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Antimicrobial activity of chicken and turkey heterophil peptides CHP1, CHP2, THP1, and THP3

Ellen W. Evans [a,*], F. Gwen Beach [a], Kristi M. Moore [b], Mark W. Jackwood [b], John R. Glisson [b], Barry G. Harmon [a]

[a] *Department of Veterinary Pathology, University of Georgia, Athens, GA, USA*

[b] *Poultry Diagnostic and Research Center, University of Georgia, Athens, GA, USA*

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Abstract

Four avian heterophil antimicrobial cationic peptides (Chicken Heterophil Peptides 1 and 2, and Turkey Heterophil Peptides 1 and 3) were evaluated for in vitro microbicidal activity against selected avian pathogens and human pathogens which are harbored by birds. At concentrations of 16–2 μg/ml, all four avian peptides effected a greater than 90% reduction in the survival of *Candida albicans*, *Salmonella enteriditis*, and *Campylobacter jejuni*. None of the peptides, including the known antimicrobial peptide protamine (used as a positive control), were able to reduce the survival of *Pasteurella multocida* by 90% at the maximum peptide concentration (16 μg/ml) tested. At 16 μg/ml, the turkey peptide THP3 did not effect a 90% reduction in survival of *Bordetella avium*, *Escherichia coli*, or *Salmonella typhimurium*, while all of the other peptides tested were effective at this concentration or less. This peptide, THP3, does not share the same homologous amino acid sequence shared by the other three peptides. Under our experimental conditions, none of the peptides neutralized Infectious Bronchitis Virus, an enveloped coronavirus of chickens.

Keywords: Beta-defensin; Avian heterophils; Chicken; Turkey; Antimicrobial peptides

1. Introduction

Cationic antimicrobial peptides have been isolated and characterized from plants (Ozaki et al., 1980; Duvick et al., 1992), insect immune hemolymph or fat body (Kimbrell, 1991), frog skin (Zasloff, 1987), mammalian tracheal and intestinal epithelium (Ouelette et al.,

* Corresponding author. Present address: Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706, USA. Tel: (608) 263-7862, Fax: (608) 263-6573, E-mail: EvansE@svm.vetmed.wisc.edu

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1989; Diamond et al., 1991; Jones and Bevins, 1993) hemocytes of horseshoe crabs (Muta et al., 1990), and leukocytes of a number of mammalian species (Lehrer et al., 1993). They have varying degrees of microbicidal activity against Gram-negative and Gram-positive bacteria, tumor cells, fungi, and enveloped viruses (Lehrer et al., 1993). This large group of peptides is an important component of innate disease resistance in all classes of animals.

We recently described the purification of five avian cationic antimicrobial peptides from turkey and chicken heterophil granules. These include Chicken Heterophil Peptides (CHP) 1 and 2, and Turkey Heterophil Peptides (THP) 1, 2, and 3. These peptides belong to a recently described group of chicken antimicrobial peptides termed "gallinacins" (Harwig et al., 1994). The chicken and turkey peptides also share considerable structural similarities with the bovine beta-defensins (Selsted et al., 1993; Harwig et al., 1994). Chicken Heterophil Peptides 1 and 2, and THP1 had bactericidal activity against a pathogenic field isolate of *Staphylococcus aureus* and a laboratory strain of *Escherichia coli* K-12 (ATCC #25922). Turkey Heterophil Peptides 2 and 3 were active against *S. aureus* but not *E. coli* (Evans et al., 1994).

Polymorphonuclear phagocytes (heterophils) of avian species lack myeloperoxidase (Breton-Gorius et al., 1978; MacRae and Powell, 1979), and have minimal ability to generate an oxidative burst with phagocytosis (Penniall and Spitznagel, 1975); therefore, nonoxidative microbicidal mechanisms such as cationic antimicrobial peptides are probably the principle antimicrobial mechanism for leukocytes of these species.

In the present study, we describe the in vitro antimicrobial activity of four avian heterophil peptides; CHP1 and 2, and THP1 and 3; against several avian pathogens including *Escherichia coli*, *Bordetella avium*, *Candida albicans*, *Mycoplasma gallisepticum*, and *Pasteurella multocida*. We have also tested the antiviral activity of the peptides against an enveloped coronavirus, Infectious Bronchitis Virus. The activity of the peptides against several human pathogens (*Salmonella enteriditis*, *Salmonella typhimurium*, and *Campylobacter jejuni*) which are harbored by birds was also tested.

