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α-Defensins from Blood Leukocytes of the Monkey *Papio hamadryas*

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Abstract—Three antimicrobial peptides named PHD1-3 (*Papio hamadryas* defensin) have been isolated from hamadryas baboon blood leukocytes using preparative electrophoresis and reverse-phase HPLC. The primary structures of these peptides have been determined by automated Edman degradation and mass-spectrometry. The results suggest that the peptides belong to the α-defensin family. Structural homology analysis reveals that among α-defensins from other animal species, PHD3 is the most closely related to RMAD5 (rhesus macaque α-defensin) (90% homology) from rhesus macaque leukocytes and also highly similar to human α-defensin HD5 (60% homology), which is produced by intestinal Paneth cells. The homology of PHD3 with human neutrophil α-defensin HNP1 (human natural peptide) was 30%. The primary structures of PHD1 and PHD2 are most similar to RED1 (rhesus enteral defensin), one of six enteral α-defensins of rhesus monkeys. PHD1-3 have been shown to be active against the Gram-positive bacteria *Listeria monocytogenes* and *Staphylococcus aureus*, the Gram-negative bacterium *Escherichia coli*, and the fungus *Candida albicans*, similarly to the human HNP1 defensin.

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Key words: antimicrobial peptides of mammals, primary structure, α-defensins, leukocytes, monkey, *Papio hamadryas*

The ability of animals to defend themselves from infection by microorganisms depends primarily on the functions responsible for recognition, killing, and elimination of potentially pathogenic microbes. An essential role in these body functions belongs to molecular protective mechanisms mobilized during the first minutes and hours of host exposure to parasites. In the course of evolution, these emergency responses to microorganisms (innate immunity) anteceded the acquired (adaptive) immunity, which is fully developed only in vertebrates. The innate immunity systems involve an elaborate complex of factors including specialized phagocytic cells, receptors for recognition of microbial components, signal molecules that coordinate different defensive responses, and effector molecules for inactivation and killing of microorganisms. One of the key roles in the latter group of factors is played by leukocytic antimicrobial peptides (AMPs), which significantly contribute to defense against infections [1, 2]. In mammals, several structurally homologous groups of such peptides have been described. The most thoroughly studied of these are of the defensin family. In numerous *in vitro* and, most importantly, *in vivo* experiments, its members have been shown to exhibit antimicrobial activity (AMA) against many Gram-positive and Gram-negative bacteria, mycobacteria, fungi, and enveloped viruses [3-5]. The wide distribution and high levels of expression of defensins in epithelial tissues and immune cells suggest their importance for realization of various host defense reactions [6-12].

Defensins are cationic peptides featuring a high content of the basic amino acids arginine and lysine and of hydrophobic amino acids [13]. Another distinguishing characteristic of defensins is the presence of six cysteine residues that form three intramolecular disulfide bridges. Mammalian defensins are categorized into three groups, α-, β-, and θ-defensins differing in their coding genes, preproprotein sizes, and disulfide bridge arrangements [14, 15]. Also, θ-defensins are macrocyclic peptides, their

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cyclic structure being formed by peptide bonds [16, 17]. Peptide products corresponding to θ-defensins have been found so far only in rhesus monkeys *Macaca mulatta*, the classical animal model widely used in biological and medical experiments [16]. In addition, seven α-defensins have been isolated from rhesus monkey leukocytes [18]. Because of the extremely broad spectrum of AMPs in rhesus monkeys and in order to better understand the role of AMPs in innate immunity in primates including humans, we have isolated and purified AMPs belonging to the defensin family from leukocytes of hamadryas baboon (*Papio hamadryas*), a species which is taxonomically close to rhesus monkeys, and carried out comparative structural and functional analysis of the three peptides thus obtained.

MATERIALS AND METHODS

Preparation of leukocyte mass enriched in neutrophilic granulocytes. Whole blood stabilized with sodium EDTA (0.15% final concentration) was obtained at the Pavlov Institute of Physiology (St. Petersburg, Russia) from two adult female hamadryas baboons. After hemolysis of erythrocytes with four volumes of 0.84% aqueous ammonium chloride (15 min exposure), the blood sample was centrifuged at 400*g* for 5 min and then the sediment was used to isolate AMPs. To check the cell preparation for heterogeneity, a smear was made, stained with Fast Green and Azure A, and examined using immersion microscopy at 1000-fold magnification.

