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Immune defenses of *Xenopus laevis* against *Batrachochytrium dendrobatidis*

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1. ABSTRACT

Amphibian populations are declining at an unprecedented rate worldwide. A number of declines have been linked to a pathogenic skin fungus, *Batrachochytrium dendrobatidis.* Although amphibians have robust immune defenses, many species seem to be very susceptible to infection by this fungus and to development of the lethal disease called chytridiomycosis. One species that is relatively resistant to *B. dendrobatidis* is *Xenopus laevis.* Because *X. laevis* has been used as a model for studies of immunity in amphibians and because it is relatively resistant to chytridiomycosis, it is a good model to examine immune defenses against *B. dendrobatidis*. Although much less is known about immune defenses in *Bufo boreas*, it serves as a second model species because it is very susceptible to *B. dendrobatidis*. Here we review what is known about innate antimicrobial peptide defenses in the skin and the development of immune responses following experimental immunization with heat-killed fungal cells. Development of an immunization protocol in *X. laevis* that induces effective defenses may suggest better strategies for protecting vulnerable species such as *B. boreas*.

2. INTRODUCTION

In 1962 Rachael Carson alerted the world to the dangers of DDT and its impact on bird populations in her carefully researched book, *Silent Spring (*1). At the present time, naturalists are concerned about a different kind of silent spring. Spring in many parts of the world is heralded by the "peeps", "chirps", "trills", and "burps" of many colorful male amphibians calling females to breed. However, beginning in the 1970s, amphibian biologists began to recognize that many familiar species of amphibians were becoming more difficult to find (2-8). In the most recent survey of global amphibian populations published in 2004, approximately 32% of known species were classified as "threatened" (9). This is likely to be an underestimate because there were insufficient data to judge the status of many species (9). Thus, it is clear that many amphibian populations and some entire species are disappearing at an alarming rate (9-11).

There is evidence to support a number of possible causes for amphibian declines including loss of habitat (12, 13, rev in 14), climate change (15-19), increased ultraviolet

(UV-B) radiation (20-24), introduced species (8, 25-29), and toxic environmental chemicals (30-32). However, accumulating evidence suggests that recent declines in amphibian populations in western North America, Central America, South America, Australia, Europe, and Africa have been caused by a chytrid fungus, *Batrachochytrium dendrobatidis* (2, 33-40, rev in 41-44). *B. dendrobatidis* infects the skin and leads to rapid death in highly sensitive species. Here we review what is known about the immune defenses of two model amphibians, *Xenopus laevis* and *Bufo boreas*, against *B. dendrobatidis* and examine what this information can tell us about the possible protective defenses in *X. laevis* and impaired defenses in the more susceptible species, *B. boreas*.

3. BIOLOGY OF *B. DENDROBATIDIS*

B. dendrobatidis is a member of the phylum *Chytridiomycota*, and this phylum occupies a basal position in the kingdom Fungi (45-47). This phylum is characterized by the production of motile zoospores, each propelled by a single flagellum. There are approximately 1000 described species in the phylum *Chytridiomycota*, but only *B. dendrobatidis* is pathogenic to a vertebrate host (45-47). The fungus infects cells of the *stratum granulosum* and *stratum corneum* of the epidermis (33-35, 48). Zoospores appear to attach to skin cells and enter the deeper viable cells of the *stratum granulosum.* The zoospores develop into zoosporangia as these cells move outward and become cornified. Thus, when the dying cornified cells are nearest the exterior of the frog, the mature zoosporangium opens and new zoospores are discharged to the surface of the skin (48). The mechanism by which the fungus causes death of frogs is not well understood. One hypothesis is that *B. dendrobatidis* produces a toxic product (33, 35). An alternative hypothesis that is gaining support is that the general disturbance of the skin resulting from *B. dendrobatidis* infection interferes with the transport of essential ions or water that are needed for life (33,35). A recent study suggests that electrolyte depletion due to disruption of normal epidermal function is correlated with disease severity and death in green tree frogs (*Litoria caerulea*) (49).

