresponding *number* written at their *side*.

TABLE II
Antibacterial activity of the dermaseptin DS 01

Experiments performed in triplicates.

Microorganisms	MIC[a]				
	DS 01	Ampicillin	Imipinem	Ceftazidime	Gentamicin
			(μM)		
S. aureus ATCC 25923	3.20	<10.38	107.02	>14.63	4.17
P. aeruginosa ATCC 27853	6.42	>83.01	>428.09	>58.54	8.34
E. coli ATCC 25922	6.42	<10.38	<13.37	<14.63	2.08
A. calcoaceticus wt	25.67	>83.01	<13.37	<14.63	>33.37
E. coli wt (MR)	6.42	>83.01	<13.37	>29.67	4.17
S. aureus (MRSA)	12.83	NDA[b]	NDA	NDA	NDA

[a] MIC, minimal peptide requerid for total inhibition of cell growth in liquid medium.
[b] NDA, no detectable activity.

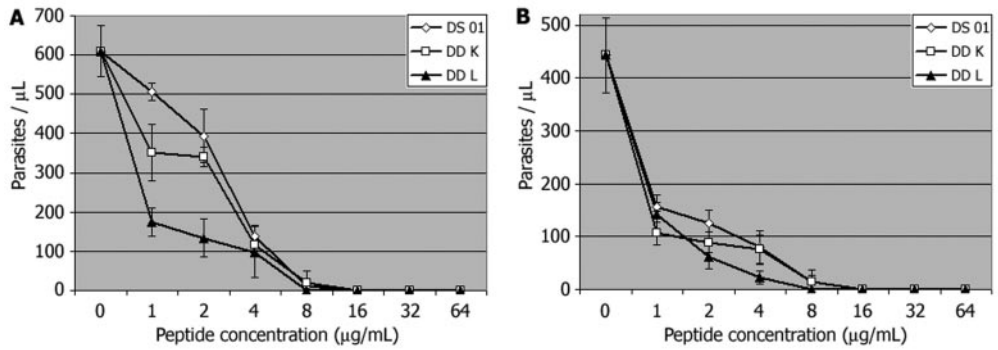

FIG. 7. **Anti-*T.cruzi* bioassays.** Lytic activity of DS 01, DD K, and DD L against *T.cruzi* trypomastigote forms grown in blood media at different incubation times, 2 (*A*) and 6 h (*B*).

air drying. The film was examined under the optical microscope, and regions with a single layer of red blood cells were marked using a lens-mounted inking device.

Atomic force microscopy (AFM) was performed in air on the blood film using a AFM (Explorer model, TopoMetrix, Santa Clara, CA) in contact mode. The glass slide carrying the blood film was mounted onto the XY scanner of the AFM, and the integral camera was used to locate the regions of interest. Contact mode silicon tips with a spring constant of ~0.3 newtons/m were used. The force applied to the sample during imaging was typically 15 nanonewtons. Repeated scanning of the same

red blood cells confirmed that no physical damage occurred during AFM. AFM images were processed and analyzed using the software SPMLab Version 4.0 (TopoMetrix).

RESULTS

Peptide Purification—The crude skin secretion fractionation obtained by RP-HPLC yielded more than 50 fractions (Fig. 1). The DS 01 fraction was submitted to further purification by using analytical RP-HPLC (Fig. 2*A*). MALDI-TOF monoisotopic mass analysis of DS 01 showed an intense ion of $[M+H]^+$ = 2793.39 (Fig. 2*B*).

Enzymatic digestion of DS 01 using endoproteinase Glu-C resulted in two fragments called DS 01a and DS 01b (Fig. 3*A*). Both fragments were separated by HPLC and showed molecular mass values of $[M+H]^+$ = 1374.49 and $[M+H]^+$ = 1437.62 Da, respectively (Fig. 3, *B* and *C*).

Primary Structure Determination and Sequence Alignment—The primary structure of the intact DS 01 and its enzymatic fragments were determined by Edman degradation and carboxypeptidase Y ladder sequencing and are shown in Figs. 4 and 5. The calculated monoisotopic mass of DS 01 and its fragments (DS 01a and DS 01b) after the N-terminal sequencing was $[M+H]^+$ = 2794.59 Da. However, MALDI-TOF/MS data revealed a discrepancy of 1.2 Da for DS01 monoisotopic molecular mass value ($[M+H]^+$ = 2793.39 Da). A similar difference in mass was also observed when the molecular mass values for the calculated DS 01b C-terminal fragment ($[M+H]^+$ = 1438.84 Da) were compared with the experimental result ($[M+H]^+$ = 1437.62 Da) shown in Fig. 3*C*.