Preparation and purification of antimicrobial peptides. The leukocyte pellet was combined with 20 ml of 10% CH_3COOH, and the cells were destroyed by homogenization at 4°C. The homogenate was centrifuged at 20,000*g* for 1 h. The supernatant was ultrafiltrated using a 10 kD cutoff filter (Amicon YM-10) (Amicon Corporation, Ireland). The filtered solution was concentrated using a 1 kD cutoff filter (Amicon YM-1). The subsequent fractionation of low molecular weight peptides was carried out by preparative acid/urea electrophoresis with acid buffer (5% acetic acid) using a Bio-Rad (USA) AG501-X8 apparatus [19]. The final purification of the fractions that showed the presence of AMPs was carried out by reverse-phase HPLC using a Beckman (USA) Gold System chromatographer with Alltech C18 column (4.6 × 250 mm) (Alltech Associates, Inc., USA) equilibrated with either 0.1% trifluoroacetic acid or 0.15 mM tetrabutyl ammonium in water. Peptides were eluted with a linear acetonitrile gradient (0 to 60% or 10 to 40% over 1 h). At every purification step, the fractions were checked with AU-PAGE [20] and Tricine-SDS-PAGE [21]. The purified peptides were dried by vacuum centrifugation using an SC 110 SpeedVac apparatus (Savant, USA), redissolved in 0.01% acetic acid, and stored at −20°C.

Determination of primary structures of peptides. The molecular mass of peptides was determined by MALDI-TOF mass-spectrometry using a Voyager DE mass-spectrometer (Perspective Biosystems Inc., USA). The amino acid sequence was determined by the Edman degradation technique using a Porton Model 2090E sequencer at UCLA, Los Angeles, USA. To determine the presence of intermolecular disulfide bridges, aliquots of PHD1-3 were incubated with 1,4-dithiothreitol (DTT) (Sigma, USA), which reduced their disulfide bridges, the consumption being 50 DTT molecules per disulfide bond.

Peptide quantification. The concentrations of PHD1-3 in 0.01% acetic acid were calculated as suggested by Pace [22] using the formula $C = A/(l \cdot \varepsilon)$, where C is molar peptide concentration, A is optical absorption at 280 nm, l is optical pathlength, and ε is molar absorption coefficient. The coefficient ε was calculated as ε = (W)(5.500) + (Y)(1.490) + (-C-C-)(125), where (W), (Y), and (-C-C-) are the amounts of tryptophan, tyrosine, and cystine residues, respectively.

Antimicrobial assays. AMA of the peptide fractions obtained as above was determined by application of polyacrylamide gel that contained peptide fractions onto agarose gels containing test microorganisms and by radial diffusion in agarose gel as suggested by Lehrer [23]. The test microorganisms used were strain ML-35p of Gram-negative bacterium *Escherichia coli*, strain EGD of Gram-positive bacterium *Listeria monocytogenes*, stain MRSA (ATCC 33591) of *Staphylococcus aureus*, and strain 820 of the fungus *Candida albicans*. The microorganisms were precultured for 16 h at 37°C in media consisting of 3% tryptic soybean broth (TSB) for bacteria (Sigma) and Sabouraud dextrose broth (SDB) for the fungus (Sigma). Aliquots of the medium containing test microorganisms were transferred separately into freshly prepared medium and incubated at 37°C for 2.5 h to obtain mid-logarithmic-phase microorganisms. Cell counts for each of the microorganisms were determined by the turbidity of cell suspensions using a Beckman DU-50 spectrophotometer at 620 nm for bacteria and 450 nm for the fungus. Suspension aliquots containing $4 \cdot 10^6$ cells were mixed with 10 ml of sterile 1% agarose solution in 10 mM sodium phosphate buffer, pH 7.4, or with the same buffer containing 0.1 M NaCl, both at 42°C. The mixtures were poured into sterile plastic 90-mm Petri plates and left at room temperature for solidification. Wells made by a 3-mm applicator were filled with 5 μl test samples representing serial peptide dilutions in 0.01% acetic acid and incubated at 37°C for 3 h. Then the dishes were covered with 1% agarose containing 6% TSB (for bacteria) or 6% SDB (for fungus) and incubated for 18 h at 37°C. The diameter of the zone of inhibited growth (the microbe-free zone around a well) was measured assuming 0.1 mm to one AMA unit and subtracting 30 such units from each result to take account of well diameter.

To detect antimicrobial peptide fractions in extracts, they were subjected to AU-PAGE [20] and then the gels were washed with 50 ml of 10 mM sodium phosphate buffer (pH 7.4) two times for 15 min in a shaker. Gel slabs were placed onto the surface of agar gel containing test microorganisms and were incubated for 3 h at 37°C. During the incubation, the peptides diffused from the polyacrylamide gel into the agar gel. To detect the foci of microbe killing, the test agar was covered with 1% agarose solution containing fresh culture medium and incubated for 18 h at 37°C.