4. IMPACT OF *B. DENDROBATIDIS* ON POPULATIONS OF *X. LAEVIS (*A RESISTANT SPECIES) COMPARED TO A MORE SENSTIVE SPECIES (*BUFO BOREAS)*

Archived specimens of *X. laevis* and related species *X. muelleri* and *X. gilli,* collected in the period 1879-1999 and preserved at South African institutions, were examined for the presence of *B. dendrobatidis* by examination of histological sections from the webbing of one hind foot. The overall prevalence of chytridiomycosis among 697 specimens examined was 2.7%, and it was unchanged over time after 1940 (50). *Xenopus* collected in the field do not show signs of illness, and there are no reports of population declines (50) or clinically apparent chytridiomycosis in captive populations of *X. laevis* (51). Thus, populations of *Xenopus* appear to persist with mild infections that are not debilitating. Experimental infection studies have also shown that this species does not develop disease even when infected with a high number of viable zoospores (52). This contrasts with the related species, *Xenopus tropicalis*, which is thought to be more susceptible to chytridiomycosis (51).

In contrast to *Xenopus, B. boreas* is very susceptible to development of chytridiomycosis following exposure to *B. dendrobatidis* in the laboratory or in nature. Boreal toads are long-lived amphibians that inhabit montane habitats in the western USA. This species suffered severe population declines in the southeastern part of its range in the late 1970s to early 1980s that are now suspected to be due to *B. dendrobatidis* (5, 37). *B. dendrobatidis* has been associated with population die-offs in Colorado (53), and the species is designated "endangered" in Colorado and New Mexico. Experimental exposure to as few as 10^4 zoospores for 1 day results in 100% mortality (37).

5. OVERVIEW OF IMMUNE DEFENSES IN *X. LAEVIS*

Because *X. laevis* has been used as a model for studies of amphibian immunity since the 1960s, much is known about immune defenses in this species. No other species of amphibian has been studied in this much detail. Like other vertebrates, *Xenopus* has a set of innate immune defenses that can be mobilized to defend against pathogens without prior exposure. Although innate defenses include recognition of pathogen-associated molecular patterns (PAMPS) that bind to toll-like receptors (TLRs), these defenses are generally not as specific as antibody-mediated and cell-mediated immune responses. However, they do provide an effective first barrier to infection. If infection is not prevented by innate defenses, *Xenopus* can mobilize adaptive antibody and cell-mediated defenses. Both innate defenses and adaptive defenses will be reviewed briefly, and then we will describe what is known about specific defenses against *B. dendrobatidis*.

5.1. Innate immune defenses in *X. laevis*

Innate immune defenses in amphibians include complement, phagocytic cells (macrophages, neutrophils, and natural killer (NK) cells), and production of lysozyme and antimicrobial peptides (AMPs) in the mucous covering the skin. In addition to these known innate mechanisms, it is likely that antigen presenting cells (dendritic cells, Langerhans cells, macrophages, and keratinocytes) in the skin can recognize PAMPs that bind to TLRs to activate both innate and adaptive immune system mediators.

5.1.1. Complement and lysozyme

Like all other vertebrates, *Xenopus* has a very effective complement system that can directly kill pathogens by activation of the alternative pathway of complement and formation of the membrane attack complex. Antibodies bound to pathogens can also activate complement via the classical pathway. Although not all of the individual protein components have been isolated and characterized, it is clear that all of the genes for the

complement components are present (54-63, rev in 64). Another innate mechanism that may be important for protection against invasive bacteria is production of lysozyme in the mucous. Lysozyme belongs to a well-characterized family of enzymes that cleave the bond between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of bacterial cell walls. A recent study of the antimicrobial activity of skin secretions of the Chinese toad *Bufo andrewsi* revealed a member of the lysozyme family. The DNA encoding the toad lysozyme had significant homology with that of lysozyme from *X. laevis*, chicken, and turtle (65). Although the gene for lysozyme was identified from *X. laevis (*66*),* the protein has not been isolated and characterized. Whether or not *B. boreas* may secrete lysozyme in the skin is not yet known. An extract of skin from *Rana pipiens* was previously shown to have an acid-stable protein with lysozyme-like characteristics (67). Thus, lysozyme may be an important bacterial defense mechanism in the mucus of many amphibians.

