

Antimicrobial Peptide Defenses in Amphibian Skin¹

LOUISE A. ROLLINS-SMITH,^{2*} LAURA K. REINERT,* CHADRICK J. O'LEARY,* LAURA E. HOUSTON,* AND DOUGLAS C. WOODHAMS*

**Departments of Microbiology and Immunology and of Pediatrics, Vanderbilt University Medical Center, Nashville, Tennessee 37232*

SYNOPSIS. One of the most urgent problems in conservation biology today is the continuing loss of amphibian populations on a global scale. Recent amphibian population declines in Australia, Central America, the western United States, Europe, and Africa have been linked to a pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, which infects the skin. The skin of amphibians is critical for fluid balance, respiration, and transport of essential ions; and the immune defense of the skin must be integrated with these physiological responses. One of the natural defenses of the skin is production of antimicrobial peptides in granular glands. Discharge of the granular glands is initiated by stimulation of sympathetic nerves. To determine whether antimicrobial skin peptides play a role in protection from invasive pathogens, purified antimicrobial peptides and natural peptide mixtures recovered from the skin secretions of a number of species have been assayed for growth inhibition of the chytrid fungus. The general findings are that most species tested have one or more antimicrobial peptides with potent activity against the chytrid fungus, and natural mixtures of peptides are also effective inhibitors of chytrid growth. This supports the hypothesis that antimicrobial peptides produced in the skin are an important defense against skin pathogens and may affect survival of populations. We also report on initial studies of peptide depletion using norepinephrine and the kinetics of peptide recovery following induction. Approximately 80 nmoles/g of norepinephrine is required to deplete peptides, and peptide stores are not fully recovered at three weeks following this treatment. Because many species have defensive peptides and yet suffer chytrid-associated population declines, it is likely that other factors (temperature, conditions of hydration, "stress," or pesticides) may alter normal defenses and allow for uncontrolled infection.

IMMUNE DEFENSES IN AMPHIBIAN SKIN

Amphibians are ancient creatures, and their immune defenses are highly evolved (reviewed in Carey *et al.*, 1999; Rollins-Smith, 2001; Rollins-Smith and Cohen, 2004). Amphibian skin is protected by both innate and adaptive immune defenses. The adaptive defenses include antibody and T lymphocyte-mediated responses that develop following the detection of pathogens by antigen presenting cells (macrophages and dendritic cells) (Du Pasquier and Flajnik, 1990; Castell-Rodriguez *et al.*, 1999, 2001). These defenses are somewhat slow to develop in cold-blooded vertebrates. In addition to the adaptive immune system, the skin is protected by innate mechanisms that may include macrophages and neutrophils (Manning and Horton, 1982; Corsaro *et al.*, 2000) complement-mediated lysis of pathogens (Green and Cohen, 1977; Grossberger *et al.*, 1989; Kato *et al.*, 1994, 1995; Lambris *et al.*, 1995), natural killer cells (Horton *et al.*, 1996, 1998, 2000, 2003), and secreted antimicrobial peptides (reviewed in Nicolas and Mor, 1995; Simmaco *et al.*, 1998; Zasloff, 2002; Rinaldi, 2002; Conlon *et al.*, 2004; Apponyi *et al.*, 2004).

ANTIMICROBIAL PEPTIDES ARE PRODUCED IN GRANULAR GLANDS

Antimicrobial peptides are synthesized and stored in the granular glands of the dermal layer of the skin

