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Short communication

Oral immunization of the African clawed frog (*Xenopus laevis*) upregulates the mucosal immunoglobulin IgX

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ABSTRACT

The frog *Xenopus laevis* is a model species for developmental biology but is also of significant interest to comparative immunologists. Amphibians are the oldest group of organisms in which both the B lymphocytes of some species undergo immunoglobulin (Ig) class switch recombination and also have a dedicated mucosal Ig isotype. The purpose of this study was to test the hypothesis that frog IgX would be produced in response to oral immunization. In order to facilitate studies of humoral, and especially mucosal immunity, in this model species, we developed a gavage technique for oral immunization. The result of this oral administration of antigen to frogs was assayed by the induction of the mucosal antibody isotype, IgX, in plasma by enzyme linked immunosorbant assay (ELISA), and a significant IgX upregulation was detected compared to frogs receiving systemic immunization into the coelom. These data are consistent with the view that IgX is the functional analog of mammalian IgA and mandate further studies of the relationship between IgX and IgA. Additionally, the gavage technique should be adaptable for functional studies of gut-associated immunology in other small aquatic vertebrates.

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1. Introduction

The frog *Xenopus laevis*, from an evolutionary standpoint, is the choice model for many studies of the adaptive immune system (reviewed in (Robert and Ohta, 2009) and (Du Pasquier et al., 1989)). Amphibians are the oldest group of animals where the capability of class switch recombination between Ig heavy chain isotypes has been observed (reviewed in (Du Pasquier et al., 2000)), allowing the transfer of a specific antibody response from IgM to other Ig isotypes with different functional abilities. Interestingly, class switch may only be in the anuran frogs and toads and has not been observed in urodele salamanders (Golub and Charlemagne, 1998) (Schaerlinger et al., 2008) (or the legless caecilians).

The most common barrier breached by pathogens of vertebrates is the mucosal surface, which comprises the greatest surface area in the body. In mammals recognition of antigen in these tissues results in B cell switching to secretory (dimeric) IgA, the dominate Ig of mucosal surfaces (Crabbe et al., 1969). However, IgA has not been clearly identified in poikilothermic vertebrates such as frogs, and study of the natural history of secretory mucosal Igs has been neglected despite their importance in host defense and homeostasis (Snoeck et al., 2006).

Other heavy chain isotypes have been described from other vertebrate groups, besides mammalian IgM, IgD, IgG, IgE and IgA. These include IgY, IgF and IgX which frogs

Abbreviations: Ig, immunoglobulin; MS-222, tricaine methane sulfonate; ELISA, enzyme linked immunosorbant assay; i.c., intracoelomic; p.o., per oral; DNP-KLH, dintriphenyl keyhole limpet hemocyanin.

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can express in addition to IgM and IgD (Hsu et al., 1985; Ohta and Flajnik, 2006; Zhao et al., 2006). The expression and function of *Xenopus* IgY is known to be similar to that of IgG (Mussmann et al., 1996b), and phylogenetically IgY is closely related to the ancestor of IgG as well as IgE (Warr et al., 1995). The induction of IgY in *Xenopus* in response to the fungus *Batrachochytrium dendrobatidis* (Ramsey et al., 2010), the lethal infectious disease linked to worldwide amphibian declines (Berger et al., 1998), is similar to IgG responses to this pathogen in mammals. IgX of *X. laevis* is structurally similar to IgM, having four constant domains and forming polymers. Unlike IgM, however, IgX is not associated with the secretory J chain is expressed by plasma cells found in the gut lamina propria (Mussmann et al., 1996a). *Xenopus* IgX is also produced in skin mucus (along with IgM and IgY to lesser extents) in response to infection (Ramsey et al., 2010), consistent with its proposed role as a mucosal isotype. IgT/Z (named T in trout and Z in zebrafish) (Danilova et al., 2005; Hansen et al., 2005) of teleost fish was shown to be a mucosal immunoglobulin. Although no J chain has been found to be associated with IgT either, it is a polymer in gut associated with a secretory component (Zhang et al., 2010). IgT is most similar to IgM in sequence, but no clear relationship to other Ig isotypes has been found, suggesting that it arose after bony fish diverged from other vertebrates. Thus, there appears to be at least two other dedicated mucosal isotypes besides IgA of birds and mammals in vertebrates: IgT in fish and IgX in amphibians.