5.1.2. Phagocytic cells and natural killer (NK) cells

Amphibian skin is also protected by phagocytic cells that can clear microorganisms that penetrate the epithelial layer. Major histocompatibility complex (MHC) class II positive cells, some with dendritic morphology, have been reported in the skin of *X. laevis* and *Rana pipiens* (68-71). Natural killer cells have been characterized in *Xenopus laevis*. They have the potential to provide an immediate cytotoxic response against virus-infected or tumor targets (72-75). Whether they may play a role in recognition and destruction of *B. dendrobatidis*-infected skin cells is unknown.

5.1.3. Toll-like receptors (TLR)

Toll-like receptor (TLR) family proteins recognize conserved patterns expressed by microbes including bacteria, viruses, protozoa, and fungi (rev in 76-78). The molecules recognized include lipoproteins, lipopeptides, lipopolysaccharide, flagellin, and nucleic acids (78). Recognition of the conserved pathogen patterns in mammals activates signaling pathways in antigen presenting cells such as dendritic cells and macrophages that result in production of cytokines, chemokines, interferons and other mediators that contribute to innate defenses as well as activate the adaptive defenses (78). Although the role of TLRs in amphibians has not been studied, a search of the *Xenopus tropicalis* genome has revealed approximately 20 TLR genes with strong homology to those of other vertebrate species that were expressed in a number of tissues including skin (79,80). These include homologs for TLR 2, 3, 4, 5, 6, 7, 8 and 9 which are involved in antifungal responses in mammals (81, rev in 82). Thus, it is likely that TLRs play important roles in amphibian immune defense against a fungus.

5.1.4. Antimicrobial peptides (AMPs)

Early histological studies identified mucous glands and granular glands (also called poison or serous glands) in the dermal layer of amphibian skin (83-86). The mucous glands produce a material rich in mucopolysaccharides that keeps the skin moist (87-89). Granular glands produce a diverse array of bioactive peptides including neuropeptides and AMPs that are thought to play a role in defense against vertebrate predators as well as microbes (rev in 90-97). Both mucous and granular glands are composed of a syncytium of epithelial cells surrounding a secretory compartment (86,98). In granular glands, the center of the gland is filled with granules packed with active peptides (99). Granular glands are surrounded by a layer of myoepithelial cells with sympathetic axons terminating between the contractile elements (85). The myoepithelial cells possess α-adrenoreceptors, and epinephrine or norepinephrine (NE) induce contraction and release of granular contents by a holocrine mechanism (98,100,101). Holocrine secretion involves loss of most of the contents of the gland; however, the multiple nuclei of the syncytial gland remain, and a new gland regenerates from remaining epithelial cells (102,103). Depending on the species, granular glands can be found all over the body with the largest ones in the dorsolateral skin (dermal plicae) and behind the eyes (paratoid glands) (83-85,102,103).

An extensive literature characterizes the amino acid sequences and activity of a large number of amphibian AMPs ranging in size from 10-50 amino acids. They are active against gram positive and gram negative bacteria, fungi, protozoa, and viruses (rev in 90-97). Although families of peptides are shared by related species, there is virtually no overlap in the individual peptides from one species to another (96). There is no consensus amino acid sequence associated with biological activity, but the peptides are usually cationic, relatively hydrophobic, and have the ability to form an amphipathic α-helix in a membrane-mimetic environment (104). This structure provides them with an ability to disturb biological membranes, and this seems to be the main mechanism of killing of their targets (rev in 92-97).

