AMPHIBIAN IMMUNE DEFENSES AGAINST *BATRACHOCHYTRIUM DENDROBATIDIS*: A WAR BETWEEN HOST AND PATHOGEN

By

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DEDICATION

To the entire Ramsey family, especially my parents, Gerald and Patricia Ramsey, and my brother, Zach Ramsey, for all of their encouragement and support

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
ABT	amphibian phosphate buffered saline supplemented with bovine serum albumin and Tween-20
ANCOVA	analysis of covariance
ANOVA	analysis of variance
APBS	amphibian phosphate buffered saline
APC	allophycocyanin
BCA	bicinchoninic acid
Bd or *Bd*	*Batrachochytrium dendrobatidis*
BSA	bovine serum albumin
C_T	threshold cycle
CD	cluster of differentiation
ConA	concanavalin A
CPF	caerulein precursor fragment
CPM	counts per minute
DLS	dorsal lymph sac
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
H&E	hematoxylin and eosin
HP	horseradish peroxidase

Ig	immunoglobulin
IL	interleukin
IP	intraperitoneal
JEL 197/275	*B. dendrobatidis* isolate identified by Joyce E. Longcore
L-15	Leibovitz-15 media
LPF	levitide precursor fragment
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
NADPH	nicotinamide adenine dinucleotide phosphate
NE	norepinephrine
NF-κB	nuclear factor kappa-B
OD	optical density
PCR	polymerase chain reaction
PE	phycoerythrin
PGLa	peptide with amino-terminal glycine and carboxyl-terminal leucinamide
PHA	phytohemagglutinin
PI	propidium iodide
pIgR	polymeric immunoglobulin receptor
SC	secretory component
SEM	standard error of the mean
TCR	T cell receptor

Th	helper T cell
TMB	3, 3’, 5, 5’-tetramethylbenzidine
UV-B	ultraviolet-B
XPF	xenopsin precursor fragment

CHAPTER I

INTRODUCTION AND RESEARCH GOALS

Global amphibian declines

Major amphibian population declines around the world became an important issue among scientists when they met at the First World Congress of Herpetology in 1989. Collected data showed that mass die-offs of amphibians began as early as the 1970s in several places, including the western United States, Puerto Rico, and northeastern Australia (37, 68, 121). In the late 1980s, more population declines occurred in Central America. As much as 40% of all amphibians disappeared quickly from one site in Costa Rica (165). At the same time, species disappearances were being recorded at other sites in Costa Rica, as well as in Ecuador and Venezuela (165, 166, 199, 240). Many of these declines occurred in habitats that were considered to be pristine, with no known explanation as to why they happened (37, 68, 121, 165, 166, 199, 240). As more reports of population declines came out in the 1990s, it became clear that amphibian populations around the world were in danger (rev. in 4, 40, 107, 210, 213, 231)

Approximately 6,300 species of amphibians have been identified (8), and more continue to be discovered (9, 125). As of 2008, nearly 32% of the known species of amphibians across the world were classified as threatened or extinct (111, 213). In contrast, 22% of the world's mammal species (112) and 12% of the world's bird species (20) were considered to be threatened or extinct. Although 38 species of amphibians are

known to be extinct, as many as 279 additional species are "possibly extinct" as they have not been found in the wild in recent years (111). Upwards of 42% of all amphibian species are experiencing population declines, with the most threatened species residing in tropical areas of Central America and the Caribbean Sea (111). Many of these declines had been considered to be enigmatic, although recent research has begun to define the causes for this extreme loss of amphibian biodiversity. The hypotheses that have been postulated to explain amphibian population declines include: the introduction of non-native predators, habitat loss, climate change, increased UV-B radiation, increased exposure to contaminants, overuse by humans, and emerging infectious diseases. Although any one of these potential causes could lead to amphibian declines, most declines are probably due to interacting factors.

Introduction of non-native species

The introduction of non-native species can have multiple effects on native amphibians. One effect is the overall decrease of amphibian numbers due to predation. A second is the increase of competition between both native and non-native species for resources. Third, non-native species could introduce new pathogens that the native species have never encountered. Lastly, non-native and native species could hybridize, which could eventually lead to the loss of both species (51). The mountain yellow-legged frog (*Rana muscosa*) of the Sierra Nevada Mountains has experienced previous population declines throughout the 20th century due to the introduction of salmonid fish to ponds (124). Removing the fish from ponds led to the recovery of *R. muscosa* populations, and caging experiments determined that the fish were predatory toward *R.*

muscosa tadpoles (229). The introduction of mosquitofish and bullfrogs (*Rana catesbeiana*) also led to the decline of the California red-legged frog (*Rana aurora draytonii*), a species classified as threatened (129).

Overall land use change

The loss of habitat or breeding sites also plays a role in some amphibian population declines. The reasons for habitat loss are twofold: a change in the way humans use land, or a physical conversion from one land type to another (141). One example of habitat loss influencing amphibian population declines came from a study of amphibian assemblages in Ontario, Canada. It concluded that deforestation and wetland drainage led to decreased habitat area and less diverse amphibian populations (97). The region studied had once been home to 13 different amphibian species, but most communities now consist of green frogs (*Rana clamitans*), northern leopard frogs (*Rana pipiens*), and American toads (*Bufo americanus*), suggesting that a change in land use or type selects against native species and in favor of those species that are highly adaptable to these altered landscapes (51, 98). In the tropics between 1990 and 1997, approximately 60,000 km^2 of humid tropical forest were converted to agricultural land, and approximately 23,000 km^2 were degraded, each year (1). The effect of this significant land change would have profound effects on amphibian biodiversity and population numbers in those areas. Thus, the loss of habitat either by land use change or physical destruction could lead to amphibian population declines as those species unable to adapt to the new landscape would die off.

Climate change

Climate changes, especially increased or decreased overall temperatures and changes in total precipitation, may play a role in declining amphibian populations. However, it should be noted that it is very hard to establish causality in regards to climate change leading to population declines (41). Some studies have found correlations between changes in certain climate factors and population declines. A study from Brazil between 1979 and 1982 hypothesized that severe frost led to the extinction of five different frog species (101). Studies in Australia linked drought conditions to declines of stream-dwelling, rain forest species, although an analysis of this data found that the correlation between drought and population declines was weak (128). Drought has also been linked to the extinction of montane *R. pipiens* populations in Colorado (54). Research in Costa Rica examining population declines and extinctions of species within the genus *Atelopus* concluded that global warming events, such as increased cloud cover and misting, led to higher nighttime temperatures and decreased daytime temperatures. This convergence of the mean maximum and mean minimum temperatures would better support the growth of *B. dendrobatidis* (167). However, this chytrid-thermal-optimum hypothesis was challenged by other studies. One suggested that the epidemics of *B. dendrobatidis* in Costa Rica were better explained by chytrid introduction events and subsequent pathogen spread as opposed to more optimal *B. dendrobatidis* growth temperatures (133). Another report reevaluating the original data determined that the temperature convergences were not significant when *Atelopus* species extinctions were increasing, but were significant only when extinctions were decreasing (183). One downside to much of the data linking climate change to amphibian population declines is

that the data were obtained over multiple decades, which may have "temporally confounded" the results in such a way that any variable that increased over that time could be correlated with amphibian losses (183). A more recent study found that the only climate-based population effect on amphibians was an increase in temperature variability, which would lead to compromised immune responses and more frequent disease outbreaks (184). Specific evidence that climate change directly causes die-offs is lacking, and it is possible that climate change has an indirect effect on amphibian communities, such as by disrupting amphibian breeding patterns or by increasing susceptibility to infectious diseases (41).

Ultraviolet radiation

UV-B radiation is an important stressor in living organisms that can cause mutations in organisms and cellular death (21, 49). Increases of UV-B radiation occur after ozone damage, which is due in large part to human-caused release of chlorofluorocarbons and other chemicals into the environment (100, 122), though transient damage can also be caused by natural disasters (48). UV-B radiation has limited capacity to directly kill developing amphibians, but it may also cause sublethal effects including increased embryo mortality, decreased tadpole survival, embryonic deformities, reduced growth, and developmental or behavioral abnormalities (21). UV-B radiation may also synergize with other environmental stressors in order to increase the incidences of these negative impacts (205). However, the overall effect of UV-B radiation on amphibian mortality in the environment is controversial. Amphibian embryos are well protected from ambient UV-B radiation in their natural environment

due to melanin, photolyase, egg jelly, and dissolved organic carbon in the water (131). Studies of high-altitude amphibian breeding sites determined that the dissolved organic carbon concentrations were sufficient to protect larvae from UV-B radiation (2, 160). Studies on embryos from lakes with very little dissolved organic carbon showed no significant effect of UV-B radiation on survival after exposure (131). In laboratory settings, egg masses are typically placed much closer to the surface of the water than eggs found in nature, which may bias experimental results (131). Furthermore, the loss of some embryos to UV-B exposure would have less impact on populations than losses in the post-metamorphic life stages (4, 19, 225). With no documented mortality in natural settings due to UV-B radiation (131), some researchers conclude that there is no link between UV-B exposure and amphibian population declines (131, 160), although a potential link continues to be studied.

Environmental contaminants

Another stressor that could affect the health of organisms is the presence of environmental contaminants such as pesticides, herbicides, fertilizers, and other pollutants (25, 211). Pesticides, which were widely used in agriculture to deter pests, can induce growth deficiencies, as well as developmental and behavioral abnormalities, in frogs that are exposed (21, 34, 211). Pollution from industries that lead to increased heavy metals in the environment or the acidification of water sources can have lethal or sublethal effects in amphibians (94, 130). Acidification of lakes may have contributed to local extinctions of the tiger salamander (*Ambystoma tigrinum*) in Colorado (94). A hypothesis explaining amphibian population declines due to environmental contamination

is that contaminants are endocrine disruptors, which leads to malformed reproductive systems (211). This can include the induction of hermaphroditism and demasculanization (96) or an increase of corticosterone release that leads to reduced stress responses and decreased reproductive performance (81, 104). One study determined that pesticide drift from upwind agricultural land was associated with declines of amphibian species in California (59). UV-B radiation and toxic chemicals can act synergistically, making the effect of environmental contaminants on amphibian declines even more complex (20). Predatory stressors may also synergize with pesticides, increasing susceptibility of amphibians, especially larvae, to death caused by environmental contaminants, although this effect tends to only be observed in laboratory settings (171-173).

Emerging infectious diseases

Perhaps the best documented reason for the decline of amphibian populations around the world is the emergence of infectious diseases in amphibians. Pathogens have been implicated as the cause of many amphibian declines in which no environmental disturbance could be linked to die-offs (32, 40, 54). Emerging diseases in amphibians can be divided into two patterns of outbreaks and declines, both typified by a particular class of pathogens. The first pattern is characterized by: 1) deaths restricted to small geographic areas; 2) deaths in larval, metamorphosing, or adult individuals; 3) frequent outbreaks in areas that are heavily impacted by humans; and 4) infections occurring at high population densities (40). This pattern is largely attributed to outbreaks of iridoviruses. The second pattern is classified by: 1) population declines occurring over a wide geographic area; 2) 50-100% mortality rates; 3) greater mortalities at higher

elevations or cooler temperatures; 4) susceptibility in only some species present at the outbreak site; 5) death largely in adult individuals; and 6) infectious disease as the cause of death (40). This pattern is largely attributed to fungal infections.

One particular genus of iridoviruses that has received attention in regards to amphibian mass mortalities is the genus *Ranavirus*. Ranaviruses are large, double-stranded DNA viruses with linear genomes that are known to infect amphibians, reptiles, and fish (58). They have been implicated as the cause of mass mortalities seen in the United States (66, 86, 88, 117), Venezuela (245), Saskatchewan and Manitoba, Canada (24), Australia (55), and the United Kingdom (56). In the common frog (*Rana temporaria*) in the United Kingdom, ranavirus-induced mortalities were due to either skin ulceration or systemic hemorrhage. Some of the frogs were also infected with an opportunistic bacteria (*Aeromonas hydrophila*), suggesting that death can be caused either directly or indirectly by secondary infections (56). Although ranaviruses are capable of inducing mass mortality events in amphibians, there is little evidence that they cause species extinctions (58). However, because ranaviruses induce high infectivity across multiple orders and species, as well as the potential for fatal opportunistic infections, they are potential agents of die-offs.

Growing evidence links a newly emerging fungal pathogen, *Batrachochytrium dendrobatidis*, to amphibian population declines around the world. This pathogen is viewed as the leading cause of enigmatic declines and extinctions, and its characteristics and pathogenesis are described in the next section.

Batrachochytrium dendrobatidis

The amphibian fungal pathogen *Batrachochytrium dendrobatidis*, initially isolated from the blue poison dart frog *Dendrobates azureus* (135), is classified within the phylum Chytridiomycota, a fundamentally basic phylum in the kingdom Fungi (102, 114, 115). The Chytridiomycota are described as having motile zoospores that utilize one flagellum for movement. *B. dendrobatidis* is the only fungus in this phylum that is a pathogen of vertebrates (102, 114, 115). A number of isolates of *B. dendrobatidis* have been collected, but at present there appears to be very little genetic variation among these isolates (5 variable nucleotides among 10 loci), suggesting that *B. dendrobatidis* is a newly emergent disease agent (146). It is not known where *B. dendrobatidis* originated, although Africa, North America, and Asia have been postulated as origin sites (84, 116, 232). The life cycle of *B. dendrobatidis* features two main stages. The zoospore is the infectious particle that disperses into its environment and moves via its flagellum (17, 135, 228). The zoospore is motile for only 24 hours before it either encysts or dies. Once it reaches a suitable host, the zoospore stops movement, resorbs its flagellum, moves into skin cells, and develops into a thallus form with thread-like rhizoids. The thallus further matures into the second main stage, the mature zoosporangium. Inside the zoosporangium, new zoospores develop after cytoplasmic cleavage and the production of new flagella. A capped discharge tube is also produced. Once the zoosporangium is completely mature, the discharge tube cap dissolves and zoospores are released into the water to either infect the same individual at a different site on the skin or a second individual. The entire life cycle from zoospore to a mature sporangium releasing new zoospores into the environment takes approximately 4 to 5 days at 22°C (17, 228).

B. dendrobatidis colonization of the skin

B. dendrobatidis colonization occurs in the epidermis of its host. It may also infect the mouthparts of tadpoles, which would become carriers and maintain infection in the environment (15, 77, 168). Once on the surface of the skin, *B. dendrobatidis* enters the epidermal cells of the *stratum granulosum* and *stratum corneum* by an unknown mechanism (17, 135, 228). One hypothesis is that the zoospores encyst on the surface of the cells then develop a germ tube to inject its genetic material into the cells (17). Maturation of the zoospore occurs within these epidermal cells. It is believed that the maturation of *B. dendrobatidis* in the skin is synchronized with the movement of epidermal cells through the skin. As the infected epidermal cells move from the deeper layers of the skin to the superficial layer, the zoospore is maturing into a sporangium and producing new zoospores. Once the epidermal cell becomes superficial, the capped discharge tube is produced and oriented toward the outside environment. The fact that *B. dendrobatidis* maturation is synchronized to epidermal cell movement in the skin suggests that the fungus is well adapted to infection of amphibian skin (17, 228).

Chytridiomycosis and *B. dendrobatidis* pathogenesis

An amphibian infected with *B. dendrobatidis* may develop clinical signs of disease or mortality. The resultant disease caused by *B. dendrobatidis* infection is termed chytridiomycosis. Signs of chytridiomycosis include: lethargy, abnormal posture, loss of appetite and righting reflex, reddening of the skin, and irregular sloughing (226-228). It has been hypothesized that the increase of skin sloughing is due to the amphibian's attempt to clear the skin of infection, not due to a process initiated by *B. dendrobatidis*

directly (61). Amphibians with severe infections show high mortality, but exactly how *B. dendrobatidis* kills infected individuals is not well understood. One potential mechanism is the secretion of a toxin or a toxic byproduct that directly kills individuals (15, 162). A second hypothesis, for which evidence is beginning to accumulate, is that *B. dendrobatidis* infection disturbs the skin, disrupting the transport of essential ions or water from the environment across the skin (226, 227). Recent studies of the green tree frog (*Litoria caerulea*) showed that individuals with clinical chytridiomycosis had significantly reduced electrolyte transport across the ventral skin. This in turn led to decreased electrolyte concentrations in the blood, especially sodium and potassium concentrations, and declining electrical functioning in the heart. Death eventually occurred due to asystolic cardiac arrest (226, 227). Regardless of the mechanism of killing, *B. dendrobatidis* has the capability of inducing mortality in amphibians, especially if the infection intensity is severe.

The temperature of the environment may play a role in the overall pathogenesis of *B. dendrobatidis*. *B. dendrobatidis* has an optimum temperature range of between 17 - 23°C, although it can grow at lower temperatures (164, 238). Temperatures at or above 30°C are lethal to the fungus (164). At lower temperatures, such as a range of 7 - 10°C, the ability of *B. dendrobatidis* zoospores to encyst and mature to produce new zoospores took a longer period of time. Although overall growth was slowed at lower temperatures, *B. dendrobatidis* maintained the capacity to grow at a wide range of temperatures (238). Temperatures may also play a role in *B. dendrobatidis* pathogenesis in the wild. In the tropics, amphibian species that had higher prevalences of *B. dendrobatidis* infection were found at lower temperatures (233), and most population die-offs occurred in upland sites

that are generally cooler (16). A hypothesis that climate change is creating more optimum temperatures for *B. dendrobatidis* growth around the world has been postulated (167), although this hypothesis is controversial (5, 133, 183, 184).

One important aspect of chytridiomycosis is that some species appear resistant to *B. dendrobatidis* whereas others are susceptible. *X. laevis* collected and assessed for *B. dendrobatidis* in the field showed no signs of chytridiomycosis, although it was determined that the prevalence of *B. dendrobatidis* was ~3%, a figure that has not changed since 1940 (232). Captive populations of *X. laevis* also have no apparent clinical signs of chytridiomycosis (161). Experimental infection of *X. laevis* failed to induce chytridiomycosis even when frogs were exposed to very high numbers of zoospores (191 and this thesis). There have been no reports of population declines of *X. laevis* due to *B. dendrobatidis* (232). In contrast to *X. laevis*, a frog of the closely related species, *Silurana* (*Xenopus*) *tropicalis*, died due to infection with *B. dendrobatidis* (161). Another amphibian, the boreal toad (*Bufo boreas*), is considered very susceptible to *B. dendrobatidis* both in the wild and in the laboratory. *B. boreas* experienced significant declines in the southeastern portion of its range between the late 1970s and the early 1980s (39, 42), as well as in Colorado (151), due to *B. dendrobatidis* infections. Exposure of *B. boreas* to as little as 10^4 zoospores for one day experimentally resulted in 100% mortality (42). The specific characteristics of each species that determines whether they are susceptible or resistant to *B. dendrobatidis* are unknown, although both innate and adaptive immune components may be important in providing resistance in amphibians.

Research objectives

The major hypothesis of my dissertation is that both innate immunity (especially antimicrobial peptides) and adaptive immune responses are required for full protection of an amphibian species from lethal *B. dendrobatidis* infections. A deficiency of one, or both, of these systems would increase susceptibility to *B. dendrobatidis* infections after exposure. In the course of this study, we developed preliminary evidence that *B. dendrobatidis* was able to inhibit the activation of lymphocytes when both cell types were co-cultured. This led to the generation of a second hypothesis; that *B. dendrobatidis* induces hyporesponsiveness in amphibian lymphocytes, which would give it a survival advantage once it successfully infects amphibian skin. The results from studies to investigate these hypotheses would help formulate a model of the overall interactions between pathogen and the host immune system.

The objectives of my dissertation studies are as follows:

1) To determine whether antimicrobial peptide defenses in a resistant species are sufficient to inhibit *B. dendrobatidis in vitro*. Previous work in the Rollins-Smith laboratory and others concluded that there is a correlation between the effectiveness of a species' antimicrobial peptides in inhibiting the growth of *B. dendrobatidis in vitro* and its survival in the wild or its resistance to experimental exposure (234-236). Thus, it would be likely that a resistant species, such as *X. laevis*, would have very strong antimicrobial peptide defenses against *B. dendrobatidis*. My work assessed the ability of both natural mixtures of *X. laevis* skin peptides as well as purified individual *X. laevis* peptides to inhibit *B. dendrobatidis* growth in a growth inhibition assay.

2) To evaluate if peptide depletion in a resistant species increases susceptibility to *B. dendrobatidis* after experimental exposure. In order to test the overall role of the antimicrobial peptide defenses in the resistance of *X. laevis* to *B. dendrobatidis* infection, my work aimed to develop a protocol for depleting the skin of its antimicrobial peptide stores. Once accomplished, frogs would be tested for increased susceptibility after both peptide depletion and *B. dendrobatidis* exposure. Both the intensity of infection on the skin over time as well as weight loss would be used as measures of overall susceptibility to *B. dendrobatidis* in peptide depleted and untreated frogs.

3) To determine if the adaptive immune system is playing a role in protecting a resistant species from *B. dendrobatidis* infection. Although no laboratory has systematically explored the role of adaptive immunity in the protection of amphibians against *B. dendrobatidis*, other studies suggested that adaptive immune responses against *B. dendrobatidis* were poor (15, 162, 175, 200, 212). My work was designed to explore the role of adaptive immunity in *X. laevis* after *B. dendrobatidis* exposure. We designed experiments to determine if inhibiting the adaptive immune system by X-irradiation would lead to an increase in susceptibility to *B. dendrobatidis* in *X. laevis*. As before, overall susceptibility would be measured both by infection intensity on the skin as well as weight loss over time. We also wanted to examine whether a robust adaptive immune response could be generated in *X. laevis* after immunization with *B. dendrobatidis* as measured by increased *B. dendrobatidis*-specific antibodies in the serum.

4) To identify adaptive immune mechanisms that may protect the skin from *B. dendrobatidis* infections. Mucus secretions on the surface of amphibian skin contain both antimicrobial peptides and lysozyme, an enzyme that can break down components of bacterial cell walls (123, 157, 243). Mammals and fish have the ability to secrete antibodies in the mucus (43, 134, 140, 198), but it was unknown if amphibian mucus also contains immunoglobulins that could help protect the skin. My work assessed whether mucosal secretions of *X. laevis* contain immunoglobulins and, if so, whether the mucosal antibodies in frogs previously exposed to *B. dendrobatidis* are pathogen-specific.

5) To assess whether *B. dendrobatidis* has the capacity to inhibit immunity in amphibians after infection. Several pathogenic fungi, including *Cryptococcus neoformans* (45), *Histoplasma capsulatum* (6), *Aspergillus fumigatus* (13, 215), and *Paracoccidioides brasiliensis* (38), are able to inhibit lymphocytes after infection. Preliminary work in the Rollins-Smith laboratory suggested that *B. dendrobatidis* inhibited lymphocytes from proliferating *in vitro*, as lymphocytes from immunized *X. laevis* were unable to become activated and proliferate in response to co-culture with *B. dendrobatidis* (196). My work aimed to elucidate whether *B. dendrobatidis* could inhibit *X. laevis* lymphocytes after stimulation with mitogens, and characterize the process by which activated lymphocytes fail to proliferate when in the presence of *B. dendrobatidis*.

