British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Immune evasion or avoidance: Fungal skin infection linked to reduced defence peptides in Australian green-eyed treefrogs, *Litoria serrata*

Douglas C. WOODHAMS[a,*], *Sara C. BELL*[b], *Nicole KENYON*[b], *Ross A. ALFORD*[b], *Louise A. ROLLINS-SMITH*[c,d,e]

[a]*Department of Ecology and Evolutionary Biology, University of Colorado, N122 Ramaley, 334 UCB, Boulder, CO 80309-0334, USA*
[b]*School of Marine and Tropical Biology, James Cook University, Townsville, Qld 4811, Australia*
[c]*Department of Pathology, Microbiology and Immunology, A-5301 Medical Center North, Vanderbilt University Medical Center, Nashville, TN 37232, USA*
[d]*Department of Pediatrics, A-5301 Medical Center North, Vanderbilt University Medical Center, Nashville, TN 37232, USA*
[e]*Department of Biological Sciences, A-5301 Medical Center North, Vanderbilt University Medical Center, Nashville, TN 37232, USA*

ARTICLE INFO

Article history:
Received 26 June 2012
Received in revised form
15 October 2012
Accepted 29 October 2012
Available online 10 November 2012
Corresponding Editor:
Michael Lorenz

Keywords:
Amphibian
Chytridiomycosis
Disease ecology
Immune evasion
Innate immunity
MALDI mass spectrometry

ABSTRACT

Many parasites and pathogens suppress host immunity to maintain infection or initiate disease. On the skin of many amphibians, defensive peptides are active against the fungus *Batrachochytrium dendrobatidis* (*Bd*), the causative agent of the emerging infectious disease chytridiomycosis. We tested the hypothesis that infection with the fungus may be linked to lower levels of defensive peptides. We sampled both ambient (or constitutive) skin peptides on the ventral surface immediately upon capture, and stored skin peptides induced from granular glands by norepinephrine administration of Australian green-eyed treefrogs, *Litoria serrata*. Upon capture, uninfected frogs expressed an array of antimicrobial peptides on their ventral surface, whereas infected frogs had reduced skin peptide expression. Expression of ambient skin peptides differed with infection status, and antimicrobial peptides maculatin 1.1 and 2.1 were on average three times lower on infected frogs. However, the repertoire of skin peptides stored in granular glands did not differ with infection status; on average equal quantities were recovered from infected and from uninfected frogs. Our results could have at least two causes: (1) frogs with reduced peptide expression are more likely to become infected; (2) *Bd* infection interferes with defence peptides by inhibiting release or causing selective degradation of peptides on the skin surface. Immune evasion therefore may contribute to the pathogenesis of chytridiomycosis and a mechanistic understanding of this fungal strategy may lead to improved methods of disease control.

Introduction

Parasites that can evade or suppress host immunity are common (Damian 1997; Chai *et al.* 2009). For example, the opportunistic fungal pathogen *Candida albicans* evades the innate complement system of humans, and can vary cell surface proteins (Rambach & Speth 2009; Verstrepen & Fink 2009). This fungus, and many other pathogenic fungi, protozoa,

* *Corresponding author*. Tel.: +1 720 245 5828.
E-mail addresses: dwoodhams@gmail.com (D. C. Woodhams), sara.bell@jcu.edu.au (S. C. Bell), nicole.kenyon@jcu.edu.au (N. Kenyon), ross.alford@jcu.edu.au (R. A. Alford), louise.rollins-smith@vanderbilt.edu (L. A. Rollins-Smith).
1878-6146/$ – see front matter © 2012 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.funbio.2012.10.005

viruses, and bacteria are inhibited by an innate immune defence that is common among eukaryotes – antimicrobial peptides (AMPs) (Zasloff 2002). Though parasites differ in sensitivity to AMPs, few studies have investigated suppression of this defence (e.g. Kraus & Peschel 2008). Parasites that can evade AMP defences may gain an advantage, particularly at colonizing mucosal surfaces or skin.

Because amphibian skin is a physiologically-active organ that regulates osmotic balance and respiration, any epidermal damage is potentially life-threatening (Voyles *et al.* 2011). Chytridiomycosis is an emerging infectious disease of amphibians that has contributed significantly to the global decline of amphibian populations. It is caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). Chytridiomycosis has also been implicated in several recent species extinctions and ecosystem alterations (Whiles *et al.* 2006; Skerratt *et al.* 2007; Connelly *et al.* 2008). In frogs with chytridiomycosis, the normal regulatory functions of the skin are severely compromised, which is thought to lead to osmotic imbalance and death due to asystolic cardiac arrest (Voyles *et al.* 2009). *Bd* colonizes keratinizing cells, or those cells fated to keratinize, in the skin tissues of adults (Berger *et al.* 1998, 1999, 2005a) and some salamander larvae (Jaime Bosch, pers. comm.), and the mouthpart tissues of larvae (Fellers *et al.* 2001).