5.2. Adaptive immune defenses in *X. laevis*

Because *X. laevis* has long been used as a model for studies of the amphibian immune system, we have a very good understanding of the tissues, cells, and molecules that contribute to an effective immune response in this species. In the following paragraphs, we will briefly review what is known about adaptive immune defenses in *Xenopus.* Much less is known about the adaptive immune responses of other anuran amphibians. Very few studies have examined immune responses of species within the genus *Bufo.* However, some basic studies of immune responses in marine toads (*Bufo marinus*) were published in the 1960s and 1970s. Toads responded to particulate antigens (bacteriophage, flagella from *Salmonella adelaide*) by production of IgM and IgG-like antibodies (IgY). The spleen and kidney were identified as major antibody forming organs (105-108). Thus, most comparative immunologists would assume that the adaptive immune responses of toads (genus *Bufo*) would be very similar to those responses described in greater detail for *X. laevis*.

5.2.1. Lymphoid organs

In *Xenopus*, the thymus is the site of development of T lymphocytes. Thymectomy early in life impairs

allograft rejection (109-111), mixed lymphocyte responses, responses to T cell mitogens but not B cell mitogens, and development of lymphocytes with rearranged T cell receptor genes (75,112,113). In adult *Xenopus*, the spleen and liver are the sites of development of B cells and other hematopoietic cells (114). Bone marrow is the site of development of granulocytes (predominantly neutrophils) but not lymphocytes (114). The spleen is the major secondary lymphoid organ where antigens from the blood, peritoneum, and tissue fluids are degraded and come into contact with T and B cells for development of an immune response. *Xenopus* and all other amphibians lack organized lymph nodes (115).

5.2.2. Major histocompatibility complex (MHC)

The major histocompatibility complex (MHC) of *Xenopus* has been characterized in detail (rev in 116-118). It consists of a well-defined class I region that encodes molecules that present peptides to and interact with $CD8^+$ T cells (119) and includes other genes that encode proteosome components PSMB8 (LMP7) (120), PSMB9 (LMP2) (121), and the peptide transporters TAP1 (122) and TAP2 (123). Adjacent to the class I genes are the class II genes encoding molecules that present peptides to helper T cells (124,125). Class III genes encode the complement components C4 and factor B and heat shock protein 70 (HSP70) (58-60,126,127). The availability of the *Xenopus tropicalis* genome has provided a new tool to search for additional MHC genes in *Xenopus*. Using this method, approximately 110 genes with significant similarity to MHC genes in other databases were recently identified including class II DM genes (128). Thus, the MHC with its many components needed for regulation of adaptive immune responses is ancient and fully functional in amphibians.

5.2.3. Antibody classes

There are three isotypes of immunoglobulin heavy chain genes in *Xenopus* (rev in 129). They are IgM (130-132), IgY (133,134), and IgX (135,136). Polymeric IgM is the first to develop in ontogeny (137) and is the predominant antibody that develops soon after experimental immunization of tadpoles and adults (129). Slightly later, the low molecular weight (IgG-like) isotype designated IgY develops (129). IgY responses are T cell-dependent (138) and reflect class switch recombination events (139). IgX is a third class of polymeric immunoglobulins found in the gut and may be important in defense of the digestive tract analogous to the function of IgA in mammals (135,136). Recently, the gene for IgD (homologous to IgW in fish) was identified (140).

5.2.4. Cytokines and chemokines

Cytokines that serve to enhance communication between subsets of leukocytes are less well-characterized in *Xenopus* than in mammals or other vertebrate groups. However, a number of cytokines have been identified including interleukin-1β (141,142), an interleukin-2 like cytokine (143,144), and TGF-β (transforming growth factor-beta) (145). Five genes for type I interferon were identified in a recent search of the *Xenopus tropicalis* genome (146). Recent studies also reveal several chemokines in *Xenopus*. Stromal cell-derived factor 1 (xSDF-1) shares 64-66% homology with human CXCL12 (147, rev in 148). Expressed sequence tags suggest molecules with homology to CXCL8 and CCL5 of mammals (rev in 148).