(also called poison glands) (Bovbjerg, 1963; Mills and Prum, 1984). Granular glands are syncytial structures (Dockray and Hopkins, 1975) surrounded by a layer of smooth muscle cells innervated by sympathetic nerves (Sjoberg and Flock, 1976). Following alarm or injury, the sympathetic nervous system is activated, adrenergic receptors are stimulated (Benson and Hadley, 1969; Holmes and Balls, 1978), and the contents of the gland are released to the surface of the skin. Antimicrobial peptides and other bioactive peptides are synthesized as larger proteins with a signal sequence and an acidic propeptide that are cleaved to release the mature active peptide before or at the time of secretion from granular glands (reviewed in Amiche *et al.*, 1999; Bowie *et al.*, 1999). Electrostimulation of the skin or exposure to adrenergic agents such as epinephrine or norepinephrine will artificially induce secretion of the contents of the granular glands (Benson and Hadley, 1969; Dockray and Hopkins, 1975; Holmes and Balls, 1978; Tyler *et al.*, 1992). The greater the degree of stimulation, the greater the amount of total peptides recovered as shown by a recent experiment using *Xenopus laevis* induced to secrete peptides by injection of norepinephrine. Approximately 80 nmoles/g of norepinephrine is required for maximal peptide secretion (Fig. 1).

RECOVERY OF PEPTIDE STORES FOLLOWING NOREPINEPHRINE-INDUCED DEPLETION

Several investigators have studied the rate of renewal of peptides in granular glands following maximal discharge induced by adrenergic agents. In *X. laevis*,

¹ From the Symposium on *EcoPhysiology and Conservation: The Contribution of Endocrinology and Immunology* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 5–9 January 2004, at New Orleans, Louisiana.