CHAPTER II

ANTIMICROBIAL PEPTIDE DEFENSES OF *XENOPUS LAEVIS* AGAINST *BATRACHOCHYTRIUM DENDROBATIDIS*

Abstract

Batrachochytrium dendrobatidis is a chytrid fungus that causes the lethal skin disease chytridiomycosis in amphibians. It is regarded as an emerging infectious disease affecting diverse amphibian populations in many parts of the world. Because there are few model amphibian species for immunological studies, little is known about immune defenses against *B. dendrobatidis*. Our work shows that the South African clawed frog, *Xenopus laevis*, is a suitable model for investigating immunity to this pathogen. Antimicrobial peptides secreted onto the surface of the skin are known to kill many pathogens, including bacteria, viruses, and fungi. Peptide effectiveness has been previously correlated with resistance to *B. dendrobatidis* infection both in the wild and following experimental exposures. *X. laevis* is a species considered to be resistant to *B. dendrobatidis* infection, but experiments evaluating the antimicrobial peptide defenses against *B. dendrobatidis* in this species are lacking. Purified antimicrobial peptides or mixtures of peptides in the skin mucus of *X. laevis* inhibited *B. dendrobatidis* growth *in vitro*. Skin peptide secretion was maximally induced by injection of norepinephrine, and significant time was required for the recovery of peptide stores after this treatment. Maximal induction resulted in sustained skin peptide depletion and increased susceptibility to infection. These data strongly suggest that the antimicrobial peptide

defenses in *X. laevis* constitute a significant barrier to infection, and they play a role in resistance to *B. dendrobatidis*.

Introduction

Although studies of innate immunity in *X. laevis* are limited, this species appears to have innate defenses that are similar to other vertebrates (182). Innate immunity includes phagocytic cells such as macrophages, neutrophils, and natural killer cells; complement proteins; and the production of lysozyme and antimicrobial peptides within the skin mucus. Although phagocytic cells, complement, and lysozyme may be important in the protection of amphibians against infections, including *B. dendrobatidis,* they will not be discussed here. Antimicrobial peptide defenses on the skin of *X. laevis* are a major part of the work in the Rollins-Smith laboratory, and so my studies were focused on these molecules.

The dermal layer of the skin of most amphibians contains two different types of glands: mucous glands and granular glands (also called poison or serous glands) (29, 142, 154, 209). Although mucous glands are important due to their role in producing a mucopolysaccharide-rich substance that moistens the skin (75, 85, 202), the granular glands play a larger role in innate immunity. Multiple bioactive peptides, including antimicrobial peptides and neuropeptides, are synthesized within the granular glands. Antimicrobial peptides in the mucus are thought to protect the amphibian skin by eradicating pathogens that attempt to colonize the skin, while neuropeptides act as deterrents against predators (11, 52, 57, 76, 153, 179, 208, 242). The granular gland is filled with granules that contain these peptides (67). Granular glands are surrounded by

myoepithelial cells that possess α-adrenoreceptors, and secretion of the contents of the glands is stimulated by epinephrine or norepinephrine, which causes the myoepithelial cells to contract and push the granules through a duct onto the surface of the skin (14, 67, 103). Granular glands are typically found all over the body, though particular glands on the dorsal side, such as the ones in the dermal plicae and behind the eyes, are the largest (29, 152, 154, 209, 220).

The antimicrobial peptides present within the granular glands typically range from 10 - 50 amino acids in length. These peptides have activity against a variety of pathogens, including gram positive and gram negative bacteria, fungi, protozoa, and certain viruses (11, 52, 76, 153, 179, 208, 242). The sequences of the antimicrobial peptides are variable with no consensus amino acid sequence, however most peptides are cationic, largely hydrophobic, and can form amphipathic α-helices (239). The overall structure of antimicrobial peptides allows them to disrupt the membranes of pathogens, which is thought to be the actual method of killing (11, 52, 153, 179, 208, 242). Between amphibian species there is very little overlap in individual shared peptides, although shared families of peptides are common among related species (52).

In the Rollins-Smith laboratory, 41 purified antimicrobial peptides from 27 amphibian species have been tested for their effectiveness in inhibiting the growth of *B. dendrobatidis in vitro*. About 83% of these peptides inhibited *B. dendrobatidis* growth at minimal inhibitory concentrations (MIC) of 100 μM or less (53, 60, 187-191, 193, 194, 235). Natural mixtures of peptides, or the total combination of all peptides secreted from granular glands onto the skin, were also tested for their capacity to inhibit *B. dendrobatidis* growth in 24 other species (60, 189, 194, 206, 234-236). The effectiveness

of the peptides in inhibiting *B. dendrobatidis* growth *in vitro* correlated with survival among groups of species in the wild (234, 235) as well as resistance of some species to *B. dendrobatidis* when experimentally infected (236). Thus, the antimicrobial peptides present in the granular glands of amphibian skin appear to be capable of protecting frogs from *B. dendrobatidis* infection, and the variation in the effectiveness of the peptides among species can be used to predict whether a species would be resistant or susceptible to *B. dendrobatidis* (234). One species not previously studied for antimicrobial peptide effectiveness against *B. dendrobatidis* is *X. laevis*, a species considered resistant to *B. dendrobatidis* (161, 191, 232). Although a number of antimicrobial peptides in *X. laevis* have been identified (10, 82, 83, 113, 127, 177, 214, 241), studies of *B. dendrobatidis* growth inhibition by these peptides was lacking. My work evaluated the growth inhibition of *B. dendrobatidis* by natural mixtures of peptides and purified peptides to determine whether these antimicrobial peptide defenses are important in protection of this species from lethal *B. dendrobatidis* infections.

Materials and Methods

Frogs

Outbred *X. laevis* frogs ranging in size from approximately 30 to 50 g were purchased from Xenopus I (Dexter, MI) and kept in polystyrene containers at a density of about 10 frogs per 16 liters of dechlorinated tap water at a temperature of between 20 to 24°C. For the peptide depletion and exposure study, the frogs were young postmetamorphic adults ranging in weight from 4 to 5 g. Three times each week, the

frogs were fed ground beef heart and their water was changed. All procedures involving animals were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

Collection and enrichment of skin peptides

Secretion of granular gland contents was stimulated by injection of norepinephrine-HCl (Sigma, St. Louis, MO) dissolved in amphibian phosphate buffered saline (APBS; 6.6 g NaCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 per 1 liter distilled water) into the dorsal lymph sac. For resting frogs, individual *X. laevis* were placed directly into collection buffer without norepinephrine stimulation. Frogs that were given a simulated "alarm" stress were removed for their tank, rinsed with clean water, and placed in a container filled with 500 ml collection buffer. The investigator placed a gloved hand into the buffer and forced the frogs to swim for 10 minutes before a 5 minute rest period, allowing secretions to accumulate in the buffer. Peptides were partially purified by pumping collection buffers through C-18 Sep-paks. Eluted peptides were quantified by microBCA assay, concentrated by centrifugation under vacuum (spin-vac), and resuspended in water at a known concentration before use. Frog weights were determined at the time of peptide induction, and total peptide concentrations were divided by weight to determine peptide recovery in µg/g. To estimate the amount of peptides in mucus, the surface area of the skin of each frog was calculated according to the method of McClanahan and Baldwin [i.e. surface area = 9.9 (weight in grams)$^{0.56}$] (137). Assuming the thickness of mucus is 50 µm (36), the volume of mucus covering one cm^2 of skin would be 5 µl. Thus, total peptides (in µg) per cm^2 x 200 = total µg/ml in mucus.

Mass spectrometry

To confirm the presence of previously described antimicrobial peptides in the samples, enriched skin peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as previously described (218, 234).

Growth inhibition assays

Growth inhibition of *B. dendrobatidis* was determined as previously described with minor modifications (187, 188, 194). Briefly, zoospores of *B. dendrobatidis* isolate JEL 197 (135) were isolated after culture for 1 week on 1% tryptone agar at 23°C and plated (5 X 10^4/50 µl, five replicates) in 1% tryptone broth in 96-well flat-bottom microtiter plates with or without the addition of 50 µl serial dilutions of a natural mixture of skin peptides or individual purified skin peptides from *X. laevis* dissolved in sterile water. The plates were incubated for 1 week at 23°C. Positive control wells received 50 µl of sterile water without peptides. No growth (negative control) wells contained zoospores treated at 60°C for 10 min. Growth after 7 days was measured as increased optical density at 490 nm (OD_{490}) with an MRS Microplate Reader (Dynex Technologies, Inc., Chantilly, VA). Magainin II was purchased from Sigma. Caerulein precursor fragment (CPF) and PGLa (for peptide with amino-terminal glycine and carboxyl-terminal leucinamide) were a generous gift from Michael Zasloff (Georgetown University, Washington DC).

Peptide depletion and exposure to *B. dendrobatidis*

Prior to treatment, all frogs were weighed and swabbed for *B. dendrobatidis* (see "Quantification of *B. dendrobatidis* Zoospores on the Skin" section below) to determine initial infection status before being placed in individual sterile containers. For peptide depletion, frogs were injected with 80 nmol/g of norepinephrine via the dorsal lymph sac on day -1. Controls were left untreated, and both groups were exposed to 10^6 zoospores of *B. dendrobatidis* isolate JEL 197 at day 0 and re-exposed on day 21 with 10^6 zoospores of *B. dendrobatidis* isolate JEL 275. The switch to JEL 275 was made because this isolate is known to be lethal for boreal toads and was isolated from a diseased frog more recently. All frogs were swabbed again on day 32 to quantify *B. dendrobatidis* infection intensity by real-time PCR. Frog were weighed approximately every 10 days throughout the course of this experiment. All frogs were observed frequently for signs of *B. dendrobatidis*-induced illness. As a control to determine if norepinephrine injection itself affected weight loss, frogs that were PCR-negative for *B. dendrobatidis* were injected with either 80 nmol/g of norepinephrine or with vehicle alone (APBS), and their weights were monitored approximately every 10 days for 50 days.

Quantification of *B. dendrobatidis* zoospores

X. laevis frogs were swabbed with a sterile cotton swab 10 times on the abdomen, legs, and each foot. DNA was extracted from swabs by adding 60 µl of PrepMan Ultra (Applied Biosystems, Foster City, CA) and 30 to 35 mg zirconium/silica beads (0.5mm diameter) (Biospec Products, Bartlesville, OK) to each swab. Swabs were homogenized in a Mini Beadbeater (MP Bio, Solon, OH) for 45 seconds before centrifugation at 15,000

x g for 30 seconds. The homogenization and centrifugation steps were repeated followed by boiling the samples for 10 minutes and cooling for 2 minutes at room temperature. Samples were centrifuged at 15,000 x g for 3 minutes, and the supernatants containing DNA were removed for real-time PCR. Real-time probe-based PCR assays were performed with an Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) using the default conditions (95°C for 10 minutes, followed by 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute) for 40 cycles according to standard protocol (30, 110). A standard curve based on the threshold cycle (C_T) values from the control zoospore DNA was generated, and the number of zoospore equivalents in each sample was calculated.

Statistical comparisons

All parameters compared were averaged, and the mean values ± standard error of the mean were compared by one-tailed or two-tailed Student's t test, one-way analysis of variance (ANOVA) with planned comparisons (Tukey post hoc tests), or analysis of covariance (ANCOVA) as detailed in the figure legends. Zoospore equivalents, peptide concentrations, and OD values were log transformed as indicated in the figure legends to meet the assumptions of normal distribution and homogeneity of variances for parametric statistics. A p value ≤ 0.05 was considered statistically significant. Error bars shown in all figures represent the standard error of the mean, except in Fig. 2-3 E and F, which show the 95% confidence intervals. If no error bar is shown, the standard error of the mean was less than the diameter of the symbol. In *B. dendrobatidis* growth inhibition assays, each data point represents the mean ± standard error of the mean of five replicate

wells. For other parameters, the number of animals or samples is shown in the figure legend.

Results

Collection of antimicrobial skin peptides

Previous experiments in the Rollins-Smith laboratory showed that *X. laevis* is a species that is resistant to lethal *B. dendrobatidis* infections (191), and we hypothesized that the antimicrobial peptide defenses provide an effective barrier to initial infections by *B. dendrobatidis*. In order to test this hypothesis, we induced secretion of the antimicrobial peptides, determined the pattern of peptides expressed within enriched secretions, and utilized both enriched natural mixtures of peptides and purified individual peptides in a *B. dendrobatidis* growth inhibition assay.

To determine the pattern of antimicrobial peptide expression in the skin secretions of *X. laevis*, enriched peptide samples were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Many antimicrobial peptides present in *X. laevis* skin had been previously identified, including PGLa (peptide with amino-terminal glycine and carboxyl-terminal leucinamide) (10), magainin I and magainin II (241), CPF (caerulein precursor fragment) (82, 83, 113, 177, 214), LPF (levitide precursor fragment) (113, 127), and XPF (xenopsin precursor fragment) (82, 83, 113, 214), and the peptide masses were known. All *X. laevis* tested routinely contained each of the previously identified antimicrobial peptides. A representative MALDI-TOF profile is shown in Fig. 2-1.

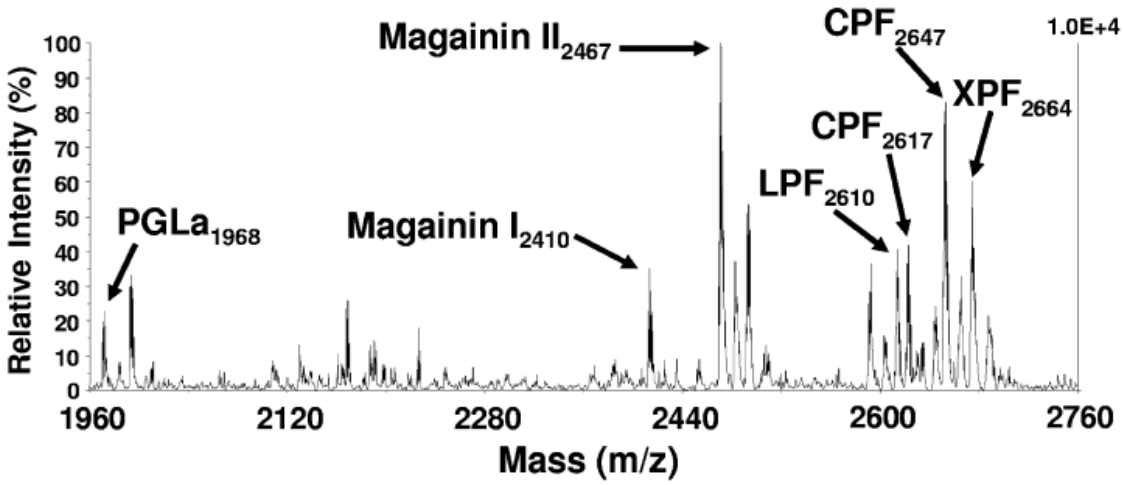

Figure 2-1. MALDI-TOF imaging profile of *X. laevis* skin secretions after induction with 80 nmol/g of norepinephrine. Previously described antimicrobial peptides are labeled, along with their mass-to-charge ratio. PGLA, peptide with amino-terminal glycine and carboxyl-terminal leucinamide; CPF, caerulein precursor fragment; XPF, xenopsin precursor fragment; LPF, levitide precursor fragment. This profile is representative of eight individuals.

Antimicrobial peptides from *X. laevis* inhibited the growth of *B. dendrobatidis in vitro*

The enriched natural mixtures of antimicrobial peptides obtained from *X. laevis* skin were tested for their ability to inhibit the growth of *B. dendrobatidis* zoospores *in vitro*. All enriched natural skin peptides mixtures tested strongly inhibited the growth of *B. dendrobatidis* zoospores after one week (Fig. 2-2A and Table 2-1). There was some variability among the samples tested in terms of their inhibitory strength, however all the samples inhibited growth at concentrations above 30 to 60 µg/ml. The MICs, or the concentration of peptides needed to abolish all zoospore growth, varied among samples, ranging from 62.5 to 500 µg/ml. At 500 µg/ml the average percent inhibition among the samples was 99.4 ± 0.4% (Table 2-1). The enriched natural peptide samples were also tested for their ability to inhibit the growth of mixtures of zoospores and mature sporangia. The peptides also showed strong inhibition of these *B. dendrobatidis* cells, although the MICs were higher compared to zoospores alone (Table 2-2). These data indicated that the peptide mixtures secreted onto the surface of *X. laevis* skin are potent inhibitors of *B. dendrobatidis* growth, and that they are likely able to prevent initial colonization of *X. laevis* skin after *B. dendrobatidis* exposure.

Three individual purified peptides from *X. laevis* skin – CPF, PGLa, and magainin II – were also tested for their ability to inhibit the growth of *B. dendrobatidis* zoospores. Although natural mixtures of skin peptides were consistently strongly inhibitory, previous work in other species of anurans showed that individual peptides differ in inhibitory strength (53, 60, 187-191, 193, 194, 235). The three individual peptides tested were strongly inhibitory but possessed different levels of inhibitory activity. CPF inhibited zoospore growth at concentrations above 1.6 µM and had an MIC of 12.5 µM

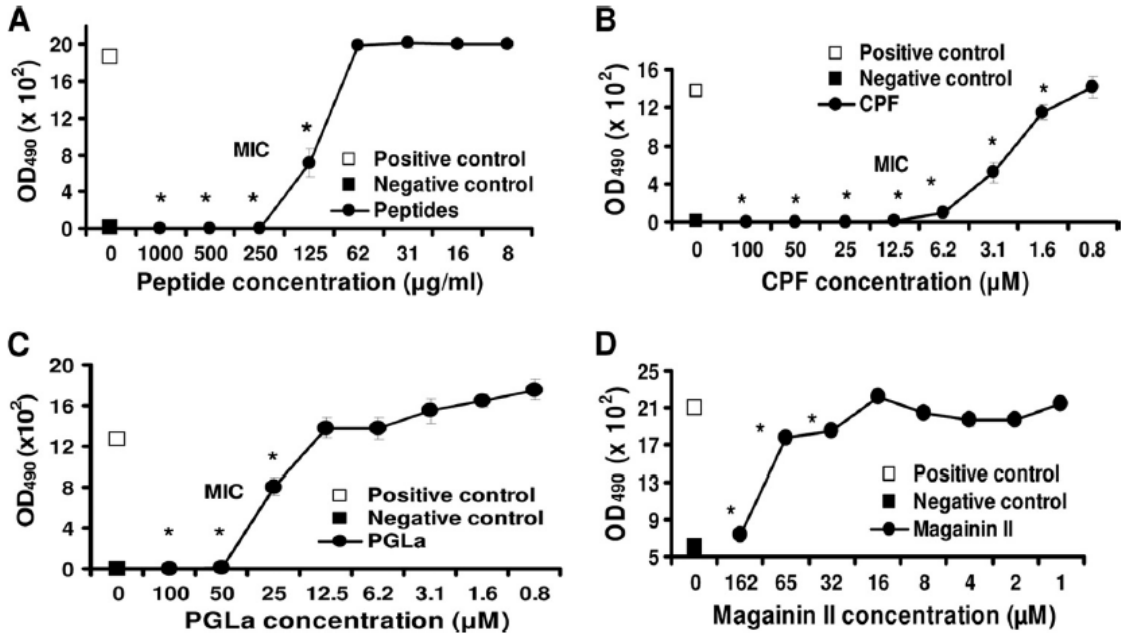

Figure 2-2. Antimicrobial peptides from *X. laevis* skin secretions inhibited *B. dendrobatidis* growth *in vitro*. *B. dendrobatidis* zoospores were cultured in the presence or absence of dilutions of (A) natural mixtures of *X. laevis* skin peptides, (B) pure synthetic CPF, (C) pure synthetic PGLa, and (D) pure synthetic magainin II. Growth was measured after 7 days as OD_{490} absorbance. Positive control denotes *B. dendrobatidis* zoospores grown in broth plus water in the absence of peptides. Negative control denotes *B. dendrobatidis* zoospores that were heat-killed by treatment at 60°C for 10 minutes in broth plus water. MIC = minimum inhibitory concentration. *, Significantly reduced growth compared to the positive control by two-tailed Student's t-test; $p \leq 0.02$.

Table 2-1. Minimum inhibitory concentrations (MIC) and percent inhibitions of growth by natural mixtures of skin peptides tested against *B. dendrobatidis* zoospores.

Sample ID*	Concentrations Tested (μg/ml)	MIC	Percent Inhibition at 500 μg/ml
1	2 - 1000	500	97.1
2	2 - 1000	62.5	100
3	2 - 1000	250	99
4	2 - 1000	250	100
5	2 - 1000	125	100
6	2 - 1000	500	100
7	2 - 1000	250	99.3
8	2 - 1000	250	100
			99.4 ± 0.4 Av

* Skin secretions were induced by injection with 80 nmol/g of norepinephrine, collected, and processed as described. Each sample was a single frog.

Table 2-2. Minimum inhibitory concentrations (MIC) and percent inhibitions of growth by natural mixtures of skin peptides tested against *B. dendrobatidis* whole cultures containing both mature sporangia and zoospores.

Sample ID*	Concentrations Tested (μg/ml)	MIC	Percent Inhibition at 500 μg/ml
1	1.6 - 500	> 500	12.4
2	12.5 - 500	500	100
3	3.1 - 2000	> 500	92.5
4	1.6 - 2000	> 2000	17.9
5	1.6 - 2000	2000	44.2
6	6.2 - 2000	2000	24.0
7	1.6 - 500	> 500	89.2
8	3.1 - 2000	2000	39.4
9	3.25 - 500	> 500	92
10	3.1 - 2000	2000	40.5
11	3.1 - 500	> 500	3.5
12	3.1 - 500	250	100
13	3.1 - 500	100	100
14	3.1 - 500	> 500	90.8
15	2 - 1000	> 1000	14.8
16	2 - 1000	125	100
17	2 - 1000	> 1000	7.4
			57.0 ± 9.5 Av

* Skin secretions were induced by injection with 80 nmol/g of norepinephrine, collected, and processed as described. Each sample was a single frog.

(Fig. 2-2B). PGLa was able to inhibit growth when concentrations were greater than 25 μM with an MIC of 50 μM (Fig. 2-2C). Magainin II was inhibitory at concentrations above 32 μM, although it hadn't yet reached an MIC at 162 μM (Fig. 2-2D). We interpreted these data to suggest that the antimicrobial peptide milieu present in the skin of *X. laevis* is strongly inhibitory to *B. dendrobatidis* growth, and the antimicrobial peptides potentially protect the frogs from *B. dendrobatidis* infection by preventing colonization.

