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IMMUNIZATION IS INEFFECTIVE AT PREVENTING INFECTION AND MORTALITY DUE TO THE AMPHIBIAN CHYTRID FUNGUS *BATRACHOCHYTRIUM DENDROBATIDIS*

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ABSTRACT: The fungal pathogen *Batrachochytrium dendrobatidis* (Bd), the causative agent of chytridiomycosis, has been implicated in amphibian declines worldwide. It has been hypothesized that low inherent immunogenicity in Bd may be related to the high rates of morbidity and mortality that are associated with Bd-infected anuran populations. To test this idea, juvenile *Rana muscosa* (mountain yellow-legged frogs) were immunized with adjuvants in combination with a formalin-killed Bd culture to determine if it is possible to stimulate a protective immune response when challenged with a live inoculum of *B. dendrobatidis*. Three groups of juvenile *R. muscosa* (6 mo postmetamorphosis) were immunized with saline, Freunds Complete (FCA) and Incomplete Adjuvant (FIA), or the adjuvants in combination with a formalin-killed culture of *B. dendrobatidis*. The effects of immunization were modeled using survival analysis and a proportional hazards model. No significant differences were found between the groups in overall mortality, time to infection, infection prevalence, or intensity. While this study suggests that immunizing anurans against chytridiomycosis will not alter rates of infection or mortality among individuals, it does raise several questions regarding the attenuation and efficacy of anuran adaptive immune responses and whether they may be protective against this disease.

Key words: *Batrachochytrium dendrobatidis*, chytridiomycosis, immunization, *Rana muscosa*.

INTRODUCTION

Since *Batrachochytrium dendrobatidis* (Bd) was first described in association with amphibian declines in Australia in 1993, this fungal pathogen has been documented on almost every continent and linked to anuran declines worldwide (Berger et al., 1998; Weldon et al., 2004; Rachowicz et al., 2006; Bosch et al., 2007). In recent years, chytridiomycosis has become an important contributor to declines of *Rana muscosa* (mountain yellow-legged frogs), a once common amphibian in the Sierra Nevada Mountains of California. The first report of Bd in *R. muscosa* was published in 2001 (Fellers et al., 2001), but analysis of preserved *Bufo canorus* specimens suggests that the fungus has been present at least since the 1970s in California (Green and Sherman, 2001). Surveys of several hundred *R. muscosa* populations in the southern Sierra Nevada from 2002 to the present have linked the presence of Bd with declines of *R. muscosa* throughout its range (Rachowicz et al., 2006). All life stages of *R. muscosa*, with exception of egg masses, have been found to be susceptible to infection with Bd (Rachowicz and Vredenberg, 2004), but only metamorphosing and adult animals die in response to infection. Disease progression in field-caught and laboratory-infected larvae shows that individuals in both groups succumbed to infection with Bd shortly after metamorphosis, and mortality rates were as high as 96% in the field (Rachowicz et al., 2006). Mortality rates in Bd-exposed adult *R. muscosa* have also been recorded as high as 95% under laboratory conditions (Andre et al., 2008).

Recent work has shown the mechanism behind the pathogenesis of chytrid infections involves a disruption in electrolyte hemostasis and normal cutaneous function (Voyles et al., 2009). Chytrid infections are localized to the outermost layer of the amphibian dermis, the stratum corneum,

which may interfere with the uptake of ions across the skin and basic osmotic requirements in blood and lymph fluid.

The number of extant uninfected *R. muscosa* populations has been decreasing each year, and the threat that chytridiomycosis poses to remaining populations of *R. muscosa* has raised concerns. Prevalence of Bd in Sierran populations was found to increase rapidly following arrival of the pathogen at a site, with rates of mortality approaching 100% in both juvenile and adult frogs (C. Briggs, personal observation). In contrast, other species of amphibians appear to be resistant to developing disease following Bd exposure (Hanselmann et al., 2004). These findings have led to questions about infection prevention and mortality from Bd in amphibian communities, and they demonstrate the need for additional work on protective immune responses.

What makes declines in *R. muscosa* and other species so difficult to understand is that the immune systems of anurans have near the same complexity as other vertebrates. Amphibians have immune responses found in mammals, including innate and adaptive responses capable of recognizing a broad spectrum of pathogens. This includes specific responses to pathogens through cell-mediated responses (Rau et al., 2001) and antibody isotype variability (Du Pasquier et al., 1989). To date, there has been no evidence to suggest either a robust inflammatory response from the skin (Nichols et al., 2001) or an innate immunity and antimicrobial peptide release is completely protective against disease with Bd (Rollins-Smith et al., 2006). The current study was motivated by the suggestion that low antigenicity of Bd allows infection and the resulting disease to progress before an effective immune response develops (Berger et al., 1999). This immunologic response during vaccination may be promoted with an adjuvant, and these have been successfully used in frogs to increase the immunogenicity of haptens (Pross and Rowlands, 1976).