5.2.5. T Cell-mediated immunity

Early thymectomy studies demonstrated the involvement of T cells in skin graft rejection (109-111), mixed lymphocyte reactions, and *in vitro* responses to classical T cell mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) (112,113). Skin allograft rejection mediated by T cells is a classical measure of cellular immunity in amphibians. It is dependent on recognition of major histocompatibility complex (MHC) or minor histocompatibility antigens (149-155 rev in 156). It is characterized by classical first- and second-set kinetics and is dependent on the incubation temperature of the hosts (156). The development of genetically identical strains of *Xenopus* (laevis-gilli hybrid strains) (157) permitted studies to demonstrate that both cytotoxic and helper T cell responses are MHC-restricted (158-160). Monoclonal reagents to recognize the CD8 subset of lymphocytes have been developed (161), and the role of CD8 cells in allograft rejection and immune responses to viral infection have been described (163, 164).

In mammalian species, the majority of circulating lymphocytes express T cell receptors (TCRs) generated by the products of TCR-α and -β genes. In contrast, T lymphocytes that home to the skin and mucosal epithelia express TCRs composed of γ/δ heterodimers. *Xenopus* TCR genes of α/β and γ/δ types have been described (165-167). Little is known about the distribution of α/β and γ/δ subsets of lymphocytes in *Xenopus*; however, reagents that are directed to conserved regions of γ/δ TCRs were able to stain a set of lymphocytes in *Xenopus* skin, suggesting that this unique subset is present there (71). In mammals, this subset of cells is thought to recognize protein antigens in an MHC-independent manner and may serve to eliminate "stressed," metabolically compromised, or transformed epithelial cells. In so doing, they promote wound healing in the skin. Whether they play a role in protection of the skin of *Xenopus* is not yet known.

6. IMMUNE DEFENSES AGAINST *B. DENDROBATIDIS*

6.1. Antimicrobial peptide defenses against *B. dendrobatidis*

Xenopus laevis secretes milky white mucus when alarmed, and this species was one of the first for which antimicrobial peptides in skin secretions were described. A number of antimicrobial peptides from this species have been isolated and characterized. They include magainin I and magainin II (168), PGLa (peptide with amino terminal glycine and carboxyl terminal leucinamide) (169), LPF (levitide precursor fragment) (170), XPF (xenopsin precursor fragment) (170,171), and several CPF (caerulein precursor fragment) family members (170,172,173). Matrix-assisted laser desorption ionization (MALDI) time-

Table 1. Serum antibodies (IgM and IgY) binding to *B. dendrobatidis* by ELISA

Treatment	IgM ($O.D._{450}$)	IgY ($O.D._{450}$)	Number of Frogs
Controls (APBS)[1]	101.2 ± 4.5	7.2 ± 0.6	10
Bd-Immunized[1]	147.7 ± 7.0[2]	169.6 ± 22.8[2]	10

[1]Serum collected at day 14 after final injection and diluted 1/100. [2]Significantly greater than control values by a one-tailed Student's t test; p-value ≤ 0.0005.

of-flight (TOF) mass spectrometry (MS) can separate complex mixtures of proteins and peptides based on their known masses. By MALDI-TOF MS it is possible to identify the known AMPs (174). The broad peak of hydrophobic peptides between mass 2600 and 2700 include a number of CPF family members with likely AMP activity, although not all of them have been tested (170,172). Our laboratory has demonstrated antimicrobial activity for three of the purified peptides against *B. dendrobatidis*. The most effective was CPF with a minimal inhibitory concentration (MIC) of 50 µM (175). The MIC for PGLa was 100 µM (175) and for magainin II was 200 µM (L. Rollins-Smith, unpublished data). Two of the peptides (magainin II and PGLa) can act synergistically to inhibit growth of bacteria and *B. dendrobatidis (*175,176). Injection of increasing concentrations of norepinephrine induces the secretion of skin peptides in a dose-dependent fashion (177), and we have observed that a total peptide concentration of 400-1000 µg/ml of enriched hydrophobic peptides effectively inhibits growth of *B. dendrobatidis in vitro* (L. Rollins-Smith, unpublished). To determine the approximate concentration of antimicrobial peptides in the skin mucus of *Xenopus* and other species, we bathed frogs in collection buffer, passed the peptides over a C-18 sep-pak cartridge to collect the hydrophobic peptides, quantified the peptides by a micro-BCA assay, and determined the total quantity of recovered peptides per gram of frog weight. The standard peptide used for quantification was a nine amino acid peptide found in the skin secretions of many amphibians (bradykinin). We express peptide concentrations as µg equivalents/ml based on the bradykinin standard. If we assume that the mucus layer is 500 µm thick, then the volume of mucus covering one cm^2 of skin would be 50 µl. The surface area of the skin can be estimated based on the weight in grams (178). Therefore, we are able to quantify the total hydrophobic peptides recovered in the mucus in µg equivalents/ml. For resting frogs (*Xenopus laevis*), the amount recovered is about 126 µg equivalents /ml (L. Rollins-Smith and D. Woodhams, unpublished data). This amount would have little effect on the viability of zoospores that encyst on the skin. However, if *X. laevis* are chased in the water to mimic the effort to escape a predator, the amount of recoverable peptides increases to about 326 µg equivalents/ml. This is in the range of concentrations that would be inhibitory for growth of the fungus (L. Rollins-Smith and D. Woodhams, unpublished data).