Determination of minimal inhibiting concentrations. The minimal inhibitory concentrations (MIC) of the peptides were determined by linear regressions of AMA vs. peptide concentration. The points of intersections of the regression lines with the X-axis were assumed to be the MIC values.

Statistical treatment. The results were treated using conventional methods of analysis of variance as realized in Statistica 5 and SigmaPlot 9.0 software packages. The data are represented as mean ± standard deviation from four experiments. Peptide homology was analyzed using BLAST and CLUSTAL software packages.

RESULTS

Preparation of leukocyte mass enriched in neutrophilic granulocytes. It was possible to obtain 200 ml of whole blood from two adult female baboons. After hemolysis of erythrocytes and leukocyte sedimentation, the amount of wet leukocyte mass was 1.9 g. Differential cell count in the smears of the leukocyte preparation showed about 70% of neutrophils, 5% of eosinophils and basophiles, and 25% of monocytes and lymphocytes.

Isolation and purification of antimicrobial peptides. The peptides were extracted with 10% acetic acid as described above. Five sequential extractions (E_1-E_5) were made. Electrophoretic analysis of the extracts in the acid buffer system revealed several highly basic peptide fractions, and AMA testing by polyacrylamide gel application showed that the fractions were active against *E. coli* and *L. monocytogenes*. Comparative analysis of the spectrum of human AMP extracted in a similar way showed that it had no such peptides (Fig. 1, lanes *4* and *5*). The extracts E_1 and E_2, which appeared to be more heterogeneous than the subsequent extracts E_3-E_5, were pooled and ultrafiltrated with Amicon YM-10 membranes. This was done for preliminary separation of high and low molecular weight peptide fractions and for removal of salts present in extracts and interfering with peptide purification. SDS-PAGE showed that 3-4-kD peptide fractions did not pass the 10-kD cutoff filter, so the subsequent fractionation was carried out with preparative electrophoresis, which made it possible to perform preliminary fractionation of peptides according to their charge and molecular weight. The final purification of highly charged low molecular weight fractions showing AMA was achieved by reverse-phase HPLC on an Alltech C18 column (Fig. 2). For better separation of peaks 2 and 3, the unresolved fractions were rechromatographed on the same column, the peptides being eluted with a linear 10 to 40% acetonitrile gradient over 1 h. This yielded three highly purified peptides PHD (*Papio hamadryas* defensin) 1 to 3, the numbers referring to the relative mobility of the peptides towards the cathode during AU-PAGE (Fig. 1, lanes *1-3*). The amounts of PHD1 and 2 were roughly similar and one order of magnitude lower than that of PHD3.

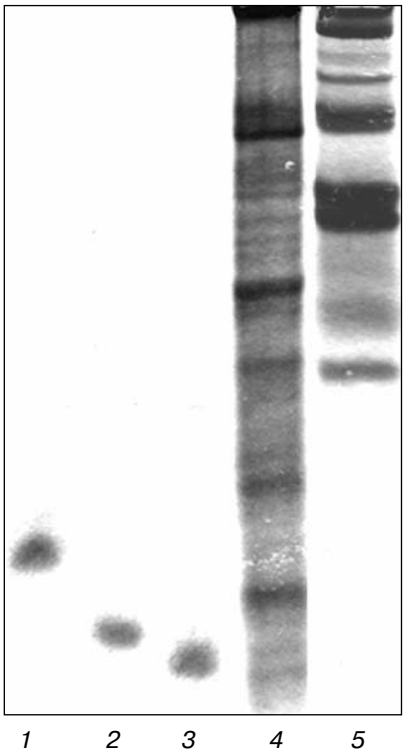

Fig. 1. Electrophoregram of purified hamadryas baboon α-defensins. The gel contained 12.5% acrylamide and 6.25 M urea; the electrode buffer contained 0.9 M CH_3COOH, pH 2.2; electrophoresis was performed in 0.75-mm plates; run time was 1 h at 150 V. Lanes: *1-3*) PHD3-1, respectively (1 μg of each); *4*) extract of hamadryas baboon leukocytes obtained with 10% acetic acid; *5*) extract of human leukocytes obtained with 10% acetic acid.

