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Research Note—

Antimicrobial Activity of Chicken NK-Lysin Against *Eimeria* Sporozoites

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SUMMARY. NK-lysin is an antimicrobial and antitumor polypeptide that is considered to play an important role in innate immunity. Chicken NK-lysin is a member of the saposin-like protein family and exhibits potent antitumor cell activity. To evaluate the antimicrobial properties of chicken NK-lysin, we examined its ability to reduce the viability of various bacterial strains and two species of *Eimeria* parasites. Culture supernatants from COS7 cells transfected with a chicken NK-lysin cDNA and His-tagged purified NK-lysin from the transfected cells both showed high cytotoxic activity against *Eimeria acervulina* and *Eimeria maxima* sporozoites. In contrast, no bactericidal activity was observed. Further studies using synthetic peptides derived from NK-lysin may be useful for pharmaceutical and agricultural uses in the food animal industry.

RESUMEN. *Nota de Investigación*—Actividad antimicrobiana del polipéptido aviar NK-lisina contra los esporozoitos de *Eimeria*.

El polipéptido aviar NK-lisina tiene propiedades antitumorales y antimicrobianas y se considera que juega un papel importante en la inmunidad innata. Este polipéptido aviar pertenece a la familia de proteínas similares a la saposina y muestra una gran actividad contra las células tumorales. Para evaluar las propiedades antimicrobianas del polipéptido aviar NK-lisina, examinamos su capacidad para reducir la viabilidad de varias cepas bacterianas y dos especies de *Eimeria*. Los sobrenadantes de cultivo de las células COS7 transfectadas con un ADN complementario del polipéptido NK-lisina y con un polipéptido purificado de las células transfectadas, ambos mostraron una alta actividad citotóxica contra los esporozoitos de *Eimeria acervulina* y de *Eimeria maxima*. Por el contrario, no se observó actividad bactericida. Estudios adicionales usando péptidos sintéticos derivados del polipéptido NK-lisina pueden ser útiles para usos farmacéuticos y agrícolas en la industria de alimentos de origen animal.

Key words: NK-lysin, antimicrobial activity, *Eimeria*, sporozoites

Abbreviations: BHI agar = brain-heart infusion agar; CFU = colony-forming units; ESTs = expressed sequence tags; IELs = intestinal intraepithelial lymphocytes; IMDM = Iscove's modified Dulbecco's medium; MIC = minimum inhibitory concentration; NK = natural killer; OD = optical density; sup = supernatant

Antimicrobial peptides such as NK-lysin are widespread in nature and have been characterized in mammalian as well as avian species (11). NK-lysin in mammals, or granulysin in humans, is an antimicrobial and antitumor polypeptide expressed by natural killer (NK) cells and T lymphocytes. It possesses lytic activities against gram-positive and gram-negative bacteria, fungi, and protozoan parasites (6,9,10,13,20). NK-lysin interacts with outer-membrane lipids (lipopolysaccharides) of gram-negative bacteria and forms pores in the cell membrane because of its α-helical structure, although this property is not generally sufficient for bacterial killing (2,6). NK-2, a shortened synthetic peptide comprising residues 39–65 derived from the cationic core region of porcine NK-lysin with homologs in human and cattle, kills cancer cells as well as a variety of gram-negative and gram-positive bacteria, but exhibits no hemolytic or cytotoxic activity against human cells (4,5,13,19).

In previous reports, we described the cloning and characterization of chicken NK-lysin, which exhibited antitumor cell activity against a retrovirus-transformed B-cell line and comprised an important part of the innate immune response during avian coccidiosis, an intestinal disease resulting from infection by *Eimeria* parasites (11,14). In the current study, we undertook an investigation of the cytotoxic activity of chicken NK-lysin against bacteria strains and *Eimeria* parasites.