² E-mail: louise.rollins-smith@vanderbilt.edu

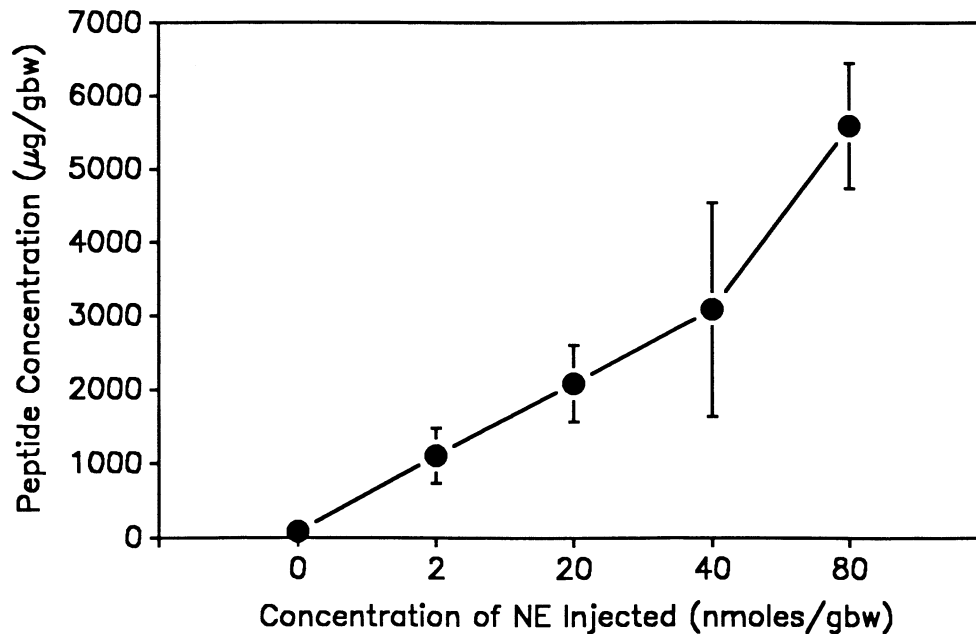


FIG. 1. Total concentration ($\mu\text{g}/\text{gram body weight}$) (gbw) of skin peptides (mean \pm SE) recovered from adult J-strain *Xenopus laevis* following injection of increasing concentrations of norepinephrine (NE). Frogs (3–5 individual frogs per dose) were injected bilaterally with 0, 2, 20, 40, or 80 nmoles/gram body weight (gbw) of [+/–]-norepinephrine hydrochloride in a volume of 0.1 ml sterile amphibian phosphate buffered saline per 10 grams of body weight via the dorsal lymph sac. Immediately following injection, the frog was placed in 100 ml of collection buffer (25 mM NaCl and 25 mM sodium acetate, pH 7.0) (buffer adapted from Giovannini *et al.*, 1987; Rollins-Smith *et al.*, 2002c), and skin secretions were allowed to accumulate in the buffer for a period of 15 minutes. Following release of skin secretions, the frog was removed, and the buffer was acidified by addition of 1.0 ml of trifluoroacetic acid (TFA) to inhibit endoproteases (Resnick *et al.*, 1991; Steinborner *et al.*, 1997). The skin secretions were partially purified by passage over a C-18 Sep-Pak cartridges (Waters Corporation, Milford, MA) as previously described (Goraya *et al.*, 1998). Protein concentration was determined using the Micro BCA[®] Assay (Pierce, Rockford, IL) following manufacturer's instructions except that Bradykinin (RPPGFSPFR) (Sigma Chemical, St. Louis, MO) was used to establish a standard curve (Rollins-Smith *et al.*, 2002c).

replenishment of peptides following a very mild norepinephrine induction (0.5 to 1 nmole/gram) was detected by fast atom bombardment mass spectrometry. The full complement of peptides was detected within 2–6 days (Giovannini *et al.*, 1987) suggesting that recovery is rapid or peptide stores were not depleted by the norepinephrine. However, following induction using a higher concentration of norepinephrine (3 nmoles/g), gland morphology was not completely restored for 2 weeks as determined by histological and electron microscopic studies of the same species (Dockray and Hopkins, 1975). This suggests that a higher concentration of norepinephrine is necessary to more completely deplete peptides, and restoration of gland morphology may be delayed.

Another group examined the process of granular gland regeneration using immunohistological methods. Following induction with epinephrine at a concentration of 0.5 nmoles/g, complete restoration of gland morphology required six weeks (Flucher *et al.*, 1986). We recently re-investigated this question in young outbred *X. laevis* induced to secrete peptides by injection of a concentration of norepinephrine designed to more completely deplete the contents of granular glands (80 nmoles/g). The ability to secrete skin peptides at concentrations equivalent to the starting population was surprisingly slow to recover following this more com-

plete depletion. In contrast to the results of other investigators using a milder stimulus (Dockray and Hopkins, 1975; Giovannini *et al.*, 1987) recovery was not yet complete at 21 days after depletion (Fig. 2). The frogs appeared to be healthy throughout the experiment. Further studies are underway to determine the length of time necessary for full recovery. Thus, skin peptide defenses in this species appear to be significantly impaired for at least three weeks following a stimulus that causes maximal granular gland discharge.

PROPERTIES OF AMPHIBIAN ANTIMICROBIAL PEPTIDES

An extensive literature characterizes the amino acid sequences, nucleotide sequences, and activity of a large number of biologically active peptides isolated from amphibian skin (reviewed in Erspamer, 1994). Among them are diverse antimicrobial peptides with sizes ranging from 10–46 amino acid residues. They exhibit potent activity against gram positive and gram negative bacteria, fungi, protozoa, and viruses (reviewed in Nicolas and Mor, 1995; Simmaco *et al.*, 1998; Zasloff, 2002; Rinaldi, 2002; Conlon *et al.*, 2004; Apponyi *et al.*, 2004). Each species appears to produce its own unique set of peptides with activity against a variety of microbes (Amiche *et al.*, 1999; Conlon *et al.*, 2004). The main families of antimicrobial skin peptides belong to a large group of linear