2. Materials and methods

2.1. Avian heterophil antimicrobial peptides

Heterophils were collected as previously described from sterile peritoneal exudates induced in 6-week-old broiler chickens and turkey poults ($n=80$) (Sabet et al., 1977; Harmon et al., 1992). The granules were separated from the heterophils and peptides were extracted in acetic acid then purified by size exclusion column chromatography and high pressure liquid chromatography as previously described (Eisenhauer et al., 1989; Yamashita and Saito, 1989; Selsted et al., 1993). Four avian peptides, CHP 1 and 2, and THP 1 and 3 (Evans et al., 1994), were used for the antimicrobial assays described in this study. Peptide purity was assessed by acid-urea polyacrylamide gel electrophoresis and by high pressure liquid chromatography (Evans et al., 1994). Protein concentrations in 0.01% acetic acid were determined by spectrophotometric absorbance at 205 nm. The absorbance was converted to protein concentration (μg/ml) according to a published procedure (Stoscheck,

1990). From 20–30 mg of crude heterophil granule protein, 80–150 μg of purified peptides were obtained.

2.2. Bacteria and fungi

E. coli strain 4206, isolated from birds with air sacculitis (Poultry Diagnostic and Research Center [PDRC], Athens, GA); *C. albicans* American Type Culture Collection (ATCC) #14053, *S. enteriditis* ATCC #13076; and *S. typhimurium* strain SR 11, 3K-3181 (Lockman and Curtiss, 1992) were grown on blood agar plates, transferred to 3% trypticase soy broth (TSB, Difco Laboratories, Detroit, MI), and incubated for 18 h at 38°C. They were then transferred to fresh TSB and grown to the desired optical density for each microorganism immediately prior to the assay.

P. multocida serotype A:3, strain 1059 and a field isolate of *B. avium* (PDRC, Athens, GA) were grown at 38°C on blood agar and transferred to 3.7% Brain-Heart Infusion (BHI) (Difco, Detroit, MI) broth for 18-h cultures.

M. gallisepticum strain A5969 (PDRC, Athens, GA) (Ahmad et al., 1988) was grown entirely in Frey's liquid medium at 37°C, and 18-h cultures were used in the assay.

C. jejuni strain 81-176, which produced diarrhea in humans experimentally (Black et al., 1988), was grown on blood agar under microaerobic conditions. Colonies were transferred to a flask containing a solid phase of Brucella agar (Difco, Detroit, MI) beneath a layer of liquid medium consisting of 2.8% Brucella broth (Scott Chemical Co., Fiskeville, RI) with 0.05% ferrous sulfate (Baker Chemical Co., Phillipsburg, NJ), 0.02% sodium bisulfite (Sigma Chemical Co., St. Louis, MO), and 0.05% pyruvic acid (Sigma Chemical Co., St. Louis, MO) added. This culture was incubated for 20 h in 10% carbon dioxide, 5% oxygen and 90% nitrogen at 42°C prior to performing the assay.

2.3. Antibacterial and antifungal assays

Antibacterial and antifungal activities of the peptides were assessed by a colorimetric assay (Peck, 1985). Overnight or log-phase cultures of bacteria or *C. albicans* were washed in sterile 10 mM sodium phosphate buffer, pH 7.4. Concentrations in the sodium phosphate buffer were estimated by spectrophotometric absorbance at 620 nm and then confirmed by standard dilutions and plate counts, with the exception of *M. gallisepticum*, for which the inoculum density was determined using the Most Probable Number (MPN) technique to measure color changing units. The inocula placed in the wells for each assay are listed in Table 1. The inoculum for each microbe was adjusted to the lowest concentration for which microbicidal activity could be measured by this assay.

A 50-μl aliquot of sodium phosphate buffer containing bacteria or *C. albicans* was pipetted into wells of 96-well tissue culture plates. Peptide solution (10 μl) was then added to these wells in two-fold dilutions from 16 μg/ml to 2 μg/ml (final concentration) in 0.01% sterile acetic acid. All peptides were tested in duplicate. As a positive control, antimicrobial activity of a known microbicidal peptide, protamine sulfate (Sigma Chemical Co., St. Louis, MO), was assessed. The wells were prepared as for the peptide assays, but the two-fold dilutions started with 67 μg/ml and ended with 0.25 μg/ml in 0.01% acetic

Table 1
Colony forming units of bacteria or fungi added to each test well prior to addition of peptide

Bacteria/fungus	Inoculum	
	Protamine assay	Heterophil peptide assay
C. albicans	5.00×10^5	1.81×10^5
B. avium	8.96×10^5	8.96×10^5
E. coli	2.40×10^5	1.48×10^5
S. enteriditis	1.30×10^5	1.99×10^5
S. typhimurium	1.46×10^5	1.65×10^5
C. jejuni	1.12×10^5	1.12×10^5
M. gallisepticum	5.15×10^7	5.15×10^7
P. multocida	1.12×10^5	1.12×10^5

acid. Negative control wells consisted of quadruplicate wells containing the bacteria or fungus to which 10 μl of 0.01% sterile acetic acid without peptides was added.