An "alarm" stress induced the secretion of antimicrobial peptides at physiologically relevant concentrations on the skin

Although the antimicrobial peptide defenses in *X. laevis* were potent *in vitro*, the frogs must have the capacity to secrete enough antimicrobial peptides on the surface of the skin to inhibit colonization. To determine whether *X. laevis* individuals were able to secrete peptides onto the skin at physiologically relevant concentrations, we determined the levels of peptides released in both resting frogs and frogs given an "alarm" stress. Resting frogs were only briefly handled as they were removed from their tank and placed directly into collection buffer. After 15 minutes in collection buffer, the frogs were removed, and the released peptides were enriched, prepared, and quantified as previously described. Resting frogs released an average concentration of peptides at 3,256 ± 345 μg/ml in mucus, which is within the range of concentrations required to inhibit *B. dendrobatidis in vitro* (Fig. 2-3A). Frogs that were given an "alarm" stress were removed from their tanks, washed with water, then placed in a separate tank filled with collection buffer. The frogs were chased around the tank by hand, forcing the frog to swim for ten

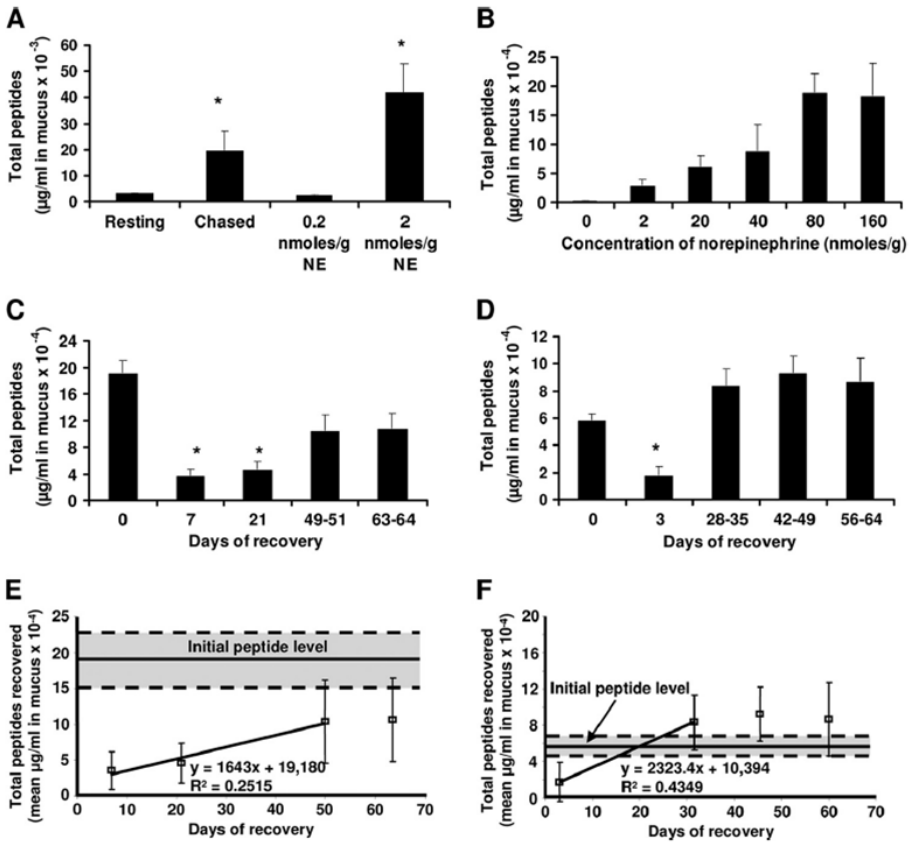

Figure 2-3. Effects of "alarm" stress of norepinephrine (NE) injection on induction of *X. laevis* skin peptides and recovery of peptide stores in the skin. (A) Effect of simulated predator stress or norepinephrine injection on the secretion of skin peptides (n = 5 in each group except 2 nmol/g of NE, n = 3). *, Significantly greater peptide concentration as compared to resting frogs by one-way ANOVA (after log transformation) with Tukey post hoc test; $p < 0.01$. (B) Dose-dependent secretion of peptides after norepinephrine injection (n = 3 to 5 frogs per dose except 160 nmol/g of NE, n = 2). (C) Peptide recovery in the skin over time after induction with 80 nmol/g of norepinephrine (n = 97 frogs at time 0, 7 to 10 frogs at all other time points). *, Significantly reduced peptide concentration compared to day 0 by one-way ANOVA (after log transformation) with Tukey post hoc test; $p < 0.05$). (D) Peptide recovery in the skin over time after induction with 20 nmol/g of norepinephrine (n = 35 frogs at day 0, 4 frogs at day 3, and 10 frogs at other time points). One outlier value was omitted at day 3. *, Significantly reduced peptide concentration compared to day 0 by one-way ANOVA (after log transformation) with Tukey post hoc test; $p < 0.01$). (E) Peptide recovery in mucus after induction with 80 nmol/g of norepinephrine (mean µg/ml ± 95% confidence interval). Approximately 19,180 µg/ml of peptides remained in the mucus after one injection. Full peptide recovery required approximately 50 to 105 days. (F) Peptide recovery in mucus after induction with 20 nmol/g of norepinephrine (mean µg/ml ± 95% confidence interval). Approximately 10,394 µg/ml of peptides remained in the mucus after one injection. Full peptide recovery occurred by 20.4 days.

minutes followed by a five minute rest period. The frog was removed, and the peptides present in the collection buffer were enriched and quantified. We found that *X. laevis* individuals stressed in this way secreted significantly greater concentrations of peptides than resting frogs, having released an average of 19,851 ± 7,340 µg/ml in mucus (Fig. 2-3A). We also determined the concentration of peptides released after norepinephrine injection. A minimal dose (0.2 nmol/g) led to the induction of peptides at a level equivalent to that seen in resting frogs (2,262 ± 623 µg/ml in mucus). A higher dose (2 nmol/g) stimulated secretion of peptide concentrations averaging 41,646 ± 11,121 µg/ml in mucus, a level significantly higher than both resting and chased frogs (Fig. 2-3A). These data suggested that both resting and stressed frogs were able to release concentrations of peptides onto the skin that could inhibit *B. dendrobatidis* growth. The handling of the resting frogs may be responsible for the induction of peptide secretion, but the possibility that resting frogs are able to continuously secrete low levels of peptides on their skin to act as an antimicrobial mantle cannot be discounted.

Norepinephrine injection depleted *X. laevis* skin of its antimicrobial peptides

Because *X. laevis* antimicrobial peptides were inhibitory to *B. dendrobatidis* and were secreted at concentrations sufficient for inhibition while resting or after stress, we investigated the overall role that these peptides play in the resistance of *X. laevis* to *B. dendrobatidis* infection. We developed a protocol for depleting the skin of its peptides, and determined whether depletion led to increased susceptibility to *B. dendrobatidis* infection after exposure. As an initial step in developing this protocol, we determined the dose of norepinephrine that stimulated the greatest release of peptides and examined the

kinetics of peptide renewal after norepinephrine injection. Norepinephine injection into the dorsal lymph sac led to a dose-dependent increase in peptide secretion, with both 80 nmol/g and 160 nmol/g inducing concentrations of peptides that were maximal and not significantly different (Fig. 2-3B). Thus, we considered 80 nmol/g the dose sufficient for maximal peptide secretion. To examine the kinetics of peptide renewal following norepinephrine injection, groups of frogs were initially given either the maximal dose (80 nmol/g) or a moderate dose (20 nmol/g) of norepinephrine. Over time a subset of frogs from each group was injected again with the same dose, and the concentration of peptides recovered was determined. At both one and three weeks after the initial induction with 80 nmol/g of norepinephrine the concentrations of peptides released were significantly reduced. By seven to nine weeks the peptide levels were still slightly decreased as compared to the initial concentration secreted, but this difference was no longer significant (Fig. 2-3C). When the data were analyzed by determining the slope of recovery, we calculated that frogs retained the ability to secrete approximately 19,180 µg of peptides/ml in mucus after the initial injection, and that full recovery of peptide stores in the skin would require between 50 and 104 days (Fig. 2-3E). In comparison to the maximal dose, the concentration of peptides recovered three days after initial stimulation with a moderate dose (20 nmol/g) of norepinephrine was significantly reduced, but full recovery occurred by four to five weeks (Fig. 2-3D). Analyzing the data by evaluating the slope of recovery showed that after that first injection with 20 nmol/g of norepinephrine frogs could still secrete approximately 10,394 µg of peptides/ml in mucus, and full recovery required around 20 days (Fig. 2-3F). These data indicated that induction of peptide secretion with 80 nmol/g of norepinephrine reduced the

concentration of peptides that remained available for secretion, and that the kinetics of peptide renewal were very slow. Thus, we determined that 80 nmol/g of norepinephrine was an appropriate dose for depleting the skin of its peptide defenses, allowing us to examine the importance of this defense in the protection of *X. laevis* from *B. dendrobatidis* infection.

X. laevis depleted of their antimicrobial skin peptides were more susceptible to *B. dendrobatidis*

To determine the infection intensity of *B. dendrobatidis* on the skin of exposed frogs, we swabbed the skin of each individual with sterile cotton swabs (30, 110). To evaluate the protection afforded by the antimicrobial peptides present in the skin, a group of frogs was injected with 80 nmol/g of norepinephrine in order to deplete peptide stores one day prior to exposure to 10^6 *B. dendrobatidis* zoospores of isolate JEL 197 (135). Control frogs were not treated, but were exposed to *B. dendrobatidis* at the same infectious dose. Both groups were exposed to *B. dendrobatidis* isolate JEL 275 (179) 21 days after the first exposure. At 32 days after the first exposure, all frogs were swabbed to determine the infection intensity on the skin. The intensity of infection was significantly greater in frogs that were injected with 80 nmol/g of norepinephrine to deplete the skin peptides compared to frogs that were untreated and exposed (Fig. 2-4A). The increase in infection intensity after peptide depletion indicated that the loss of antimicrobial peptide defenses rendered the frogs more susceptible to *B. dendrobatidis* infection. As a second measure of increased susceptibility after peptide depletion, we monitored weight changes in both the norepinephrine-injected and untreated *B. dendrobatidis*-exposed groups. The peptide-depleted frogs lost significantly more weight

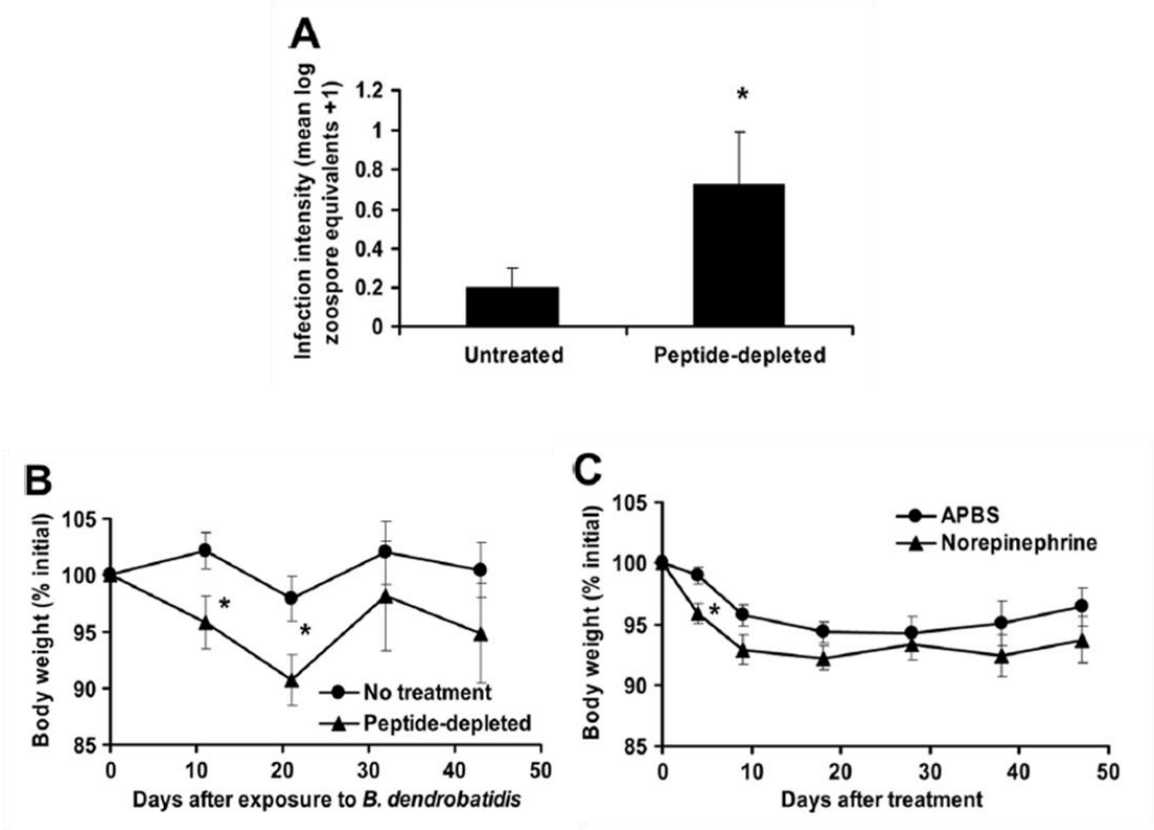

Figure 2-4. Peptide depletion induced by norepinephrine increased susceptibility of *X. laevis* to *B. dendrobatidis*. (A) Intensity of infection on the skin of frogs that were either untreated (n = 10) or injected with 80 nmol/g of norepinephrine (peptide-depleted) (n = 10) on day -1. Both groups were exposed to *B. dendrobatidis* zoospores on days 0 and 21, and the infection intensity was measured on day 32 by real-time PCR. The data represents the mean log zoospore equivalents + 1 ± SEM. *, Significantly greater number of zoospore equivalents present on the skin as compared to untreated frogs by one-tailed Student's t test (after log transformation); $p = 0.0395$. (B) Percentage of initial weight over time in untreated or peptide-depleted frogs exposed to *B. dendrobatidis*. Treatments were significantly different by ANCOVA; $p = 0.028$. *, Significantly increased percentage of weight loss compared to untreated frogs by two-tailed Student's t test; $p \leq 0.05$. (C) Percentage of initial weight over time in APBS-injected or norepinephrine-injected (80 nmol/g) frogs left unexposed to *B. dendrobatidis*. Treatments were significantly different by ANCOVA; $p < 0.001$. *, Significantly increased percentage of weight loss compared to APBS-injected frogs by two-tailed Student's t test; $p = 0.0077$. Lines connect data points for ease of interpretation only.

compared to the untreated control frogs as determined by ANCOVA ($p = 0.028$). Significantly greater weight loss in peptide-depleted frogs was observed up to three weeks after exposure as compared to untreated frogs (Fig. 2-4B). These data further suggested that peptide depletion resulted in *X. laevis* that were more susceptible to *B. dendrobatidis*. It is possible that the injection of the high dose of norepinephrine alone could have caused the weight loss instead of *B. dendrobatidis* infection. Therefore, with the help of an undergraduate, Laura Harper, we tested the effect of norepinephrine injection on weight loss in individuals that tested negative for an existing *B. dendrobatidis* infection. One group of *X. laevis* individuals was injected with 80 nmol/g of norepinephrine, while a second group was injected with amphibian phosphate buffered saline (APBS) as a control. Both groups were left unexposed, and weight loss was measured over time. Although both groups differed significantly by ANCOVA ($p < 0.001$), norepinephrine-injected frogs only showed significant weight losses at day 4 when compared to APBS-injected controls (Fig. 2-4C). This suggested that norepinephrine injection led to temporary weight loss. Thus, *B. dendrobatidis* is more likely to be the cause of the sustained weight loss seen in peptide-depleted and exposed frogs. These results were clear evidence that antimicrobial peptides present in mucosal secretions play a major role in limiting the degree of *B. dendrobatidis* infection on the skin.

Discussion

This study was the first to systematically explore the immune defenses of a model amphibian, *X. laevis*, against the emerging infectious fungal pathogen, *B. dendrobatidis*.

Because *B. dendrobatidis* is a skin pathogen, understanding the immune defenses that are present in the skin is critical for determining the characteristics of disease resistance versus susceptibility. The data from this chapter strongly suggest that innate immune defenses, in particular antimicrobial peptides, are involved in the resistance to lethal *B. dendrobatidis* infections in *X. laevis*.

Antimicrobial peptides secreted by the skin mucus constitute one of the most important innate immune defenses in amphibian skin. Many studies have demonstrated that the skin peptides from various amphibian species can inhibit the growth of *B. dendrobatidis in vitro* (53, 187-195, 234-236). In this study, we show that the peptides present in skin secretions of *X. laevis* are also able to strongly inhibit *B. dendrobatidis* zoospore growth. The most active purified peptide, CPF, had an MIC of 12.5 μM, while enriched mixtures of skin peptides had MICs that ranged from 62.5 to 500 μM when tested against zoospores (Fig. 2-2 and Table 2-1). In the life cycle of *B. dendrobatidis*, the zoospore is the infectious stage, and it is responsible for establishing infection on the surface of amphibian skin. Once on the skin, the zoospore colonizes epithelial cells, where it further matures and replicates (15, 17, 135, 162). Antimicrobial peptides have the capability of preventing the initial infection and colonization of epithelial cells by killing zoospores in the mucus. By constantly secreting small concentrations of antimicrobial peptides onto the skin, frogs can inhibit reinfection of the skin by newly emerging zoospores in an established infection. Frogs that were placed under stress that mimicked predator attack secreted significantly increased amounts of skin peptides than resting, unmanipulated frogs (Fig. 2-3A). However, both groups secreted amounts of peptides that were within the range of concentrations necessary to inhibit zoospore

growth (Fig. 2-2A). These data indicate that frogs that are exposed to *B. dendrobatidis* in the wild can secrete skin peptides at concentrations that could prevent initial infections. To put this in perspective, previous studies with stream-dwelling Australian amphibian species have shown that there was a correlation between the effectiveness of skin peptides in inhibiting *B. dendrobatidis* growth *in vitro* and population declines. Those species with the greatest peptide effectiveness did not decline, while those with the poorest effectiveness did (235). This was recapitulated in the laboratory, where species with the greatest *in vitro* peptide effectiveness showed significantly better survival after experimental exposure to *B. dendrobatidis* (236). The ability to predict the susceptibility of a species to *B. dendrobatidis* based on antimicrobial peptide effectiveness may be useful in conservation efforts. A study of stream-dwelling amphibians in Panama suggested that two out of the nine species tested would be predicted to experience population declines after *B. dendrobatidis* exposure due to their weak peptide effectiveness (234).

The peptide stores in the skin of amphibians require time to recover after they are secreted onto the skin surface. The Rollins-Smith lab previously determined that *X. laevis* injected with the maximal dose of norepinephrine (80 nmol/g) had not fully recovered their peptides after 3 weeks (193). The data from this study suggest that peptide levels had recovered by 7 to 9 weeks (Fig. 2-3C). Analysis of the slope of recovery further suggested that 50 to 104 days were required for complete peptide recovery (Fig. 2-3E). After a more moderate dose of norepinephrine (20 nmol/g), about 4 weeks are necessary for the recovery of peptides in the skin of *X. laevis* (Fig. 2-3D), and these animals would be expected to have full peptide stores within 20.4 days (Fig. 2-3F).

Thus, induction of skin peptide release using the maximal dose of norepinephrine would allow for peptide depletion lasting for several weeks. After depletion with 80 nmol/g of norepinephrine, exposure of *X. laevis* to *B. dendrobatidis* resulted in a significantly higher intensity of infection compared to untreated and exposed control frogs at day 31 after first exposure (Fig. 2-4A). Peptide-depleted frogs also lost significantly more weight than the controls (Fig. 2-4B). This weight loss was not due to the norepinephrine itself (Fig. 2-4C), suggesting that frogs were losing weight due to the effects of *B. dendrobatidis*. *B. dendrobatidis* exposure in more susceptible species, such as *Pseudacris triseriata* (174) and *Rana muscosa* (93), also resulted in significant weight losses. This is the clearest evidence that antimicrobial peptide defenses play a role in limiting *B. dendrobatidis* infection on the skin. It is possible that norepinephrine injection could be causing other immunosuppressive effects, such as corticosterone release (207), but the most direct effect of injection was the depletion of peptides stores in the skin.

Future studies will be designed to investigate whether one injection of norepinephrine at the maximal dose is sufficient to cause complete depletion. After injection with norepinephrine, frogs still retain the ability to secrete concentrations of peptides that are within the range of concentrations needed to inhibit *B. dendrobatidis* growth (Figs. 2-3E and F). Recent histological studies in the Rollins-Smith lab have suggested that more than one injection with 80 nmol/g of norepinephrine may be necessary to completely deplete the skin of its peptides.

CHAPTER III

ADAPTIVE IMMUNE DEFENSES OF *XENOPUS LAEVIS* AGAINST *BATRACHOCHYTRIUM DENDROBATIDIS*

Abstract

Batrachochytrium dendrobatidis is a chytrid fungus that causes the lethal skin disease chytridiomycosis in amphibians. It is regarded as an emerging infectious disease affecting diverse amphibian populations in many parts of the world. Because there are few model amphibian species for immunological studies, little is known about immune defenses against *B. dendrobatidis*. The studies described in this chapter show that the South African clawed frog, *Xenopus laevis*, is a suitable model for investigating immunity to this pathogen. After an experimental exposure, a mild infection developed over 20 to 30 days and declined by 45 days postexposure. Sublethal X-irradiation of frogs decreased leukocyte numbers in the spleen and resulted in greater susceptibility to infection. Immunization against *B. dendrobatidis* induced elevated pathogen-specific IgM and IgY serum antibodies. Mucus secretions from *X. laevis* previously exposed to *B. dendrobatidis* contained significant amounts of IgM, IgY, and IgX antibodies that bind to *B. dendrobatidis*. These data strongly suggest that adaptive immune defenses are involved in the resistance of *X. laevis* to lethal *B. dendrobatidis* infections.

Introduction

X. laevis is used as a model to study adaptive immunity in amphibians, and as a result, much is known about the cells, tissues, and molecules that play a role in adaptive immunity (40, 72, 182). In general, amphibian adaptive immunity is fundamentally similar to mammals, although some features of the adaptive immune system are lacking. One major difference between mammalian and amphibian adaptive immune systems is that amphibians lack lymph nodes (136). In amphibians, the major secondary organ in which adaptive immunity develops is the spleen (136). Another difference is that bone marrow, which is absent from some species, is responsible for developing granulocytes but not lymphocytes in *X. laevis*. Instead, B lymphocytes and other hematopoietic cells develop in the spleen and liver (T lymphocytes develop in the thymus) (91). However, the remaining aspects of amphibian adaptive immunity, including cell types, complement proteins, cytokines, and chemokines are very similar to the mammalian adaptive immune system.

In *X. laevis* there are three main isotypes of immunoglobulin heavy chains (74): IgM (90, 203, 204), IgY (7, 150), and IgX (109, 149). The gene for IgD, which is homologous to fish IgW, has been recently identified (156). A fifth isotype, IgF, has been characterized in *Silurana (Xenopus) tropicalis* (244). IgM, a polymeric immunoglobulin, is the first isotype to develop during ontogeny, as well as the first produced after experimental immunization (74). The second isotype, IgY, is generated shortly after IgM. IgY is considered the amphibian equivalent of IgG, and develops after T cell-dependent class switch recombination (74). IgX, a polymeric (hexamer) immunoglobulin analogous to IgA, is found largely in the gut and plays a role in the

defense of the digestive tract and other mucosal surfaces (109, 149). It is known that immunoglobulins can be secreted in mucus in mammals and fish (43, 134, 140, 198), but the ability of amphibians to release antibodies via mucosal secretions has not been determined.

T cell-mediated responses are also critical for the production of effective adaptive immune responses in amphibians. Thymectomy studies show that T cells play a role in skin graft rejection, mixed lymphocyte reactions, and responses to the T cell mitogens phytohemagglutinin (PHA) and concanavalin A (ConA) (71, 89, 105, 178, 219). Much is known about T cell-mediated responses as a result of studies of skin graft rejection. Major histocompatibility complex (MHC) or minor histocompatibility antigens are required for graft rejection, and this rejection is illustrated by classical first- and second-set kinetics (12, 44, 50, 64, 65, 69, 70, 155). Previous work shows that both cytotoxic and helper T cell responses are MHC-restricted (23, 78, 95). The role of $CD8^+$ cells in graft rejection and viral immunity has also been characterized (145, 181). The T cell receptor (TCR)-α and -β genes, as well as the less common TCR-γ and -δ genes, have been described (47, 92, 170). In *X. laevis*, the distribution of α/β and γ/δ cells is unknown, although reagents directed against conserved regions of γ/δ TCRs stained cells in the skin, suggesting that γ/δ T cells may play a role in skin immunity (139).

