Antimicrobial Peptide Defenses in Amphibian Skin


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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 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 immune 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; Lambiris et al., 1995), natural killer cells (Horton et al., 1996, 1998, 2000, 2003), and secreted antimicrobial peptides (reviewed in Nicolas and Mor, 1995; Sime macro 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 propiece 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,*
replenishment of peptides following a very mild nor-
epinephrine induction (0.5 to 1 nmole/gram) was de-
tected by fast atom bombardment mass spectrometry.
The full complement of peptides was detected within
2–6 days (Giovannini et al., 1987) suggesting that re-
covery is rapid or peptide stores were not depleted by
the norepinephrine. However, following induction
using a higher concentration of norepinephrine (3
nmole/g), gland morphology was not completely re-
stored 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 concentra-
tion of 0.5 nmole/g, complete restoration of gland
morphology required six weeks (Flucher et al., 1986).
We recently re-investigated this question in young out-
bred X. laevis induced to secrete peptides by injection
of a concentration of norepinephrine designed to more
completely deplete. In contrast to the results of other in-
vestigators using a milder stimulus (Dockray and Hop-
kins, 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 experi-
ment. Further studies are underway to determine the
length of time necessary for full recovery. Thus, skin
peptide defenses in this species appear to be signifi-
cantly 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 (re-
viewed 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 antimicro-
bial skin peptides belong to a large group of linear
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).

**RODE OF ANTIMICROBIAL SKIN PEPTIDES IN PROTECTION FROM PATHOGENS THAT AFFECT 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 form *X. laevis*; dermaseptin from *Phylomedusa sauvagii*; and ranalexin from *R. catesbeiana*) were tested against another fungal pathogen, *Basidibolus 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
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 DBI-0213851 (James Collins, PL) 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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