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MATERIALS AND METHODS

Cell culture and NK-lysin transfection. COS7 cells (ATCC, Manassas, VA) were cultured in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and transiently transfected at 80%–90% confluence with the chicken NK-lysin cDNA cloned in the pTriEx4 vector as described (11). At 18 hr posttransfection, the cells were cultured for 24 hr in IMDM containing 10 mM **i**nsulin-transferrin-sodium selenite supplement (Sigma, St. Louis, MO) and recombinant histidine-tagged NK-lysin was purified from cell-conditioned culture medium or cell lysates on a Ni^{2+}-NTA His-bind resin column (Novagen, Madison, WI) according to the manufacturer's protocol.

Bactericidal test of NK-lysin. To evaluate the bactericidal activity of NK-lysin, a standardized broth microdilution assay against *Escherichia coli* strain P4 (gift of Dr. A. J. Bramley, Institute for Animal Health, Compton Laboratory, Newbury, England) and *Staphylococcus aureus* strain 305 (ATCC) was performed and the minimum inhibitory concentration (MIC) values of the purified recombinant protein were determined.

Tetracycline MIC values against the bacteria were determined in parallel as positive controls. The assays were performed in accordance with Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) guidelines (17,18). Briefly, a single colony of each fresh bacterial inoculum was diluted in cation-adjusted Mueller-Hinton broth (BD Biosciences, Franklin Lakes, NJ) and the suspension adjusted to achieve a transmittance equivalent to a 0.5 McFarland standard (10^8 colony-forming units [CFU]/ml) using a

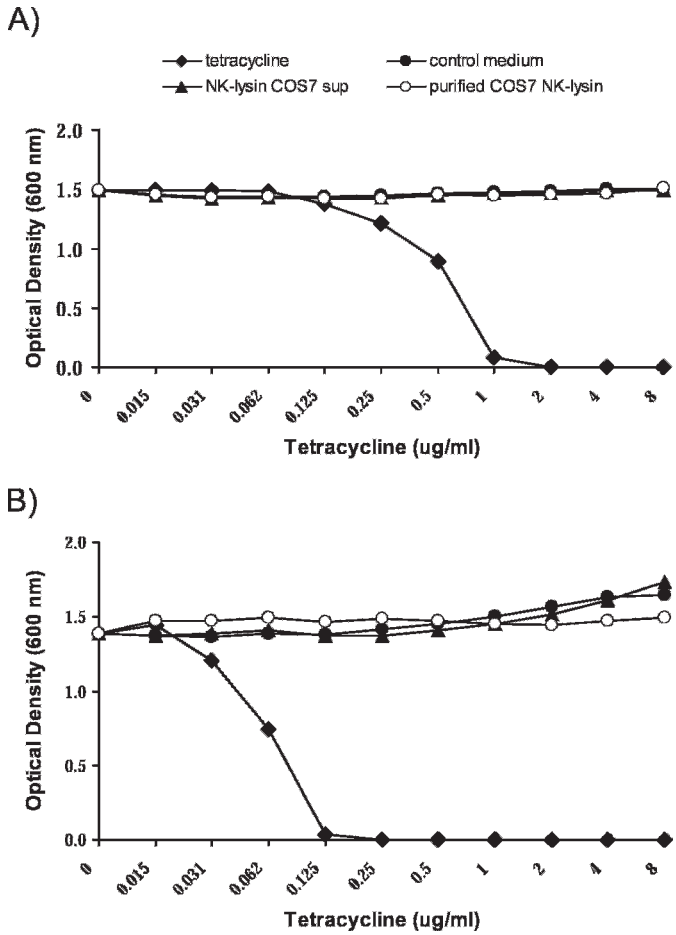

Fig. 1. Inhibition-zone assay for chicken NK-lysin against *E. coli* (A) and *S. aureus* (B). Tetracycline (0–8 μg/ml), chicken NK-lysin–transfected cell supernatant (sup) (1:2 serial dilutions), or His-tag purified NK-lysin (0–30 μg/ml) were added to cultures of the bacteria and incubated for 18 hr at 35 C; bacterial growth was determined by measuring OD_{600}. The X-axis is showing the tetracycline concentrations only.

