Antimicrobial Activity of a Bovine Hemoglobin Fragment in the Tick Boophilus microplus*

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Antifungal and antibacterial activities were detected in the hemolymph and gut contents of the cattle tick, Boophilus microplus. A peptide with antibacterial activity from the tick gut contents was purified to homogeneity by reversed-phase chromatography. The molecular mass of the purified peptide was 3,205.7 Da, measured by matrix-assisted laser desorption/ionization mass spectrometry. The amino acid sequence was obtained by Edman degradation and showed that the peptide was identical to a fragment of the bovine α-hemoglobin. A synthetic peptide based on the sequence obtained showed characterization data identical to those of the isolated material, confirming its structure. The synthetic peptide was active in micromolar concentrations against Gram-positive bacteria and fungi. These data led us to conclude that the antibacterial activity detected in tick gut contents is the result of enzymatic processing of a host protein, hemoglobin. This activity may be used by ticks as a defense against microorganisms.

Ticks are obligatory blood-sucking arthropods found throughout the world and are among the most important vectors of human and animal diseases. The diversity of pathogenic organisms transmitted by ticks includes fungi, viruses, rickettsiae, bacteria, and protozoa and exceeds that found in all other arthropods (1). It is intriguing how many different organisms survive inside the ticks, and little is known about the mechanisms they use to avoid recognition by the defense system of the vector.

Animals defend themselves against microorganisms and parasites through a number of cellular and humoral mechanisms. Antimicrobial peptides belong to the latter class, and despite the fact that these peptides are very well characterized in insects and limulus (2, 3), they are poorly studied in arachnids. The first biochemical study of antimicrobial peptides in arachnids showed the presence of an antibacterial peptide in the hemolymph of the scorpion, Androctonus australis (5). Two of them are new peptides, androctonin and buthinin, and the other one is a representative of the insect defensin. Recently, lyctoxins I and II, which have been identified in the venom of the spider, Lycosa carolinensis, have shown potent antimicrobial activity against both prokaryotic and eukaryotic cells (6). Two hemolymph factors of the tick, Dermacentor variabilis, have activity against Bacillus subtilis (7). One of these factors has a molecular mass of 14.5 kDa and was tentatively identified as lysozyme, in agreement with the work of Kuhn and Haug (8) which showed a lysozyme-like immunoactivity in tick hemocytes. This antibacterial activity was increased significantly after challenge with bacteria (7).

We studied antimicrobial active compounds originating in the tick, Boophilus microplus. This tick is an ectoparasite of cattle and a vector of pathogens such as Babesia bigemina, Babesia bovis, and Anaplasma marginale. In addition, B. microplus causes severe production losses because its bites usually result in bovine blood loss and leather damage (1). We propose that a knowledge of these antimicrobial compounds will lead to better control of ticks and of the pathogens they transmit. Here we report that the antimicrobial activity present in gut contents of the tick is a fragment of the bovine α-hemoglobin.

EXPERIMENTAL PROCEDURES

Ticks—The B. microplus colony (Porto Alegre strain, Babesia sp. free) was obtained from the Biotechnology Department and Center of Rio Grande do Sul, Brazil. Ticks were reared on Holstein-Friesian calves in the Animal Facilities at the Biomedical Sciences Institute of São Paulo University, Brazil. Partially engorged adult females were removed from the host after approximately 21 days post-infection and were used for all subsequent hemolymph collections and inoculation experiments. Fully engorged adult females dropped from the host were used for gut contents collection.