While innate responses (i.e., antimicrobial peptides) have been shown to be efficacious at killing Bd in *R. muscosa* (Rollins-Smith et al., 2006), primary responses in general are short lived, have a finite limit to clearing an infection, and exhibit no memory to pathogens. An adaptive immune response is more specific, can be sustained for longer time periods, and mounts a memory response to antigens upon re-exposure.

In this study, we tested the assertion that low immunogenicity in Bd infections leads to high rates of infection and mortality in juvenile *R. muscosa*. We hypothesized that immunization with a killed chytrid adjuvant mixture would lead to lower rates of infection and mortality when animals were subsequently exposed to live cultures of Bd.

MATERIALS AND METHODS

Rana muscosa egg masses were collected from Sixty Lake Basin in Sequoia Kings National Park in the summer of 2002 and raised in the Office of Laboratory Care facility at the University of California at Berkeley. Animals were housed at 17 C for the duration of their care and all experiment procedures. Tanks for tadpoles were cleaned twice weekly, and tadpoles were fed Purina Rabbit Chow ad libitum at each cleaning. At front leg emergence (FLE), metamorphs were switched to tanks with approximately 250 ml of water. At Gosner stage 45, tank water was reduced again to 150ml, and a folded stack of unbleached paper towels measuring 15×12×0.5 cm was added as a substrate. Tanks for postmetamorphic frogs were changed weekly. Beginning at Gosner stage 46, juvenile frogs were fed 15–20 crickets and dusted with calcium carbonate once weekly. Juvenile frogs were maintained until 6 mo postmetamorphosis before beginning any experimental procedures. At the termination of this study, animals were assigned a health score based on the following criteria; 1=jumping and righting responses normal and no accumulation of shed skin in tank; 2=jumping and righting responses normal, accumulation of shed skin in tank; and 3=abnormal jumping and righting responses and accumulation of shed skin in tank. All animal identification was coded prior to the termination of the study to prevent bias in this score.

Animals were randomly assigned into three groups at the start of the experiment and housed individually for its duration. Adjuvants used in this study have been used successfully in fish and other anurans (Olivier et al., 1985) and in promoting the recognition of haptens by the anuran immune system (Pross and Rowlands, 1976).

Control animals received a saline immunization prior to exposure to live chytrid. The adjuvant-only group received a 1:1 immunization of saline to Freunds Complete Adjuvant (FCA) and 1 mo later received a 1:1 immunization of saline with Freunds Incomplete Adjuvant (FIA). The final group received a 1:1 mixture of formalin-killed chytrid in FCA and 1 mo later received a formalin-killed chytrid preparation in FIA. All injections were restricted to 0.05 cm^3 and were administered into the dorsal lymph sac.

Cultured Bd (LJR 119), originally isolated from one larval *R. muscosa* collected in 2002, was used for immunizations and live exposures in all three groups. Animals in each group received exposure to the same number of live zoospores (10^5 zoospores on the same day as counts were performed). This one-time exposure occurred 1 mo after the final immunization with either saline, adjuvant only, or adjuvant and Bd. Plates were flooded with 1 ml of filtered water (the same water source as that used for animal care) and allowed to sit for 20 min. Zoospores were pooled, and counts were determined according to methods in Daszak et al. (2004). For the adjuvant and Bd immunizations, an aliquot containing 10^5 zoospores was dispensed into Eppendorf vials, followed by 1 ml of 10% formalin (Fisher Scientific, Pittsburgh, Pennsylvania, USA), and vials were allowed to sit at room temperature overnight. Vials were spun for 10 min at 15,996 × G. All but 50 µl were removed from vials after centrifugation, 1 ml of saline (Abbott Laboratories, Abbott Park, Illinois, USA) was added, and vials were centrifuged for 10 min at 7,000 × G. This step was repeated twice more to remove the formalin from the aliquot of Bd. After the third wash, all but the last 50 µl were removed, and to this, 50 µl of FCA or ICA were added and used for injections. The Bd and adjuvant combinations were mixed via syringe ten times prior to their administration. An aliquot delivering 10^5 zoospores was administered to each animal in all groups. The inoculum was mixed by inversion twice before being drawn and delivered to the next animal. Animals remained in tanks for 24 hr after the inoculum was added and before fresh water was replaced in the tanks. Each individual was monitored every week for infection intensity (Bd load) using swabs of the ventral surface of each animal as described previously (Hyatt et al., 2007). Targeted areas for swabs included the drink patch (lower ventral surface of abdomen), inner portions of both hindlimbs, and webbing of each foot. Swabs were processed using a real-time polymerase chain reaction (PCR) protocol developed by Boyle et al. (2004). Zoospore load determined from the real-time PCR protocol represents the total number of copies of Bd DNA on the swab. All analyses were performed using JMP 5.1 statistical software.