colorimeter. The bacterial suspension was diluted 100-fold in cation-adjusted Mueller-Hinton broth to 1.0×10^6 CFU/ml. Fifty microliters was added to the wells of a 96-well round-bottom plate containing 50 μl of either broth alone, or broth containing serially diluted NK-lysin or tetracycline (Sigma). A negative control consisted of an eluate of a mock-loaded His-bind resin column with 100 μl of broth alone. The wells were incubated for 18 hr at 37 C with ambient air circulation and without shaking, and were subsequently inspected for cloudiness and pellet formation using a magnifying glass plate reader. The lowest concentration at which no pellet or cloudiness was apparent in a given well was identified as the MIC. In addition to visualization by eye, the optical density (OD) of each well was measured on a Synergy HT™ multimodal plate reader (Bio-Tec Instruments, Winooski, VT) at 600 nm. Background values, derived from noninoculated wells containing broth alone, were subtracted from all readings.

For the inhibition-zone assay, 50-μl samples of 16 broth-cultured bacteria strains (1.0×10^6 CFU/ml) were spread on brain-heart infusion (BHI) agar (Anaerobe Systems, Morgan Hill, CA), allowed to briefly dry and settle, and 5 or 10 μl of chicken NK-lysin–transfected COS7 cell supernatant (sup) or negative control medium was spotted. The plates were incubated for 18 hr at 37 C, 42 C (*Campylobacter*), or 30 C (*Vibrio*).

Antimicrobial test to *Eimeria* sporozoites. *Eimeria acervulina* and *Eimeria maxima* sporozoites were obtained by excystation of sporulated oocysts (8). After breaking the oocyst membrane by a Potter-Elvenheim tissue grinder tube, sporocysts were purified by isopycnic centrifugation on a Percoll gradient and washed in cold phosphate-buffered saline, then treated with excystation solution (0.25% trypsin, 0.014 M taurocholic acid) for 60 min at 41 C to release sporozoites. Excystation fluid was filtered and the sporozoites were washed three times with cold Hank's balanced salt solution at 3000 rpm for 10 min at 4 C and resuspended to 1.0×10^6/ml in 10% RPMI-1640 medium (Sigma). One milliliter of each sporozoite solution (1.0×10^6/ml) was incubated with 1 ml of NK-lysin–transfected COS7 cell supernatants (sup) or COS7 cell purified NK-lysin (0.17ng/ul) for 24 or 48 hr at 41 C in 5-ml polystyrene round-bottom tubes (BD Bioscience) in triplicate and stained with trypan blue; viable sporozoites were counted microscopically.

Statistical analysis. Mean ± SD values for each group ($n = 3$) were calculated and analyzed with the Dunnet multiple comparison test using InStat® software (Graphpad, San Diego, CA) and considered significant at $P < 0.05$.

RESULTS

Bactericidal test of chicken NK-lysin. Neither cell culture supernatant from chicken NK-lysin–transfected COS7 cells nor purified His-tagged recombinant NK-lysin protein inhibited the growth of *E. coli* or *S. aureus* at any concentration tested (Fig. 1). The MIC values of tetracycline against *S. aureus* (0.125 μg/ml) and *E. coli* (1.0–2.0 μg/ml) were within the reported quality control ranges of 0.12–1.0 μg/ml and 0.5–2.0 μg/ml, respectively (17,18). Moreover, by the inhibition zone assay, NK-lysin containing COS7 cell supernatant (sup) demonstrated no visible antimicrobial activity against a panel of 16 bacterial strains (Table 1).

Cytotoxic activity to *Eimeria* sporozoites. By contrast, incubation of *E. acervulina* or *E. maxima* sporozoites for 24 hr with NK-lysin cell culture supernatant (sup) or purified protein significantly decreased parasite viability compared with the medium-only negative control (Fig. 2). Interestingly, the number of sporozoites in the control also was drastically reduced at 24 hr incubation, from about 25,000 to 11,000 in the case of *E. acervulina* and from about 25,000 to 5000 in *E. maxima*.

This cytotoxic effect persisted following 48 hr incubation using *E. maxima*, but not in the case of *E. acervulina* due to overall loss of sporozoite viability for this extended time period.

