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Immunological responses and protection in Chinese giant salamander *Andrias davidianus* immunized with inactivated iridovirus

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ABSTRACT

Chinese giant salamander hemorrhage is a newly emerged infectious disease in China and has caused huge economic losses. The causative pathogen has been identified as the giant salamander iridovirus (GSIV). In this study, the immunological responses and protection in Chinese giant salamander immunized with β-propiolactone inactivated GSIV are reported. Red and white blood cell counting and classification, phagocytic activity, neutralizing antibody titration, immune-related gene expression and determination of the relative percent survival were evaluated after vaccination. The red and white blood cell counts showed that the numbers of erythrocytes and leukocytes in the peripheral blood of immunized Chinese giant salamanders increased significantly on days 4 and 7 post-injection ($P < 0.01$). Additionally, the differential leukocyte count of monocytes and neutrophils were significantly different compared to the control group ($P < 0.01$); the percentage of lymphocytes was $70.45 \pm 7.52\%$ at day 21. The phagocytic percentage and phagocytic index was $38.78 \pm 4.33\%$ and 3.75 ± 0.52, respectively, at day 4 post-immunization which were both significantly different compared to the control group ($P < 0.01$). The serum neutralizing antibody titer increased at day 14 post-immunization and reached the highest titer (341 ± 9.52) at day 21. The quantitative PCR analysis revealed that the immunization significantly up-regulated the expression of immune related genes TLR-9 and MyD88 the first two weeks after immunization. The challenge test conducted at day 30 post-injection demonstrated that the immunized group produced a relative survival of 72%. These results indicate that the inactivated GSIV could elicit significant non-specific and specific immunological responses in Chinese giant salamander that resulted in significant protection against GSIV induced disease.

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1. Introduction

Chinese giant salamander (*Andrias davidianus*), belonging to the family *Cryptobranchidae*, is the biggest amphibian species in the world and one of the protected animals in China listed in Annex I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and in class II of the national protected animals in China (Morescalchi et al., 1977; Zhang et al., 2002). In the last decade, the artificial breeding and culture of this animal has developed significantly and has become an important industry with great economic value in China. However, with the increase of intensive culture of this species, the prevalence of infectious diseases, especially viral diseases, has become a great concern (Ariel et al., 2009).

Iridoviruses have been identified as one of the most important pathogens in many amphibians and reptiles. These pathogens are considered one of the major causes in the decline of the amphibian and reptile resources (Daszak et al., 1999). As an emerging pathogen affecting the Chinese giant salamander, an iridovirus associated with morbidity and mortality was first reported in farmed Chinese giant salamanders in 2011 (Dong et al., 2011; Geng et al., 2011). The clinical signs of virus infected giant salamanders included skin hemorrhage, limb swelling and ulceration, and internal organ hemorrhaging and necrosis. The mortality associated with this disease can reach 90–100% (Dong et al., 2011; Geng et al., 2011). Jiang et al. (2011) investigated cell lines for virus replication and biological characterization. In November 2010, an iridovirus was isolated from a diseased giant salamander in Hubei Province. This iridovirus was identified as the causative agent of Chinese giant salamander hemorrhage disease based on the results of experimental infection, electron microscopy, cell culture, histopathology and molecular epidemiological analysis (Meng et al., 2014).

Vaccination has been proven to be an effective method in fish for preventing diseases. To date, many vaccines for fish have been developed and are widely used for the reducing diseases in aquaculture (Corbeil et al., 2000; Kai and Chi, 2008; Kibenge et al., 2012). There have been several reports that examined inactivated iridovirus vaccines in the laboratory or in field trials. Ou-yang (Ou-yang et al., 2012) reported that a whole, inactivated iridovirus vaccine could stimulate an immune response in fish and observed a good protective effect against the Singapore grouper iridovirus (SGIV) under laboratory conditions. Nakajima et al. (1999) and Dong et al. (2013) reported on the effectiveness of an inactivated iridovirus vaccine in protecting fish against the red seabream iridovirus (RSIV) in field trials. Currently, there are no effective treatment methods or vaccines available for the control of the Chinese giant salamander hemorrhage disease which initiated this investigation.

In this study, the immunological responses and protection in Chinese giant salamander that were immunized with inactivated GSIV were evaluated. The results showed that the inactivated GSIV vaccine could induce significant non-specific and specific immunological responses in Chinese giant salamander, resulting in significant protection against Chinese giant salamander hemorrhage disease. The results of this study provide a basis for the development of prevention and control strategies of giant salamander hemorrhage disease for the future.

2. Materials and methods

2.1. Animals

Healthy Chinese giant salamander (mean weight, 70 g) were obtained from the experimental farm of the Yangtze River Fisheries Research Institute, which has no record of Chinese giant salamander hemorrhage disease. The animals were maintained at 18–20 °C in tanks and fed daily with diced meat of bighead carp for two weeks before the experiment was started.

2.2. Cell line, virus and bacterial strain

The epithelioma papilloma cyprini (EPC) cell line was provided by the China Center for Type Culture Collection (CCTCC), Wuhan University and grown at 25°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The GSIV was originally isolated and identified from diseased Chinese giant salamanders by our laboratory and this isolate was used in these studies (Meng et al., 2014). *Staphylococcus aureus* was cultured in Luria broth at 37°C and inactivated with 0.4% (V/V) formalin (F-SA) for the phagocytic activity assay.

2.3. Inactivation of GSIV

The GSIV was inoculated onto confluent EPC monolayer at a multiplicity of infection (MOI) of 0.05 and the cytopathic effect (CPE) was checked daily by microscopy. When the CPE reached about 70–80%, virus was harvested and freeze-thawed three times before centrifugation at $2500 \times g$ for 25 min at 4°C. The supernatant was collected and stored at −80°C and used for inactivating the virus and for the challenge study. The virus was diluted in a 10-fold series in DMEM without FBS and the titer ($TCID_{50}\,ml^{-1}$) was determined according to the method of Reed and Muench (1938). The inactivation of GSIV was conducted with β-propiolactone (BPL) as described previously (Sun et al., 2013). Briefly, GSIV was treated with BPL at a final concentration of 0.1% (V/V) and incubated at 4°C for 72 h. The residual BPL was hydrolyzed at 37°C for 2 h. The inactivated vaccine was tested for safety by cell culture assays and in fish.

2.4. Immunization and collection of blood samples

The healthy Chinese giant salamanders were randomly assigned into two groups and each group was composed of 60 animals. The animals in the immunized group were inoculated intraperitoneally with 0.5 ml of inactivated GSIV at a dose of $1 \times 10^6\,TCID_{50}\,ml^{-1}$ while the control group received the same volume of Dulbecco's phosphate buffered saline (DPBS, Sigma, USA). The experimental animals were maintained in tanks at 20°C and fed with