Microorganisms—Bacillus megaterium (ATCC 10778), B. subtilis (ATCC 6633), Saccharomyces cerevisiae (ATCC 26108), Serratia marcescens (ATCC 4112), and Staphylococcus epidermidis (ATCC 12228) were obtained from the American Type Culture Collection. Pseudomonas aeruginosa (IAL1025) was obtained from Adolfo Lutz Institute Culture Collection, São Paulo, Brazil. Escherichia coli D31 and Enterobacter cloace K12 were provided by Dr. H. G. Boman, Stockholm University, Sweden. E. coli SBS 363, Micrococcus luteus A270, and Neurospora crassa were obtained from Dr. P. Bulet, CNRS, Strasbourg, France. M. luteus was obtained from the Pharmaceutics Sciences Institute of São Paulo University, Brazil. Aspergillus nidulans (UT448) was obtained from the Utrecht University Collection, Holland. Candida albicans (MD56) was obtained from the Microbiology Department Collection, Biomedical Sciences Institute of São Paulo University, Brazil.

Hemolymph and Gut Contents Collections—For hemolymph collections, a small cut was made with a hypodermic needle on the dorsal cuticle at the posterior region of the ticks. Hemolymph was recovered with a glass capillary after gentle pressure to the body of the tick.
Antimicrobial Hemoglobin Fragment

Hemolymph samples were stored at −20 °C. The gut contents were obtained from ticks within 24 h from the time they had dropped from the calves. The gut contents were collected using a Pasteur pipette, diluted 1:1 (v/v) with 0.15 M ammonium acetate buffer, pH 7.0, and stored at −20 °C.

Bacteria Inoculation—One μl of bacterial suspension (10^5–10^6 cells) was injected into the spiracles, anus, or genital apertures of partially engorged ticks with a Hamilton syringe. Different bacteria strains, M. luteus, E. cloacae, or E. coli, were inoculated. Hemolymph collected at 2, 24, 48, and 72 h post-inoculation was either streaked onto agar plates for bacterial growth detection or stored at −20 °C for antibacterial activity analysis.

Acidic Extract of Neutrophil Granules—Granulocytes were isolated from bovine blood as described by Carlson and Kaneko (9). Neutrophil granules were obtained according to Geno et al. (10) and extracted for 2 h in an ice bath with 0.2 M sodium acetate buffer, pH 4.0, containing 5 mM EDTA. The pH was adjusted to 7.0 with 6 N NaOH, and the insoluble material was sedimented by centrifugation for 10 min at 10,000 × g. The resulting supernatant was analyzed for antibacterial activity.

Erythrocyte Lysis in Vitro—Erythrocytes were isolated from EDTA-anticoagulated bovine blood using a Ficoll-paque (Amersham Pharma Biotech) gradient according to the manufacturer. After centrifugation (400 × g, 30 min, 22 °C), the cell suspension was washed and suspended in phosphate-buffered saline (2.6 × 10^6 cells/ml). Erythrocytes were lysed as described by Carlson and Kaneko (9), and the supernatant was concentrated 2.5 times in a vacuum centrifuge.

Antimicrobial Assays—Two different approaches were used to evaluate the antibacterial activity, an inhibition zone assay (11), and a liquid growth inhibition assay (12). The liquid growth inhibition assay was used for the determination of the minimal inhibitory concentration of the synthetic peptide. The lowest concentration that caused 100% of growth inhibition was recorded. The antifungal activity was detected using the liquid growth inhibition assay described in Felbaum et al. (13). The concentrations tested for synthetic peptide were in the range of 0.65–21 μM.

Bactericidal Assay—A M. luteus culture was incubated with different concentrations of gut contents or synthetic peptide using the assay described by Bulet et al. (12). The culture that had total growth inhibition at the lowest concentration of the samples was plated on nutrient agar.

The number of colony-forming units was determined after an overnight incubation at 37 °C. The synthetic peptide was tested at the concentration in the range of 0.65–21 μM, and the gut contents were diluted from 80 to 1280 times.