TABLE III
Hemolytic activity of the dermaseptin DS 01

DS 01 concentration	% Hemolysis of human red blood cells
(μg/mL)	
1	0.00
2	0.00
4	0.41
8	0.54
16	0.81
32	1.08
64	1.36
128	2.17

Carboxypeptidase Y ladder sequencing of the intact DS 01 yielded seven C-terminal residues: (112.16) Ala-Gly-Leu/Ile-Ala-Ala-Lys/Gln (Fig. 5), suggesting that the C-terminal Leu-29 of DS01 was amidated (Fig. 4). Sequence similarity searches were performed using FASTA 3 program that runs under the Expasy Molecular Server. This procedure revealed up to 76% sequence identity with the adenoregulin precursor of *Phyllomedusa bicolor* (14) (Table I).

Secondary Structure Prediction and Helix-Wheel Plot—The secondary structure prediction (performed by SOPMA) of the three peptides investigated in this work (DS 01, DD K, and DD L) indicates a strong propensity to assume helical conformation. This tendency toward an α-helical arrangement, obtained upon contact with some organic solvents or the lipid bilayer (44), also gives the peptides a strong amphiphilic character, as demonstrated by the helix-wheel plot (Fig. 6).

Antimicrobial Activity—Synthetic DS 01 exhibited a broad spectrum antibacterial activity given that it inhibited the growth of most of the bacterial strains studied, which included Gram-positive and Gram-negative strains (Table II). However, significant differences in the growth inhibition of the different bacterial strains were observed, in accordance with the literature published for other dermaseptins (9–12, 45). The DS 01 minimal inhibitory concentration was compared with those obtained by testing with some conventional antibiotics (gentamicyn, ampicillyn, imipenem, and ceftazidime), and with minor exceptions, DS 01 was shown to be far more effective as an antibacterial agent.

Anti-T. cruzi Assay—Bioassays revealed that DS 01 and the synthetics DD K and DD L are potent anti-*T. cruzi* trypomastigote agents. Fig. 7*A* shows that when incubated for 2 h with the protozoan cells in concentrations up to 4 μg/ml, the peptides are able to lyse most of the cell population. When the