DISCUSSION

In previous reports, we identified a set of overlapping chicken-expressed sequence tags (ESTs) homologous to mammalian NK-lysin, cloned a full-length chicken NK-lysin cDNA, and observed high expression of the endogenous gene in intestinal intraepithelial lymphocytes (IELs), a population well enriched with NK cells (11,16). Although chicken NK-lysin showed relatively low amino acid sequence identity to mammalian NK-lysins or granulysin (<20%), it possessed the characteristically conserved six cysteine and one proline residues, hallmarks of the saposin protein family, and necessary to form three disulfide bonds required for antimicrobial activity (1,3). Therefore, it was reasonable to assume, like the mammalian proteins, that chicken NK-lysin possessed a broad-spectrum antimicrobial activity.

Although our prior studies did demonstrate an antitumor effect of chicken NK-lysin, preliminary unpublished results revealed that it lacked antibacterial activity. Therefore, the current study was undertaken to examine this apparent paradox in more detail. Our results showed that NK-lysin had no demonstrable bactericidal activity against a wide range of gram-positive and gram-negative microorganisms tested (Fig. 1, Table 1). Similarly, Linde *et al.* (15)

Table 1. Antibacterial activity of chicken NK-lysin supernatant (sup) using the inhibition-zone assay. COS7 cell culture supernatant was harvested from pTriEx4-NK-lysin–transfected cells. Each culture of 16 test bacterial strains (1.0×10^6 CFU/ml) was spread on BHI agar, 5 or 10 μl of supernatant (sup) or control medium was spotted, and the plates were incubated for 18 hr under appropriate conditions at 37 C, 42 C (*Campylobacter*), or 30 C (*Vibrio*).

Organism	Strain	5-μl Spot result	10-μl Spot result
Bacillus subtilis	STOP	neg[A]	neg
Salmonella Montevideo	S1	neg	neg
Salmonella Heidelberg	S2	neg	neg
Clostridium perfringens	A. 1113	neg	neg
Clostridium difficile	#3	neg	neg
Clostridium difficile	27.1	neg	neg
Campylobacter jejuni	C3 Dreeson	neg	neg
Campylobacter jejuni	C2 Dreeson	neg	neg
Enterobacter sakazaki	A. 51329	neg	neg
Vibrio harveyi	TAES2	neg	neg
Klebsiella pneumoniae	A. 13883	neg	neg
Escherichia coli O157:H7	265RC1	neg	neg
Enterobacter cloacae	A. 23355	neg	neg
Enterococcus faecium	50-52	neg	neg
Staphylococcus aureus	A. 25923	neg	neg
Proteus vulgaris	A. 13315	neg	neg

[A]neg = No visible zone of inhibition.

reported that a synthetic peptide (cNKLF2) corresponding to the core sequence of chicken NK-lysin did not inhibit the growth of *E. coli* D21, although antimycobacterial activity was observed against *Bacillus megaterium* and *S. aureus* when a buffer-diluted medium was used instead of Luria-Bertani medium. Chicken recombinant NK-lysin, however, was cytotoxic for *E. acervulina* and *E. maxima* parasites. It is suggests that the massive reduction in numbers of sporozoites in the control from 0 to 24 hr, especially with *E. maxima* sporozoites, may be species-dependent because of the natural loss as a result of *in vitro* maintenance. Interestingly, *E. tenella* sporozoites were relatively more resistant to the effects of NK-lysin supernatant (sup) compared with these other two species (data not shown).