Purification of Antibacterial Peptide from Gut Contents—The gut contents (10 ml) collected from fully engorged ticks was mixed with 0.15 M ammonium acetate buffer, pH 7.0 (1:1, v/v), and concentrated in a vacuum centrifuge. The dried material was suspended in 0.1% trifluoroacetic acid (TFA) and heated to 80 °C for 10 min. After centrifugation at 8,000 × g for 30 min at 4 °C, the supernatant was loaded onto reversed-phase Sep-Pak C_18 cartridges equilibrated with 5% acetonitrile (ACN) in 0.1% TFA. The elution was performed with different concentrations (20, 30, 40, and 95%) of ACN in 0.1% TFA. The antibacterial peptide eluted in 30% ACN was purified to homogeneity by reversed-phase chromatography in HPLC. The column effluent was monitored by absorbance at 214 nm, and the concentration of antibacterial activity was determined by the inhibition zone assay.

Mass Spectrometry Analysis—The peptides dissolved in acidified water (0.1% TFA) were analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry using the matrix α-cyano-4-hydroxycinnamic acid. Positive ion MALDI mass spectra were acquired on a Bruker BIFLEX time-of-flight mass spectrometer (isolated peptide from the tick) and on a Micromass spectrometer model TOFSpec S.E. (synthetic peptide).

N-terminal Amino Acid Sequencing—Automated Edman degradation of the pure isolated peptide and detection of phenylthiohydantoin derivatives were performed on a pulse liquid automatic sequenator (Applied Biosystems, Model 473A).

Peptide Synthesis—the peptide was synthesized manually by using the t-Boe strategy on a methylbenzhydrolamine (MBHA) resin (substitution of 0.79 mmol/g) (14, 15). The t-Boe amino acids were protected as follows: Lys (ε-2-Cl-Z), Ser (Bzl), Tyr (2-Br-Z), Thr (Bzl), His (Tos) iDCHA, and Asp (β-OctHex). After peptide chain assembly was completed, the resulting peptide resin was treated with HF, 1% anisole for 90 min at 45 °C on Peptide Institute Inc. Teflon equipment. The crude peptide was precipitated with anhydrous diethyl ether and extracted with 5% acetic acid, and the resulting solution was lyophilized. Purification of 360 mg of the crude peptide was achieved on a semipreparative Vydac C_18 column using a linear gradient of 15–33% ACN in 0.1% TFA at a flow rate of 8 ml/min. The fractions containing the peptide as determined by analytical reversed-phase HPLC under isocratic conditions (1.4 ml/min, 28% ACN in 0.1% TFA) were pooled and lyophilized. Peptide purity and identity were confirmed by reversed-phase HPLC, amino acid analysis, and MALDI-TOF mass spectrometry measurement. Amino acid analysis was performed on a 7300 Beckman analyzer after 24 h 6 N HCl hydrolysis in a PicoTag workstation (Waters).

RESULTS

Antimicrobial Activity of Tick Hemolymph and Gut Contents—Antibacterial and antifungal activities were detected in the hemolymph and gut contents of noninfected adult ticks, B. microplus (Table I). The antibacterial activity was detected only against Gram-positive strains. The antifungal activity was observed against two filamentous fungi, A. nidulans and N. crassa, and against the yeast, C. albicans.

The level of antibacterial activity found in the hemolymph does not increase after inoculations of 10^5–10^6 of M. luteus, E. cloacae, or E. coli cells via injection into the spiracle, anus, or genital aperture (data not shown). We interpret this to indicate that the antibacterial activity in the tick is constitutive and does not respond to bacterial inoculation.

We analyzed the number of bacteria in inoculated adult ticks by streaking hemolymph onto agar plates. The number of bacteria in the hemolymph markedly decreased after 24–48 h inoculation (data not shown).

Neutrophil granules have a marked antibacterial activity (10, 16). We investigated the possibility that the antibacterial activity found in tick hemolymph and gut contents originated from cells present in the host blood. Using the infection zone assay, we showed that bovine cells are active against Gram-positive and Gram-negative bacteria while the tick preparations are active only against Gram-positive bacteria, indicating that the tick antibacterial activity most likely does not come from the bovine neutrophils (Table I).