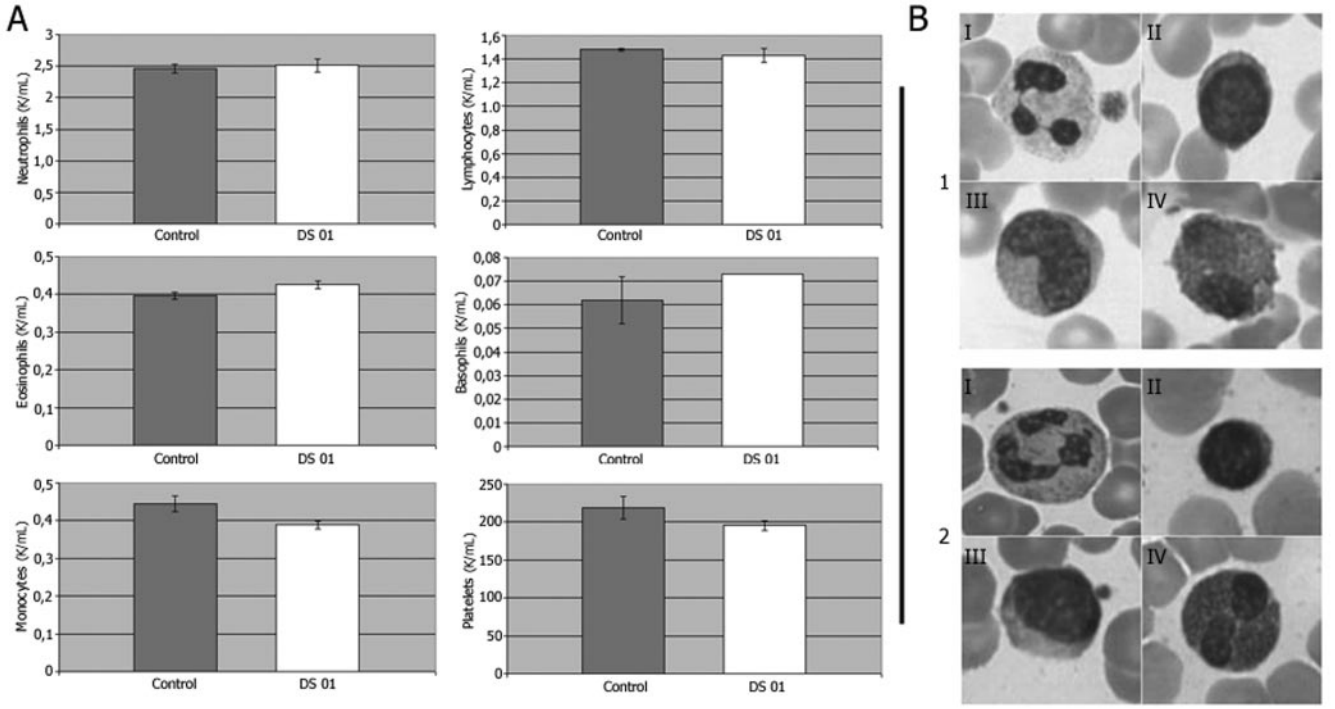

FIG. 8. **Hemograme.** *A*, bar graphs showing white blood cell populations subjected to DS 01 at 128 μg/ml and corresponding standard deviations. *B*, morphological monitoring of neutrophyls (*I*), lymphocytes (*II*), monocytes (*III*), and eosinophyls (*IV*) of the control (*panel 1*) and experimental cell samples (*panel 2*).