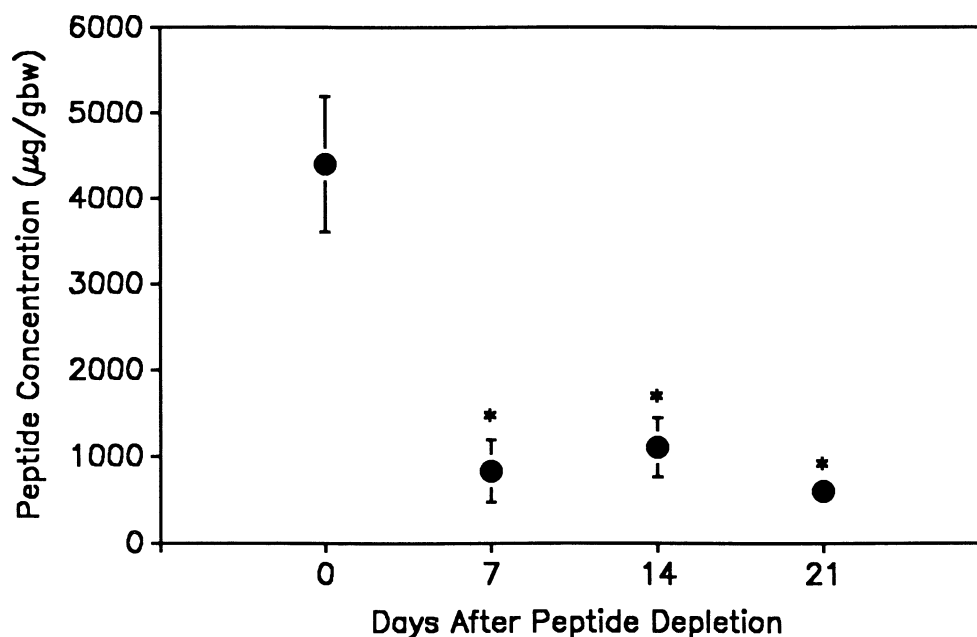


FIG. 2. Kinetics of restoration of skin peptide synthesis and release following norepinephrine-induced peptide depletion in *Xenopus laevis* juveniles. Young outbred frogs were injected with 80 nmoles/gbw of norepinephrine; peptides were collected, partially purified, concentrated, and total peptide concentration was determined as described in the legend of Figure 1. Twenty four individual frogs were peptide-depleted by NE-induction at day 0. At days 7, 14, and 21, three different sets of five individual frogs were re-stimulated with 80 nmoles/gbw and peptides were collected again. *Significantly different from peptide concentration recovered at day 0 by Student's t-test ($P = 0.05$).

amphipathic helical peptides. They are cationic, containing a variable number of positively charged residues and hydrophobic regions. These characteristics provide them with an ability to bind to negatively-charged molecules and/or membrane lipids and disturb the membrane structure. This seems to be the main mechanism of induction of death of their targets (reviewed in Nicolas and Mor, 1995; Simmaco *et al.*, 1998; Zasloff, 2002; Conlon *et al.*, 2004).

ROLE OF ANTIMICROBIAL SKIN PEPTIDES IN PROTECTION FROM PATHOGENS THAT AFFLICT AMPHIBIANS

Most publications describing novel antimicrobial peptides isolated from amphibian skin begin with the accepted generalization that these peptides have a role to play in protection of the amphibian from environmental pathogens. However, studies to support this generalization are very limited. Most of the described peptides are routinely assayed against pathogens of mammalian origin in a search for novel antimicrobial substances for use in the treatment of human disorders. Few have been tested against amphibian pathogens. To investigate this question, we have tested the activity of twenty antimicrobial peptides derived from nine amphibian species against the lethal chytrid fungus *Batrachochytrium dendrobatidis* (Rollins-Smith *et al.*, 2002a, b, c, 2003). Because zoospores of *B. dendrobatidis* are the infectious stage, we tested most of these peptides against isolated zoospores. Results for peptides isolated from a number of species of the genus *Rana* are shown in Table 1. The majority of the peptides were active at concentrations at or below 25 µM.