The relationship between frog IgX and mammalian IgA is not clear. The three abundant antibody classes of *Xenopus* provide a tractable model to study the evolution of humoral and mucosal adaptive immunity in tetrapods. Monoclonal antibodies specific for these frog isotypes of IgM, IgY and IgX (Hsu and Du Pasquier, 1984; Mussmann et al., 1996a) were used to study the systemic humoral immune responses in *X. laevis* after intracoelomic injection with antigen (frogs have no peritoneum). The authors found increases in both IgM and IgY but not IgX (Mussmann et al., 1996a), and noted the need for oral immunization studies to better assess IgX function.

The purpose of this study was to test the hypothesis that frog IgX would be produced in response to oral immunization. This methodology had to be developed for *Xenopus* and the detailed procedure is also described here. We report the first demonstration of upregulation of a mucosal isotype upon oral immunization in amphibians, the oldest vertebrates that employ the Ig heavy chain class switch mechanism.

2. Materials and methods

2.1. Animal husbandry

Outbred *X. laevis* were initially purchased from *Xenopus* Express (Brooksville, FL). Subsequent generations were bred in-house using human chorionic gonadotropin hormone to prime for egg and sperm maturation (Sigma–Aldrich, St. Louis MO). Animals were housed in two XenoPlus (Tecniplast, Buguggiate Italy) recirculating systems. Juvenile and adult frogs were provided a 40% protein sinking pellet diet, while tadpoles were fed a powdered yeast diet (Xenopus Express), but frogs were fasted for 24 h before immunization. The colony was visually monitored daily for symptoms of disease. All frogs in the present study displayed none and were considered pathogen free. The frog room was maintained on a 12:12 h light:dark cycle and the ambient air and system water temperature kept at 21 °C. Frogs in this study were siblings of mixed sex, were not manipulated prior to this study, and the immunization experiment was conducted in winter. Maintenance, anesthesia and recovery tanks were all maintained at the stated temperature, pH of 7.2–7.6 and conductivity of 1200–1600 microsiemens. Each frog bred or used in an experiment was identified with an identification chip (AVID, Norco, CA) inserted intracoelomicly at the time of immunization. Procedures for animal management, reproduction, anesthesia and immunization were performed in accordance to a protocol approved by the Texas A&M Institutional Animal Care and Use Committee (AUP#2008-33).

2.2. Animal sedation and blood collection

Previous literature reported euthanasia dosage in adult frogs weighing 100–125 g (Torreilles et al., 2009). The average size of animal used in this immunization study was 20–30 g. Using MS-222 (Finquel, Argent Laboratories, Redmond, WA), a range of published concentrations was tested to sedate the frogs quickly, yet provide a wide safety margin against any suffering before euthanasia and to maintain unresponsiveness during the immunization (Green, 2009; Wright and Whitaker, 2001). One frog was immersed to test each dose (500 mg/L, 1 g/L, 2 g/L and 5 g/L) and observed for movement and righting reflex at 5 min and 10 min post-immersion. All animals were allowed to revive in a separate shallow recovery tank without complications. The results of this pilot study also assisted in determination of euthanasia concentrations. All frogs euthanized in MS-222 were confirmed dead with a secondary method; pithing or decapitation.