Although it is unclear how cell entry is achieved, Longcore *et al.* (1999) hypothesized that zoospores encyst (stop movement and settle, resorb the flagellum, and form a cell wall) on the exterior surface of an epidermal cell and insert *Bd* nuclear material *via* a germ tube. The pathogen moves from the surface to the intracellular environment of the stratum granulosum skin layer where it grows and develops. The zoospore transforms into a growing thallus with fine, thread-like rhizoids. The thallus transforms into an urn-shaped zoosporangium in which the zoospores develop (Berger *et al.* 1998, 1999, 2005a; Longcore *et al.* 1999; Pessier *et al.* 1999). Thus, *Bd* growth and development occurs within epidermal cells (Barr 1980). As new layers of skin are added, infected cells move to the surface stratum corneum, the zoosporangium matures and extends discharge tubes through the membrane of the host epithelial cell, then the discharge papilla opens, and mature zoospores swim out (Berger *et al.* 1998, 1999; Longcore *et al.* 1999; Pessier *et al.* 1999). Water-borne zoospores attach to keratinized cells to initiate another cycle.

Infectivity, which includes the process of attachment, is thought to be influenced by environmental factors such as temperature. In culture, the rate of encystment increases with temperature from 4 to 28 °C (Woodhams *et al.* 2008), consistent with the temperature range at which most amphibians are active and at risk of exposure. Furthermore, *Bd* appears to exhibit chemotaxis towards favourable substrates (Moss *et al.* 2008), or away from unfavourable substrates and antifungal metabolites produced by bacteria (Lam *et al.* 2011). In addition to life-cycle coordination with host activity and skin dynamics (Berger *et al.* 2005a, b), keratinolytic enzyme activity of *Bd* suggests an adaptation to amphibian skin and a potential mechanism of pathogenesis (Piotrowski *et al.* 2004; Symonds *et al.* 2008). Differential gene expression profiles between the infectious zoospore stage and the growing sporangium stage of the fungus suggest that fungalysin, metallopeptidases, and serine proteases may be important factors in pathogenesis (Rosenblum *et al.* 2008). These proteolytic enzymes are key virulence factors in other pathogenic fungi (Huang *et al.* 2004; Jousson *et al.* 2004).

Several host factors may reduce infectivity by inhibiting zoospore attachment or directly killing zoospores. These include AMPs, mucosal antibodies or other mucosal factors produced by the host including fatty acids, alkaloids, lysozyme, mast cells, or mucosal factors produced by symbiotic microbes, such as small antibiotic molecules (Brucker *et al.* 2008a, b; Rollins-Smith 2009; Ramsey *et al.* 2010). The chemical environment of the skin, including pH, redox potential, nutrient availability, and availability of attachment sites (Wilson 2005), may also influence infectivity of *Bd*, although these factors have not been studied in detail. AMPs may directly influence infection status by killing zoospores or inhibiting the process of infection, or indirectly through causing changes in the composition of symbiotic microbiota, as suggested by Conlon (2011). Because AMPs are major components of amphibian skin secretions (Rollins-Smith 2009), and therefore influence the chemical and microbial microenvironment of amphibian skin, we focused our study on these important immune effectors against *Bd*. We have previously shown that there is variation in skin peptides among populations of the Australian green-eyed treefrogs, *Litoria serrata*, (formerly *Litoria genimaculata*, Richards *et al.* 2010) which is correlated with variation in *Bd* infection prevalence (Woodhams *et al.* 2010).

In this study, we measure several aspects of the skin peptide defence system including synthesis, storage, and expression of AMPs, and examine whether levels of skin peptides are correlated with *Bd* infection on individual hosts. We examine the hypothesis that infection by *Bd* may regulate the production or release of amphibian skin defence peptides. These studies culminate in a conceptual model of the amphibian skin peptide defence.

Materials and methods

Study species and site

Green-eyed treefrogs, *Litoria serrata*, were sampled from Birthday Creek, Paluma Range National Park, Queensland, Australia as described by Woodhams *et al.* (2010). Twenty-eight adult males were sampled on 7–8 September 2005 during the cool dry season, which is associated with peaks in the prevalence of *Bd* infection (Woodhams & Alford 2005).

Detection of Batrachochytrium dendrobatidis *by Taqman real time PCR and histology*

After sampling the ventral skin surface for peptides (see below), frogs were swabbed twice across the ventral surface and on all four feet with a sterile rayon swab (Medical Wire and Equipment, Corsham, Wilshire, UK). DNA was extracted from the swabs, and total *Bd* infection load (zoospore genome equivalents) was determined at James Cook University using qPCR according to Boyle *et al.* (2004). Swab samples were collected after ambient peptide sampling and before peptide induction.