Developmental and Comparative Immunology 47 (2014) 52–58

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Negative effects of low dose atrazine exposure on the development of effective immunity to FV3 in *Xenopus laevis*

Jason Sifkarovski [a,1], Leon Grayfer [a,1], Francisco De Jesús Andino [a], B. Paige Lawrence [b,a], Jacques Robert [a,*]

[a] Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, USA
[b] Department of Environmental Medicine, University of Rochester Medical Center, Rochester, USA

ARTICLE INFO

Article history:
Received 13 May 2014
Revised 21 June 2014
Accepted 22 June 2014
Available online 28 June 2014

Keywords:
Water pollutants
Ranavirus
Antiviral immunity
Immune toxicant

ABSTRACT

The recent dramatic increase of the prevalence and range of amphibian host species and populations infected by ranaviruses such as Frog Virus 3 (FV3) raises concerns about the efficacies of amphibian antiviral immunity. In this context, the potential negative effects of water contaminants such as the herbicide atrazine, at environmentally relevant levels, on host antiviral immunity remains unclear. Here we describe the use of the amphibian *Xenopus laevis* as an ecotoxicology platform to elucidate the consequences of exposure to ecologically relevant doses of atrazine on amphibian antiviral immunity. *X. laevis* were exposed at tadpole and adult stages as well as during metamorphosis to atrazine (range from 0.1 to 10.0 ppb) prior to infection with FV3. Quantitative analysis of gene expression revealed significant changes in the pro-inflammatory cytokine, TNF-α and the antiviral type I IFN gene in response to FV3 infection. This was most marked in tadpoles that were exposed to atrazine at doses as low 0.1 ppb. Furthermore, atrazine exposure significantly compromised tadpole survival following FV3 infections. In contrast, acute atrazine exposure of mature adult frogs did not induce detectable effects on anti-FV3 immunity, but adults that were exposed to atrazine during metamorphosis exhibited pronounced defects in FV3-induced TNF-α gene expression responses and slight diminution in type I IFN gene induction. Thus, even at low doses, atrazine exposure culminates in impaired development of amphibian antiviral defenses.

1. Introduction

As the result of increasing global prevalence of infections of poikilothermic vertebrates by pathogens belonging to the Ranavirus genus (RV, Iridoviridae) and the alarming spread of RVs such as Frog Virus 3 (FV3) to new hosts, these pathogens are now receiving considerable attention as commercially and ecologically-relevant etiological agents (Bandin and Dopazo, 2011; Chinchar, 2002; Chinchar et al., 2009; Greer et al., 2005; Jancovich et al., 2010). While this recognition has focused scientific attention on amphibian host immunity, it also highlights the growing concerns that other stressors, such as water contamination may increase host susceptibility to disease, thus providing an additional explanation for the emergence of infectious agents such as RVs (Collins and Storfer, 2003).

Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) is a broad-spectrum herbicide that has been globally distributed and has a propensity to reach water sources via agricultural runoffs. The U.S. Environmental Protection Agency (EPA) maintains a maximum contaminant level (MCL) for atrazine at 3 parts-per-billion (ppb; 3 μg/L) in drinking water (Environmental Protection Agency, 2012), but atrazine levels exceed this limit in certain water sources (Environmental Protection Agency, 2013; Shipitalo and Owens, 2003). Moreover, exposure to ecologically relevant atrazine concentrations has been linked to pathological conditions across several vertebrate classes. Such exposure in rodents, for example, affects the population dynamics and function of several immune cell subsets (Filipov et al., 2005; Rooney et al., 2003; Rowe et al., 2006; Zhao et al., 2013). Given their aquatic nature, amphibians are likely to be exposed regularly to any water contaminants and therefore are more susceptible to potential adverse health effects resulting from these exposures. Indeed, atrazine has previously been described as an amphibian endocrine disruptor at concentrations below the MCL (Hayes et al., 2002, 2010; Rooney et al., 2003). These observations raise the question of whether there are other possible physiological consequences of exposure

* Corresponding author. Address: Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642, USA. Tel.: +1 (585) 275 1722; fax: +1 (585) 473 9573.
E-mail address: Jacques_Robert@urmc.rochester.edu (J. Robert).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.dci.2014.06.012
0145-305X/

including alterations to host defense mechanisms. Several studies support this idea. For instance, a 2010 meta-analysis performed in 2010 on 27 studies of amphibian and fish species revealed consistent sub-lethal deleterious effects on immune function (Rohr and McCoy, 2010). Particularly notable were associations between levels of exposure atrazine approaching, at, or below current standards and diminished peritoneal leukocyte number, reduced phagocyte activities, and increased susceptibility to pathogens (Brodkin et al., 2007; Christin et al., 2013; Forson and Storfer, 2006).

In addition to gaps in understanding the direct immunomodulatory effects of atrazine, the long-term implications of exposure to atrazine and many other waterborne environmental contaminants remain unclear. Much of aforementioned work was conducted using immunologically mature animals. While this approach is valuable and informative, several recent studies suggest that exposure to low levels of environmental agents during critical periods of development do not cause obvious immediate effects; rather, they set the stage for altered pathophysiology later in life. Given its strength as an FV3 infection model (Chen and Robert, 2011; De Jesus Andino et al., 2012; Morales et al., 2010), the frog *Xenopus laevis* further lends itself as an ecologically, economically, and physiologically relevant platform with which to study the consequences of atrazine exposure at different developmental periods on aquatic vertebrate antiviral immunity. Thus, in the work reported herein, we tested the hypothesis that tadpole exposure to current environmental levels of atrazine result in long lasting, deleterious effects on the development of amphibian antiviral immunity, thus increasing susceptibility to pathogens such as FV3.