2.3. Gavage technique

The optimal sedation concentration for the post-metamorphosis frogs was 2 g/L of MS-222 buffered solution. At 2 g/L, frogs ranging from 20–30 g were adequately sedated to handle without extensive animal movement which minimized trauma and risk of dropping. Sedation was adequate at 10 min, but not at 5 min duration which was assessed by cessation of movement, especially swimming motion of the rear feet and delayed righting reflex if turned over. At 500 mg/L and 1 g/L, the frogs continued to move 20–30 min after immersion. At 5 g/L, some frogs stopped moving after 2 min and the righting reflex returned after 60 min in the recovery tanks. Based on these observations, 2 g/L was determined to be an adequate euthanasia dose (Supplemental Table 1). This dose was effective for subsequent sedation for oral gavage of frogs ranging from 20 to 30 g.

The optimal method of oral antigen delivery to frogs in the 20–30 g size range was empirically determined as using a 20 gauge 1.5 inch stainless steel feeding needle (Fig. 1A).

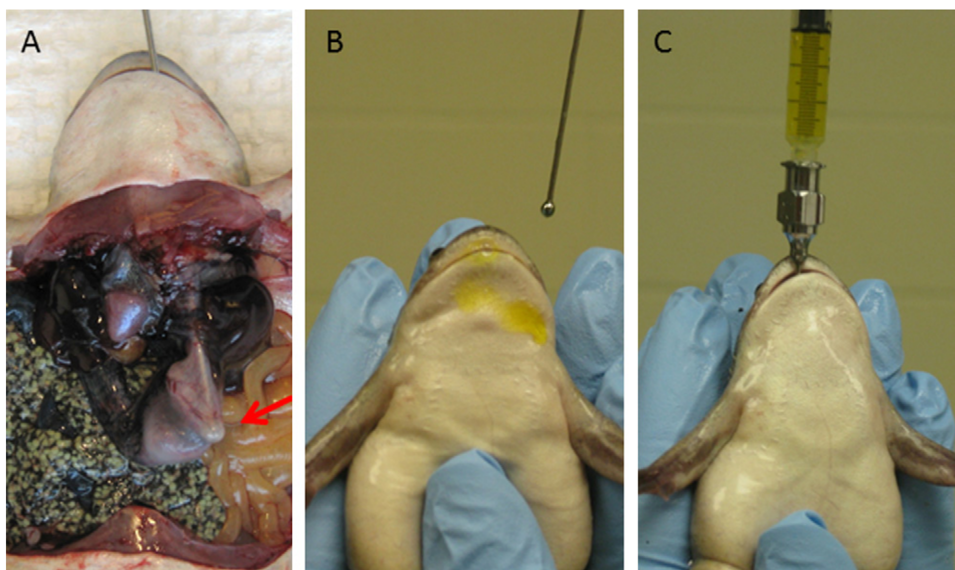

Fig. 1. Optimized oral gavage in *Xenopus laevis*. (A) Euthanized frog used in determination of best placement of 1.5 inch steel feeding needle. The ball of the needle can be seen through the stomach. Red arrow is pointing to the gavage needle terminus. (B) Incomplete anesthesia, improper needle size or material, and failure to properly negotiate pharynx all can result in immunogen leakage and regurgitation, as shown by the yellow inoculant on this frog. (C) Demonstration of proper gavage technique. After teasing jaw open at corner of mouth, needle is moved to the rostrum and gently inserted through pharynx and into the stomach until the hilt touches the frog's mouth. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Although disposable plastic gavage needles were adequate for the testing during necropsy, they were too flexible to be easily guided into the mouth at the commissure of the jaw in live frogs. Longer three inch needles increased the risk of over insertion and stomach perforation. A more heavily sedated frog was ideal for antigen delivery. The jaw closure and swallow reflexes were still intact in sedated frogs immersed in 2 g/L MS-222 for 10 min and antigen was not consistently delivered or maintained in the stomach. Frogs of 20–30 g sedated for 15 min in 2 g/L MS-222 relaxed their jaw and allowed insertion of the feeding needle and proper delivery of antigen. Additionally, these more heavily sedated animals were less likely to regurgitate the immunogen (Fig. 1B), and still had no complications in recovery from the anesthesia.