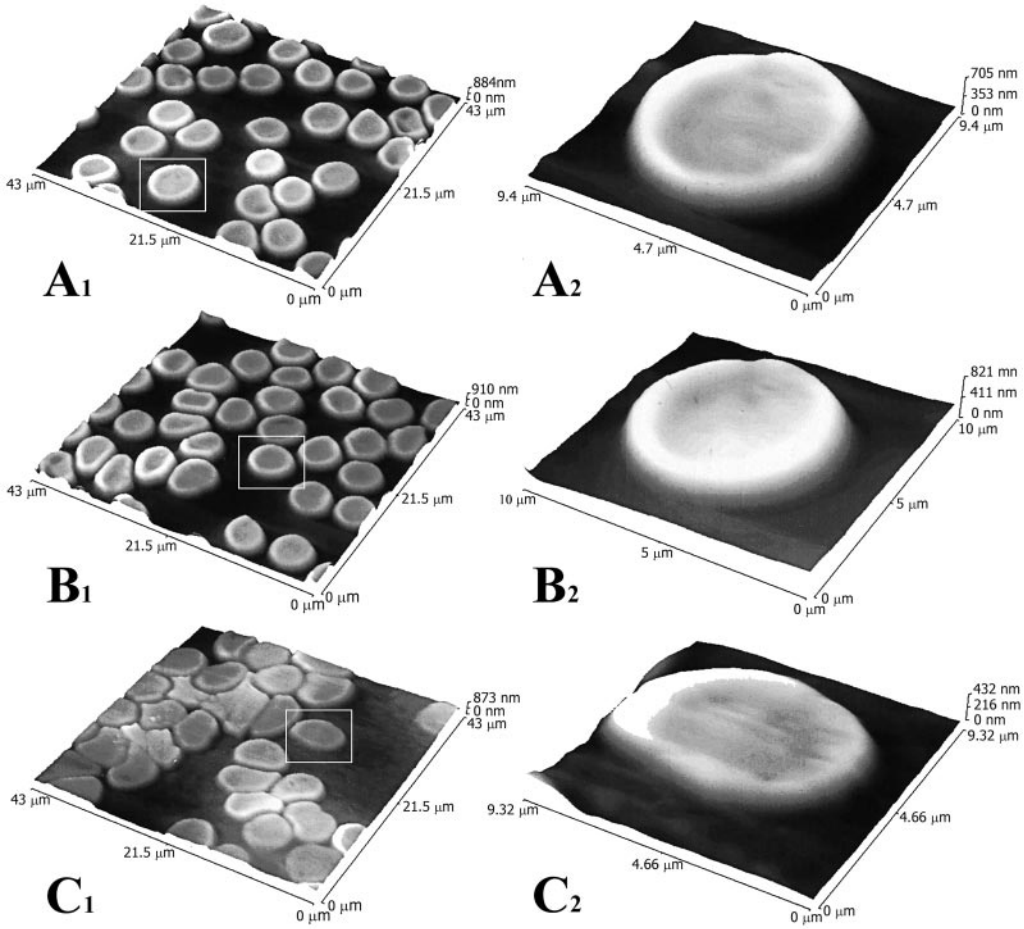

FIG. 9. **AFM monitoring of red blood cells.** Three-dimensional view AFM images (43 × 43 μm) of a normal blood film (*A*), peptide-treated blood film (*B*), and Triton X-100-treated blood film (*C*) are shown. Three-dimensional view high-magnification AFM images of isolated normal erythrocyte (*D*), isolated peptide-treated erythrocyte (*E*), and isolated Triton X-100-treated erythrocyte (*F*) are also shown.

incubation time is increased to 6 h (Fig. 7*B*), the same effect is seen with much lower concentrations (1 μg/ml), regardless of the amino acid composition of the peptides.

Evaluation of DS 01 Hemolytic Activity and Effect on Leukocytes—Investigation of the synthetic DS 01 hemolytic activity against human red blood cells (Table III) showed similar lytic levels to other dermaseptins (9–12). Even in the highest peptide concentration used in the assay (128 μg/ml), only about 2% of the red blood cells were lysed. DD K and DD L hemolytic activities are described elsewhere (9).

The interaction of the synthetic DS 01 with white blood cells was assessed in the hemogram assay. Fig. 8*A* shows the comparison of the total cell count of the blood sample incubated with synthetic DS 01 (128 μg/ml) and the control, in which the blood was submitted to the same procedures, except for the addition of the peptide. The bar graph shows that there are no significant differences between populations found in the control and the experimental group for any of the cell types analyzed (neutrophils, eosinophils, monocytes, lymphocytes, basophils, and platelets). Subsequent investigation on leukocyte morphology was carried out by using light microscopy (Fig. 8*B*). Once again, when compared with the control sample (Fig. 8*B*, *panel 1*), the cells subjected to high peptide concentrations (Fig. 8*B*, *panel 2*) showed no detectable morphological alterations.

Atomic Force Microscopy—DS 01 at a concentration of 30 μg/ml did not cause detectable morphological alterations on the surface of red blood cells under the experimental conditions used (Fig. 9, B_1 and B_2). The erythrocytes maintained their biconcave discoidal shape and appeared unchanged after the incubation period when compared with the control sample (Fig. 9, A_1 and A_2). Triton X-100-treated erythrocytes, used as positive control, showed disruption of the cell surface (Fig. 9, C_1 and C_2), and many of them were severely misshapen.

DISCUSSION

The ever growing number of resistant microorganism strains due to the abuse of the commercially available antibiotics has stimulated research on new alternatives for therapy of infections in the area of novel drugs. Cationic antimicrobial peptides have been considered by many to be an important component of the so-called primitive immune system (46). Therefore, these molecules, which play a key role in host defense against microorganism invasion, seem to be a suitable basis for further studies. Many drug companies are engaged in systematic screening for naturally occurring compounds that could be used as a starting point for new drug design and development. Among these molecules are the well known dermaseptins, which are present in the granular glands of the *Phyllomedusinae* subfamily of frogs and which display considerable antimicrobial activity against various kinds of microorganisms. These peptides also have different levels of activity against Gram-negative and Gram-positive bacteria, fungi (including yeasts), and protozoa (including the *Leishmania* genus) at micromolar concentrations. Importantly, most of the dermaseptins have little or no hemolytic activity against mammalian cells at antimicrobial concentrations.