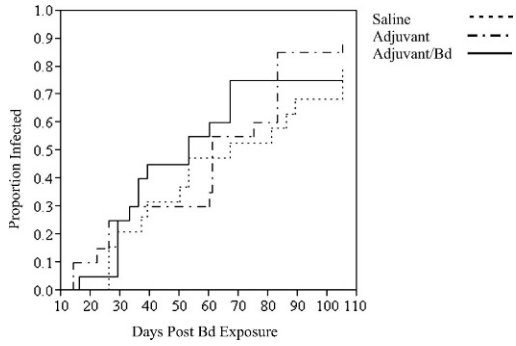

FIGURE 1. Failure of immunizations to prevent the development of *Batrachochytrium dendrobatidis* (Bd) infections. Number of days post exposure to live Bd until individuals were noted as positive, measured by real-time PCR. There was no difference between groups in the proportion of individuals that became positive for Bd (log rank $\chi^2=1.29$, $p>0.05$).

RESULTS

Immunization had no significant effect on the proportion of frogs that became infected with Bd (Table 1; one-way analysis of variance [ANOVA] between groups, $P>0.4$) or on the time to Bd infection (Fig. 1). A failure analysis was performed to determine if immunization affected the time to Bd detection in each individual, censoring for individuals who remained negative for the duration of the experiment. No significant difference was found between immunization group using either the Wilcoxon or log-rank tests ($P>0.5$ for both tests).

Immunization had no effect on the proportion of Bd-infected individuals that died prior to the end of the experiment at day 108 postexposure (Table 1; one-way

TABLE 1. Effects of experimental treatment on infection prevalence and mortality. Shown is the fraction of frogs in each group that tested positive at some point during the experiment (fraction infected) and the fraction of individuals that died prior to the end of the experiment (day 108). No difference in Bd growth nor for the max zoospore load was found between groups, 1 way ANOVA ($p>0.2$ and $p>0.4$ respectively).

Immunization group	Saline/control	FCA/FIA saline	FCA/FIA:killed Bd
Sample size (*n*)	19	20	20
Fraction infected	0.79	0.9	0.75
Fraction dead	0.21	0.35	0.4
Maximum Bd load (mean±SE)	5,106±6,487	17,831±12,922	53,990±78,843
Bd growth rate (mean±SE)	0.089±0.017	0.077±0.036	0.078±0.017

ANOVA between treatments, $P>0.9$). A Kaplan Meier curve was generated using days survived as the response variable and grouping animals according to treatment (Fig. 2). Data were censored for animals that were euthanized at the termination of the experiment. One animal in the adjuvant group and two in the adjuvant/Bd group were euthanized prior to the termination of the experiment because they were observed to be lethargic, anorexic, and had an accumulation of shed skin in their tanks. These animals were positive for Bd and were euthanized because they were unlikely to recover from Bd based on similar laboratory studies in *R. muscosa* (Rachowicz et al., 2006; Andre et al., 2008). No significant difference was found between groups using either the log-rank or Wilcoxon tests ($P>0.2$ for both tests). The majority health score recorded for all animals was 2, which represented an animal that had normal jumping and righting behavior and an accumulation of shed skin in the tank at the final reading. A health score of 2 was assigned to animals in each treatment group in the following proportions: saline-only immunizations (0.9), adjuvant-only (0.5), and adjuvant Bd combination (0.8). The other score assigned to animals was recorded as 1 and describes normal behavior and no abnormal shedding present in tanks. No animals in any group received a health score of 3, which denotes an animal unable to right itself or jump and a large amount of shed skin in its tank at the time of observation.