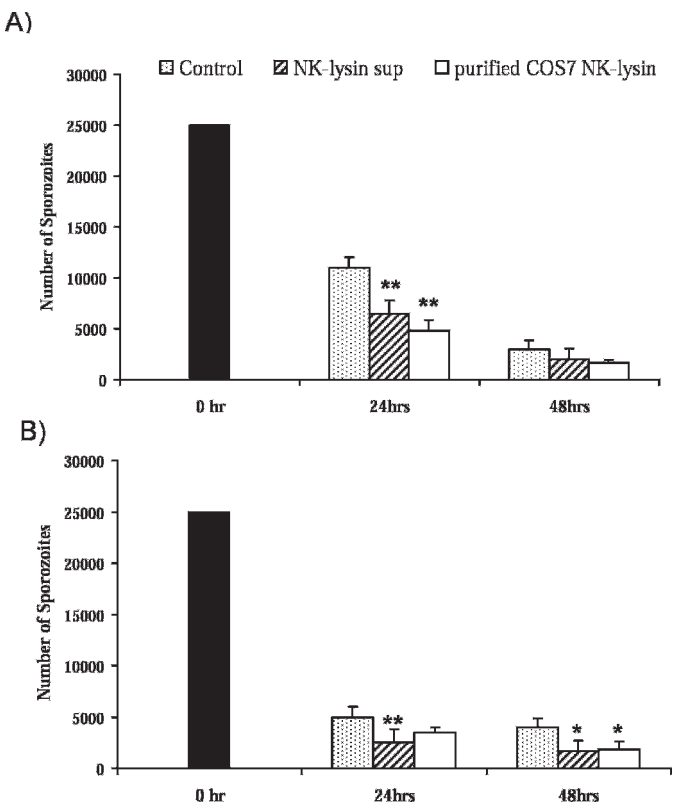

Fig. 2. Cytotoxic activity of chicken NK-lysin for *E. acervulina* (A) and *E. maxima* (B) sporozoites. Sporozoites were incubated with control media, which came from COS7 culture, NK-lysin–transfected COS7 cell culture supernatant (sup), or purified NK-lysin (0.17ng/ul) for 24 or 48 hr at 41 C; sporozoite viability was assessed by trypan blue exclusion. * indicates $P < 0.05$; ** indicates $P < 0.01$.

The microbial plasma membrane is the target of NK-lysin or derived synthetic peptides such as NK-2, and cell death is accompanied by the formation of pores in the phospholipid bilayer. Such a molecular mechanism may explain how the NK-2 peptide kills parasites, such as intracellular trypanosomes, without destroying host cells (13). Moreover, these residues are directly related to association of peptides with pathogen membranes that ultimately leads to membrane disruption. Current studies are underway in our laboratory to determine the ability of chicken NK-lysin to form pores in the membranes of bacteria and *Eimeria*.

Min *et al.* (16) reported that chicken NK-lysin was one of the most highly expressed transcripts detected in an *Eimeria*-induced intestinal IEL cDNA library, being represented by 87 individual ESTs. These results directly correlated with high NK-lysin mRNA levels in gut IELs as assessed by quantitative reverse transcription–polymerase chain reaction as well as intestinal NK cell activity following *Eimeria* infection (7,12). Furthermore, NK-lysin expression in the intestinal jejunum following *E. maxima* infection, a species that preferentially infects this region of the gut (12), was higher than those of the duodenum or caeca. Thus, it is clearly apparent that chicken NK-lysin plays an important role in intestinal innate immunity.

In summary, chicken NK-lysin displayed direct cytotoxic activity against *Eimeria* sporozoites, but had no bactericidal effect. Future studies using purified recombinant NK-lysin or synthetic peptides derived from its amino acid sequence are expected to shed new light on antiparasite peptides and proteins that may hold promise for pharmaceutical and agricultural uses in the food animal industry (21).

REFERENCES

1. Andersson, M., T. Curstedt, H. Jornvall, and J. Johansson. An amphipathic helical motif common to tumourolytic polypeptide NK-lysin

and pulmonary surfactant polypeptide SP-B. FEBS Lett. 362:328–332. 1995.

2. Andersson, M., R. Girard, and P. Cazenave. Interaction of NK lysin, a peptide produced by cytolytic lymphocytes, with endotoxin. Infect. Immun. 67:201–205. 1999.

3. Andersson, M., A. Holmgren, and G. Spyrou. NK-lysin, a disulfide-containing effector peptide of T-lymphocytes, is reduced and inactivated by human thioredoxin reductase. Implication for a protective mechanism against NK-lysin cytotoxicity. J. Biol. Chem. 271:10116–10120. 1996.

4. Andra, J., M. H. Koch, R. Bartels, and K. Brandenburg. Biophysical characterization of endotoxin inactivation by NK-2, an antimicrobial peptide derived from mammalian NK-lysin. Antimicrob. Agents Chemother. 48:1593–1599. 2004.