Very little is known about the potential adaptive immune responses in amphibians after *B. dendrobatidis* infection. The prevailing view before these studies were undertaken is that adaptive immune responses do not play a role in protecting amphibians from chytridiomycosis. Previous studies show limited lymphocytic infiltrate in histological skin sections of exposed frogs (15, 162). Furthermore, immunization of

Rana muscosa juveniles with formalin-killed *B. dendrobatidis* cells did not protect the frogs from developing chytridiomycosis after exposure to live *B. dendrobatidis* (212). During the course of our work, two microarray studies were published that found little evidence of the initiation of adaptive immune responses after *B. dendrobatidis* infection in *S. tropicalis* (175, 200). Nevertheless, systematic studies of adaptive immunity in the protection of amphibians against *B. dendrobatidis* are lacking, and my work focused on determining whether adaptive immune responses could be generated against *B. dendrobatidis* and whether adaptive immunity plays a role in protection.

Materials and Methods

Frogs

Outbred *X. laevis* frogs ranging in size from approximately 30 to 50 g were purchased from Xenopus I (Dexter, MI) and kept in polystyrene containers at a density of about 10 frogs per 16 liters of dechlorinated tap water at a temperature of between 20 to 24°C. Three times each week, the frogs were fed ground beef heart and their water was changed. All procedures involving animals were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

Collection of mucus secretions

X. laevis frogs that were previously exposed to *B. dendrobatidis* approximately 5 months earlier were injected with 80 nmol/g of norepinephrine and placed in 50 ml of collection buffer for 15 minutes. Buffers were immediately frozen on dry ice, then

lyophilized and resuspended in lysis buffer (8.77 g NaCl, 1.68 g EDTA, 1.58 g Tris-Cl, and 10 ml Triton X-100 per 1 liter of distilled water, supplemented with 1 ml each of 5 mM dithiothreitol, 100 µM phenylmethylsulfonyl fluoride in isopropanol, and 5 mM ε-aminocaproic acid). Sample concentrations were assessed by microBCA assay performed according to manufacturer's instructions. The presence of each immunoglobulin class, the total concentration of each class, and the relative amounts of antibodies binding to *B. dendrobatidis* were determined by ELISA as described below.

Irradiation and exposure to *B. dendrobatidis*

Prior to treatment, all frogs were weighed and swabbed for *B. dendrobatidis* (see "Quantification of *B. dendrobatidis* zoospores" section below) to determine initial infection status before being placed in individual sterile containers. Frogs were subjected to irradiation with 9 Gy X-rays or injected with 80 nmol/g of norepinephrine and also irradiated with 9 Gy X-rays on day -1 (a third group was also peptide depleted without irradiation; see Chapter 2). Control frogs were left untreated, and all groups were exposed to 10^6 JEL 275 zoospores in 500 ml water supplemented with 0.005 IU/ml penicillin and 0.005 µg/ml streptomycin at days 0 and 21. Each frog was swabbed for *B. dendrobatidis* and weighed approximately every 10 days. Infection intensity was determined by real-time PCR. All frogs were observed frequently for signs of *B. dendrobatidis*-induced illness.

Effects of irradiation on leukocytes and on peptide secretion

Frogs were either irradiated with 9 Gy X-rays or left untreated, and the spleens were removed 5 days later. Total leukocytes were enriched over Ficoll, counted, and expressed as total leukocytes per gram of body weight. Skin squares from both groups were fixed in 10% formalin and stained by H&E staining. In a second experiment, frogs were either irradiated with 9 Gy X-rays or left untreated, injected with 2 nmol/g of norepinephrine at 3 or 10 days after treatment, and placed in collection buffer for 15 minutes to collect and quantify peptides as described in Chapter 2.

Quantification of *B. dendrobatidis* zoospores

X. laevis frogs were swabbed with a sterile cotton swab 10 times on the abdomen, legs, and each foot. DNA was extracted from swabs by adding 60 µl of PrepMan Ultra (Applied Biosystems, Foster City, CA) and 30 to 35 mg zirconium/silica beads (0.5mm diameter) (Biospec Products, Bartlesville, OK) to each swab. Swabs were homogenized in a Mini Beadbeater (MP Bio, Solon, OH) for 45 seconds before centrifugation at 15,000 x g for 30 seconds. The homogenization and centrifugation steps were repeated followed by boiling the samples for 10 minutes and cooling for 2 minutes at room temperature. Samples were centrifuged at 15,000 x g for 3 minutes, and the supernatants containing DNA were removed for real-time PCR. Real-time probe-based PCR assays were performed with an Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) using the default conditions (95°C for 10 minutes, followed by 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute) for 40 cycles according to established protocols (30, 110). A standard curve based on the threshold cycle (C_T) values from the control

zoospore DNA was generated, and the number of zoospore equivalents in each sample was calculated.

Immunization and blood collection

Prior to immunization, 20 *X. laevis* individuals were anesthetized, and blood was drawn by cardiac puncture to obtain pre-immune plasma samples. Immunized frogs were injected intraperitoneally (10 µl per gram body weight) with 5 x 10^7 heat-killed (60°C for 20 minutes) JEL 197 whole culture cells (mature sporangia and zoospores) at days -42, -28, and -0 prior to the collection of serum samples. Control frogs were injected with vehicle (APBS) alone (10 µl per gram body weight). Frogs were anesthetized, blood was drawn by cardiac puncture on days 7, 14, 21, and 28 after the final immunization, and IgM and IgY antibodies that bind *B. dendrobatidis* were quantified by ELISA (see "ELISA" section below). *B. dendrobatidis*-specific IgY titers in sera from APBS-injected or *B. dendrobatidis*-immunized frogs were also determined by ELISA (see "ELISA" section below).

ELISA

B. dendrobatidis antigen-coated 96-well plates were prepared by adding 5 x 10^4 JEL 197 cells at 50 µl per well as either zoospores alone or as whole cultures (mature sporangia and zoospores). Plates were centrifuged at approximately 200 x g, and the cells were fixed by the addition of 0.25% glutaraldehyde in APBS. Fixative was removed, and the plates were washed with APBS supplemented with 0.5% bovine serum albumin and 0.1% Tween-20 (ABT) and stored at 4°C. *X. laevis* sera (1/100 dilution) or

lyophilized mucus (at 1 mg/ml) in ABT were added to the wells, and *B. dendrobatidis*-specific antibodies were detected using anti-*Xenopus* monoclonal antibodies specific for IgM (10A9), IgY (11D5) (108), or IgX (410D9) (149), followed by horseradish peroxidase-conjugated goat anti-mouse antibodies. Reactions were visualized with 3, 3', 5, 5'-tetramethylbenzidine substrate. After about 60 minutes, the reaction was stopped by the addition of 2 M H_2SO_4, and the plates were read at 450 nm (OD_{450}) in an ELISA plate reader.

To test for the presence of total IgM, IgY, or IgX in mucosal secretions, lyophilized mucus samples were diluted to a concentration of 5 mg/ml in ABT and plated in a 96-well microtiter plates (4 replicates). Plates were incubated at 37°C for 1 hour. Control wells were coated with 1% normal rabbit serum in ABT. After incubation, all of the wells were blocked with 1% BSA in ABT at 37°C for 2 hours. The plates were washed with ABT, and IgM, IgY, and IgX were detected using antibodies specific for IgM (10A9), IgY (11D5) (108), or IgX (410D9) (149), followed by horseradish peroxidase-conjugated goat anti-mouse antibodies. Reactions were visualized by the addition of substrate as described above. Using purified IgM, IgY, and IgX standards, a standard curve was generated, and total antibodies of each class were quantified.

Statistical comparisons

All parameters compared were averaged, and the mean values ± standard error of the mean were compared by one-tailed or two-tailed Student's t test, one-way analysis of variance (ANOVA) with planned comparisons (Tukey post hoc tests), or analysis of covariance (ANCOVA) as detailed in the figure legends. Zoospore equivalents, peptide

concentrations, and OD values were log transformed as indicated in the figure legends to meet the assumptions of normal distribution and homogeneity of variances for parametric statistics. A p value ≤ 0.05 was considered statistically significant. Error bars shown in all figures represent the standard error of the mean. If no error bar is shown, the standard error of the mean was less than the diameter of the symbol. For all parameters, the number of animals or samples is shown in the figure legend.

Results

Natural kinetics of *B. dendrobatidis* infection over time

To determine whether adaptive immunity played a role in protection of a resistant species such as *X. laevis* from *B. dendrobatidis* infection, we first examined the kinetics of infection in a naïve frog. Naïve individuals were exposed to *B. dendrobatidis* isolate JEL 275 at two time points (days 0 and 21), and infection intensity on the skin was monitored over 60 days. All *X. laevis* individuals used in this protocol tested negative for *B. dendrobatidis* prior to the first exposure. After exposure, 90% of the exposed frogs tested positive for low level infection of *B. dendrobatidis* on day 11, with an average infection intensity of 7.4 ± 3.4 zoospore equivalents. The intensity of infection increased significantly to 272.5 ± 124.2 zoospore equivalents by day 20. Day 30 did not significantly differ from day 20, but by day 44 the infection intensity had decreased to a low of 5.2 ± 1.7 zoospore equivalents (Fig. 3-1A, no treatment group). These data suggested that immune defenses in naïve frogs were activated within 30 days after

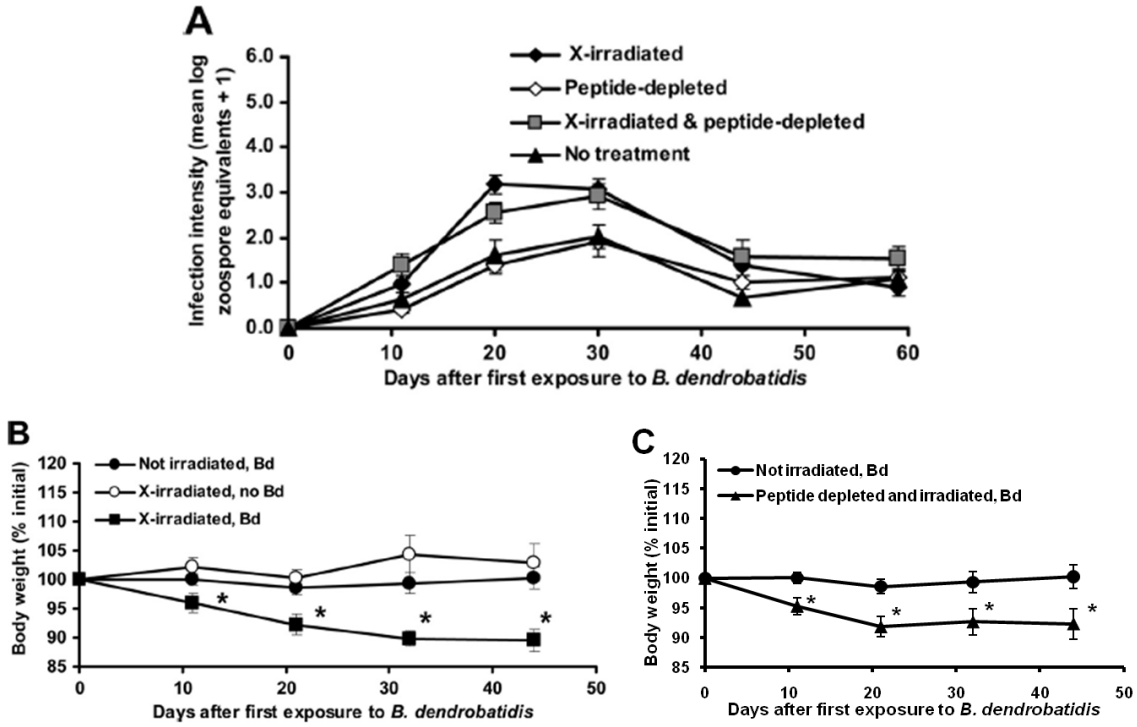

Figure 3-1. X-irradiation increased susceptibility to *B. dendrobatidis* infections in *X. laevis*. (A) Intensity of infection on the skin of frogs treated as listed in the figure legend before exposure to *B. dendrobatidis* zoospores on days 0 and 21. Intensities were measured by real-time PCR on days 0, 11, 20, 30, 44, and 59. The X-irradiated frogs both with or without peptide depletion by injection with 80 nmol/g of norepinephrine had significantly higher infection intensities as compared to untreated and exposed frogs by ANCOVA ($p < 0.001$). In this experiment, peptide depletion alone did not lead to increased infection intensity after exposure. Each data point represents the mean log zoospore equivalents + 1 ± SEM (n = 8 to 10 frogs). (B & C) Percentage of initial weight over time in frogs that were (B) not irradiated but exposed to *B. dendrobatidis*, X-irradiated and left unexposed, X-irradiated and exposed; or (C) not irradiated but exposed and peptide depleted plus X-irradiated and exposed. The change in weight over time differed significantly over time by ANCOVA ($p < 0.001$). *, Significantly greater weight loss compared to not irradiated and exposed frogs by two-tailed Student's t test for each time point ($p \leq 0.05$). The data for panels B & C were pooled from two similar experiments (n = 19 or 20 per group). Lines connect data points for ease of interpretation only.

infection, and that they were able to control *B. dendrobatidis* infections within 45 days of exposure.

Effects of X-irradiation on susceptibility to *B. dendrobatidis* infection

One way to suppress the adaptive immune system in vertebrates is to irradiate with either X-rays or gamma-rays, which destroys the lymphoid cells in the body. A group of *X. laevis* individuals was irradiated with a sublethal dose of X-rays (9 Gy) one day before exposure to *B. dendrobatidis* isolate JEL 275. As before, the irradiated frogs were exposed a second time at day 21, and infection intensity was measured over time by real-time PCR. The intensities of infection in the irradiated and *B. dendrobatidis*-exposed frogs were significantly greater in comparison to those of nonirradiated and exposed controls (ANCOVA, $p \leq 0.001$). There was a two-log increase in infection intensity after X-irradiation at day 20 and a one-log increase at day 30. Like the control group, the infection intensity declined by day 44 in the irradiated frogs (Fig. 3-1A). As a measure of morbidity to *B. dendrobatidis* infection, weight changes in both the irradiated and nonirradiated groups were monitored over the course of the experiment after exposure. Those frogs that were irradiated and exposed to *B. dendrobatidis* experienced much greater weight loss over time than the group that was nonirradiated and exposed (ANCOVA, $p < 0.001$). The weight loss was not attributed to the X-ray irradiation, as a group of frogs that was irradiated but not exposed maintained their weight throughout the time course of this experiment (Fig. 3-1B). These data demonstrated that suppression of the adaptive immune system via X-irradiation resulted in greater susceptibility of *X.*

laevis to *B. dendrobatidis* infection. This was the clearest evidence that adaptive immunity plays a role in the protection of a resistant species from *B. dendrobatidis*.

We also evaluated a group of frogs that were both X-irradiated and peptide-depleted by injection with 80 nmol/g of norepinephrine before exposure to determine if the loss of both innate and adaptive defenses would lead to a further increase of infection intensity on the skin. These doubly-treated frogs displayed infection intensity kinetics similar to those seen with frogs that were irradiated and exposed to *B. dendrobatidis* (Fig. 3-1A). The peptide-depleted and irradiated frogs also lost significantly more weight compared to controls (Fig. 3-1C). Both the increased infection intensity and weight loss suggested that frogs that were irradiated and peptide-depleted were more susceptible to *B. dendrobatidis* infection than controls, though not more so than frogs that were only irradiated and exposed. In this experiment, we also tested frogs that were peptide-depleted only before exposure, and those frogs did not show an increase in susceptibility after exposure (Fig. 3-1A). Nevertheless, both innate and adaptive immune responses appear to be involved in the protection of *X. laevis* against *B. dendrobatidis* infections.

Effects of X-irradiation on splenic leukocytes and skin peptide secretion

To determine whether the irradiation protocol affected adaptive immune cells, we removed the spleens from a group of irradiated frogs, quantified the total number of leukocytes isolated, and compared the numbers to those of frogs that were not irradiated. X-ray irradiation led to a significantly decreased number of leukocytes that could be isolated from the spleen (Fig. 3-2A), suggesting that the sublethal dose of X-rays severely impacted the frog's ability to mount an adaptive response. Irradiation of animals can

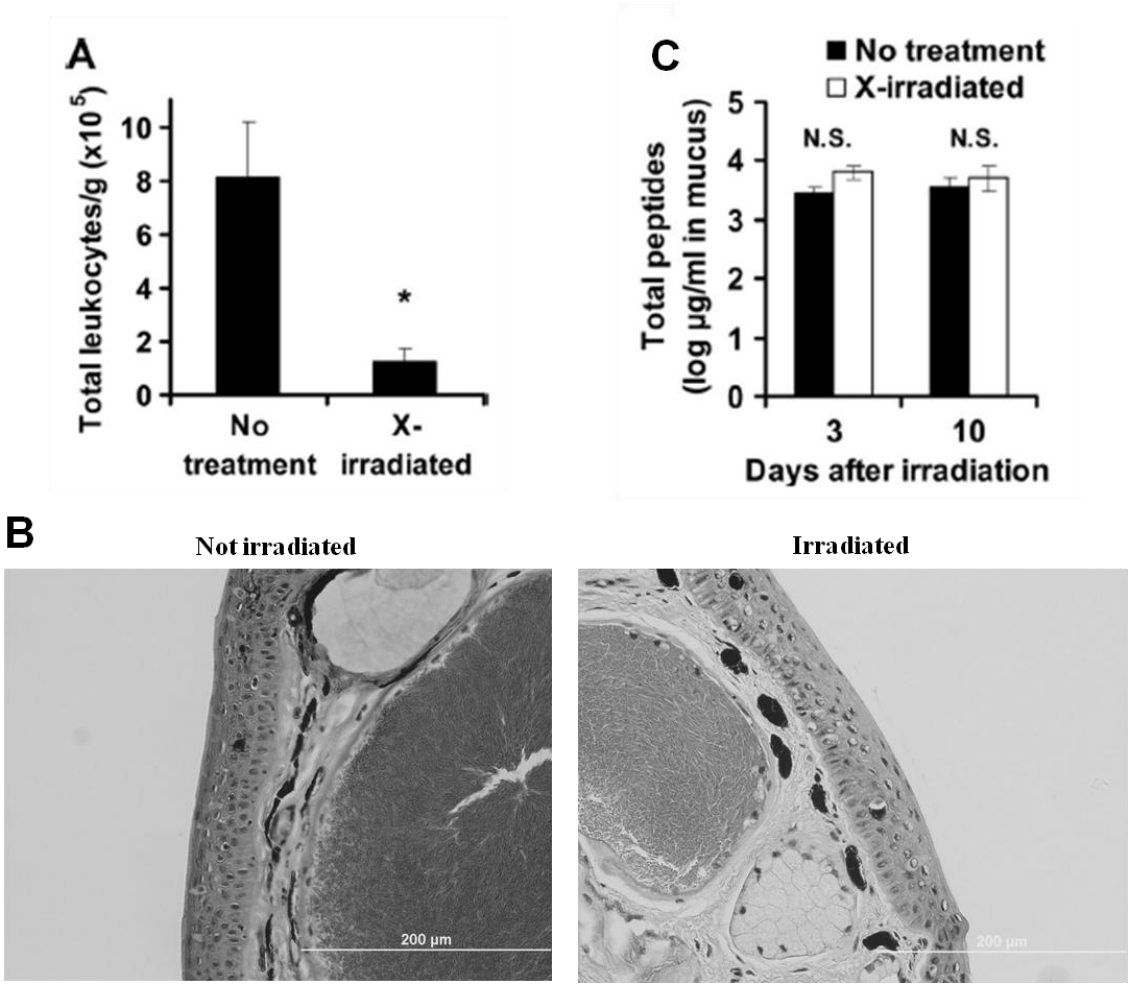

Figure 3-2. X-ray irradiation reduced leukocyte numbers in the spleen but did not damage the skin nor alter the capacity to secrete skin peptides. (A) Effects of X-irradiation (9 Gy) on total leukocyte numbers in the spleen 5 days after treatment (*n* = 3 for each treatment). *, Significantly reduced numbers of leukocytes as compared to untreated controls by two-tailed Student's t test ($p = 0.0314$). (B) Dorsal skin sections stained by H&E staining. The epidermal cells look healthy and intact, regardless of treatment status. Granular glands are also healthy and remain filled with granules. (C) Effects of X-irradiation on capacity to secrete peptides onto the skin. X-irradiated (9 Gy; *n* = 10) or not treated (*n* = 10) frogs were injected with 2 nmol/g of norepinephrine at 3 or 10 days after treatment (*n* = 5 in each treatment at both time points). N.S., no significant different in the peptide concentrations recovered by two-tailed Student's t test after log transformation ($p = 0.0601$ at day 3; $p = 0.6178$ at day 10).

cause other effects besides the destruction of lymphoid cells, and it is possible that the X-ray irradiation could have caused damage to the skin. If this were true, then peptide defenses could have been impaired and could have caused the increased susceptibility observed in irradiated and exposed frogs. We checked all frogs in the previous study over time for visual signs of skin damage after irradiation and exposure to *B. dendrobatidis,* and there was never any evidence of obvious skin damage. Histological sections of irradiated skin also showed no signs of damage, as epithelial cells and granular glands were intact (Fig. 3-2B). We also evaluated the concentrations of skin peptides that are secreted after irradiation and injection with 2 nmol/g of norepinephrine as compared to frogs that were not irradiated. There was no significant difference between the induced peptide concentrations after norepinephrine stimulation between irradiated and nonirradiated controls (Fig. 3-2C), further suggesting that irradiation did not impair innate immunity in the skin. Taken together, we interpreted the increased susceptibility to infection observed after *B. dendrobatidis* exposure and X-ray irradiation in *X. laevis* as evidence of impaired adaptive immune responses.

Immunization of *X. laevis* against *B. dendrobatidis* induced a high-titer systemic antibody response

With the knowledge that adaptive immunity appeared to be involved in protection of *X. laevis* from *B. dendrobatidis* infections, we next determined whether a systemic response could be generated in *X. laevis* after immunization with *B. dendrobatidis*. *X. laevis* individuals were immunized three times over a six week period with heat-killed *B. dendrobatidis* isolate JEL 197 cells, while control frogs were injected with vehicle alone (APBS). Every week for four weeks after the last immunization, serum samples were

taken by heart puncture from a subset of frogs and evaluated for *B. dendrobatidis*-specific antibodies in an ELISA assay. Preimmune serum samples were taken from a small group of frogs before the immunization protocol, and most frogs were bled only once throughout this experiment. Immunization with *B. dendrobatidis* led to significantly elevated IgM and IgY responses that were specific to *B. dendrobatidis*, regardless of whether serum samples were tested against whole cultures (mature sporangia and zoospores together) or zoospores alone. Significantly greater IgY binding to *B. dendrobatidis* was observed in immunized frogs compared to control individuals at all time points, except at day 28 in the assay against whole cultures. There was a peak antibody response at 14 days after the final immunization, with the *B. dendrobatidis*-specific IgY responses waning beginning at day 21 (Fig 3-3A). Frogs injected with APBS alone showed low *B. dendrobatidis*-specific IgY titers across all time points (<1/800). However, in *B. dendrobatidis*-immunized frogs, IgY titers ranged from 1/800 to 1/6400 at day 14, and remained high (up to 1/3200) at days 21 and 28 (Fig. 3-3B). These data demonstrated that a robust systemic antibody response can be generated in *X. laevis* that are immunized with *B. dendrobatidis*, and that this response can persist for at least one month.