Infection intensity (zoospore load) was measured by real-time PCR performed on weekly skin swabs from each animal (Figs. 3–5). Many animals lacked a complete swab sampling record, and one animal was excluded in the graphs and survival analysis because at day 0, its swab result was positive for Bd. There was no significant difference between the groups in the maximum zoospore load observed per frog (Table 1; one-way ANOVA, $P>0.4$). The maximum zoospore load usually occurred at the time of death. There was no significant difference between the groups in the rate of increase in zoospore load for each individual (calculated as the slope of a straight line fitted to the time trajectory of ln [Bd load]; Table 1; one-way ANOVA, $P>0.2$).

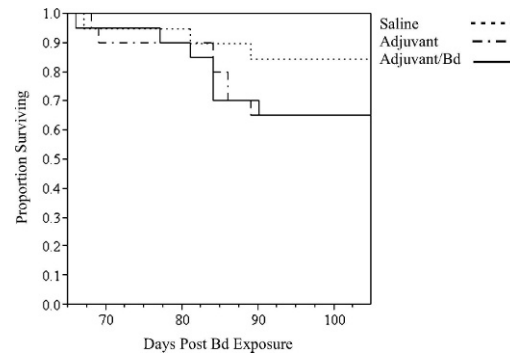

FIGURE 2. Survival analysis of immunization and subsequent mortality in *R. muscosa*. Mortality amongst groups receiving saline, adjuvant only or adjuvant and killed chytrid immunizations is modeled using a Kaplan Meier curve. No difference in mortality nor the rate at which animals died was found between (log rank $\chi^2=0.53$, $P>0.05$).

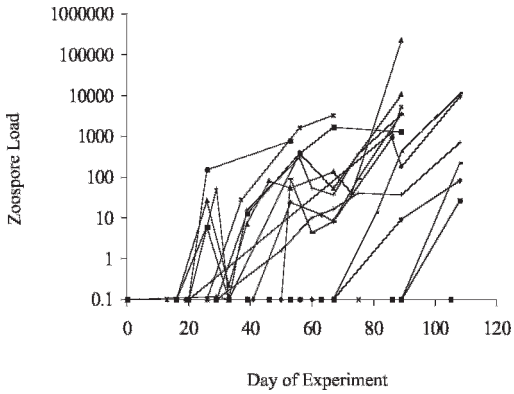

FIGURE 3. Infection prevalence over time in animals receiving saline immunization, measured via qPCR. Swab results for nineteen individuals examining the rate of Bd infection represented by "zoospore load" after saline immunization. Approximately 21% of controls died before the experiment ended; one of which was found dead at the end of the experiment, at day 108, which had remained negative for Bd infection for the duration of this study. Seventy-nine percent of animals in this group were positive for Bd.

DISCUSSION

In this study, we found no evidence that immunization against Bd, using killed Bd and/or adjuvant, protects animals against infection or mortality due to chytridiomycosis. Thus, stimulation of the adaptive immune response is unlikely to be an effective conservation strategy to protect threatened amphibian populations from this lethal disease. However, several factors may have affected these results. Our study did not include a negative control group, and there is the possibility that mortality may have been related to other infections. The presence of additional mortality factors is unlikely because no individual at the termination of the study received an abnormal health score; this should have been observed if other infectious amphibian diseases were present (Densmore and Green, 2007). During the course of these experiments, common amphibian bacterial infections were managed by housing animals individually and by coordinating the feeding and cleaning schedules to minimize any bacterial growth in tanks.

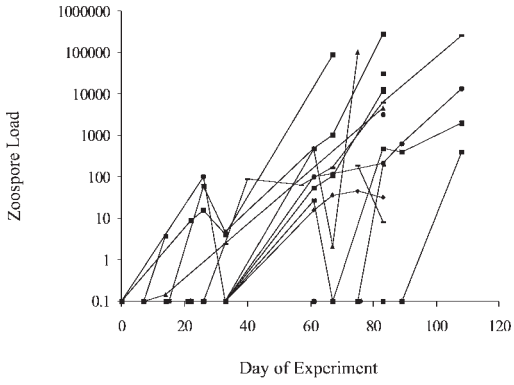

FIGURE 4. Bd infection prevalence measured via qPCR, in animals receiving adjuvant immunizations. Animals in this group received a primary immunization with Freund's Complete Adjuvant and a secondary immunization with Freund's Incomplete Adjunvant one month later. Thirty-five percent of animals either died ($n=6$) or were euthanized ($n=1$) before the end of the experiment at 108 days and 90% were infected with Bd.

