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Generation of a long-lasting, protective, and neutralizing antibody response to the ranavirus FV3 by the frog Xenopus ☆

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Abstract

Xenopus serves as an experimental model to evaluate the contribution of adaptive immunity in host susceptibility to emerging ranaviral diseases that may contribute to amphibian population declines. It has been previously shown that following a secondary infection with the ranavirus frog virus 3 (FV3), adult *Xenopus* more rapidly clear FV3 and generate specific anti-FV3 IgY antibodies. We have further evaluated the potency and persistence of the *Xenopus* antibody response against FV3. Frogs inoculated with FV3 (without adjuvant) up to 15 months after priming produce specific, thymus-dependent anti-FV3 IgY antibodies detectable from 10 days to 8 weeks post-infection. These antisera from boosted frogs are neutralizing in vitro and provide partial passive protection to susceptible larvae when they are injected a few minutes before FV3 inoculation. These results with *Xenopus* suggest that other anuran amphibians are likely to develop effective long-lasting protective humoral immunity after an initial viral exposure.

Keywords: Xenopus laevis; Amphibians; Humoral immunity; Antibody; IgY. FV3

1. Introduction

Ranaviruses (family *Iridoviridae*) infect a wide range of vertebrate species, including fish and both anuran (frogs and toads) and urodele (salamanders) amphibians, and are thought to contribute to the world-wide decline of amphibian populations [rev. in 1]. Frog Virus 3 (FV3), a large (120–200 nm) DNA virus, was first isolated from the North American leopard frog *Rana pipiens*; FV3 or FV3-like viruses are now found in diverse amphibian species throughout the world [1–4]. Indeed, ranaviruses have been causally implicated in several amphibian die-offs world-wide [5,6], and the prevalence of ranaviral diseases affecting amphibian populations' world wide is increasing [1,4,7; http://lsvl.la.asu.edu/irceb/amphibians/]. Whereas several species of amphibians are highly susceptible to ranavirus infections, *Xenopus laevis* is relatively resistant [8]. Specifically, infection of adult outbred *Xenopus* by i.p. injection of FV3 is mildly pathogenic (only ~10–20% of adults infected with 10^7 pfu die within a month) unless the host's immune system has been compromised experimentally by sub-lethal irradiation or CD8 T cell depletion [9]. In contrast, *Xenopus* larvae are highly susceptible [8]. Interestingly, specific anti-FV3 IgY antibody cannot be detected following a primary infection of adults by an

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i.p. injection of FV3, but is easily detectable 2 weeks after secondary injection of virus [8]. The additional fact that virus clearance is accelerated after a repeated injection with FV 3 suggests the occurrence of a secondary (memory) adaptive immune response. Although a critical protective role of CD8 T cells in this secondary response has been demonstrated by anti-CD8 mAb depletion [9], the importance of humoral response in viral elimination needs to be evaluated. In mammals, anti-viral antibodies play an important role in the early phase of infection by neutralizing the virus; they may also play a role at the onset of infection, by promoting antibody-dependent cell-mediated cytotoxicity [rev in 10,11].

The *Xenopus* immune system is fundamentally similar to that of mammals (e.g. rearranging TCR and Ig genes, MHC class I- and class II-restricted T-cell recognition; [12]). In particular, the organization and usage of Ig gene loci with combinatorial joining of V, D, J elements can generate a IgH and IgL repertoire as diverse as mammals. In addition, the Ig switch from IgM to IgY (IgY is the functional equivalent of mammalian IgG), and somatic hypermutations at the Ig locus, two events typical of affinity maturation, do occur during the course of an antibody response in *Xenopus* [13,14]. This Ig switch is thymus-dependent [15], requires T–B collaboration [16] and is likely to involve activation-induced deaminase (AID [17,18]). Despite these similarities, however, the affinity of *Xenopus* IgY antibody against DNP-coupled proteins increases by less than 10 times in contrast to a more than a 10,000-fold affinity increase in mammals [13,14]. Similarly, poor antibody affinity has been found in other amphibians, including *Rana catesbeiana* [19], *R. pipiens* [20,21] and *Bufo marinus* [22,23]. Analysis of V genes expressed upon immunization in *Xenopus* has revealed a strong bias toward alterations at GC pairs by somatic mutation [24]. This suggests an absence of an effective selection mechanism presumably related to the observed absence of germinal centers, and as such, would explain the limitation of affinity maturation [24].

Although molecular and functional studies with model antigens have clearly revealed fundamental differences in the antibodies generated by ectothermic and endothermic vertebrates, they shed little light on the protective potential of humoral responses against natural pathogens in amphibians. This issue taking advantage of FV3 has been explored, and the ability of *Xenopus* to generate long-term B cell memory leading to a potent neutralizing anti-viral antibody response that can provide passive protection to virus-susceptible larvae has been reported.

2. Materials and methods

2.1. Animals, cells and, virus

Two-year-old outbred *Xenopus* (~6 cm length) were obtained from our in-house breeding colony. Randomized groups of frogs were inoculated by i.p. injection of 5×10^7 pfu of FV3 in 300 μl of PBS modified to amphibian osmolarity (APBS). The A6 *Xenopus* fibroblast cell line [25] was maintained in our lab as previously published [26]. FV3 was grown in and purified from A6 cells as previously described [8]. Viral titers were determined using A6 cells by the 50% endpoint dilution method [27].

2.2. Flow cytometry

Fresh splenocytes (5×10^5) were stained with undiluted hybridoma supernatants specific for *Xenopus* MHC class II [28], CD5 (pan T cell, 2B1, [29]), CD8 [28], IgM [30], or isotype-matched isotype controls followed by fluorescein-labeled goat anti-mouse antibody. Stained cells (10,000) were analyzed on a flow cytometer (FACSCalibur, Beckton-Dickinson) and gated on live (propidium iodide-negative) lymphocytes by forward- and side-scatter parameters.

2.3. ELISA

Virus particles (either fresh or heated in boiling water for 1 h to denature virus) diluted in amphibian PBS (10^7 pfu/ml) were adsorbed onto ELISA plates (NUNC-Immuno plate) overnight at 4 °C, followed by diluted anti-sera. Specific (anti-FV3) antibodies were detected by using anti-IgY mAb (11D5, [30]) or anti-IgM (10A9, [30]) supernatants followed by biotin-conjugated rabbit anti-mouse IgG (Sigma Chemicals, #B-8560) and HRP-conjugated streptavidin (Pierce, No. 21126). The reactions were visualized by adding ABTS substrate (Pierce No. 37615), incubating at 37 °C for 20 min and reading at 405 nm in an ELISA plate reader. Total IgY and IgM were measured by adsorbing diluted sera directly to the plate and continuing as above.

2.4. Virus neutralization and passive protection

For neutralization, aliquots of 20 μl FV3 (1×10^3 pfu) that had been pre-incubated, on ice, with different sera for 1 h, were added to A6 cell monolayers cultured in 96-well flat-bottom plates. Each culture well (triplicate for each condition) was evaluated daily by the