Immunoglobulins in skin mucus

In response to *B. dendrobatidis* immunization, *X. laevis* was capable of developing a systemic antibody response, however *B. dendrobatidis* infects the skin of amphibians, and it is possible that a systemic response may not be productive in clearing the infection. An adaptive response in the mucus would be more beneficial to control or

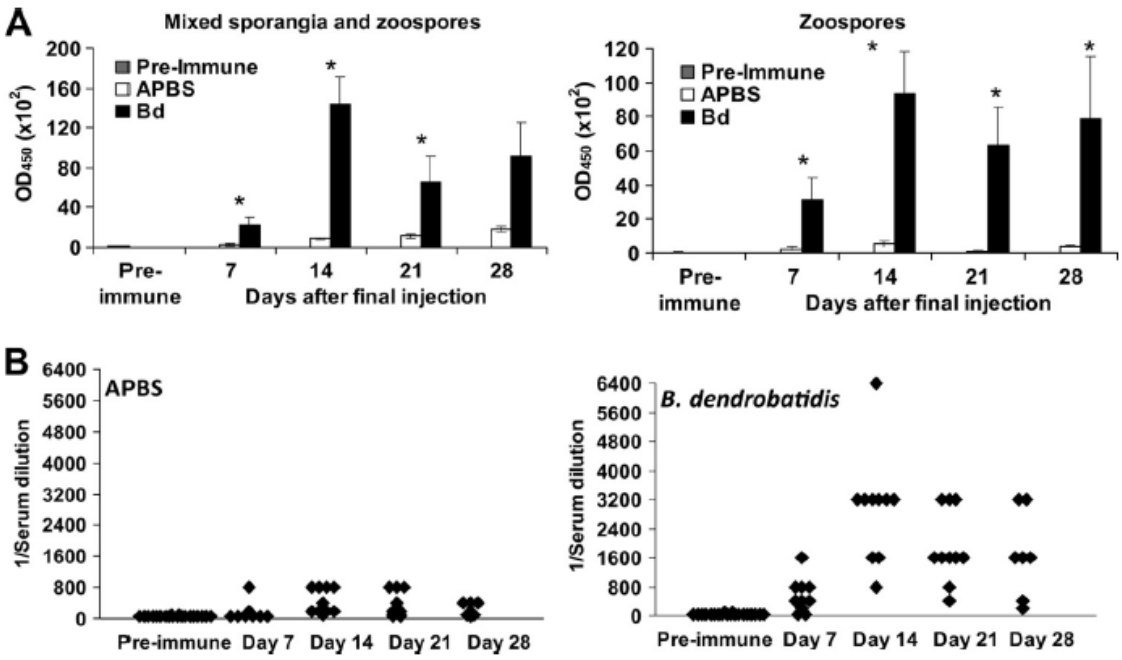

Figure 3-3. Immunization of *X. laevis* against *B. dendrobatidis* (Bd) induced production of a high-titer antibody response. Frogs were immunized three times over a six week period. After the final immunization, blood was drawn each week for 4 weeks by cardiac puncture. Preimmune controls were drawn prior to immunization (n = 20). *B. dendrobatidis*-specific IgY serum antibody binding activity (OD_{450}) was measured by ELISA in frogs either immunized with heat-killed *B. dendrobatidis* or injected with vehicle alone (APBS). (A) *B. dendrobatidis*-specific IgY in the serum (1/100 dilution) of APBS-injected or *B. dendrobatidis*-immunized frogs at day 7, 14, 21, and 28 (n = 10 at all time points except day 28 where n = 7) after the last injection. The left panel shows binding activity against a mixture of mature sporangia and zoospores. The right panel shows binding activity against zoospores alone. Mean OD_{450} values were significantly different from preimmune serum values at all time points after log transformation by one-way ANOVA with Tukey post hoc test ($p < 0.01$). *, Significantly higher binding values compared to APBS controls after log transformation by two-tailed Student's t test ($p \leq 0.05$). (B) *B. dendrobatidis*-specific IgY titers for APBS-injected (left panel) or *B. dendrobatidis*-immunized (right panel) frogs in preimmune (n = 20) and day 7, 14, 21, and 28 (n = 10 at all time points except day 28 where n = 7) serum samples as determined by ELISA.

clear the pathogen. To evaluate whether *X. laevis* skin mucus contained immunoglobulins, mucosal secretion was induced by injecting 80 nmol/g of norepinephrine into the skin of *X. laevis* individuals that had previously been exposed to *B. dendrobatidis* approximately 5 months earlier. Mucosal secretions were collected in collection buffer, lyophilized, and resuspended in a buffer containing protease inhibitors before being tested for the presence of IgM, IgY, and IgX immunoglobulins by ELISA. All five individual *X. laevis* tested had significant levels of all three immunoglobulin classes in their mucosal secretions, with the exception of IgX in frog #5, when compared to an irrelevant serum control (1% normal rabbit serum) by one-way ANOVA with Tukey post hoc test ($p < 0.01$) and two-tailed Student's t test ($p \leq 0.025$) (Fig. 3-4A). To calculate the concentration of immunoglobulins being secreted onto the surface of the skin, we used purified IgM, IgY, and IgX at known concentrations to create a standard curve in an ELISA assay. Using these purified standards, we determined that, on average, IgM is secreted at approximately 98 µg/ml in mucus, IgY at approximately 801 µg/ml in mucus, and IgX at approximately 1671 µg/ml in mucus (Fig. 3-4B). As IgX is considered the equivalent of mammalian IgA (149), it may not be surprising that it is the most concentrated immunoglobulin in the skin mucus.

X. laevis that were previously exposed to *B. dendrobatidis* had high concentrations of immunoglobulins present in the mucus, but the question remained as to whether any antibodies secreted were specific for *B. dendrobatidis*. To test this, the reconstituted mucosal secretions were tested for binding activity against *B. dendrobatidis* in an ELISA assay. All mucus samples were diluted to 1 mg/ml, and all showed significant binding to *B. dendrobatidis* as compared to an irrelevant serum control (1%

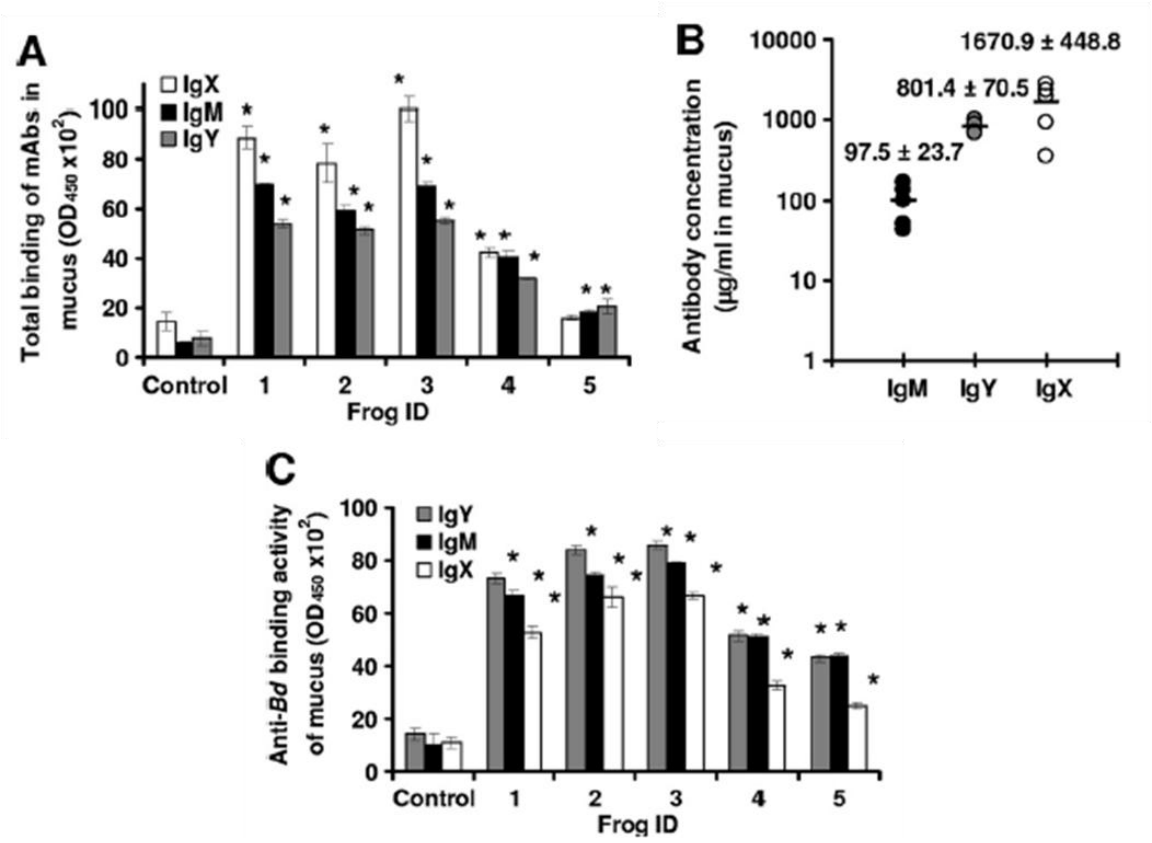

Figure 3-4. Total and pathogen-specific antibodies in *X. laevis* mucosal secretions. (A) Total binding activity of monoclonal antibodies specific for *X. laevis* immunoglobulins in mucosal secretions (5 mg/ml concentration of total proteins; n = 5) compared to an irrelevant serum control (1% normal rabbit serum) as evaluated by ELISA. (B) Using purified *X. laevis* IgM, IgY, and IgX as standards, total antibodies of each class in the mucus of the same frogs as in panel A were quantified by ELISA. (C) *B. dendrobatidis*-specific immunoglobulin binding activity in the mucus of the same frogs as in panels A and B compared to an irrelevant serum control (1% normal rabbit serum). *, For panels A and C, significantly higher OD_{450} values for each frog and each immunoglobulin class after log transformation by one-way ANOVA with Tukey post hoc test ($p < 0.01$). All values were also significantly higher from the irrelevant serum control after log transformation by two-tailed Student's t test ($p \leq 0.025$).

normal rabbit serum) by one-way ANOVA with Tukey post hoc test ($p < 0.01$) and two-tailed Student's t test ($p \leq 0.025$) (Fig. 3-4C). These data suggested that *X. laevis*, and potentially other amphibian species, were capable of secreting immunoglobulins onto the surface of the skin via mucosal secretions. After exposure to *B. dendrobatidis*, some of these immunoglobulins can bind to *B. dendrobatidis* and may play a role in resistance against this pathogen on the skin. However, future studies need to be performed to determine whether immunoglobulins specific for *B. dendrobatidis* are increased after exposure, and whether these immunoglobulins are protective.

Discussion

Along with the work described in the previous chapter, these studies were the first to systematically explore the immune defenses of *X. laevis* against the newly emerging fungal pathogen *B. dendrobatidis*. These studies are necessary to understand resistance or susceptibility to *B. dendrobatidis* infection. The data from this chapter strongly suggest that a component of adaptive immunity is playing a role in the protection of *X. laevis*, a resistant species, from lethal *B. dendrobatidis* infections.

Although *X. laevis* is resistant to *B. dendrobatidis* and does not die after exposure, the skin becomes infected. After experimental exposure, zoospores can be observed in skin swabs from untreated frogs at 11 days postinfection, a time that corresponds to approximately three cycles of replication at optimal *B. dendrobatidis* temperatures. By days 20 and 30, the intensity of infection significantly increases before waning to very low levels within 44 days (Fig. 3-1A). The kinetics of infection and clearance in untreated but exposed *X. laevis* suggests that immune defenses are activated within 30

days. The same kinetics are observed in individuals that have been irradiated with X-rays at a dose known to inhibit skin allograft rejection, tumor rejection, and ranavirus resistance (106, 180, 181, 201). However, in irradiated frogs, the infection intensity is almost two-logs higher than the intensity seen in untreated frogs at day 20 and one-log higher at day 30 (Fig. 3-1A). X-irradiation impacted the number of leukocytes present in the spleen (Fig. 3-2A), but did not damage the skin (Fig. 3-2B) nor impair the ability to secrete peptides onto the surface of the skin (Fig. 3-2C). Thus, the most direct impact of X-irradiation was the reduction of the number of leukocytes available for an adaptive immune response, although it is possible that the irradiation induced a stress response that resulted in the release of corticosteroids that would also suppress the immune system. The individuals that were irradiated then exposed also lost significantly more weight compared to untreated and exposed controls. This weight loss was not attributed to the irradiation protocol, as irradiated frogs that were left unexposed maintained their weights (Fig. 3-1B). Although no frogs died due to exposure to *B. dendrobatidis*, the increased infection intensities and weight loss in irradiated and exposed frogs suggest that an irradiation-sensitive component of the adaptive immune system is involved in clearance of *B. dendrobatidis* infections in *X. laevis*.

Contrary to data previously discussed (Fig. 2-3 C, D, E, and F), peptide depletion by norepinephrine injection did not increase susceptibility to *B. dendrobatidis* after exposure in this experiment. One injection with norepinephrine is enough to severely reduce peptide defenses in the skin of *X. laevis*, however they still retain a small amount of peptides in the skin. Recent work in our lab by Scott Fites and Whitney Gammill has suggested that at least two injections with norepinephrine are necessary to fully deplete

the skin of its peptide stores. The data that one injection of norepinephrine would not necessarily fully deplete the frog of its peptides may explain why we didn't see any increased susceptibility in these frogs.

When these studies were initiated little was known about the adaptive immune responses in amphibians against *B. dendrobatidis*. Our studies have shown that an irradiation-sensitive component of adaptive immunity plays an important role in inhibiting *B. dendrobatidis* infection. Experimental immunization of *X. laevis* to *B. dendrobatidis* led to the production of a high-titer class-switched IgY response that was specific to *B. dendrobatidis* (Figs. 3-3A and B). This study did not determine the T cell contribution to this response, but it is known that the induction of high-titer antibody responses in *X. laevis*, as well as the switch from IgM to IgY, is T cell-dependent (23, 222). Thus, the generation of high-titer antibody responses after immunization suggests that T cells are being activated alongside B cells.

B. dendrobatidis is a skin pathogen, and it is unknown if a systemic adaptive immune response would be generated after infection. In order to determine how adaptive immunity could be protecting the skin of amphibians, we decided to investigate whether immunoglobulins were present in the skin mucus. Immunoglobulins are known to be a component of mucus in fish (134, 198) and mammals (43, 140). In mammals, secretion of IgM and IgA is achieved when polymeric IgM and IgA bind to polymeric Ig receptors (pIgR) present on mucosal epithelial cells (33, 99). After binding to pIgR on the basolateral side of the epithelial cells, the antibody/pIgR complexes are endocytosed and transported via endosomes to the apical plasma membrane. At the apical plasma membrane, pIgR is cleaved into secretory component (SC) and the endosome fuses with

the membrane, releasing both SC and secretory IgM and IgA into the mucus (119, 120, 147). No reports have previously determined whether antibodies could be found in amphibian mucus. However, pIgR from *X. laevis* has been isolated and characterized (31), suggesting that antibodies could be secreted into the mucus of amphibians.

This work found that antibodies of all three major classes (IgM, IgY, and IgX) were present in the skin mucus of *X. laevis* that had been previously exposed to *B. dendrobatidis*. IgX is considered to be the amphibian equivalent of human IgA, since IgX-secreting B cells were determined to be found in high quantities in the gut epithelium of *X. laevis* but barely detectable in the liver and spleen (149). The presence of all three classes in the mucosal secretions suggests that all three classes may play a role in protecting the skin (Fig. 3-4). In an ELISA assay, mucosal antibodies from the previously exposed *X. laevis* individuals bound specifically to *B. dendrobatidis* (Fig. 3-4C), although it is possible that these antibodies may bind nonspecifically to many pathogens. Future experiments should be performed to determine whether experimental exposure to *B. dendrobatidis* induces an increase in pathogen-specific antibodies in the mucus. Research also needs to be conducted into whether these mucosal immunoglobulins are protective. One potential method of protection afforded by mucosal antibodies is the opsonization of pathogens for destruction by phagocytes or complement proteins. If the immunoglobulins present in mucus are determined to be protective, it may be beneficial to develop a method of immunizing across the skin of amphibians to increase resistance against *B. dendrobatidis* in susceptible species.

CHAPTER IV

INHIBITION OF AMPHIBIAN LYMPHOCYTE RESPONSES BY *BATRACHOCHYTRIUM DENDROBATIDIS*

Abstract

Batrachochytrium dendrobatidis is a chytrid fungus that causes the lethal skin disease chytridiomycosis in amphibians. Chytridiomycosis is an emerging infectious disease affecting amphibian populations around the world. Our previous studies of the effects of X-irradiation in *Xenopus laevis* suggested that adaptive immunity is an important component of resistance to *B. dendrobatidis* infection in this species. However, lack of extensive lymphocyte infiltration in diseased skin suggests an impaired immune response. A mechanism to explain impaired immunity has not been described. We show here that *B. dendrobatidis* mature sporangia, but not zoospores, have the capacity to inhibit the proliferation of mitogen-stimulated B and T lymphocytes from *X. laevis*. This inhibition occurs even when the cells are separated from the lymphocytes by a cell-impermeable membrane in a transwell assay suggesting the release of a soluble inhibitory factor or factors. Activated lymphocytes from *Rana pipiens* are also inhibited after co-culture with the pathogen suggesting that the inhibitory effect is not limited to one amphibian species. The mechanism of inhibition appears to be induction of lymphocyte apoptosis. Although both B and T cell responses are inhibited by *B. dendrobatidis in vitro*, T lymphocytes appear to be more sensitive to the induction of apoptosis. Taken together, these data define a specific mechanism by which *B.*

dendrobatidis can impair development of a protective immune response. They strongly suggest that *B. dendrobatidis* has evolved strategies to resist adaptive immune defenses in order to survive in the skin of amphibians.

Introduction

Many species of fungi are capable of inducing a wide variety of diseases. These infections have become more relevant to human health in the recent past due to an increase in the population of immunocompromised hosts (197). Hosts with competent immunity are able to stave off fungal infections, utilizing both innate and adaptive immune responses. Initial recognition of fungal pathogens is achieved by pattern recognition receptors, including toll-like receptors, on both phagocytes and dendritic cells. Both cell types become activated, leading to the secretion of pro-inflammatory cytokines, release of antimicrobial peptides or reactive oxygen intermediates, and activation of cells involved in adaptive immunity. T lymphocytes become primed, with Th1 cells stimulating phagocytosis or neutrophil degranulation, Th2 cells activating antibody responses in B lymphocytes, and cytotoxic T cells secreting antimicrobial factors. The antibodies produced by B cells can opsonize fungal cells either for increased phagocytosis or for complement-mediated killing. Both innate and adaptive responses are required for full protection from fungi in mammals, although the extent to which both systems are activated is dependent on the fungal species (rev. in 197). Although hosts appear to have sufficient defenses against fungal pathogens to successfully eliminate infection, certain fungi have evolved methods to evade immunity by suppressing adaptive immune responses.

Two metabolites produced by fungi that have the capability to modulate adaptive immunity are cyclosporine and gliotoxin. Cyclosporine was initially identified as a metabolite of the fungus *Tolypocladium inflatum* Gams (27), although it was also found to be produced by *Cylindrocarpon lucidum* Booth (163). Cyclosporine is an 11 amino acid cyclical peptide with a unique unsaturated C-9 amino acid (163). It possesses not only mild antifungal activity, but also immunosuppressive properties without being toxic to lymphocytes (26-28). Cyclosporine has the capacity to inhibit the production of antibodies against T-dependent antigens, to impede cell-mediated cytolysis, to prevent rejection of skin grafts, and to suppress delayed-type hypersensitivity responses (rev. in 87). All inhibitory properties were more marked in T cells than in B cells (87). Cyclosporine prevents the activation and proliferation of T cells by binding to cyclophilin and forming a heterodimeric complex. This complex binds to calcineurin, which is only present in T cells, and inhibits the production of IL-2 which reduces T cell differentiation and activation (80). However, lymphocytes already activated are not affected by cyclosporine, and cyclosporine effects are reversible (87).

Gliotoxin is a metabolite produced by multiple fungal species within the genera *Aspergillus*, *Penicillium*, and *Trichoderma*. Gliotoxin is an epipolythiodioxopiperazine immunosuppressive mycotoxin that is regulated by LaeA, a transcription factor that regulates secondary metabolism in *Aspergillus fumigatus* (13). Transcription of gliotoxin genes is upregulated after the onset of aspergillosis in *A. fumigatus*-infected hosts (138). The toxin has multiple effects on mammalian cells, including downregulating the oxidative burst pathway in polymorphonuclear leukocytes via disrupting the production of NADPH oxidase complexes (221), as well as inhibiting the activation of NF-κB in B

and T lymphocytes (159). By preventing NF-κB activation, all the cytokines necessary for an effective adaptive immune response are inhibited, abrogating B and T cell stimulation (159). Mice treated with gliotoxin *in vivo* show significant apoptosis of B cells (215), although gliotoxin may also activate apoptosis in polymorphonuclear leukocytes, peripheral blood mononuclear cells, and macrophages (13).

The work so far presented in this thesis gives evidence that the adaptive immune system of amphibians is necessary in order to fully protect species from lethal *B. dendrobatidis* infections. Although this work was the first to fully explore adaptive immunity against this pathogen, other studies have been conducted to determine that adaptive immunity is required for protection. In two New Zealand amphibian species, *Litoria ewingii* and *Litoria raniformis*, both of which are susceptible to *B. dendrobatidis*, frogs that were initially exposed to *B. dendrobatidis* and treated with chloramphenicol were able to clear the fungus within 35 days after re-exposure in the absence of the drug (176). Boreal toads (*Bufo boreas*) that survived an initial exposure to *B. dendrobatidis* in situations in which they could choose a dry environment were able to survive longer after re-exposure in a constant wet environment, suggesting the involvement of acquired immunity in protection (C. Carey, personal communication). However, the lack of extensive lymphocyte infiltration within the skin of infected frogs has been interpreted to suggest that adaptive immune responses against *B. dendrobatidis* are poor (15, 162). In a recent microarray study, it was determined that several adaptive immune system genes were downregulated after *B. dendrobatidis* infection, including B-cell translocation gene, cutaneous T-cell lymphoma associated antigen, and complement factors. This downregulation may suggest that *B. dendrobatidis* has the capacity to suppress activation

of adaptive immune responses after infection (175). No studies have been published evaluating whether *B. dendrobatidis* can interfere with adaptive immunity, and my work focused on characterizing the effects of *B. dendrobatidis* on the activation of *X. laevis* lymphocytes *in vitro*.

Here we show that *X. laevis* lymphocytes activated by a mitogen or killed bacteria fail to proliferate when cultured with live or dead *B. dendrobatidis* cells (mixed zoospores and mature sporangia). The inhibition of proliferation is maintained even when *B. dendrobatidis* cells are physically separated from the stimulated lymphocytes by a 0.4 µm pore-size membrane in a transwell assay. Purified zoospores alone are also capable of inhibiting proliferation of activated lymphocytes over the three days of culture; however, the effect is lost if zoospores are heat-killed preventing further maturation, and it cannot be demonstrated when zoospores are separated from the lymphocytes by a cell-impermeable membrane. The inhibitory activity of *B. dendrobatidis* is not specific to *X. laevis* as proliferation of stimulated lymphocytes from another amphibian species, *Rana pipiens*, is also inhibited in the presence of *B. dendrobatidis*. The apparent mechanism of inhibition by the fungal factor is induction of lymphocyte apoptosis. Characterization of the fungal factor or factors responsible for the inhibition and apoptosis is an active area of continuing research. Collectively, these data strongly suggest that *B. dendrobatidis* has evolved mechanisms to evade adaptive immune responses giving it a survival advantage in amphibian skin once an infection is established.