Using this same rodent technique as a model, a frog was euthanized in 5 g/L of MS-222 and dissected to observe all major organs. A feeding needle was inserted into the mouth and observed to pass into the stomach (Fig. 1A). We tested the technique with stainless steel and plastic feeding needles of various diameters and lengths. A 20 gauge, 1.5 inch (3.81 cm) stainless steel feeding needle (Popper and Sons, New Hyde Park, NY) provided the best method of oral inoculant delivery. During actual immunizations, the frogs were sedated in 2 g/L buffered MS-222. The bulb of the feeding needle was placed at the joint of the upper and lower jaw. Slight pressure was applied to open the mouth then the needle was directed towards the center into the stomach. The full gavage needle was inserted up to the hub for proper delivery into the stomach (Fig. 1C). The frogs received 10 μg of cholera toxin (List Biological Laboratories, Campbell, CA) resuspended in PBS in each oral inoculant.

2.4. Immunizations

Intracoelomic immunization was performed with a one inch 23 gauge needle in the ventral caudal half. The group of immunized frogs contained nine frogs (three orally immunized (p.o.), two intracoelomicly immunized (i.c.) and four non-immunized controls). Intracoelomicly immunized animals received 200 μg of DNP-KLH (dinitophenyl conjugated keyhole limpet hemocyanin, CalBiochem, San Diego, CA) and 200 μl of complete Freund's adjuvant (Rockland Immunochemicals, Gilbertsville, PA) for the first inoculation and 200 μg of antigen with the same volume of incomplete Freund's adjuvant (Rockland Immunochemicals) for the remaining boost inoculations. To prepare the emulsion for i.c. injection, two 1 ml syringes and one 18 gauge double hub emulsifying needle were used. Initial immunizations were followed by boosts at one week intervals and sacrifice two weeks after last boost (four weeks after initial administration of antigen).

2.5. ELISA

Serum was separated from whole blood collected from frogs. Using a 96 "U" well microtiter plate (Becton Dickenson, Franklin Lakes, NJ), 100 μl of tenfold serially diluted (1:10–1:10^{11}) frog sera of one frog was placed in each row. The plate with sera was incubated at 37 °C for 1 h. The contents of the plate were dumped, washed twice with a-PBS, blotted dry and blocked with 2% casein overnight at 4 °C. Anti-*Xenopus* Ig hybridoma supernatants (kind gifts from Louis Du Pasquier and Martin Flajnik) and their properties have been described. Antibodies 4110D5,

4110B3 and 408A10 are directed against IgX (Hsu and Du Pasquier, 1984). The next day the plate was washed with PBS and 100 μl of mouse anti-frog IgX hybridoma supernatant at 1:100 dilution was added to the wells. The plate was incubated overnight at 4 °C. ELISA controls that contained no primary (mouse anti-frog IgX, 408A10) or no secondary antibodies were selected from the immunized group. 100 μl of sheep anti-mouse IgG conjugated to horseradish peroxidase (Sigma–Aldrich) was added after washing with PBS. After incubation for an hour at room temperature, the plate was washed twice with a-PBS, and blotted to remove residual liquid. A substrate solution of 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma–Aldrich) was made by dissolving one tablet in one ml of dimethyl sulfoxide and adding 9 ml of 0.05 M phosphate-citrate buffer, pH 5.0 (Sigma–Aldrich). Two μl of 30% hydrogen peroxide (EMD Chemicals, USA) per 10 ml of substrate buffer solution was added immediately prior to use. The reaction was stopped by the addition of 100 μl of 2 M sulfuric acid (VWR, West Chester PA, 13.9 ml of 18 M stock sulfuric acid solution was diluted in 86 ml of de-ionized water to prepare a 2 M solution) per well. Absorption was detected at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad, Berkeley, CA).

2.6. Statistical analysis

The OD_{450} values of ELISA for total serum IgX were averaged and the values were compared by the unpaired *t*-test. A *P* value of ≤ 0.05 was considered to be statistically significant. Error bars in Fig. 2 represent standard error.