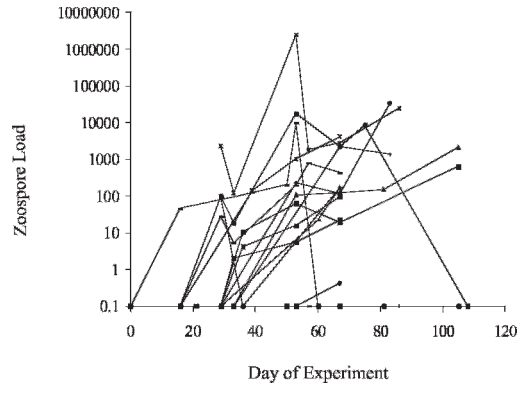

FIGURE 5. Bd infection prevalence measured via qPCR in animals receiving adjuvant and killed Bd immunizations. Animals in this group received a primary and secondary immunization with Freund's Complete Adjuvant one month apart from each other. Forty percent of animals either died ($n=6$) or were euthanized ($n=2$) before the termination of the experiment and 75% of animals test positive for Bd at some point during the experiment.

Given the adverse reactions associated with Freunds adjuvant (Powers et al., 2007), it was assumed that this particular adjuvant would be extremely irritating. For this reason, the amount of adjuvant used was kept to a minimum (less than half of what had been used previously in anurans and had been successful; Pross

and Rowlands, 1976). We conducted a 60 day pilot study using varied amounts of adjuvant (25–100 μl) and observed no adverse effects. Future studies investigating tolerable amounts of adjuvant in ectothermic vertebrates may be justified because increased adjuvant concentration may promote a more protective immune response. The type of adjuvant and site of inoculation may also be important factors in facilitating an immune response in anurans, and these should also be considered in future studies. Adjuvants other than Freund's, such as Ribi and Titermax adjuvants, have been formulated to allow for less toxicity and more direct induction of cell-mediated and humoral responses; however, these have not been tested in ectotherms.

Temperature also may have influenced our results; in our experiments, animals were kept at 17 C. It has been shown that Bd can be eliminated in *R. muscosa* at temperatures of 22 C (Andre et al., 2008). Because 22 C is within the range of optimal Bd growth, this suggests that disease was eliminated due to a host response rather than limited growth of the pathogen. Low temperatures (4 C) have been shown to cause the loss of lymphocytes in the spleen and thymus of adult *Rana pipiens* (Cooper et al., 1992). Temperature also has been shown to be an important factor in susceptibility to *Bd* infections (Woodhams et al., 2003). Based on work done in the most basal members of the order Anura, we know they have immune systems that include both adaptive (Du Pasquier et al., 1989) and innate responses (Conlon et al., 2004). Work is just beginning to look at the ways in which both innate and adaptive responses are involved in natural infections, particularly those associated with amphibian declines.

There is no evidence to date that adaptive immunity plays any part in resolving chytrid infections. Histologic examination of anuran skin in chytridiomycosis infections in *Dendrobatid* spp. has revealed only low numbers of neutrophils and macrophages (Nichols et al., 2001). Survival of Bd-infected anuran species may be most dependent on the innate immune response and antimicrobial peptide production rather than any adaptive immune response; however, Bd is an intracellular pathogen (Berger et al., 1998), and anurans can mount protective adaptive responses to intracellular pathogens (Morales and Robert, 2007).

Rana muscosa antimicrobial peptides are capable of inhibiting Bd growth in vitro (Rollins-Smith et al., 2006), but the species is highly susceptible to infection with Bd. The work by Rollins-Smith suggests that innate immunity and the repertoire of antimicrobial peptides produced by *R. muscosa* may not be the determining factor in limiting Bd infections. The role of adaptive immunity in limiting this disease is not currently understood but may be important.

Our study investigated the net effect of a putative adaptive immune response on frog infection and survival, but we did not look directly for increased antibody production following immunization. Existing ELISA protocols to determine antibody titers in anurans have been developed for *Xenopus laevis* and have exhibited little or no cross reactivity in some anuran species like *R. muscosa* (Jeremy Ramsey, personal comm.). Our laboratory is currently developing a *R. muscosa*–specific ELISA protocol to measure humoral responses to chytridiomycosis. Although the results of our study do not offer any new methods for protecting *R. muscosa* and other threatened species from Bd, they do suggest that anurans may not become immune when exposed to inactive Bd, and they present a starting point for future work on establishing effective ways to combat infections in threatened amphibian populations.

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