The present study deals with the purification and characterization of a new antimicrobial peptide named dermaseptin 01

(DS 01) from the skin secretion of the newly described Brazilian species *P. oreades* (31). DS 01 possesses all the structural features present on the dermaseptin polypeptide family (9–12), including amidation at the C-terminal (Figs. 4 and 5). This peptide is similar in length to DS V (Table I) isolated from *Phyllomedusa sauvagii* (12), and the pairwise sequence alignment of DS 01 and DS V revealed only 59% amino acid positional identity. A comparison with the adenoregulin precursor from *P. bicolor* reveals that the amino acid positional identity is about 76% (Table I).

When plotted as an α-helical wheel, DS 01 showed the distinguishable polar and apolar domains (Fig. 6). The propensity of small-sized cationic peptides to form α-helical amphiphilic structures in apolar medium has been proposed to be a prerequisite for their membranolytic activity (16–20).

Antimicrobial activity of DS 01 was assessed against bacterial strains, toward which the effectiveness of the dermaseptin family of antimicrobial peptides was already known, and against the pathogenic protozoa *T. cruzi*. The minimal inhibitory concentrations obtained in the antibacterial tests shown in Table II are slightly higher than those reported in the literature (7–11). However, there is no reason to believe that DS 01 has comparatively lesser antimicrobial activity than other dermaseptins since there are no standards and the experimental conditions used in the tests were more severe (on the order of 10^7 colony forming units/ml and Mueller-Hinton medium).

The anti-*T.cruzi* assay reveals that DS 01, DD K, and DD L are remarkable trypanocidal agents. The protozoan cell population was reduced to a non-detectable level by these antimicrobial peptides in concentrations close to 16 μg/ml (~6 μM) after 2 h of incubation. Further incubation only accentuated dermaseptin-mediated cell lysis, probably due to the greater diffusion of the peptide through the blood medium. The anti-*T.cruzi* activity of the dermaseptins toward the trypomastigote and epimastigote forms was also investigated in culture and showed very similar results (data not shown). The effect of antimicrobial peptides on *T. cruzi* has already been investigated by the use of a cationic undecapeptide. After the incubation of a 20 μg/ml solution, there was a decrease of 54.7–10.7% in the infection rate of HeLa cells (47). In general, protective agents commonly used in blood supplies show little or no lytic activity against *T.cruzi* blood forms; their main effect is limited on the reduction of the mobility of the parasite. On the other hand, the dermaseptins assayed in this study (DS 01, DD K, and DD L) induced killing of *T.cruzi*, most likely by membrane disruption and cell leakage. Indeed, little or no intact protozoan cells were found under optical microscopy (data not shown). The assays performed show that the trypanocidal activity of the dermaseptins might be a general feature of this family of antimicrobial peptides.

Since the usefulness of DS 01 as a blood treatment agent depends not only on its trypanocidal activity but also on its lack of toxicity for the blood cells, some experiments were performed to detect possible cell alterations upon contact with the peptide. The conventional hemolysis test, shown in Table III, reveals that DS 01 at concentrations up to 128 μg/ml has little hemolytic effect. Although that is clearly an important property for a novel drug candidate, there is also the need to probe peptide interactions with other cell types. The effect of DS 01 on leukocytes was assessed through the total cell count of a blood sample incubated with DS 01 for 4 h. The results, shown in Fig. 8*A*, demonstrated that there was no significant alteration in the frequencies of blood cell populations in either treated or control samples. Also, when the morphology of the investigated cells was compared, no detectable differences were found (Fig. 8*B*).

AFM was applied as an additional step in the evaluation of hemolysis and to monitor the morphology of erythrocytes treated with DS 01. The AFM results not only confirmed that DS 01 was not hemolytic for erythrocytes, as already stated by the conventional analysis, but also provided an elegant evaluation of the morphological integrity of the erythrocytes after the exposure to the peptide. Indeed, the AFM result also suggested that the dermaseptins did not associate to the erythrocyte membrane in a manner that could damage cell functions or its overall architecture. This procedure introduces a more refined step of control on the conventional methods to access cell damaging by drugs in toxicity bioassays. To the best of our knowledge, this is the first time that AFM has been used for this purpose.

As stated previously, the dermaseptin family of peptides may have potential use as therapeutic drugs provided that they are not toxic to animals or plants. Consistent with the data reported above, where the antibacterial and trypanocidal activities of DS 01, DD K, and DD L were demonstrated, combined with their harmless properties to red and white blood cells, it is clear that these peptides should be considered important candidates as blood treatment agents.

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