A standard curve was obtained by preparing duplicate wells with serial two-fold dilutions of microbes and no peptide (Fig. 1). The plates were incubated for 1 h at 38°C. Then 50–100 μl of liquid medium was added to all wells, and the plates were incubated for 3 h, or 10 h in the case of *C. jejuni*. After the incubation in media, 10 μl of 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) was added to the wells, and the plates were incubated for 10 to 120 min. Dehydrogenases of viable cells cleave the tetrazolium ring of MTT, which produces formazan, a purple dye. Optical densities at 570 nm were read by an automated micro-plate reader (MR-700; Dynatech Laboratories, Chantilly, Va.) which constructed standard curves and calculated the relative bacterial or fungal concentrations in the test wells. Serial dilutions of samples from selected test wells were cultured on blood agar plates or, for *M. gallisepticum*, in liquid medium, to confirm the results of the colorimetric assay. For comparison of antimicrobial activity among peptides,

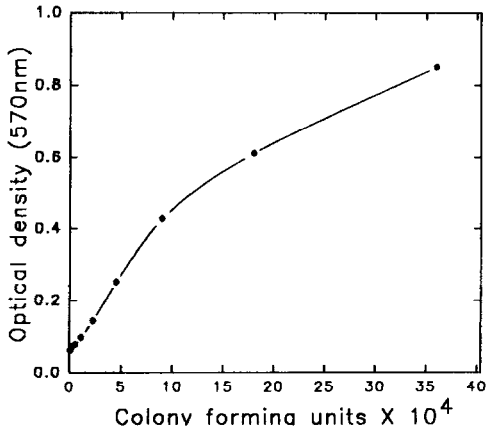

Fig. 1. Standard curve for *Candida albicans*. The average optical density of duplicate wells (Y-axis) is plotted against the number of bacteria in each well (X-axis). The color change (increase in optical density) is due to

a minimum inhibitory concentration was defined as the concentration of peptide required to reduce the bacterial concentration to less than 10% of the original inoculum.

2.4. Antiviral assay

The antiviral assay was conducted with the Arkansas DPI strain of Infectious Bronchitis Virus, serotype Arkansas (Gelb et al., 1981). Antiviral activity was determined by incubating virus with peptide, followed by observing cytopathic effects of the virus on chicken embryo kidney cells in vitro. Monolayers of chicken embryo kidney cells were prepared in 96-well tissue culture plates according to established procedures (Schat and Purchase, 1989). The peptides were dissolved in sterile 0.01% acetic acid at a concentration of 100 μg/ml and two-fold dilutions to 0.38 μg/ml were made with maintenance medium: F-10-M199 Medium (Gibco, Grand Island, NY). The peptide solutions were then incubated for one hour at room temperature with the virus; 50 μl of each peptide solution was incubated with 100 μl of virus solution containing 2×10^5 tissue culture infectious dose-50's ml^{-1} of maintenance medium, calculated for the highest dilution causing cytopathic effects in cell culture. The peptide/virus solutions were added, 75 μl/well, to a chicken embryo kidney cell monolayer with 125 μl of F-10-M199 media containing 3% calf serum. The plates were then incubated at 37°C and 5% CO_2. Peptides were tested in duplicate. The plates were examined daily by light microscopy for cytopathic effects (King and Cavanagh, 1991).

3. Results

3.1. Antibacterial and antifungal assays

At a concentration of 16 μg/ml, all four avian peptides possessed some degree of microbicidal activity against all of the microorganisms tested (Table 2). A minimum inhibitory concentration could not be demonstrated at 16 μg/ml for any peptide against *P. multocida* (Table 2). However, all of the peptides produced a 70–80% reduction in the concentration of *P. multocida* at 16 μg/ml (data not shown). At the maximum concentration, THP3 did not cause a 90% reduction in the survival of *B. avium*, *E. coli*, or *S. typhimurium* (Table 2). Of the avian peptides tested, THP1 required the lowest concentration for inhibition. Protamine inhibited at lower concentrations than the avian peptides against all microorganisms except *P. multocida* and *M. gallisepticum*. Even within the narrow range of peptide concentrations tested, a concentration-dependent reduction in bacterial survival was demonstrated (Table 3).