5. Andra, J., and M. Leippe. Candidacidal activity of shortened synthetic analogs of amoebapores and NK-lysin. Med. Microbiol. Immunol. 188:117–124. 1999.

6. Andreu, D., C. Carreno, C. Linde, H. G. Boman, and M. Andersson. Identification of an anti-mycobacterial domain in NK-lysin and granulysin. Biochem. J. 344(Pt. 3):845–849. 1999.

7. Chai, J. Y., and H. S. Lillehoj. Isolation and functional characterization of chicken intestinal intra-epithelial lymphocytes showing natural killer cell activity against tumour target cells. Immunology 63:111–117. 1988.

8. Dulski, P., and M. Turner. The purification of sporocysts and sporozoites from *Eimeria tenella* oocysts using Percoll density gradients. Avian Dis. 32:235–239. 1988.

9. Ernst, W. A., S. Thoma-Uszynski, R. Teitelbaum, C. Ko, D. A. Hanson, C. Clayberger, A. M. Krensky, M. Leippe, B. R. Bloom, T. Ganz, and R. L. Modlin. Granulysin, a T cell product, kills bacteria by altering membrane permeability. J. Immunol. 165:7102–7108. 2000.

10. Gansert, J. L., V. Kiessler, M. Engele, F. Wittke, M. Rollinghoff, A. M. Krensky, S. A. Porcelli, R. L. Modlin, and S. Stenger. Human NKT cells express granulysin and exhibit antimycobacterial activity. J. Immunol. 170:3154–3161. 2003.

11. Hong, Y. H., H. S. Lillehoj, R. A. Dalloul, W. Min, K. B. Miska, W. Tuo, S. H. Lee, J. Y. Han, and E. P. Lillehoj. Molecular cloning and characterization of chicken NK-lysin. Vet. Immunol. Immunopathol. 110:339–347. 2006.

12. Hong, Y. H., H. S. Lillehoj, E. P. Lillehoj, and S. H. Lee. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria maxima* infection of chickens. Vet. Immunol. Immunopathol. 114:259–272. 2006.

13. Jacobs, T., H. Bruhn, I. Gaworski, B. Fleischer, and M. Leippe. NK-lysin and its shortened analog NK-2 exhibit potent activities against *Trypanosoma cruzi*. Antimicrob. Agents Chemother. 47:607–613. 2003.

14. Lillehoj, H. S., W. Min, and R. A. Dalloul. Recent progress on the cytokine regulation of intestinal immune responses to *Eimeria*. Poult. Sci. 83:611–623. 2004.

15. Linde, C. M., S. Grundstrom, E. Nordling, E. Refai, P. J. Brennan, and M. Andersson. Conserved structure and function in the granulysin and NK-lysin peptide family. Infect. Immun. 73:6332–6339. 2005.

16. Min, W., H. S. Lillehoj, C. M. Ashwell, C. P. van Tassell, R. A. Dalloul, L. K. Matukumalli, J. Y. Han, and E. P. Lillehoj. EST analysis of *Eimeria*-stimulated intestinal intraepithelial lymphocytes in chickens. Mol. Biotechnol. 30:143–150. 2005.

17. National Committee for Clinical Laboratory Standards. Methods for determining bacterial activity of antimicrobial agents; approved guideline. Wayne, PA. 1999.

18. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard. In: The National Committee for Clinical Laboratory Standards (NCCLS). Wayne, PA. 2002.

19. Schroder-Borm, H., R. Bakalova, and J. Andra. The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increased surface levels of negatively charged phosphatidylserine. FEBS Lett. 579:6128–6134. 2005.

20. Stenger, S., D. A. Hanson, R. Teitelbaum, P. Dewan, K. R. Niazi, C. J. Froelich, T. Ganz, S. Thoma-Uszynski, A. Melian, C. Bogdan, S. A. Porcelli, B. R. Bloom, A. M. Krensky, and R. L. Modlin. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 282:121–125. 1998.

21. Zhang, G., C. R. Ross, and F. Blecha. Porcine antimicrobial peptides: new prospects for ancient molecules of host defense. Vet. Res. 31:277–296. 2000.

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