Materials and Methods

Frogs

Outbred *Xenopus laevis* frogs ranging in size from approximately 50 to 100 g were purchased from Xenopus I (Dexter, MI) and held in polystyrene containers at a density of about 10 frogs per 16 liters of dechlorinated tap water. Three times each week, the frogs were fed ground beef heart and their water was changed. Outbred *Rana pipiens* adults ranging in size from approximately 20 to 40 g were purchased from Connecticut Valley Biological (Southampton, MA) and held in polystyrene containers at a density of about 5 frogs per tank. Containers were tilted slightly, and enough dechlorinated tap water was added to cover one end of the tank leaving the other end dry to allow the frogs access to dry space. Three times each week, *R. pipiens* were fed live crickets and their water was changed. All containers were kept between 20 - 24°C. All animal procedures were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

Lymphocyte culture

Lymphocytes were isolated from the spleen and cultured as previously described (185) in Leibovitz (L-15) culture medium (Sigma, St. Louis, MO) diluted to amphibian tonicity and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 X 10^{-2} M sodium bicarbonate, 5 X 10^{-5} M 2-mercaptoethanol, 2-mM L-glutamine, and 1% heat-inactivated fetal calf serum (Atlanta Biologicals, Lawrenceville, GA). After sacrifice of the frogs, spleens were removed using sterile technique and placed in a small volume of

L-15. Each spleen was dissociated between two sterile frosted slides and washed, and lymphocytes were enriched by centrifugation over Ficoll-Hypaque cushion (ρ = 1.119; Sigma, St. Louis, MO), counted, and resuspended at a known concentration in L-15.

Lymphocyte inhibition assays

Lymphocytes from *X. laevis* or *R. pipiens* were plated in a 96-well round-bottom plate at 1 X 10^5 cells per well (5 X 10^5/ml). PHA (2 µg/ml) or heat-killed (100°C for 30 minutes) *Aeromonas hydrophila* cells originally isolated from a diseased Wyoming toad (216, 217) (2.5 X 10^6 cells/ml) were added to stimulate T or B cell proliferation, respectively, as previously described (144, 185). *B. dendrobatidis* whole cultures (mature sporangia and zoospores) or zoospores alone from either isolate JEL 197 (135) or JEL 275 (42) were added to the wells following re-suspension in L-15. Zoospores were isolated as previously described (187). Briefly, zoospores were harvested by flooding the agar surface of 7-day old cultures on 1% tryptone agar (1 g tryptone, 1 g agar, 100 ml water) using about 3.0 ml of sterile 1% tryptone broth (1 g tryptone, 100 ml water) twice. The broth containing zoospores was passed over sterile nylon spectra/mesh filters (20 µm mesh opening; Spectrum Laboratories, Rancho Dominguez, CA) to remove mature cells, and zoospores were re-suspended in L-15. For experiments in which *B. dendrobatidis* was heat-killed, *B. dendrobatidis* was treated at 60°C for 10 minutes before addition to culture. Plates containing lymphocytes were incubated at 26°C in an atmosphere of 5% CO_2/95% air for 3 days before harvesting (185). All wells were pulsed with 0.5 µCi ^{3}H-thymidine (5 µCi/ml, specific activity 2 Ci/mmole) (PerkinElmer, Waltham, MA) 24

hours prior to harvesting, and overall proliferation, measured as ^{3}H-thymidine uptake, was quantified as counts per minute (CPM) using a scintillation counter.

Transwell assays

Lymphocytes from *X. laevis* (5 X 10^5 cells per well) were plated in a 24-well transwell plate with 0.4 µm pore-size transwell inserts (Corning, Corning, NY) at a final density of 8.3 X 10^5 cells/ml. PHA was added to achieve a final concentration of 2µg/ml. Mixed cultures of *B. dendrobatidis* sporangia and zoospores (8.3 X 10^5 cells/ml) or zoospores alone (1.7 X 10^7 cells/ml) of isolate JEL 197 were added above the transwell inserts (8.3 X10^5 cells/ml). In co-culture control wells, *B. dendrobatidis* cells were added directly to lymphocytes, and media was added above the insert. In one experiment, 5 X 10^5 lymphocytes were plated along with zoospores above the insert to determine whether cell contact was necessary for zoospore production of an inhibitory factor. Plates were incubated at 26°C for 3 days before harvesting. Wells containing lymphocytes were pulsed with ^{3}H-thymidine 24 hours prior to harvesting. The lymphocytes were transferred to a 96-well round bottom plate for ease of harvesting, and overall proliferation was measured as CPM using a scintillation counter.

Apoptosis assays

Lymphocytes from *X. laevis* were plated in a 24-well transwell plate with 0.4 µm pore-size transwell inserts at 1.7 X 10^6 cells/ml. Mixed sporangia and zoospores of *B. dendrobatidis* isolate JEL 197 were added above the transwell inserts at a density of 3.3 X 10^7 cells/ml. Control inserts were filled with media alone. Cultures were incubated for

24, 48, 72, or 96 hours at 26°C before use in flow cytometry. To analyze the percent of apoptotic cells, cells were stained with a FITC-conjugated Annexin V Apoptosis Detection Kit according to manufacturer's instructions (BD Pharmingen, San Diego, CA). Briefly, lymphocytes were washed with APBS and resuspended in 1X binding buffer at 1 X 10^6 cells per ml. Approximately 1 X10^5 lymphocytes were stained with 5 µl Annexin V-FITC and 1 µl propidium iodide (PI) (50 µg/ml). Control preparations of cells were left unstained or stained with Annexin V only or PI only. Cells were analyzed by flow cytometry with the assistance of the Vanderbilt Flow Cytometry core. Cells were gated based on FITC and PI positivity, and those cells that were $FITC^+/PI^-$ were considered to be undergoing apoptosis (223).

For analysis of apoptosis in B or T cell populations, lymphocytes were plated in 24-well plates (no transwell inserts) at 1.7 X 10^6 cells/ml. Mixed sporangia and zoospores of *B. dendrobatidis* isolate JEL 197 were heat-killed at 60°C for 10 minutes and plated with lymphocytes at 1.7 X 10^7 cells/ml. Plates were incubated for 48 hours at 26°C before use in flow cytometry. All lymphocytes were stained with anti-*Xenopus* monoclonal antibodies specific for MHC class II (14A2) (186), followed by polyvalent allophycocyanin (APC)-conjugated goat anti-mouse antibodies (0.5 mg/ml). [All lymphocytes are MHC class II positive in adults of this species (73, 79, 186).] This allowed us to select for lymphocytes while gating out *B. dendrobatidis* cells. After washing, the MHC II^+ cell population was divided and stained either with monoclonal antibodies specific for IgM (6.16) (22) to identify B cells or with monoclonal antibodies specific for CD5, a pan T-cell marker in *Xenopus* (2B1) (118), to identify T cells. Both populations were further stained with polyvalent phycoerythrin (PE)-conjugated goat

anti-mouse antibodies (0.5 mg/ml). All antibody stocks contained 24 µg/ml of 7-aminoactinomycin D (7-AAD) to gate out dead cells. The IgM^+ fraction and the $CD5^+$ fraction were washed and resuspended in 1X binding buffer before staining with 5 µl FITC-conjugated Annexin V according to manufacturer's instructions. Control populations of lymphocytes were unstained, stained with 7-AAD only (24 µg/ml), with APC only (0.5 mg/ml), with PE only (0.5 mg/ml), or with FITC only. Cells were analyzed by flow cytometry with the assistance of the Vanderbilt Flow Cytometry core. B cells were gated on the IgM-PE positive population while T cells were gated on the CD5-PE positive population. Both B and T cells were then analyzed for dual staining for Annexin V (FITC) and 7-AAD, and those cells that were FITC positive and 7-AAD negative were considered to be apoptotic. To determine the extent of apoptosis regardless of cell type, lymphocytes were also gated on MHC II-APC positive population before being analyzed for Annexin V (FITC) and 7-AAD fluorescence.

Statistical comparisons

All parameters compared were averaged, and the mean values ± standard error of the mean were compared by one-tailed or two-tailed Student's t test or one-way analysis of variance (ANOVA) with planned comparisons (Tukey post hoc tests) as detailed in the figure legends. A p value ≤ 0.05 was considered to be statistically significant. Error bars shown in all figures represent the standard error of the mean. If no error bar is shown, the standard error of the mean was less than the diameter of the symbol.

Results

Lymphocytes from *X. laevis* immunized against *B. dendrobatidis* failed to proliferate in response to culture with the fungus

As previously shown, *X. laevis* immunized against *B. dendrobatidis* had high-titer IgY antibodies in the serum that were specific for the pathogen, suggesting that a robust adaptive immune response had been generated (Fig. 3-3). To further show that adaptive responses had been activated, we isolated lymphocytes from immunized frog spleens and cultured them with *B. dendrobatidis* cells. The lymphocytes should recognize their respective antigens on *B. dendrobatidis* and proliferate in response to culture with the pathogen. The lymphocytes from the immunized frogs failed to proliferate in response to culture with *B. dendrobatidis*, as determined by significantly higher ^{3}H-thymidine uptake compared to lymphocytes alone. This increase in ^{3}H-thymidine counts per minute was not due to *B. dendrobatidis* taking up the isotope, as *B. dendrobatidis* cells alone showed very low counts per minute (Table 4-1). There was no inherent defect in proliferation of these lymphocytes, as they all responded and proliferated significantly when stimulated with the T cell mitogen phytohemagglutinin (PHA) (Table 4-1). These data led to our hypothesis that *B. dendrobatidis* is capable of inhibiting activation and proliferation of *X. laevis* lymphocytes, which would give the fungus a survival advantage in the skin after infection.

B. dendrobatidis inhibited proliferation of activated T and B cells

To determine whether *B. dendrobatidis* can inhibit the proliferation of lymphocytes that had been activated, live or heat-killed *B. dendrobatidis* cells (mature

Table 4-1. Spleen cell proliferation following immunization with *B. dendrobatidis*.

Identifier	CPM Cells Only[1]	CPM Cells + PHA[1]	S.I.[2] PHA	CPM Cells Only[3]	CPM Cells vs. Bd[3]	S.I *Bd*
[4]APBS 1	70.1 ± 8.1	4613 ± 264*	65.8	139 ± 34	190 ± 45	1.4
APBS 2	440.0 ± 32.4	5110 ± 149*	11.6	356 ± 164	2217 ± 184*	6.2
APBS 3	442.2 ± 64.0	29096 ± 377*	65.8	183 ± 48	2906 ± 558**	15.9
APBS 4	262.3 ± 100.5	7223 ± 1634**	27.5	1986 ± 548	1289 ± 211	0.6
APBS 5	153.9 ± 22.2	5425 ± 660*	35.2	113 ± 13	342 ± 82***	3.0
APBS 6	104.1 ± 18.4	10456 ± 805*	100.4	287 ± 108	331 ± 83	1.1
[5]*Bd* 1	86.4 ± 5.3	3490 ± 114*	40.4	70 ± 10	88 ± 12	1.3
Bd 2	756.0 ±144.0	9030 ± 257*	11.9	1742 ± 456	1947 ± 204	1.1
Bd 3	436.6 ± 30.0	33089 ± 895	75.8	447 ± 262	2218 ± 536***	5.0
Bd 4	75.5 ± 5.2	7028 ± 249*	92.8	60 ± 11	50 ± 12	0.8
Bd 5	75.7 ± 6.1	5118 ± 513*	67.6	33 ± 2	48 ± 7	1.4
Bd 6	82.6 ± 15.4	5105 ± 1017**	61.8	80 ± 18	45 ± 8	0.6
Bd 7	703.6 ± 172.8	8545 ± 980*	12.14	2456 ± 536	1338 ± 500	0.5

[1]Cells harvested at day 3 after *in vitro* culture. [2]S. I.: Stimulation index = stimulated counts divided by background counts. [3]Cells harvested at day 5 after *in vitro* culture. [4]APBS control frogs were injected with amphibian phosphate buffered saline at days 0, 14, and 28. Cells were harvested at day 33. [5]*Bd* frogs immunized with heat-killed *B. dendrobatidis* at days 0, 14, and 28. Cells were harvested at day 33.

*, Significantly greater than CPM of cells only, $p \leq 0.0005$. **, Significantly greater than CPM of cells only, $p \leq 0.005$. ***, Significantly greater than CPM of cells only, $p \leq 0.025$.

sporangia and zoospores) were co-cultured with lymphocytes stimulated with either PHA (T cell stimulation) or heat-killed *Aeromonas hydrophila* bacteria (B cell stimulation) as described in materials and methods with the assistance of Scott Fites and Whitney Gammill. As expected, lymphocytes stimulated with either PHA or *A. hydrophila* in the absence of *B. dendrobatidis* proliferated well. When *B. dendrobatidis* was added to the stimulated lymphocytes, there was a significant dose-dependent reduction in proliferation as the numbers of fungal cells increased (Figs. 4-1A and B). Live *B. dendrobatidis* inhibited PHA-stimulated proliferation significantly at all pathogen-to-lymphocyte ratios, whereas heat-killed *B. dendrobatidis* was less inhibitory showing consistent inhibition only at the two highest concentrations of added cells (Fig. 4-1A). Heat-killed *B. dendrobatidis* also inhibited proliferation of lymphocytes stimulated with *A. hydrophila* (data not shown). These data suggested that *B. dendrobatidis* was able to inhibit proliferation of B and T cells, and the inhibitory factor can be released by either live or dead cells.

B. dendrobatidis secreted a soluble inhibitory factor

To better understand the nature of the lymphocyte inhibitory factor, we tested whether inhibition required cell-to-cell contact. *B. dendrobatidis* sporangia and zoospores were separated from PHA-stimulated lymphocytes by a transwell filter. *B. dendrobatidis* cells were prevented from coming into contact with lymphocytes; however, any molecules secreted by *B. dendrobatidis* could pass through the filter and interact with lymphocytes. Lymphocytes stimulated with PHA in the absence of *B. dendrobatidis* showed a high level of proliferation. When PHA-activated lymphocytes

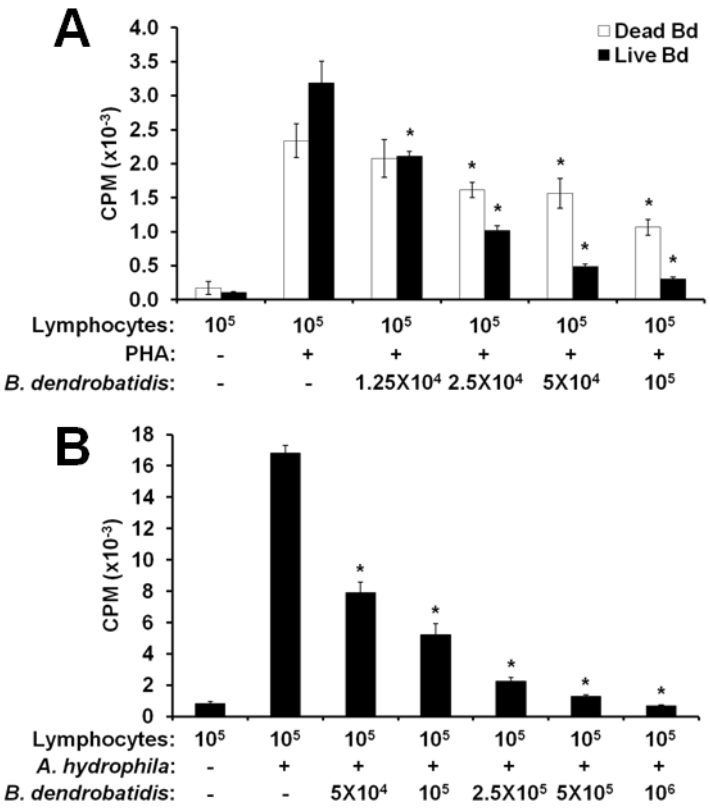

Figure 4-1. *B. dendrobatidis* (Bd) cells inhibited proliferation of *X. laevis* lymphocytes. (A) Lymphocytes from frogs were isolated, stimulated with PHA (2 µg/ml), and cultured with increasing numbers of either live or heat-killed *B. dendrobatidis* cells (isolate JEL 197). Cell proliferation was measured as ^{3}H-thymidine uptake determined as CPM in a scintillation counter. *, Significantly reduced ^{3}H-thymidine uptake compared to PHA-stimulated lymphocytes in the absence of *B. dendrobatidis* by two-tailed Student's t test ($p \leq 0.05$). Data are representative of three similar experiments. (B) Lymphocytes from frogs were isolated, stimulated with heat-killed *A. hydrophila*, and cultured with increasing numbers of live *B. dendrobatidis* cells. Cell proliferation was measured by ^{3}H-thymidine uptake. *, Significantly reduced ^{3}H-thymidine uptake compared to *A. hydrophila*-stimulated lymphocytes in the absence of *B. dendrobatidis* by two-tailed Student's t test ($p \leq 0.0001$). Data are representative of three similar experiments.

were cultured in the presence of live *B. dendrobatidis* (equal numbers of *B. dendrobatidis* sporangia and lymphocytes) without being separated by a transwell filter, there was a severe reduction in proliferation. When separated by a transwell filter, *B. dendrobatidis* retained the ability to inhibit lymphocyte proliferation at the same equal *B. dendrobatidis*-to-lymphocyte ratio, although the magnitude of inhibition was diminished in comparison to that observed when both cell types were cultured together (Fig. 4-2). These data suggested that the inhibition induced by *B. dendrobatidis* was likely due to a soluble factor, as the fungus was able to inhibit proliferation in activated lymphocytes even when separated from them by a transwell filter. However, close proximity to the lymphocytes was necessary for maximal inhibition of proliferation.

B. dendrobatidis zoospores did not inhibit lymphocyte proliferation

There are two discernible life stages of *B. dendrobatidis.* The zoospore is the infectious life stage, and following infection, they mature into sporangia. The experiments presented in Figs. 4-1 and 4-2 showing that *B. dendrobatidis* causes lymphocyte inhibition used cells from broth cultures containing both zoospores and maturing sporangia. To determine if the ability to inhibit lymphocytes was specific to a particular life stage, we evaluated the ability of zoospores alone to inhibit proliferation. *B. dendrobatidis* zoospores of isolates JEL 197 and JEL 275 were purified as described in materials and methods and cultured directly with lymphocytes stimulated to proliferate with PHA. During the three-day cultures, zoospores appeared to significantly inhibit the PHA-stimulated lymphocyte proliferation in a dose-dependent manner, regardless of the

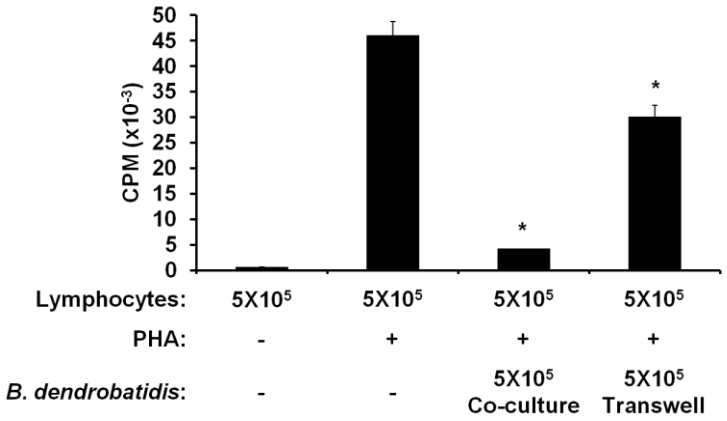

Figure 4-2. *B. dendrobatidis* inhibited *X. laevis* lymphocyte proliferation even when physically separated by a transwell filter. Lymphocytes were isolated, stimulated with PHA (2 µg/ml), and cultured with *B. dendrobatidis* either together (co-culture) or separated by a 0.4 µm pore-size filter (transwell) for three days. Cell proliferation was measured as ^{3}H-thymidine uptake using a scintillation counter. *, Significantly reduced ^{3}H-thymidine uptake in comparison with stimulated lymphocytes in the absence of *B. dendrobatidis* by one-way ANOVA with Tukey post hoc test ($p < 0.01$). The data are representative of three similar experiments.

isolate tested (Fig. 4-3A). It should be noted that greater numbers of zoospores were necessary to achieve the same inhibitory effect as that seen with *B. dendrobatidis* mixed cultures (Fig. 4-1A). Because zoospores can mature rapidly at 26°C, we tested whether heat-killed zoospores that were unable to develop to mature sporangia would inhibit. In contrast to the live zoospores, heat-killed zoospores were incapable of inhibiting lymphocyte proliferation (Fig. 4-3B). We next tested whether live zoospores could inhibit lymphocytes when separated from them by a transwell filter with the assistance of Sophia Gayek. Even when zoospores vastly outnumbered the lymphocytes (a ratio of twenty zoospores to one lymphocyte), zoospores were unable to inhibit proliferation of PHA-stimulated lymphocytes. The same numbers of zoospores, however, completely reduced proliferation when cultured directly with lymphocytes (Fig. 4-3C). To determine whether zoospores needed to directly contact lymphocytes to release an inhibitory factor, an experiment was designed to place lymphocytes along with zoospores in the top chamber of the transwell and follow the effects on lymphocytes stimulated with PHA in the bottom chamber. Although the developing zoospores were strongly inhibitory when cultured with lymphocytes in the lower chamber, they failed to release a factor that could diffuse into the lower chamber even when in direct contact with lymphocytes in the upper chamber (Fig. 4-3D). These data suggested that zoospores alone were unable to inhibit activated lymphocytes unless they mature to sporangia. Zoospores cultured alone in L-15 for three days matured to a rounded-looking thallus with rhizoids. Because zoospores were unable to inhibit lymphocyte proliferation when separated from lymphocytes by a cell-impermeable filter, they appeared to require additional time to develop to a stage that

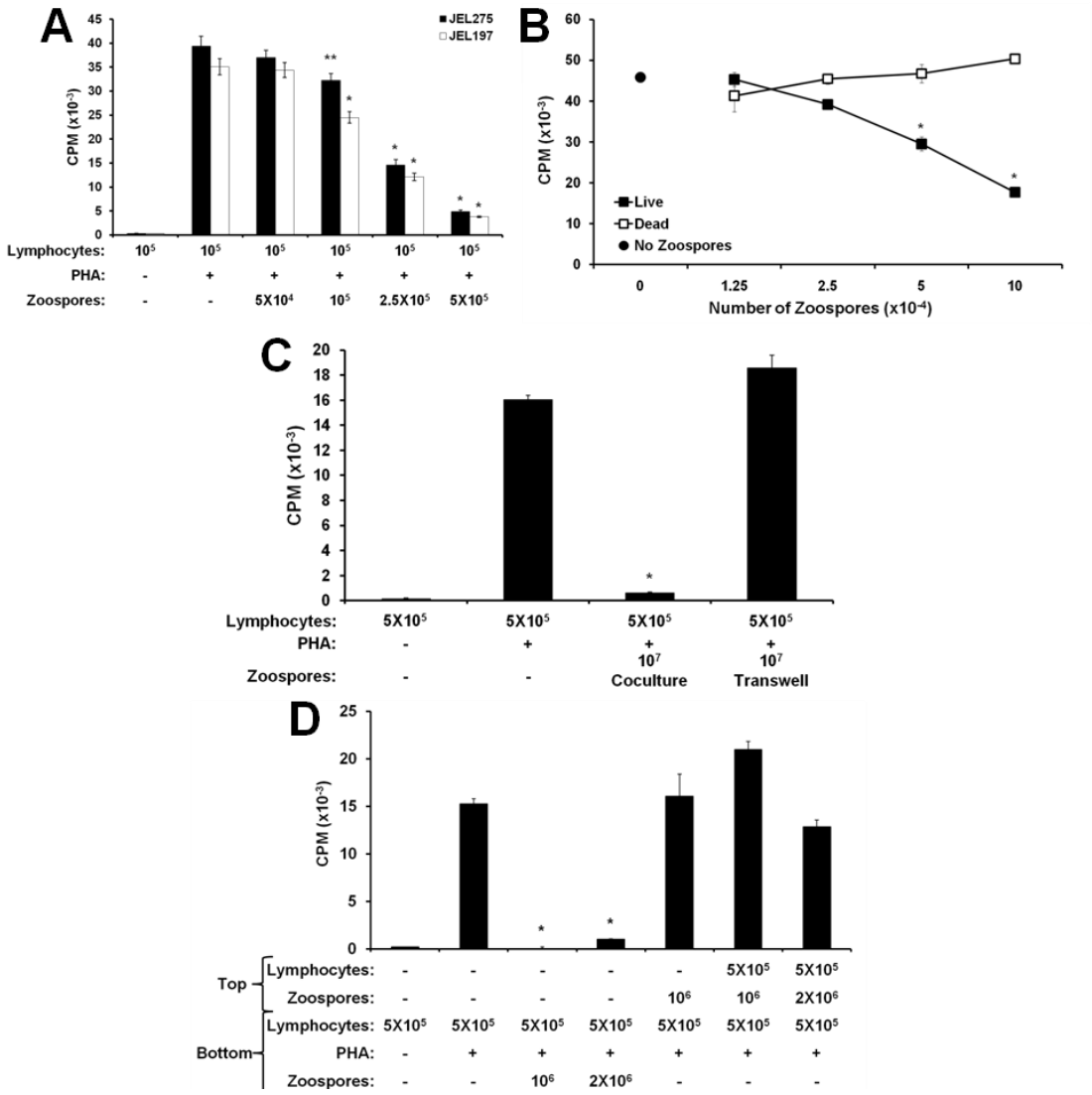

Figure 4-3. Live, but not dead, *B. dendrobatidis* zoospores inhibited proliferation of stimulated *X. laevis* lymphocytes in culture. (A) Lymphocytes were isolated, stimulated with PHA (2 µg/ml), and cultured with increasing numbers of zoospores of isolate JEL 197 or JEL 275 for three days. Proliferation was measured by ^{3}H-thymidine uptake. Zoospore inhibition of proliferation was observed in three additional co-culture experiments. (B) Isolated lymphocytes were stimulated with PHA and increasing numbers of live or heat-killed (60°C, 10 min) zoospores were added. Proliferation was measured by ^{3}H-thymidine uptake. (C) Isolated lymphocytes were stimulated with PHA and cultured with zoospores either together (co-culture) or separated by a transwell filter for 3 days. Cell proliferation was measured by ^{3}H-thymidine uptake. The data are representative of three similar experiments. (D) Lymphocytes (5 x 10^{5}) were stimulated in the top chamber of a transwell culture plate and lymphocytes alone with PHA in the bottom chamber as shown. In some wells, zoospores were cultured along with lymphocytes in the upper chamber to determine whether the interaction would result in inhibition of PHA-stimulated lymphocyte proliferation in the bottom chamber. A-C, The symbols indicate significantly reduced CPM as compared to stimulated lymphocytes in the absence of zoospores by one-way ANOVA with Tukey post hoc test (**, $p < 0.05$; *, $p < 0.01$).

was actively releasing sufficient inhibitory factors to affect lymphocyte proliferation at a greater distance.