Purification and Characterization of an Antimicrobial Peptide from Gut Contents—to isolate the active material from the...
gut contents, 10 ml of a sample collected from fully engorged females were loaded onto reversed-phase Sep-Pak C18 cartridges equilibrated with 5% acidified ACN. The active fraction eluted with 30% ACN was analyzed on a Resource-RPC (Amersham Pharmacia Biotech) using a linear gradient of 12–36% ACN in 0.1% TFA over 30 min (Fig. 1A). The active fraction eluted with 31.5% ACN was then purified on the same column with a different linear gradient of ACN described in the previous step (C). The active fraction eluted with 27% of ACN was finally purified on a Vydac C18 reversed-phase column with a linear gradient of ACN in 0.1% TFA (D). Elution was performed at a flow rate of 1.0 ml/min. The black area indicates the fractions active against *M. luteus* using inhibition zone assay.

The molecular mass of the purified peptide, eluted with 40.8% acetonitrile, was 3,205.7 Da measured by MALDI-TOF mass spectrometry. The peak at 1,601.7 Da corresponds to the double charged ion (Fig. 2A). Edman degradation yielded the following peptide sequence of 29 amino acids: FLSFPTTKTY-FPHFDLSHGSAQVKGHGAK. The theoretical molecular mass and pI are 3,206.6 and 9.53, respectively, calculated by the pI/Mw program at ExPASy (Molecular Biology Server of the Geneva University Hospital and the University of Geneva, Switzerland). The comparison of this peptide with a Swiss Protein Database showed 100% identity to the bovine α-hemoglobin fragment 33–61.

Synthetic Peptide—With the aim of confirming the identity and biological properties of the isolated peptide, we synthesized this peptide corresponding to the α-hemoglobin fragment (33–61). Purity of the synthetic peptide was confirmed by reversed phase-HPLC (90–95% pure), mass spectrometry measurement, and amino acid analysis. The molecular mass obtained by MALDI-TOF was 3,204.3 Da (Fig. 2B). The amino acid analysis results, with the expected values given in parentheses, were: Asp, 1.03 (1.00); Glu, 1.06 (1.00); Gly, 3.01 (3.00); Ala, 2.25.
Antimicrobial Activity of Erythrocytes Lysed in Vitro—We investigated whether erythrocytes lysed in vitro have antibacterial activity because it is known that extensive proteolytic degradation of hemoglobin takes place inside these cells (17). The sample containing lysed erythrocytes was analyzed in liquid growth inhibition assay (12). No activity against the bacterial strain tested (M. luteus) was observed.

**TABLE II**

| Microorganism | MIC (µM) |
|---------------|----------|
| *M. luteus* A270 | 5 |
| *M. luteus* | 5 |
| *S. epidermidis* | 21 |
| *C. albicans* | 5 |
| *S. cerevisiae* | 11 |
| *A. nidulans* | 1.3 |

**DISCUSSION**

Antimicrobial proteins and peptides constitute an important part of the humoral immune system of arthropods. In this study, we detected antimicrobial activity in the hemolymph and gut contents of noninfected ticks. This activity is not increased in the hemolymph of experimentally bacteria-infected ticks, and we conclude that it is constitutive as opposed to the induction observed in insects. Similar results were reported in other arachnids, such as the scorpion, *A. australis* (5). In contrast, Johns et al. (7) observed an increase of antibacterial activity in the hemolymph of the bacteria-infected tick *D. variabilis*.

A peptide with activity against *M. luteus* was purified from the gut contents to homogeneity by reversed-phase chromatography. The amino acid sequence showed that it was a fragment (33–61) of the bovine α-hemoglobin. The synthetic peptide showed characterization data identical to those of the isolated native peptide, confirming its identity by mass spectrometry measurement and amino acid analysis. It was active against Gram-positive bacteria and fungi in micromolar concentrations, being bactericidal on our tested bacteria strain (*M. luteus*). The predicted secondary structure of the peptide does not display amphipathic alpha-helix character typical of basic antimicrobial pore-forming peptide structure (18, 19). An investigation of the structural requirements of the α-hemoglobin fragment 33–61 would be important for the understanding of its antimicrobial activity.