Very little is known about the concentrations of individual peptides present in skin secretions of amphibians at rest or engaging in normal activities. If the concentration of individual peptides in the mucous layer is 25 µM or above, the majority of peptides tested in this genus would be expected to interfere with the colonization of the skin by zoospores present in the local environment.

Activity against *B. dendrobatidis* was tested, and additionally, six peptides (magainin I and II, PGLa, and CPF from *X. laevis*; dermaseptin from *Phyllomedusa sauvagii*; and ranalexin from *R. catesbeiana*) were tested against another fungal pathogen, *Basidiobolus ranarum*. All of the peptides completely inhibited growth of this pathogen at concentrations of about 30 µM or lower (Rollins-Smith *et al.*, 2002a). Two of the peptides (magainin II and PGLa) from *X. laevis* act synergistically to inhibit growth of both *B. dendrobatidis* and *B. ranarum* (Rollins-Smith *et al.*, 2002a), and we believe that mixtures of peptides, as they would be secreted on the skin surface, may be more effective than individual peptides. The peptides are more effective against the zoospore transmission stage of the chytrid fungus than against mature stages (Rollins-Smith *et al.*, 2002b, c). Seven amphibian peptides (magainin I, magainin II, and PGLa from *X. laevis*; dermaseptin from *P. sauvagii*; temporin A from *R. temporaria*; esculentin-2P and ranatuerin-2P from *R. pipiens*) were tested against the iridovirus, frog virus 3 (FV3) and the herpesvirus, channel catfish virus (CCV). Most showed some degree of inhibition of FV3 and CCV plaque formation (*i.e.*, infectivity), and

TABLE 1. Minimal inhibitory concentration (MIC) of peptides from ranid frogs in growth inhibition assays against zoospores of *B. dendrobatidis*.*

Peptide	Species of origin	MIC (μM)	Reference
Brevinin-1TRa	<i>Rana tarahumarae</i>	12.5	Rollins-Smith <i>et al.</i> , 2002c
Brevinin-2Ob	<i>Rana ornativentris</i>	6.25	Rollins-Smith <i>et al.</i> , 2002b
Esculentin-1A	<i>Rana areolata</i>	12.5	Rollins-Smith <i>et al.</i> , 2002b
Esculentin-2L	<i>Rana luteiventris</i>	12.5	Rollins-Smith <i>et al.</i> , 2002b
Palustrin-3A	<i>Rana areolata</i>	6.25	Rollins-Smith <i>et al.</i> , 2002b
Ranalexin	<i>Rana catesbeiana</i>	12.5	Rollins-Smith <i>et al.</i> , 2002b
Ranateurin-1	<i>Rana catesbeiana</i>	12.5	Rollins-Smith <i>et al.</i> , 2002b
Esculentin-2P	<i>Rana pipiens</i>	25	Rollins-Smith <i>et al.</i> , 2002b
Temporin-1Ob	<i>Rana ornativentris</i>	25	Rollins-Smith <i>et al.</i> , 2002b
Ranateurin-2TRa	<i>Rana tarahumarae</i>	50	Rollins-Smith <i>et al.</i> , 2002b
Temporin-1P	<i>Rana pipiens</i>	50	Rollins-Smith <i>et al.</i> , 2003
Temporin A	<i>Rana temporaria</i>	66	Rollins-Smith <i>et al.</i> , 2003
Ranateurin-2P	<i>Rana pipiens</i>	100	Rollins-Smith <i>et al.</i> , 2002b
Ranateurin-6	<i>Rana catesbeiana</i>	>100	Rollins-Smith <i>et al.</i> , 2003