3. Results and discussion

The frog oral gavage method was used in an immunization experiment to assess IgX response to mucosal immunization (p.o.) compared to systemic (i.c. delivery). Cholera toxin was used for mucosal immunization (Sanchez and Holmgren, 2011). Serum from individual animals was assayed by ELISA and the values obtained were averaged for each experimental set. Results from immunized frogs were compared to those of control frogs that received neither treatment. ELISA for total IgX in serum revealed no significant changes in the levels of this isotype in blood between i.c. and control animals, but oral immunization resulted in a significant increase of IgX compared to control animals at the 1/100 dilution of serum ($p = 0.0335$) (Fig. 2). Additionally, at the 1/1000 serum dilution IgX levels in the p.o. set were significantly higher than those receiving i.c. immunization ($p = 0.0277$). Together these data show IgX induction in response to oral immunization.In order to explore adaptive immunity in an ancestral model of Ig class switch recombination, we developed a gavage technique for oral antigen delivery in the anuran amphibian *X. laevis*. A gavage method has been reported for urodele newts, although used to deliver food into their stomachs under microgravity, not oral administration of defined antigen (Boxio et al., 2005). This new method has allowed for the testing of the hypothesis that oral immunization will induce a response of a dedicated mucosal antibody isotype. IgX is suspected to be a functional analog of mammalian IgA in amphibians. Upon gavage immunization, IgX levels measured by serum ELISA were significantly higher than those in nonimmunized frogs, and as compared to those receiving intracoelomic antigen delivery. The finding of IgX induction upon oral immunization is consistent with this isotype functioning as a mucosal Ig like mammalian IgA. The gavage technique employed here should be adaptable for application in immunology studies of other similarly sized aquatic vertebrates, especially those whose buccal cavity and pharynx have evolved to employ inertial suction feeding. This is common in teleost fish, aquatic turtles and salamanders, pipid frogs such as *Xenopus* combine this technique with forelimb scooping (Carreno and Nishikawa, 2010).

Using a straight 20 gauge 1.5 inch stainless feeding needle provided the best delivery method for gavage of 20–40 g *X. laevis*. Emesis behavior is also a common sign of illness in amphibians, especially frogs and toads (Naitoh et al., 2000). Importantly, frogs sedated for 15 min in 2 g/L MS-222 recovered without complications or regurgitation of stomach contents. Problems reported in gavage of small mammals include perforation of the esophagus and gavage into the trachea or lungs. Sequelae of these events include sepsis or even death (Atcha et al., 2010). There were no such adverse effects observed in *Xenopus* from the optimized gavage method described here. In honing this method in aquatic species, it is helpful to have the immunogen colored to easily recognize regurgitation or inadequate delivery, either immediately or in the recovery tank. This can be achieved with simple food coloring, although we used DNP-KLH that was bright yellow when resuspended in PBS (Fig. 2).

The results of the first trial of oral immunization in cold blooded vertebrates, supports the hypothesis that IgX, a proposed analog of IgA, would be specifically produced with oral immunization compared to systemic immunization or non-immunized controls. This is the first evidence that oral immunization elicits an IgX response, adding to the characteristics shared by anuran IgX and IgA of birds and mammals. It is important to note that in a urodele amphibian (the Mexican axolotl, *Ambystoma mexicanum*) IgX has been found to have high expression in the intestine (Schaerlinger and Frippiat, 2008), yet IgY expression co-localizing with the secretory component has been seen there as well (Fellah et al., 1992). Another urodele (the Iberian ribbed newt, *Pleurodeles waltl*) has yielded an isotype distinct from IgM, IgY or IgX that is expressed early in development (Schaerlinger et al., 2008). Additionally, an isotype has been identified in a reptile that shows identity with IgM, IgX, IgY and IgA (Deza et al., 2007). Oral immunization trials should shed light on the functions of these isotypes in these species, and with the wealth of comparative immunogenetic data now available from diverse vertebrates, the phylogenetic relationship between amphibian IgX and IgA should be studied through amphibians, reptiles, birds, and non-placental mammals.