Whole hemoglobin did not show antibacterial activity in our experiments although an early report by Hobson and Hirsch (20) showed such activity in vitro. However, according to these authors an antimicrobial function in vivo is unlikely because the conditions used in the assays are far from those existing in tissues.

In a recent review, Ivanov et al. (17) discussed the role of hemoglobin as a source of biologically active peptides. Apparently, the generation of hemoglobin fragments starts inside the erythrocytes. The primary proteolysis gives rise to peptides with 30 amino acid residues. The next processing step occurs with the excretion of newly formed shorter peptides from the erythrocytes. The function of these peptides would be regulatory and complementary to the conventional hormonal and neuromodulatory systems.

More than 150 established amino acid sequences of endogenous hemoglobin fragments are available; however, in no case has an antibacterial activity been mentioned. A literature search for hemoglobin fragments that have some structural identity to the peptide isolated from the tick gut contents revealed six peptides: one isolated from pig hypothalamus (21), three others from human cerebellum (22), and two from rat hippocampus (23) (Table III). The pig hypothalamus peptide (PHP) includes the amino acid residues corresponding to fragment 33–46 of the α-hemoglobin and has an activity related to release of corticotropin in vitro (21). The human cerebellum peptides (HCP1, HCP2, and HCP3) have sequences starting at amino acid residue 33 of α-hemoglobin, but the C-terminal amino acids are not known (they have been used as peptide markers of Alzheimer’s disease) (22). The rat hippocampus peptides (RHP1 and RHP2) sequences are not completely defined and have been used as markers of ischemia (23).

Because we were not able to detect any antibacterial activity against *M. luteus* in lysed erythrocytes, we suppose that the active hemoglobin fragment is originated inside the tick gut, probably through enzymatic cleavage at phenylalanine residue. It has been reported that globin in the *Schistosoma mansoni*...
The function of hemoglobin as source of amino acids for blood-sucking insects and ticks is well known. In addition, hemoglobin may be utilized as a regulatory molecule. Azambuja et al. (25) showed that hemoglobin is important for edcsynergism production and for the establishment of the edcsynergism process in the hematophagous triatomine insect Rhodius prolixus. Fraidenraich et al. (26) purified a peptide corresponding to residues 1–40 of the aD globin chain of chicken from Triatoma hindgut. This peptide induced the differentiation of T. cruzi epimastigotes to metacyclic trypomastigotes in vitro, through activation of adenyl cyclase. Garcia et al. (27) showed that hemoglobin and synthetic peptides corresponding to 30–49 and 35–73 of the aD globin chain induced T. cruzi metacyclogenesis in the R. prolixus gut. It would be interesting to study the effect of the synthetic peptide (α-hemoglobin fragment 33–61) on the development of B. bovis and B. bigemmina, the protozoa transmitted by B. microplus to the cattle (1).

In conclusion, we propose that the ticks utilize a host protein, hemoglobin, to its own defense against microorganisms. The utilization of host proteins for microorganism defense could be a mechanism used by other ticks, because a rabbit lysozyme in the tick Ornithodorus hemolymph has been purified.2 We do not know if the hemoglobin fragment that we have isolated from the gut contents and characterized in this study is also present in the hemolymph. It is known, however, that proteins from the host blood pass directly from the gut contents into the tick hemolymph, either intact or separated into subunits (28). Because of this, we are now investigating the possible recovery of this peptide from tick hemolymph. Finally, we are considering whether the hemoglobin fragment can have antimicrobial function in vertebrates, this could be an important effector of the innate immune response to kill microbial invaders.

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