In contrast to the expression of a number of effective antimicrobial peptides in the skin secretions of *X. laevis*, *B. boreas* skin secretions appear to contain no conventional antimicrobial peptides. However, there appear to be hydrophobic molecules in the mass range of about 700 to 750 that can inhibit *B. dendrobatidis in vitro* (174).

6.2. Antibody-mediated defenses against *B. dendrobatidis*

This section summarizes preliminary studies from our laboratory that will be published in greater detail when they are more complete. To investigate whether *X. laevis* could develop an effective immune response against *B. dendrobatidis*, we immunized outbred adults (N= 37) obtained from commercial suppliers with heat-killed *B. dendrobatidis* cells (a mixture of zoospores and mature cells) via the intraperitoneal route. Controls (N= 37) received amphibian phosphate buffered saline (APBS) alone. Blood was drawn by cardiac puncture from 7-10 frogs at one to four weeks after the final immunization (each animal was bled once after immunization), and IgM and IgY antibodies that bind *B. dendrobatidis* cells fixed to a microtiter plate were quantified by ELISA. Immunization resulted in the development of a high-titer antibody response to *B. dendrobatidis* by day 14 that exceeded non-specific binding observed with serum from control frogs (Table 1). Results are shown for serum diluted at 1/100 for animals bled at day 14. The IgY response of *Bd*-immunized frogs was significantly greater than that of controls at all days tested. However, the IgM response was significantly increased only at day 14. These results show that it is possible to generate an antibody response to *Bd* that exceeds non-specific binding in the control frogs. The IgY antibody titers ranged from 1/800 to 1/6400 at day 14 (N = 10; data not shown).

6.3. T Cell-mediated defenses against *B. dendrobatidis*

It is likely that effective immune responses to *B. dendrobatidis* will involve T cell-mediated responses (138,158, 179-183; see below). To begin to investigate T-cell mediated defenses against *B. dendrobatidis*, adult outbred *Xenopus* from our laboratory colony were immunized with heat-killed *B. dendrobatidis* via the dorsal lymph sac at days 0 and 14. Controls were injected with APBS. At day 28, they were given a final injection of the same number of killed *B. dendrobatidis* cells or APBS via the intraperitoneal route in an effort to induce antigen reactive cells to move into the spleen. At day 33, the frogs were sacrificed and spleen cells cultured with phytohemagglutinin (PHA) as a general stimulator of T cells or with freshly killed *B. dendrobatidis* cells. Spleen cells from one of seven *B. dendrobatidis*-immunized frogs showed significant proliferation against *B. dendrobatidis* as well as PHA. The stimulation index against *B. dendrobatidis* was 5.0. The other six responded significantly to PHA but not *B. dendrobatidis.* The stimulation indices against PHA for all seven *B. dendrobatidis*-immunized frogs ranged from 11.9 to 92.8 indicating that T cells from all of the frogs were competent to respond to a T cell target *(*Table 2). At the same time, splenocytes from three of six APBS-injected control frogs demonstrated significant proliferation in response to freshly killed *B. dendrobatidis* as well as PHA. All six responded significantly to PHA (stimulation indices ranged from 11.6 to 100.4). The stimulation indices against *B. dendrobatidis* were 3.0, 6.2, and 15.9 (Table 2). These proliferation