* Growth inhibition assays were conducted as previously described (Rollins-Smith *et al.*, 2002b). Briefly, 5×10^5 zoospores in a volume of 50 μl of broth were plated in replicates of three or more in a 96-well flat bottom microtiter plate (Costar 3596, Corning Inc., Corning NY, USA) with or without addition of 50 μl serial dilutions of each peptide in broth. The plates were incubated on a laboratory bench at 23°C. Positive control wells received 50 μl of broth without peptide, and negative control wells (on a separate plate) received 50 μl of broth containing 0.4% paraformaldehyde. Growth was determined at days 1–7 by measuring increased optical density at 492 nm (O.D.₄₉₂) with an ELISA plate reader. Minimal inhibitory concentration (MIC) is defined as the lowest concentration at which no growth was detectable. That is, the O.D.₄₉₂ was not significantly greater than that observed for negative control wells containing paraformaldehyde.

several very strongly inhibited FV3 and CCV plaque formation at concentrations ranging from 5–500 μM . Furthermore, the peptides act directly on the virus and not by influencing events in virus-infected cells (Chinchar *et al.*, 2001, 2004). Other investigators have shown that bathing frogs in a culture of bacteria induced an increase in the synthesis of antimicrobial peptides (Miele *et al.*, 1998) that was prevented by glucocorticoid treatment of the frogs. It has also been reported that the freeze-tolerant wood frog, *Rana sylvatica*, does not have antimicrobial peptide activity in skin during winter. However, it begins to synthesize and release an active peptide after acclimation to warmer laboratory temperatures (Matutte *et al.*, 2000). Thus, environmental factors, such as cold temperature may have a profound effect on synthesis and secretion of antimicrobial peptides.

ANTIMICROBIAL ACTIVITY OF NATURAL MIXTURES OF SKIN PEPTIDES

The studies reviewed in the preceding paragraphs argue convincingly that antimicrobial skin peptides should play a role in protection from pathogens such as *B. dendrobatidis* in the wild. Most of the studies described above examined the antimicrobial activity of a single purified peptide. In order to examine the anti-chytrid repertoires of additional species more rapidly, we have developed a method to partially purify, concentrate, and test natural mixtures of skin peptides (Rollins-Smith *et al.*, 2002c). Recent studies of the anti-chytrid activity of natural mixtures of skin peptides collected from a number of species of Australian amphibians support the general hypothesis that antimicrobial skin peptides do play a role in protection from pathogens such as *B. dendrobatidis* in wild populations (Woodhams, 2003). Continuing studies of the anti-chytrid potency of natural mixtures of peptides

from a much larger set of amphibian species correlated with their known susceptibility to chytridiomycosis will provide a more complete answer to the question of whether antimicrobial peptides in the skin are protective against this emerging pathogen (Carey *et al.*, 1999; Daszak *et al.*, 1999, 2003).

CONCLUDING REMARKS

Amphibian skin is a remarkable organ that serves the multiple roles of fluid balance, respiration, and transport of essential ions. Protection of the skin from microbial invaders is essential for survival of individuals and populations. Understanding whether antimicrobial peptides can protect the skin from skin-invasive pathogens is an important goal from a conservation biology point of view in order to predict the survival of individual species of amphibians at a time when this ancient class of vertebrates is suffering global population declines. Our studies suggest that antimicrobial peptides produced in the skin are, indeed, an important defense against skin pathogens and do affect survival of populations. Future studies will need to examine the potency of the antimicrobial peptide repertoire of additional species, especially in growth inhibition assays against *B. dendrobatidis*. We will also need to address the question of how production and release of skin peptides is integrated with other essential skin functions and how environmental factors such as seasonal temperature changes, hydration stress, toxic chemicals, and other environmental stressors may change the normal pattern.

ACKNOWLEDGMENTS

Research from the author's laboratory was supported by NSF IRCEB grants IBN-9977063 and DEB-0213851 (James Collins, P.I.) and NSF grant IBN-0131184 (to L.R-S.). DW was supported by Doctoral

Research Scholarship, Supplementary Internal Research Account Scholarship, Sigma-Xi, The Scientific Research Society Grant-in-Aid-of-Research, and an International Post-Graduate Research Scholarship from James Cook University.

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