Primary structures of PHD1-3. The amino acid sequences of PHD1-3 determined by Edman degradation using an automated sequencer are shown in Fig. 3. The calculated total charges of defensin molecules were +8 for PHD1 and +7 for PHD2 and PHD3. The molecular weights of PHD1-3 determined by mass-spectrometry were 3938.9, 3780.8, and 3727.1 daltons, respectively. MS-analysis of the reduced forms of PHD1-3 showed that their molecular masses increased by 6 daltons, indicating the presence of three disulfide bonds in the molecules of PHD1-3. The close match between the calculated molec-

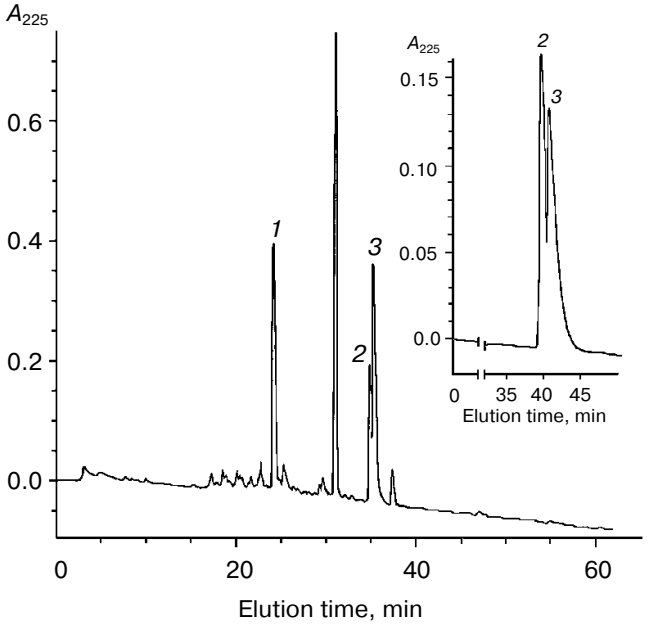

Fig. 2. Purification of hamadryas baboon α-defensins by reverse-phase HPLC. Elution profile of peptides from an Alltech C18 column developed using a linear 0 to 60% acetonitrile gradient over 1 h. Peaks *1-3* contain PHD3, PHD1, and PHD2, respectively. The inset shows additional HPLC run for separation of second and third peaks using a linear 10 to 40% acetonitrile gradient over 1 h. A_{225}, absorbance at 225 nm.

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PHD1    RRICRCRIGRCLGLEVYFGVCFLHGRLARRCCR
PHD2     RICRCRIGRCLGLEVYFGVCFLHGRLARRCCR
PHD3     RTCRCRLGRCSRRESYSGSCNINGRIYSLCCR
RED1     RTCRCRIRRCRGLESSFGNCILHGQFAKLCCR
RMAD5    RTCRCRFGRCFRRESYSGSCNINGRIFSLCCR
HD5      ATCYCRTGRCATRESLSGVCEISGRLYRLCCR
HNP1      ACYCRIPACIAGERRYGTCIYQGRLWAFCC
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Fig. 3. Primary structures of hamadryas baboon α-defensins PHD1-3. The primary structures of human defensins (HNP1, HD5) and rhesus monkey defensins (RED1, RMAD5) are presented for comparison. Invariant amino acid residues are shown by bold letters.

ular weights (3936.8, 3780.6, and 3724.2 daltons, respectively, for PHD1-3) and the respective experimental values suggests that the determined sequences are complete.

Antimicrobial activity of PHD1-3. AMA analysis has shown that PHD1-3 produce antimicrobial effects towards all test microorganisms, albeit to different extents. To determine MIC, six serial twofold dilutions were used starting from 50 μM. MIC values for PHD1-3 and HNP1 against four test microorganisms are shown in the table. The comparison of MIC of PHD1-3 and HNP1 shows that, on the whole, they are quantitatively and qualitatively similar. PHD1-3 are most active against *L. monocytogenes* and least active against *S. aureus*, both microbes being Gram-positive. The addition of 0.1 M NaCl to agarose gels resulted in a significant reduction in the antimicrobial activity against *E. coli*, *C. albicans*, and *S. aureus*, this effect being less pronounces with *L. monocytogenes*. This phenomenon, which is rather typical for most of α-defensins studied to date, may be explained by poorer peptide sorption by membranes of microorganisms under increased ionic strength.