3.2. Antiviral assay

Cytopathic effects including syncytia formation, clustering and swelling of epithelial cells, and grainy cytoplasm were seen in all test wells, indicating minimal to no neutralization of the virus at any concentration by any of the four peptides tested.

Table 2
Average minimum inhibitory concentrations of peptides for each bacterium and fungus assayed

Bacteria/fungus	Peptide				
	protamine	CHP1	CHP2	THP1	THP3
C. albicans[a]	2[b]	8	16	8	16
B. avium	2	16	16	8	(>16)
E. coli	2	16	16	8	(>16)
S. enteriditis	0.5	16	8	8	8
S. typhimurium	0.5	4	8	4	(>16)
C. jejuni	2	2	4	2	16
P. multocida	(>16)[c]	(>16)	(>16)	(>16)	(>16)
M. gallisepticum	8	16	16	8	8

[a]The number of bacteria and fungi placed in each well at the beginning of the assay is listed in Table 1.
[b]Final concentration of peptide (in μg/ml).
[c]Minimum inhibitory concentration was not demonstrated at the maximum concentration (16 μg/ml) tested.

Table 3
Average percent survival of bacteria following addition of CHP1 at each concentration tested

Concentration of peptide	Bacteria	
(μg/ml)	*E. coli*[a]	*M. gallisepticum*
16	0%	7%
8	60	25
4	100	57
2	100	57

[a]The numbers of *E. coli* and *M. gallisepticum* placed in wells at the beginning of the assay are listed in Table 1.

4. Discussion

The four peptides derived from avian heterophil granules had a wide spectrum of microbicidal activity, being effective against fungi, a mycoplasma, and a variety of Gram-negative bacteria. Their effectiveness against *S. aureus* was demonstrated in a previous report (Evans et al., 1994). The results of these in vitro studies suggest that the avian antimicrobial peptides are important components of heterophil defenses in vivo.

Three peptides, CHP1, CHP2 and THP1, are very similar in primary structure and they are similar in spectrum of microbicidal activity. The two chicken peptides, CHP1 and 2, differ only by 4 amino acids in the known N-terminal amino acid sequences. Of the 35 known N-terminal residues for THP1, only 12 are different from the 33 known N-terminal residues of CHP2 (Evans et al., 1994). Despite the similarities in primary structure and spectrum of activity, these peptides varied somewhat in their relative potencies for different microorganisms, suggesting that slight differences in primary structure may influence interactions with specific target microorganisms. The fourth peptide, THP3, shares less primary structural homology with the other three and appeared less potent against the particular microbes tested. The presence of a variety of antimicrobial granulocyte peptides may be

necessary for a broad spectrum of antimicrobial activity. It is also possible that the heterophil peptides may act synergistically in killing microbes. It would be interesting to conduct studies comparing the activity of individual peptides with that of a combination of peptides. These peptides appeared least effective against *P. multocida*. This finding supports the findings of a previous study which showed that heterophil lysates were not very effective against *P. multocida*, yet they were effective against *S. aureus* and *E. coli* (Harmon et al., 1992). However, intact heterophils readily kill *P. multocida* (Harmon et al., 1992). The action of other heterophil components may be required to kill some bacteria.

The avian peptides were quite effective against the strains of *S. typhimurium* and *C. jejuni* which are pathogenic in humans, and were effective against the laboratory strain of *S. enteriditis*. Future investigations should compare the effectiveness of avian antimicrobial peptides with that of human enteric defensins against these bacteria and more virulent human pathogens. The susceptibility of human pathogens to avian antimicrobial peptides may be a component of the bacteria's ability to exist in the intestinal tracts of poultry without causing disease in their avian hosts.

The peptides killed laboratory strains of *C. albicans* and *M. gallisepticum*. To establish the importance of antimicrobial peptide resistance as virulence factors for *C. albicans* and *M. gallisepticum*, it will be necessary to assess the peptides' activity against highly virulent field strains of these organisms as well.

The inability of the four peptides to neutralize Infectious Bronchitis Virus may represent viral resistance to peptide neutralization. It is also possible that the higher ionic concentration of the cell medium vs. the buffers used in other assays may have influenced microbicidal activity. Defensins can be less active against bacteria and fungi under high-ionic-strength conditions (Selsted et al., 1984, 1985).

In summary, CHP1, CHP2, THP1, and THP3 were bactericidal and fungicidal, but were unable to neutralize Infectious Bronchitis Virus under these experimental conditions. Differences in antimicrobial activity among the peptides may be related to peptide structure, and the presence of a variety of peptides may be necessary for a competent heterophil defense system. The importance of resistance to these peptides as virulence factors for pathogens should be the subject of future studies.

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