Inhibition of proliferation by *B. dendrobatidis* was not specific to *X. laevis*

In order to demonstrate that the ability of *B. dendrobatidis* to inhibit lymphocytes was not limited to one amphibian species (*X. laevis*), we tested whether *B. dendrobatidis* could inhibit proliferation of lymphocytes from another amphibian species, *Rana pipiens*, a species that is susceptible to *B. dendrobatidis* infections and develops chytridiomycosis (158, 224, 237). This work was completed with the assistance of Scott Fites and Whitney Gammill. Live *B. dendrobatidis* (maturing sporangia and zoospores) were added directly to lymphocytes from *R. pipiens* that had been stimulated with either PHA or *A. hydrophila*, and lymphocyte proliferation was quantified. Both B and T cells from *R. pipiens* proliferated well when stimulated with their respective mitogens and showed significantly decreased proliferation when *B. dendrobatidis* was added at a ratio of ten *B. dendrobatidis* cells to one lymphocyte (Figs. 4-4A and B). These data suggested that *B. dendrobatidis* had the capacity to inhibit lymphocyte proliferation in more than one amphibian species.

***B. dendrobatidis* induced apoptosis in resting lymphocytes**

We next investigated the mechanism by which *B. dendrobatidis* inhibited lymphocyte proliferation. Other pathogenic fungi, including *Cryptococcus neoformans* (45, 46, 143), *Histoplasma capsulatum* (6), and *Aspergillus fumigatus* (13, 215) induce hyporesponsiveness by activating apoptosis signaling pathways. To determine if *B. dendrobatidis* induced apoptosis in lymphocytes, *X. laevis* lymphocytes were cultured

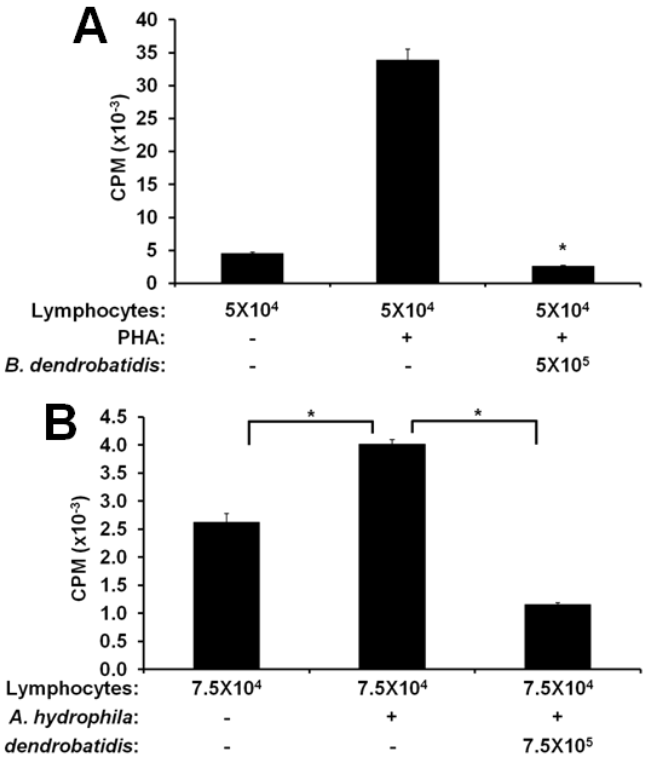

Figure 4-4. *B. dendrobatidis* cells inhibited proliferation of mitogen-stimulated *R. pipiens* lymphocytes. (A) Lymphocytes were isolated, stimulated with PHA (2 µg/ml), and cultured with *B. dendrobatidis* (mixed sporangia and zoospores) for three days. Proliferation was measured by ^{3}H-thymidine uptake. *, Significantly reduced ^{3}H-thymidine uptake as compared to stimulated lymphocytes in the absence of *B. dendrobatidis* by one-way ANOVA with Tukey post hoc test ($p < 0.01$). The data are representative of two replicate experiments. (B) Lymphocytes were isolated, stimulated with heat-killed *A. hydrophila* (5×10^5 cells/well; 2.5×10^6 cells/ml), and cultured with *B. dendrobatidis* for three days. Proliferation was measured by ^{3}H-thymidine uptake. *, Significantly reduced ^{3}H-thymidine uptake by one-way ANOVA with Tukey post hoc test ($p < 0.05$). The data are representative of two similar experiments.

with live *B. dendrobatidis* in a transwell system, then analyzed for propidium iodide (PI) and Annexin V staining by flow cytometry every 24 hours for 96 hours after culture. Lymphocytes cultured in the absence of *B. dendrobatidis* showed low percentages of apoptotic cells (PI^- , Annexin V^+) at all time points tested, although by 96 hours the percentage of apoptotic cells was beginning to increase due to cellular fatigue (Fig. 4-5). When cultured with *B. dendrobatidis*, the percentage of apoptotic lymphocytes was not different from control cells at 24 hours (Figs. 4-5A and 4-5E). However, the percentage of apoptotic lymphocytes was significantly greater at both 48 and 72 hours of culture in comparison with control populations (Figs. 4-5B-C and 4-5E). By 96 hours, the percentage of apoptotic lymphocytes was no longer significantly greater than controls (Figs. 4-5D and 4-5E). Taken together, these data suggested that *B. dendrobatidis* induced hyporesponsiveness in lymphocytes by activating apoptosis signaling pathways rather than by disturbance of the membrane and direct induction of necrosis.

B. dendrobatidis induced apoptosis largely in T cells

Many pathogenic fungi that induce apoptosis in lymphocytes largely affect T cells, including *Cryptococcus neoformans* (45, 46) and *Histoplasma capsulatum* (6). However, certain fungal pathogens, such as *Aspergillus fumigatus*, induce apoptosis in B cells or other antigen presenting cells (13, 215). To determine if either B cells or T cells, or both, are affected by apoptosis after incubation with *B. dendrobatidis*, lymphocytes from *X. laevis* were cultured directly with heat-killed *B. dendrobatidis* cells at a ratio of ten *B. dendrobatidis* cells to one lymphocyte. After 48 hours of culture, the lymphocytes were analyzed for apoptosis by flow cytometry. Cells were gated based on either IgM-

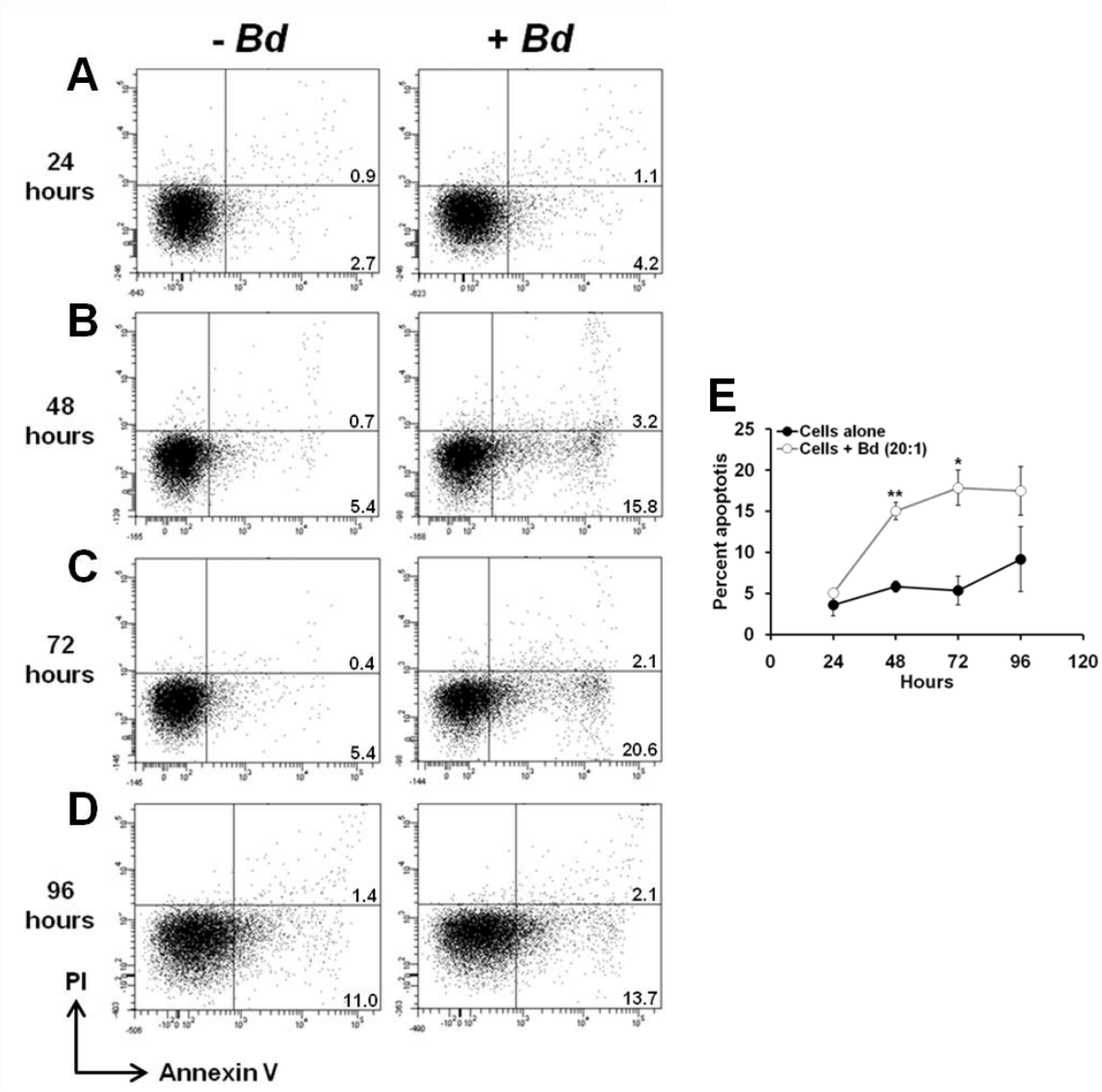

Figure 4-5. *B. dendrobatidis* induced apoptosis in *X. laevis* lymphocytes. Lymphocytes were isolated and cultured with *B. dendrobatidis* separated by a transwell filter (twenty *B. dendrobatidis* cells to one lymphocyte) and analyzed for percent apoptotic cells by flow cytometry. Lymphocytes that were Annexin V^+ and PI^- were considered to be apoptotic, whereas cells that were Annexin V^+ and PI^+ were considered to be dead (43). (A-D) Flow cytometry plots when lymphocytes were stained with Annexin V and PI at (A) 24 hours, (B) 48 hours, (C) 72 hours, or (D) 96 hours of incubation in the absence or presence of *B. dendrobatidis*. The plots shown are representative of three independent experiments. (E) Overall apoptosis in lymphocytes cultured in the absence or presence of *B. dendrobatidis* for 96 hours (n = 3 frogs per time point). The asterisks indicate significantly increased percentages of apoptotic cells as compared to lymphocytes cultured in the absence of *B. dendrobatidis* by two-tailed Student's t test (*, $p < 0.025$; **, $p < 0.01$).

positivity (B cells) or CD5-positivity (T cells) before determining 7-AAD and Annexin V staining. In the absence of *B. dendrobatidis*, both IgM^+ and $CD5^+$ cells showed low percentages of apoptotic ($7\text{-}AAD^-$, Annexin V^+) cells (Figs. 4-6A and B). When cultured with *B. dendrobatidis*, the percentage of apoptotic IgM^+ B cells only increased modestly compared to control cells (Fig. 4-6A). In contrast, the percentage of apoptotic $CD5^+$ T cells increased significantly after culture with *B. dendrobatidis* (Fig. 4-6B). The percentage of $CD5^+$ T cells that were $7\text{-}AAD^+$, Annexin V^+ after incubation with *B. dendrobatidis* also increased without an increase of necrotic ($7\text{-}AAD^+$, Annexin V^-) cells (Fig. 4-6B), suggesting that these cells underwent apoptosis. These data indicated that the apoptosis induced by *B. dendrobatidis* upon culture with lymphocytes largely affected T cells, although some B cells may also be affected.

Discussion

Understanding the pathogenesis of chytridiomycosis and immune defenses in the skin of amphibians against *B. dendrobatidis* infection is critical for developing a mitigation strategy to manage amphibian populations or protect amphibians in captivity. The skin is protected by both innate and adaptive immunity. Antigens from skin pathogens are transported to the spleen where an immune response involving both B and T cells can develop (40, 72, 182). It is likely that antigen presenting cells that reside in the skin would encounter an invasive pathogen, transport antigens to the spleen, and splenic lymphocytes would migrate back to the skin to respond locally in an effort to clear the pathogen. Although an inflammatory response in skin consisting of infiltration of neutrophils, lymphocytes, and macrophages (162) can sometimes be detected, it is

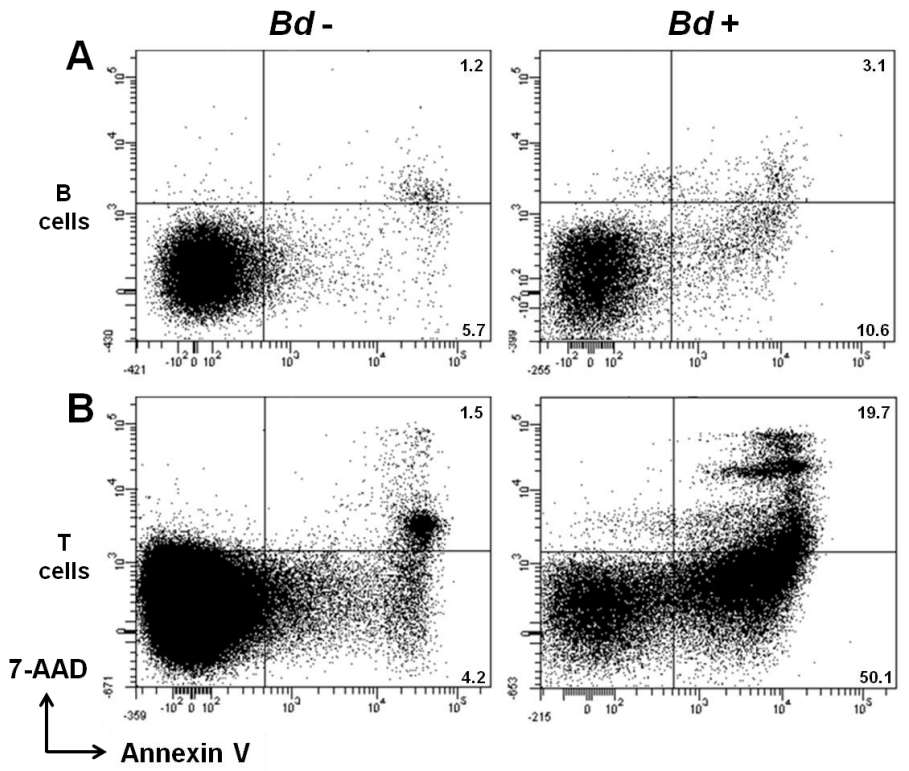

Figure 4-6. *B. dendrobatidis* induced apoptosis largely in *X. laevis* T cells, but modestly in B cells. Lymphocytes were isolated and cultured with heat-killed *B. dendrobatidis* at a ratio of ten *B. dendrobatidis* cells to one lymphocyte for 48 hours. Lymphocytes were analyzed for percent apoptotic cells by flow cytometry as described in materials and methods. Lymphocytes that were Annexin V^+ and 7-AAD^- were considered to be apoptotic, whereas lymphocytes that were Annexin V^+ and 7-AAD^+ were considered to be dead. (A) B cells were gated out as IgM^+ cells. (B) T cells were gated out as $CD5^+$ cells. Lymphocytes were cultured either in the absence (left plots) or presence (right plots) of *B. dendrobatidis*. This figure is representative of two independent experiments.

inconsistent (15, 162). This suggests the absence of an effective immune response to *B. dendrobatidis* (15, 162). In addition, two global microarray studies in a closely related species, *Silurana* (*Xenopus*) *tropicalis*, showed little evidence for upregulation of immune-related genes in the tissues examined following exposure to *B. dendrobatidis* (175, 200). In contrast to these observations, sublethal X-irradiation of *X. laevis* prior to exposure to *B. dendrobatidis* resulted in greater susceptibility to infection. Irradiated frogs had significantly higher infection intensities on their skin and lost more weight compared to untreated and exposed controls (169). This was correlated with reduced lymphocyte numbers and no apparent alteration of mucosal antimicrobial peptide defenses (169). Thus, it appears that an irradiation-sensitive compartment of the adaptive immune system contributes to protection of *X. laevis* from *B. dendrobatidis* infection.

Our previous studies showed that *X. laevis* immunized with heat-killed *B. dendrobatidis* cells can develop a robust pathogen-specific IgM and IgY response (169). However, splenic lymphocytes from immunized frogs failed to proliferate after stimulation when co-cultured with killed *B. dendrobatidis* (Table 4-1). We hypothesized that *B. dendrobatidis* is able to inhibit the proliferation of stimulated lymphocytes. Proliferation of lymphocytes activated with PHA and co-cultured with either live or heat-killed *B. dendrobatidis* sporangia was significantly impaired in comparison with control PHA-stimulated cultures (Fig. 4-1A). Similarly, lymphocytes stimulated by heat-killed *A. hydrophila*, an agent that induces vigorous B cell proliferation (144), became hyporesponsive when cultured in the presence of live *B. dendrobatidis* cells (Fig. 4-1B). These data suggest that *B. dendrobatidis* directly inhibits lymphocytes by a previously unknown mechanism *in vitro*. We suggest that this capacity to inhibit lymphocytes may

help to explain the lack of evident lymphocyte infiltration in heavily infected skin (15, 162).

Whole cultures of *B. dendrobatidis* with a mixture of zoospores and maturing sporangia retain the capacity to inhibit the proliferation of activated lymphocytes when physically separated from the lymphocytes by a 0.4 μm pore-size transwell filter. This inhibition, however, is diminished in magnitude in comparison with that observed when lymphocytes are cultured in the same well with *B. dendrobatidis* (Fig. 4-2). This suggests that factors produced by the fungus (small molecules or proteins) diffuse freely into the lymphocyte culture through the transwell filter when *B. dendrobatidis* is prevented from coming into contact with the lymphocytes. Ongoing studies in our laboratory have demonstrated that supernatants of *B. dendrobatidis* cultured in sterile water overnight also inhibit stimulated B and T lymphocytes (S. Fites, unpublished observation). These data suggest that *B. dendrobatidis* secretes a water-soluble molecule that interacts with lymphocytes and induces a signaling cascade that leads to hyporesponsiveness. Studies to characterize the molecule responsible for this phenomenon are currently underway.

The zoospore life stage is the infectious stage of the *B. dendrobatidis* life cycle, and it is responsible for initiating the infection on the surface of frog skin. To what extent zoospores come into direct contact with cells of the immune system is not clear. The process by which a zoospore moves from the surface of the skin to enter the cells of the *stratum granulosum* and *stratum corneum* are not yet well understood (228). If they encounter lymphocytes, it is possible that the zoospores would be able to inhibit the proliferation of activated lymphocytes in order to increase their chances of entering the

more protected environment of epidermal cells. Our work shows that purified live *B. dendrobatidis* zoospores can inhibit lymphocytes but only when they are cultured for three days in the presence of the lymphocytes (Figs. 4-3A). Because heat-killed zoospores are unable to inhibit stimulated lymphocytes (Fig. 4-3B), we suggest that the cultured zoospores are developing to a more mature stage during the three days of culture, and the more mature cells are synthesizing and secreting the inhibitory factor. This interpretation is supported by the failure of purified zoospores to inhibit across a 0.4 μm filter in a transwell assay even when they are co-cultured with lymphocytes in the upper chamber (Figs. 4-3C and D). These data suggest that the maturing zoospores are able to directly inhibit lymphocytes in co-culture, but they are not producing the inhibitory molecule at concentrations sufficient for inhibition at a distance. Because zoospores lack a cell wall (135, 162), we speculate that the inhibitory factor produced by sporangia may be part of the cell wall structure.

X. laevis is regarded as resistant to the lethal effects of *B. dendrobatidis* infection. *B. dendrobatidis* has been detected in archived specimens of *X. laevis* since approximately 1938 and appears to be endemic in wild populations (232). It is possible that *B. dendrobatidis* has co-evolved with *X. laevis* for a long period of time. To test whether the ability of *B. dendrobatidis* to impair lymphocyte function is specific only to *X. laevis*, we determined if stimulated lymphocytes from another species, *R. pipiens*, would be unable to proliferate when co-cultured with *B. dendrobatidis*. Both stimulated B and T cells from *R. pipiens* showed significantly reduced proliferation when co-cultured with *B. dendrobatidis* (Fig. 4-4). These data suggest that the hyporesponsiveness induced by *B. dendrobatidis* is not specific to one species of

amphibians, and that it may utilize this mechanism to more effectively colonize the skin of all amphibians.