Table 2. Spleen cell proliferation following immunization with *B. dendrobatidis*

Identifier	CPM[1] Cells Only	CPM Cells + PHA[2]	S.I.[3] PHA	CPM Cells Only	CPM Cells vs. Bd	S.I.[3] *Bd*
APBS1[4]	70 ± 8	4613 ± 264[5]	65.9	139 ± 34	190 ± 45	1.4
APBS 2	440 ± 32	5110 ± 149[5]	11.6	356 ± 164	2217 ± 184[5]	6.2
APBS 3	442 ± 64	29096 ± 377[5]	65.8	183 ± 48	2906 ± 558[6]	15.9
APBS 4	262 ± 100	7223 ± 1634[6]	27.6	1986 ± 548	1289 ± 211	0.6
APBS 5	154 ± 22	5425 ± 660[5]	35.2	113 ± 13	342 ± 82[7]	3.0
APBS 6	104 ± 18	10456 ± 805[5]	100.5	287 ± 108	331 ± 84	1.1
Bd 1[8]	86 ± 5	3490 ± 114[5]	40.6	70 ± 10	88 ± 12	1.3
Bd 2	756 ± 144	9030 ± 257[5]	11.9	1742 ± 456	1947 ± 204	1.1
Bd 3	464 ± 30	33089 ± 895[5]	71.3	447 ± 263	2218 ± 536[7]	5.0
Bd 4	76 ± 5	7028 ± 249[5]	92.5	60 ± 11	50 ± 12	0.8
Bd 5	76 ± 6	5118 ± 513[5]	67.3	33 ± 2	48 ± 7	1.4
Bd 6	83 ± 15	5105 ± 1017[6]	61.5	80 ± 18	45 ± 8	0.6
Bd 7	704 ± 173	8545 ± 980[5]	12.1	2456 ± 536	1338 ± 500	0.5

Abbreviations: [1] CPM, Counts per minute. [2]PHA, Phytohemagglutinin. [3] S. I., Stimulation index = stimulated counts divided by background counts. [4]APBS control frogs were injected with amphibian phosphate buffered saline at days 0, 14, and 28. Cells were harvested at day 33. [5]Significantly greater than CPM of cells only, $p \leq 0.0005$. [6]Significantly greater than CPM of cells only, $p \leq 0.005$. [7]Significantly greater than CPM of cells only, $p \leq 0.025$. [8]*Bd* frogs were immunized with heat-killed *B. dendrobatidis* at days 0, 14, and 28. Cells were harvested at day 33. Cells cultured with PHA were harvested at day 3. Cells cultured with *B. dendrobatidis* were harvested at day 5.

Table 3. Survival of immunized or control *B. boreas* following exposure to infectious *Bd*

Treatment	Number of Toads	Percent Survival Day 35
Controls[1] not exposed to *Bd*	42	100
Not injected, *Bd* exposed	14	0
APBS injected, *Bd* exposed	14	0
Bd immunized, *Bd* exposed	15	0

[1]Controls consisted of 14 APBS-injected toads, 14 *Bd*-immunized toads, and 14 toads that were not manipulated. None of these controls were exposed to *B. dendrobatidis*. All other toads were exposed to a lethal dose of *B. dendrobatidis (*10^6 zoospores).

responses in non-immunized frogs may suggest that the control frogs have been exposed to *B. dendrobatidis* or related pathogens, and their splenocytes can respond significantly *in vitro.* We did not determine whether the test animals were infected with *B. dendrobatidis*. The relatively weak proliferation responses to *B. dendrobatidis* following immunization may suggest that *B. dendrobatidis* is capable of inhibiting development of a protective response. Further studies are underway to evaluate the cell-mediated immune responses to this pathogen.