To address the effects of low dose atrazine exposure on amphibian antiviral immune responses, three life stages (tadpoles, metamorphic, and adults) of *X. laevis* were exposed to this herbicide and the capacities of exposed animals to upregulate expression of immuno-relevant genes (TNF-α, Type I IFN, Mx1, IL-1β, IFN-γ, IL-10, CSF-1) following infection with FV3 was assessed.

2. Materials and methods

2.1. Animals

All outbred *X. laevis* tadpoles and adult frogs were acquired from the *X. laevis* research resource for immunology at the University of Rochester (http://www.urmc.rochester.edu/mbi/resources/Xenopus/). For tadpole survival and expression experiments, stage 50 and 56 tadpoles were used, respectively (Nieuwkoop and Faber, 1967). One-year-old frogs were used for all adult experiments. All animals were handled in accordance with stringent laboratory and University Committee on Animal Research regulations (Approval number 100577/2003-151).

2.2. Atrazine

Atrazine (Chem Service Inc.) was dissolved in DMSO to create an initial stock solution from which subsequent working solutions were prepared. A concentration of DMSO equivalent to the highest dose of atrazine was used as a control in all experiments. Fresh atrazine (or DMSO control) was reapplied with each water change for exposures lasting longer than 7 days.

2.3. Frog Virus 3 stocks and infection

Fathead minnow cells (FHM; American Type Culture Collection, ATCC No. CCL-42) used for virus production were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), streptomycin (100 μg/ML), and penicillin (100 U/mL) at 30 °C with 5% CO_2. FV3 was grown using a single passage through FHM cells and was subsequently purified by ultracentrifugation on a 30% sucrose cushion. Plaque assays on a FMH monolayer were used to quantify FV3. For tadpole infections by water bath, tadpoles were exposed to 5×10^6 plaque forming units (PFU)/mL of FV3 in 4 mL of clean water for 1 h. For intraperitoneal (i.p.) infection, tadpoles were injected with 5×10^4 plaque forming units (PFU) of FV3 in 10 μL aliquots of amphibian phosphate buffered saline (APBS). Post-metamorphic froglets were infected by i.p. injection of 1×10^5 PFU in 20 μL, while adult frog infections were performed using 1×10^6 PFU FV3 in 100 μL. For all i.p. infections, uninfected control animals were mock-infected (i.p.) with an equivalent volume of APBS.

2.4. Atrazine exposure and FV3 challenge

Stage 54–56 tadpoles were exposed to 0.0, 0.1, 1.0, 10.0 ppb atrazine in 1.5 L containers; 12 animals per treatment (Fig. 1). Adult frogs were exposed to 0.0, 1.0, 10.0 ppb atrazine in 400 mL of water for 1 week and either injected with APBS (mock-infection; 3 animals/treatment) or infected with FV3 (5 animals/treatment). Six days post infection (dpi), animals were euthanized by overdose of MS-222 and kidneys were extracted. For metamorphosis studies, stage 56 tadpoles were exposed to atrazine (1.0 ppb) atrazine or DMSO control in 1.5 L water until the completion of metamorphosis, at which point the resulting froglets were removed and acclimatized in clean water for 3 weeks before mock or FV3 infection. Animals were euthanized using MS-222 at 6 dpi for kidney extraction (Fig. 1, bottom panel).

2.5. Tadpole survival studies

Two independent FV3 survival experiments were conducted, in which animals were infected by either water bath or i.p. injection. In both experiments, 40–44 stage 50 tadpoles were distributed equally across 4 treatments; 0.0, 0.1, 1.0, 10.0 ppb atrazine in 1.5 L of water. Following one week of exposure, tadpoles were infected with FV3, either via water bath infections (in 4 mL of water containing 5×10^6 PFU/mL FV3 for 1 h) or by i.p. inoculation and moved to 1.5 L of clean water for monitoring. Tadpoles were checked daily; dead animals were immediately removed, frozen, and stored at −20 °C.

2.6. Quantitative gene expression analyses

RNA and DNA were extracted from frog kidneys using Trizol reagent, as per the manufacturer's protocol (Invitrogen). DNA

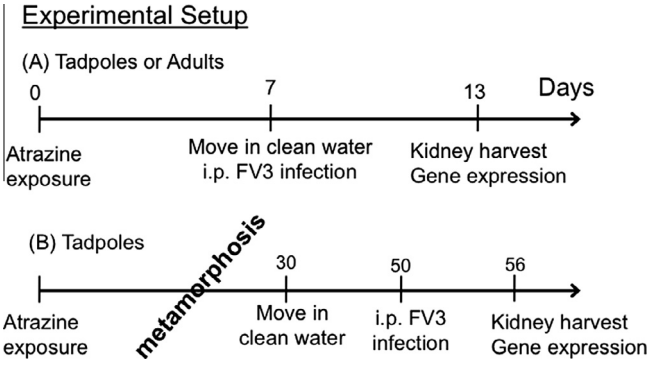

Fig. 1. Schematic of treatment strategy. (A) Acute treatment of tadpoles or adults. (B) Treatment of animals during metamorphosis.