Pathogenic fungi, including *Cryptococcus neoformans* (45, 46, 143), *Histoplasma capsulatum* (6), and toxigenic species of *Aspergillus* (3, 13, 215) modulate adaptive immune responses and render lymphocytes, especially T cells, hyporesponsive by inducing apoptosis. We evaluated whether *B. dendrobatidis* similarly induces apoptosis in amphibian lymphocytes. Unstimulated lymphocytes cultured in the presence of *B. dendrobatidis* cells separated by a transwell filter showed significantly increased numbers of apoptotic cells (Annexin V^+, PI^-) over time in comparison with lymphocytes cultured in the absence of the pathogen (Fig. 4-5). An evaluation of apoptosis in B and T cell populations after lymphocytes were co-cultured with heat-killed *B. dendrobatidis* cells indicated that both cell populations had increased percentages of apoptotic cells as compared to lymphocytes cultured in the absence of the pathogen, although the effect on B cells was modest compared to that seen in T cells (Fig. 4-6). These data suggest that *B. dendrobatidis* induces apoptosis in lymphocytes, greatly reducing the number of lymphocytes that are available to proliferate after stimulation, and that apoptosis largely affects T cells.

The hyporesponsiveness induced by *B. dendrobatidis* in lymphocytes from multiple species may help to explain why some species are very susceptible to fatal infections by this pathogen. Innate and adaptive defenses within the skin may both be required for full resistance to *B. dendrobatidis*. Both adaptive immune responses against *B. dendrobatidis* and antimicrobial peptides in the skin play a role in protecting *X. laevis* from infection (169, 191, and this thesis). *X. laevis* has very effective antimicrobial

peptide defenses in the skin (169), which may help to limit the initial colonization by *B. dendrobatidis* zoospores. The antimicrobial peptides present in the mucus may limit *B. dendrobatidis* to a degree that prevents the inhibitory molecule from reaching critical concentrations that would inhibit local lymphocyte proliferation. However, in more susceptible amphibian species, many of which have very poor skin peptide defenses (234-236), *B. dendrobatidis* may continue to grow unchecked in the skin. If the infection progresses quickly, the pathogen may produce and release sufficient inhibitory factors to diminish the capacity of lymphocytes to respond and control the infection. The uncontrolled infection may then lead to the disruption of skin functions and death (226-228).

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

My dissertation work is the first to systematically explore the immune responses of amphibians responding to infection with *B. dendrobatidis*. It also provides the first experimental evidence that *B. dendrobatidis* has evolved a mechanism to evade adaptive immunity to give itself a survival advantage in the skin. This interplay between the host's immune responses against the pathogen, and the pathogen's ability to evade these defenses, may explain a species' overall resistance to *B. dendrobatidis*. Understanding what defenses are being mobilized, or whether *B. dendrobatidis* inhibits them, may allow scientists to create protocols that will increase resistance to *B. dendrobatidis* in the laboratory and eventually aid conservation efforts.

Antimicrobial peptide defenses are critical for protection against *B. dendrobatidis*

X. laevis is considered resistant to *B. dendrobatidis* infections because infected individuals show no signs of chytridiomycosis (161) and the disease cannot be induced in frogs after experimental infection with a high dose of *B. dendrobatidis* zoospores (191). Previous work by the Rollins-Smith lab and others has shown that the effectiveness of an amphibian species' antimicrobial peptide defenses in inhibiting *B. dendrobatidis* growth *in vitro* is correlated with survival of that species in the wild or its resistance in laboratory exposure (235, 236). My work shows that *X. laevis* skin secretions contain previously

identified *X. laevis* antimicrobial peptides, and that these natural mixtures of peptides are very effective at inhibiting *B. dendrobatidis* growth *in vitro*. Purified antimicrobial peptides from *X. laevis* skin are also inhibitory alone with varying degrees of effectiveness. These data show that *X. laevis* has very strong antimicrobial peptide defenses in the mucus that would protect it from initial infections or re-infections of *B. dendrobatidis* after exposure.

Although we demonstrate that the antimicrobial peptide defenses in *X. laevis* are robust, it is not known if individuals can secrete concentrations of peptides onto the surface of their skin at concentrations sufficient to inhibit *B. dendrobatidis*. Natural mixtures of peptides are inhibitory at concentrations above 30 - 60 µg/ml. My work concludes that *X. laevis* is capable of secreting mixtures of antimicrobial peptides well above these concentrations, both when chased in a simulated predator attack and when resting. The resting frogs are only minimally handled, and thus the concentrations may be slightly higher than true resting frogs. Nevertheless, *X. laevis* has the capability to secrete antimicrobial peptides at levels that are inhibitory to *B. dendrobatidis* and appears to secrete smaller, though still inhibitory, concentrations of peptides continually to protect the skin from infection.

In order to assess whether antimicrobial peptide defenses are necessary for the protection of frog skin from chytridiomycosis, we developed a protocol for depleting the skin of its peptides during the course of my work. Frogs secrete their antimicrobial peptide stores when injected with norepinephrine. There is a dose-dependent response of peptides secreted onto the surface of the skin after norepinephrine injection, with 80 nmol/g of norepinephrine being the dose necessary for maximal secretion of *X. laevis*

peptides. Injection with that dose significantly depletes the peptide stores in the skin for 50 - 103 days, although frogs still have the capacity to secrete approximately 19,180 µg of peptides per milliliter in mucus. In comparison, a moderate dose (20 nmol/g) of norepinephrine depletes peptides for approximately 20 days, and the frogs retain a capacity to secrete about 10,394 µg of peptides per milliliter in mucus. *X. laevis* individuals that have been peptide depleted with 80 nmol/g of norepinephrine are more susceptible to *B. dendrobatidis* as measured by an increase of infection intensity on the skin and an increase in weight loss after exposure. This work demonstrates the importance of the antimicrobial peptide defenses in the skin for protection against chytridiomycosis, as depletion of the peptides leads to increased susceptibility to this pathogen.

Adaptive immune responses play an important role in resistance to *B. dendrobatidis* infection

The adaptive immune responses in *X. laevis* against *B. dendrobatidis* are largely unknown, and my work aims to determine whether adaptive immunity is necessary for resistance against *B. dendrobatidis*. The prevailing view in the literature is that adaptive immunity does not play a significant role in the protection of amphibians from chytridiomycosis. This view is based on reports that there are limited lymphocytic infiltrates in the skin after exposure (15, 162), and that *R. muscosa* juveniles immunized with formalin-killed *B. dendrobatidis* were not protected after exposure to the fungus (212). However, there is no evidence that the immunization protocol induced a response (212). A systematic study of adaptive immunity against *B. dendrobatidis* is lacking. My work concludes that adaptive immunity is likely to be involved in protection of *X. laevis*

individuals from chytridiomycosis after exposure to *B. dendrobatidis*. A sublethal dose of X-irradiation greatly impacts the number of leukocytes present in the spleen for adaptive responses without perturbing the skin or interfering with the ability to secrete peptides. This irradiation also leads to increased infection intensity on the skin and greater weight loss after exposure to *B. dendrobatidis*. The increased susceptibility suggests that an irradiation-sensitive compartment of the *X. laevis* adaptive immune system is playing a role in protecting this species from chytridiomycosis.

To determine whether *X. laevis* is capable of mounting an effective adaptive immune response to *B. dendrobatidis*, we immunized individuals intraperitoneally with heat-killed *B. dendrobatidis* and assayed the production of pathogen-specific antibodies in the serum. My work suggests that individuals immunized against *B. dendrobatidis* have significantly higher levels of pathogen-specific IgM and IgY in the serum compared to controls. These results demonstrate that *X. laevis* individuals are able to mount a robust adaptive antibody response to *B. dendrobatidis*. Although immunization leads to a productive systemic response, it is unclear whether elevated antibodies in circulation would be protective in frogs. We suggest that adaptive immune responses in the skin would be key to protection, contradicting earlier reports that suggest that adaptive immunity is not protective against *B. dendrobatidis*. My work also evaluates a potential mechanism for adaptive immune protection in the skin after infection with *B. dendrobatidis*. Frogs that were previously exposed to *B. dendrobatidis* have pathogen-specific antibodies in their mucosal secretions of all three major amphibian immunoglobulin classes (IgM, IgY, and IgX). These individuals also have high levels of

all three classes when assessing total levels of antibodies, suggesting that antibodies secreted onto the surface of the skin may aid in protection against chytridiomycosis.

Taken together, both innate and adaptive immune responses are necessary to protect species from chytridiomycosis after exposure to *B. dendrobatidis*. Antimicrobial peptides secreted onto the skin would prevent the initial colonization of the skin or re-infection after the release of newly emerging zoospores. Adaptive responses may be mobilized to help control or eliminate infections that the antimicrobial peptides could not clear. Thus, a deficiency in either response could increase the susceptibility to fatal *B. dendrobatidis* infections. With this knowledge, we aimed to answer a second question that arose during the course of my work. Does *B. dendrobatidis* possess a mechanism for inhibiting adaptive immune responses in infected amphibians?

B. dendrobatidis modulates adaptive immunity by inducing apoptosis in lymphocytes

Some species of fungal pathogens are capable of inducing hyporesponsiveness in lymphocytes, especially T cells, to suppress adaptive immune responses and to gain a survival advantage after successful colonization (6, 13, 38, 45, 215). In the process of determining the adaptive immune responses in *X. laevis* after immunization with *B. dendrobatidis*, we evaluated the proliferation of lymphocytes from immunized individuals after culture with *B. dendrobatidis* cells. Lymphocytes cultured with *B. dendrobatidis* are unable to proliferate as measured by increased ^{3}H-thymidine uptake. However, there is no inherent defect in the capability of these cells to proliferate, as lymphocytes from all frogs proliferate significantly when cultured with a mitogen (PHA) (196). Thus, we hypothesize that *B. dendrobatidis* has the capacity to inhibit the

activation of adaptive immune responses in amphibians. *X. laevis* in the wild have consistent prevalence of *B. dendrobatidis* infections since the 1940s (232), and it is possible that it has evolved a method of increasing its survival on the skin.

My work evaluates the hypothesis that *B. dendrobatidis* has the ability to inhibit amphibian lymphocytes. Lymphocytes isolated from *X. laevis* and cultured with either heat-killed bacteria or PHA are unable to proliferate when co-cultured with *B. dendrobatidis* mature sporangia and zoospores. This effect is dependent on the numbers of *B. dendrobatidis* cells present in the culture, and proliferation is completely abolished in lymphocytes at a ratio of one *B. dendrobatidis* cell to one lymphocyte. Both live and heat-killed *B. dendrobatidis* cells are able to inhibit this proliferation, though live cells inhibited more. Two different *B. dendrobatidis* isolates (JEL 197 and JEL 275) inhibit proliferation similarly. These data demonstrate that *B. dendrobatidis* is able to prevent the full activation and proliferation of lymphocytes by an unknown mechanism.

Because both live and heat-killed fungal cells can inhibit lymphocyte proliferation, *B. dendrobatidis* might induce hyporesponsiveness by a cell contact-dependent mechanism. It is also possible that *B. dendrobatidis* could produce a secreted molecule that could perform this function. My work assesses whether cell contact is required for *B. dendrobatidis* to inhibit proliferation. When mitogen-stimulated lymphocytes are cultured directly with *B. dendrobatidis* whole cultures (both present in the same well), the lymphocytes are unable to proliferate. When lymphocytes are physically separated by a transwell insert, *B. dendrobatidis* is still able to significantly inhibit, although at a diminished capacity. This suggests that *B. dendrobatidis* releases a

factor that can diffuse across a transwell filter, interact with activated lymphocytes, and prevent their proliferation.

The previous work was conducted using a mixture of *B. dendrobatidis* mature sporangia and zoospores. However, the zoospore is the infectious life stage responsible for establishing an infection on the surface of amphibian skin. To what extent zoospores come into direct contact with cells of the immune system during an initial infection is not clear. It is possible that zoospores could encounter lymphocytes within the skin and would be able to inhibit the proliferation of activated lymphocytes in order to increase their chances of entering epidermal cells to mature. Live *B. dendrobatidis* zoospores can inhibit lymphocyte proliferation but only when they are cultured for three days in the presence of lymphocytes. Heat-killed zoospores, however, are incapable of inhibiting lymphocytes, suggesting that live zoospores are developing into a more mature stage during the three days of culture, and the more mature cells are synthesizing and secreting the inhibitory factor. This is supported by the failure of zoospores to inhibit proliferation in lymphocytes across a 0.4 μm filter in a transwell assay even when they are co-cultured with lymphocytes in the upper chamber of the transwell system. These data suggest that maturing zoospores are able to directly inhibit lymphocytes in co-culture, but they are not producing the inhibitory molecule at concentrations sufficient for inhibition at a distance. We speculate that the inhibitory factor produced by sporangia may be part of the cell well present on mature *B. dendrobatidis* cells, as zoospores lack a cell wall (135, 162).

As mentioned, *X. laevis* has been showing steady *B. dendrobatidis* prevalence levels in the wild since the 1940s, and it is possible that the immune suppression would have arisen due to co-evolution between the fungus and this species. My work aims to

determine whether *B. dendrobatidis*-specific prevention of proliferation could occur in another species. Lymphocytes from *R. pipiens* stimulated with either heat-killed bacteria or PHA are unable to proliferate when co-cultured with *B. dendrobatidis*. This suggests that the hyporesponsiveness is not species-specific. It is possible that *B. dendrobatidis* could use this mechanism to increase its chances of survival on the skin of all amphibian species, allowing it to affect many amphibian populations as it spreads across the globe.

My work gives definitive evidence that *B. dendrobatidis* prevents the proliferation of lymphocytes, thus attenuating the adaptive immune response against it. However, the mechanism by which *B. dendrobatidis* interferes with lymphocyte proliferation is unknown. It is possible that *B. dendrobatidis* could induce anergy, or could signal the cells to undergo apoptosis. Using flow cytometry to determine the mechanism of action, we find that lymphocytes cultured in the presence of *B. dendrobatidis* have significantly higher percentages of apoptotic cells over time. This apoptosis is more pronounced in T cells, although a modest amount of apoptosis also occurs in B cells. These data show that *B. dendrobatidis* induces apoptosis in lymphocytes, especially in T cells, thus interfering with a frog's ability to mount an effective adaptive immune response. Though we see inhibition of B cell proliferation when lymphocytes were stimulated with heat-killed bacteria and cultured with *B. dendrobatidis*, the percentages of B cells undergoing apoptosis are low.

Determining resistance or susceptibility to *B. dendrobatidis* infections

The results from my dissertation work hint at a complex relationship between the fungal pathogen *B. dendrobatidis* and its amphibian host. The interplay between innate

and adaptive defenses in the frog to prevent infection and inhibitory responses by the fungus may determine whether an amphibian species is resistant or susceptible to fatal chytridiomycosis. When a zoospore attempts to infect the skin of a frog, it must first overcome the innate defenses on the skin surface. These defenses include antimicrobial peptides (53, 60, 187-191, 193, 194, 206, 234-236), lysozyme (123, 157, 243), metabolites produced by the resident skin bacteria that can be inhibitory to *B. dendrobatidis* growth (36, 93), and mucosal immunoglobulins (169). If *B. dendrobatidis* can survive these initial attacks, it will establish an infection and continue to propagate. Over time, adaptive immune responses would be mobilized to help ward off, and potentially eliminate, infection. However, *B. dendrobatidis* can resist by releasing a molecule that can kill lymphocytes via apoptosis, thus making it harder to fully eradicate.

There are three possible outcomes of infection with *B. dendrobatidis*. In amphibian species that have robust antimicrobial peptides, such as *X. laevis*, most zoospores that attempt to begin an infection would be killed. Those few that do survive may be able to establish a light infection (low intensity of infection), but the population on the skin would be too low to attenuate adaptive immunity to a significant degree. The adaptive immune system will become activated and would easily control or eliminate the infection. These species would be considered resistant. In the second case, the antimicrobial peptide defenses are very weak, and the invading zoospores would be able to quickly establish a heavy infection (high intensity of infection). Growing unchecked on the skin, a large amount of the inhibitory molecule would be produced, preventing the adaptive immune system from activating. The infection intensity on the skin would eventually reach critical levels, and the infected frog would die due to the disruption of

the skin's normal function, leading to cardiac arrest. These species would be considered highly susceptible. The third outcome is largely dependent on whether or not individual antimicrobial peptide responses are effective. A study in *R. pipiens* shows that geographically distinct populations had differing peptide effectiveness against *B. dendrobatidis* (218). In this sense, it would be possible for certain individuals in a population to be susceptible (weak peptide effectiveness) while others are resistant (strong peptide effectiveness), and the same could be true for different populations of the same species. These species would be considered moderately susceptible.

The work contained within this dissertation highlights the importance of both innate immunity, especially antimicrobial peptides, and adaptive immune responses in the protection of amphibian species resistant to *B. dendrobatidis*. By understanding the role both play, researchers may be able to devise ways to protect susceptible frog species in the laboratory before releasing them back into the wild. Methods to increase peptide effectiveness or strengthen adaptive responses in the skin may be considered. It is hoped that this research will help aid conservation efforts to protect these very valuable members of our ecosystem.

Future Directions

Although this dissertation presents significant new information about innate and adaptive immune responses in amphibians against *B. dendrobatidis*, much work remains in order to fully understand the involvement of both systems. Some key questions remain to be answered for antimicrobial peptide defenses, adaptive immunity, and *B. dendrobatidis*-induced lymphocyte hyporesponsiveness.

Current work in the Rollins-Smith laboratory is underway to determine the effectiveness of one injection of the maximal dose of norepinephrine in fully depleting peptide stores in the skin of amphibians. Work completed by Scott Fites and Whitney Gammill, which is in concordance with work presented in this dissertation, suggests that after one injection with 80 nmol/g of norepinephrine, *X. laevis* still possesses some capacity to secrete antimicrobial peptides. Histology of skin from frogs injected once show that, although most of the granular glands are empty, many still contain secretory granules. This may explain why a peptide depletion experiment in larger frogs failed to show any increased susceptibility in *X. laevis* to *B. dendrobatidis*. However, two injections with 80 nmol/g of norepinephrine in three days left virtually all of the granular glands empty. A third injection did not lead to any significant peptide release, suggesting that two injections leads to full depletion of antimicrobial peptide stores. It would be of interest to repeat the peptide depletion studies in *X. laevis* after two injections of the maximal dose of norepinephrine. It is expected that these frogs would have higher intensities of infection on the skin after exposure to *B. dendrobatidis*, as well as more consistent infections.

Because immunoglobulins are present in mucosal secretions, injecting frogs with norepinephrine when depleting the skin of its peptides may also reduce the amount of antibodies available in the mucus. Future studies are needed to determine whether norepinephrine injections also deplete the frog's skin of its immunoglobulin reserves. The concentration of antibodies present in mucosal secretions would be compared between frogs that had been injected only once and frogs that had been injected a second time within three days. If it is found that antibodies are also depleted, the kinetics of

antibody renewal will be investigated as frogs are re-injected at various time points after the initial induction. Immunohistochemistry could also be performed to show that antibodies are present in the skin, and whether the levels of antibodies are reduced after norepinephine injection.

Irradiation of *X. laevis* with a sublethal dose of X-rays increases susceptibility to *B. dendrobatidis* after exposure. Although it can be concluded that an irradiation-sensitive compartment of adaptive immunity plays a role in resistance, the identity of this compartment is not known. It would be beneficial to determine whether B cell responses or T cell responses, or both, are necessary for resistance to chytridiomycosis. *X. laevis* individuals could be injected with reagents directed against B cells or T cells to deplete those cell subsets *in vivo*. Infection intensities and weight losses would be measured over time in control (untreated), B cell depleted, T cell depleted, or doubly depleted frogs after exposure to test which compartment plays a role in resistance to *B. dendrobatidis*. Also, *X. laevis* tadpoles can be thymectomized to rear T-cell deficient frogs (71, 89, 105, 178, 219), and their susceptibility to *B. dendrobatidis* can also be measured. It is expected that one or both of these compartments would be responsible for protection of frogs from chytridiomycosis.

The work presented in this dissertation is the first to identify the presence of mucosal antibodies in amphibians. However, it is not known if the immunoglobulins in the mucus play a role in protecting frogs from chytridiomycosis. First, experiments to determine whether mucosal antibody concentrations can be significantly increased in the skin after exposure to *B. dendrobatidis* should be conducted. Frogs would be experimentally infected with the fungus, and a subset of frogs would be induced to

secrete mucosal antibodies by norepinephrine injection at various time points after exposure. It is possible that the concentration of mucosal antibodies would increase after exposure to *B. dendrobatidis*. Second, whether the antibodies are functionally protective would need to be evaluated. Mucosal secretions containing antibodies would be tested for their ability to inhibit *B. dendrobatidis* growth *in vitro* by complement-mediated destruction. If the antibodies are protective, they should be able to inhibit *B. dendrobatidis* growth.

Due to lack of reagents in amphibian species other than *X. laevis*, it is very difficult to determine whether an effective adaptive immune response is being generated in other species either after experimental exposure or immunization to *B. dendrobatidis*. By developing reagents to B cells, T cells, and all three immunoglobulin classes, it would be possible to expand the research presented in this dissertation to other species of amphibians. This is especially important when testing whether immunization of a susceptible species would increase resistance to *B. dendrobatidis* infection. If immunization fails to protect, it would be possible to evaluate whether an adaptive immune response was even generated in the immunized individuals. Though there is a possibility to use alternate methods to demonstrate activated adaptive immune responses in other species, such as the antibody-mediated growth inhibition assay, using specific reagents would be more reliable.

B. dendrobatidis has the capability of inhibiting the proliferation of activated lymphocytes by inducing apoptosis in these cells. Evidence presented in this dissertation suggests that the molecule is released by *B. dendrobatidis*. Further characterization of the molecule in question needs to be performed in order to determine what type of

molecule it is. Experiments to determine if it is stable in heat, affected by acid, or susceptible to proteases are currently under way. Once the characterization of the molecule is concluded, research can be undertaken to identify the factor. Once identified, it would be of interest to determine whether the *B. dendrobatidis* factor can be inhibited or manipulated experimentally to produce a less virulent isolate of the fungus for vaccination studies.

APPENDIX A

LIST OF PUBLICATIONS

I. **Ramsey JP**, Fites JS, Gammill WM, Reinert LK, Gayek AS, and Rollins-Smith LA. Inhibition of amphibian lymphocyte responses by *Batrachochytrium dendrobatidis,* a fungus linked to global amphibian declines. *In Preparation.*

II. **Ramsey JP**, Reinert LK, Harper LK, Woodhams DC, and Rollins-Smith LA. 2010. Immune defenses against *Batrachochytrium dendrobatidis,* a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. *Infect. Immun.* 78:3981-92.

PMID: 20584973

III. Rollins-Smith LA, **Ramsey JP**, Reinert LK, and Woodhams DC. Amphibian immune defenses against chytridiomycosis: impacts of changing environments. *In Preparation.*

IV. Rollins-Smith LA, **Ramsey JP**, Reinert LK, Woodhams DC, Livo LJ, and Carey C. 2009. Immune defenses of *Xenopus laevis* against *Batrachochytrium dendrobatidis*. *Front. Biosci. (Schol. Ed.)* 1:68-91.

PMID: 19482684

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