7. CAN AMPHIBIANS BE IMMUNIZED TO PROTECT THEM FROM INFECTION BY *B. DENDROBATIDIS*?

Based on our preliminary results demonstrating the development of high-titer antibodies to *B. dendrobatidis* in *Xenopus*, we initiated experiments to determine whether the boreal toad (*Bufo boreas*) could be immunized and protected from experimental *B. dendrobatidis* infection. The Carey laboratory obtained young boreal toads that had been raised in a *B. dendrobatidis*-free hatchery by the Colorado Division of Wildlife, and the animals were immunized in our facility according to a similar immunization protocol that had resulted in high-titer antibody responses in *Xenopus*. After the final immunization, immunized toads, APBS injected control toads, and a third group of uninjected toads were returned to the Carey laboratory for exposure to *B. dendrobatidis*. Each group was divided, and half of the animals were exposed to 10^6 zoospores (37) while the remaining frogs were not exposed to the pathogen. In spite of our efforts to immunize them, toads injected with the heat-killed pathogen and exposed to live zoospores did not survive better than APBS-injected or uninjected controls that were exposed to *B. dendrobatidis*. In contrast, all toads that were maintained free of exposure to *B. dendrobatidis* survived (Table 3). These results suggest that the immunization protocol that generates effective antibody responses in *X. laevis* is not effective in protection of a susceptible species from a normal (epidermal) route of infection.

8. FUTURE DIRECTIONS FOR RESEARCH

8.1. Alternative immunization strategies

Although our immunization protocol appears to be adequate for induction of antibodies to *B. dendrobatidis*, the antibodies were not protective against this pathogen in *B. boreas*. As is the case for mammalian species, development of effective antibody responses to complex cellular antigens or large proteins in *Xenopus* requires T cell help (138,160, 179-181). Therefore, because we showed strong antibody responses, we conclude that our immunization protocol resulted in the activation of T cells and production of cytokines necessary for antibody production. However, protective immunity to fungal pathogens in mammalian hosts is complex involving mediators of the innate immune system (macrophages, neutrophils, natural killer cells, and γδ-T cells) and the adaptive immune system (T cells of both $CD4^+$ and $CD8^+$ phenotype and B cells) (rev in 182,183). Protective T cell responses depend on the predominance of the T-helper cells of type 1 (Th1) pathway which involves production of

interferon-γ, interleukin-12, and interleukin-18. These cytokines stimulate production of activated macrophages, cytotoxic T cells, and opsonizing antibodies (183). Although antibodies are raised to fungal pathogens in mammalian systems, there is limited evidence that they are protective (183). Therefore, we believe that it is essential to develop methods to induce more effective T-cell mediated immunity in our model system. Additional studies will test the effectiveness of use of adjuvants to induce more effective T cell-mediated immunity.

8.2. Focus on the skin

Although we have successfully induced a systemic immune response resulting in development of high-titer antibodies against *B. dendrobatidis* in *X. laevis*, chytridiomycosis is an infection that remains confined to the skin. Therefore, it is likely that a successful immunization strategy must be targeted to antigen presenting cells and immune effectors that home to the skin. Studies to introduce *B. dendrobatidis* antigens directly into the skin compartment with adjuvants are planned.

9. PERSPECTIVE AND CONCLUDING REMARKS

Xenopus laevis and *Bufo boreas* have been invaluable as models to study immune defenses against *B. dendrobatidis*. Our current view is that both innate skin defenses and adaptive immune responses are required for control and elimination of this pathogen. *X. laevis* appears to have very effective antimicrobial peptide defenses in the skin, and immunized animals can develop an immune response. In contrast, *B. boreas* has less effective antimicrobial peptide defenses and may succumb if infected with a sufficient number of zoospores prior to development of an effective adaptive immune response. Protection of vulnerable species such as *B. boreas* may depend on our capacity to develop an effective immunization (vaccine) or exposure strategy that allows for adult breeders to become immune in captivity so that they may be released to enable populations to persist until the pathogen declines.

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