DISCUSSION

Three novel AMPs have been isolated from leukocytes of hamadryas baboons. Their localization has not been studied by immunocytochemistry; however, they are likely to be components of the granular apparatus of neutrophilic granulocytes, of which leukocyte preparation is enriched. The analysis of the primary structures of the isolated peptides showed conservative patterns of six cysteines, two glycines, two arginines, and one glutamic acid typical of α-defensins studied earlier. This allows referring the newly isolate peptides to the α-defensin family. Further studies of the antimicrobial activities of PHD1-3 will make it possible to perform a more detailed analysis of their interrelationships and their relationships with defensins from other primate species. Experiments performed so far suggest that PHD1-3 are similar to HNP1 with regard to antimicrobial activities in media lacking NaCl. When 0.1 M NaCl is added to agarose gels, MIC

Minimal inhibitory concentrations (μM) of antimicrobial peptides

Peptide	*E. coli*		*L. monocytogenes*		*S. aureus*		*C. albicans*	
	low salt	0.1 M NaCl	low salt	0.1 M NaCl	low salt	0.1 M NaCl	low salt	0.1 M NaCl
HNP1	2.1 ± 0.7	10.2 ± 1.7	1.3 ± 0.2	2.1 ± 0.6	3.5 ± 1.0	>50	3.2 ± 0.9	>50
PHD1	1.6 ± 0.2	3.5 ± 0.7	1.3 ± 0.1	1.8 ± 0.2	3.8 ± 1.0	>50	2.3 ± 0.5	4.5 ± 1.0
PHD2	1.7 ± 0.2	2.9 ± 0.8	2.1 ± 0.7	1.9 ± 0.5	7.8 ± 1.5	>50	4.0 ± 1.2	7.5 ± 1.7
PHD3	2.0 ± 0.7	3.1 ± 0.7	1.2 ± 0.2	1.9 ± 0.4	4.5 ± 1.4	7.8 ± 1.4	2.6 ± 0.8	3.8 ± 0.8

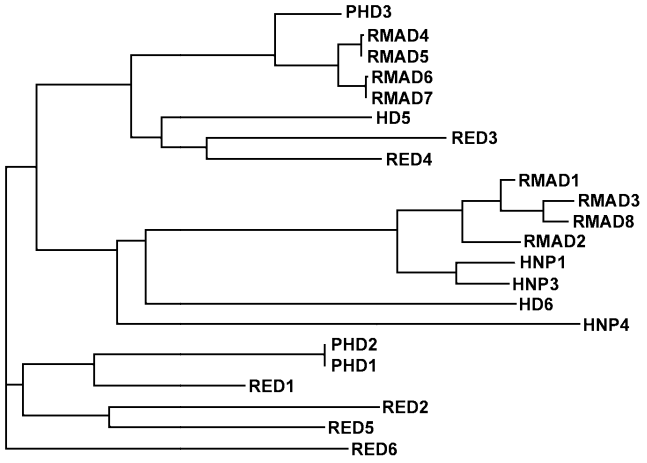

Fig. 4. Dendrograms of primate α-defensins. PHD1-3: defensins of *Papio hamadryas* (leukocytes); RMAD1-8: defensins of *Macaca mulatta* (leukocytes); RED1-6: defensins of *Macaca mulatta* (Paneth cells); HNP1, 3, 4: defensins of *Homo sapiens* (leukocytes); HD5 and 6: defensins of *Homo sapiens* (Paneth cells). The cellular localization of peptides is indicated in brackets.

values of PHD1-3 against *E. coli* and *C. albicans* are markedly lower than those of HNP1.

It is known that seven α-defensins from rhesus monkey leukocytes form two subfamilies, the representatives of one of which (RMAD1-3) showing a high homology with human leukocytes α-defensin HNP1, whereas those of the other (RMAD4-7), to HD5 (human α-defensin produced by Paneth cells in the small intestine) [18]. In addition, six α-defensins (RED1-6) have been recently isolated from rhesus monkey small intestine and characterized [24]. As seen in Fig. 3, the homology of PHD3 to one of the rhesus monkey α-defensins, i.e., RMAD5, is 90% and to HD5, is 60%, whereas to HNP1, is <30%. The homologies of PHD1 and 2, which differ by one additional arginine in PHD1, is 50% to both, RMAD5 and HD5. The greatest similarity of the primary structures of PHD1 and 2 (69% homology) is with RED1, one of six enteral α-defensins of rhesus monkeys. In rhesus monkeys, the pair RMAD4 and RMAD5 also differs from the pair RMAD6 and RMAD7 by one additional arginine. The analysis of cDNA of the precursors of RMAD4-7 has shown that this residue results from alternative posttranslational modification [18]. It can be hypothesized that, in hamadryas baboons, the same occurs in the course of processing of the precursors of PHD1 and 2. The dendrogram of known α-defensins from rhesus monkey, hamadryas baboon, and human is shown in Fig. 4 and graphically demonstrates the relatedness of their primary structures.

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