Additional future work will employ the gavage method to interrogate the effect of route of immunization on other isotypes in addition to IgX in larger cohorts, as well as with and without T cell help via the *Xenopus* larval thymectomy model (Horton and Manning, 1972). Analysis of the

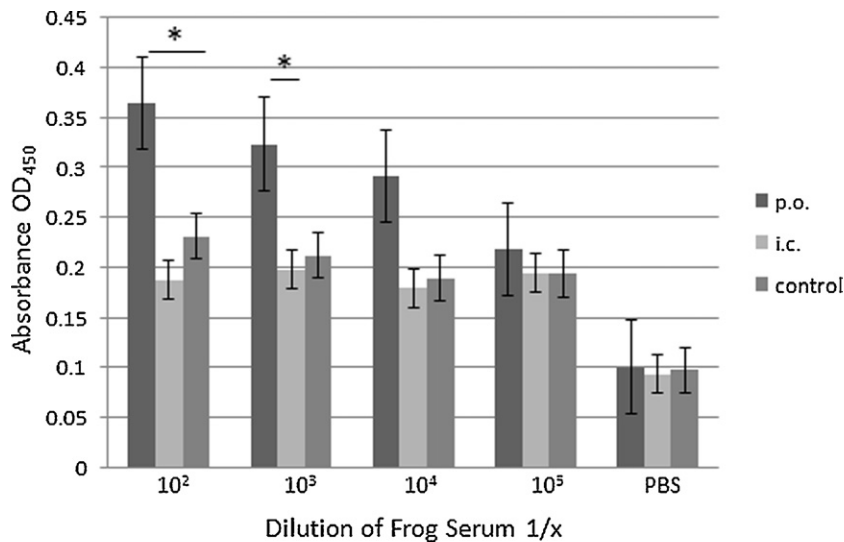

Fig. 2. IgX induction by oral immunization. ELISA for total IgX in frog serum was measured by absorbance at an optical density of 450 nm. Results from several dilutions of frog serum show a titratable effect of the IgX serum concentration in the ELISA. The far right bars are assay controls where PBS instead of frog serum is bound to plate before primary and secondary antibodies. Shaded bars represent orally immunized (p.o.), intracoelomicly immunized (i.c.) or non-immunized control frogs. Asterisks mark statistically significant ($p \leq 0.05$) comparisons between points linked by bars.

isotypes made by B lymphocytes harvested from distinct anatomic sites such as gut, gill and spleen will shed more light on the natural history of anatomic antibody specialization. The apparent lack of class switch but presence of somatic hypermutation in urodeles but presence of both mechanisms in anurans should be explored in the mucosal compartment. IgX has been reported to be a thymus independent isotype (Turner and Manning, 1974). But even if T cell help was found to be required for some IgX responses, the physiological relationship to IgA will not be simple to connect. Mammalian IgA has been found to be generated in pathways other than through Peyer's patches and mesenteric lymph nodes (Cerutti and Rescigno, 2008) and can occur independent of T cell help in responses to gut bacteria (Macpherson et al., 2000).

We hope that this study serves as a springboard for more work exploring the interface of mucosal immunity, class switch recombination to distinct functional antibody isotypes, and the natural history of adaptive immunity. Additionally, oral vaccination is an underexplored area of research in aquaculture. IgT has given fish a new tool and diagnostic in vaccine development (reviewed in Zhang, Salinas and Sunyer, in press at *FSI*), perhaps IgX could do likewise for amphibians.

Conflict of interest statement

The authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetimm.2011.10.019.

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Frog ID	Weight (g)	Concentration (g/L)	Time to sedation (min)
1	20	0.5	25
2	28	1	> 30
3	22	2	10
4	26	5	2

Supplemental Table 1. Anesthesia dose determination pilot study.