Fish & Shellfish Immunology 30 (2011) 1152–1158

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Elicited cross-protection and specific antibodies in Mozambique tilapia (*Oreochromis mossambicus*) against two different immobilization serotypes of *Cryptocaryon irritans* isolated in Hawaii

Ichiro Misumi [a,*], Teresa D. Lewis [a], Akihiro Takemura [b], Jo-Ann C. Leong [a]

[a] Hawai'i Institute of Marine Biology, School of Ocean & Earth Science & Technology, University of Hawai'i, PO Box 1346, Kāne'ohe, Hawai'i 96744, USA
[b] Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Motobu, Okinawa 905-0227, Japan

ARTICLE INFO

Article history:
Received 28 February 2009
Received in revised form
1 March 2011
Accepted 1 March 2011
Available online 6 March 2011

Keywords:
Cryptocaryon irritans
Oreochromis mossambicus
Serotype
Cross-protection
Immobilization

ABSTRACT

The objective of this study was to determine whether immunization of Mozambique tilapia with different *Cryptocaryon irritans* i-antigen serotypes elicited cross-protection against challenge infection by both serotypes. Fish were directly exposed to live theronts of isolate W1 or isolate K1, that express different surface i-antigens. There was no significant difference in the number of trophonts infecting the fish between the two isolates, W1 and K1, following primary exposure. Serum from immunized fish exposed to live theronts showed higher immobilization titres and ELISA values against homologous isolates than to heterologous isolates after the primary exposure. However, mucus antibody did not immobilize theronts although the ELISA results clearly indicated that mucus antibodies recognizing *C. irritans* were generated. In a study with Western blot analyses, serum antibodies recognized only an antigen of the corresponding serotype and no proteins common to both serotypes were identified. Sequence analyses of 754 bases of rDNA nucleotide sequence including complete nuclear ribosomal ITS-1 –5.8S rDNA–ITS-2 region were conducted and found to be identical for W1- and K1-isolates. These findings confirmed that both isolates were members of the species, *C. irritans*, and that rDNA analysis would not distinguish the two isolates. In conclusion, despite the fact that the immobilization assays and ELISA detected two serotypes *in vitro*, challenge assays provided evidence for only one type of *C. irritans*.

1. Introduction

The parasitic ciliate, *Cryptocaryon irritans*, infects many species of marine fish and causes significant losses in the aquaculture industry [1]. It can be found in temperate and tropical regions worldwide [2,3] and thus, can be a major roadblock for marine aquaculture development. Intraspecific variations of *C. irritans* in gene sequence, morphology, and development have been reported [3–6]. However, there is little information on serotypic variations between isolates of *C. irritans*.

The life cycle of *C. irritans* is similar to that of *Ichthyophthirius multifiliis*, a parasite belonging to a taxonomically different phylum [7,8]. *I. multifiliis* also has a direct life cycle consisting of an infective theront, a parasitic trophont and a reproductive tomont [9]. The mechanism of immunization against *C. irritans* is also similar to that of *I. multifiliis* [10]. *I. multifiliis* expresses abundant glycosyl phosphatidylinositol (GPI)-anchored membrane proteins referred to as immobilization antigens (i-antigens) [11,12]. Different serotypes of *I. multifiliis* have been identified using antibody-specific immobilization assays [13]. Intraperitoneal (IP) injection with purified i-antigens from *I. multifiliis* elicited serotype-specific protection and serotype-specific antibodies that immobilized live theronts *in vitro* [14,15]. Thus, it is clear that i-antigens themselves have a direct role in eliciting protective immunity in fish and can be the principal target antigens for vaccine development. However, when fish were immunized with live *I. multifiliis* theronts by either IP injection or direct exposure, these fish were conferred cross-serotype protection despite development of immobilization antibodies against the corresponding serotype [16,17]. These results strongly indicate the existence of other antigens which are common to all *I. multifiliis* and can elicit cross-serotype protection [17].

Previous studies have also shown that fish surviving an infection of *C. irritans* were protected against reinfection [18–20]. The mechanism of this protection appears to be mediated by serum and mucosal antibodies that recognize and immobilize *C. irritans*

* Corresponding author.
E-mail address: imisumi@hawaii.edu (I. Misumi).

1050-4648/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2011.03.004

[19–22]. Hatanaka et al. [22] isolated the integral membrane protein expressed on the surface of theronts, and reported evidence that this membrane protein may be an i-antigen. A more recent study by this same group has shown that serotypic variations based on immobilizations assays exist among *C. irritans* isolates [23]. However, no information was provided that confirmed serotypic differences based on challenge infection assays.

We report here the identification of two serotypes of *C. irritans* by immobilization assays in tilapia in Hawai'i. Both isolates were confirmed as *C. irritans* by rDNA sequence analysis. We also confirmed the identification of the two serotypes by fish challenge experiments. Fish were immunized by direct exposure to either of the two parasite serotypes and challenged with homologous or heterologous parasite serotype. Immobilization of the parasite and the specific antibody level were assessed for the fish serum and cutaneous mucus. The objective of this study was to determine whether the immunization by the different parasite serotypes elicited the production of serotype-specific immobilizing antibodies and cross-protective immune protection.

2. Materials and methods

2.1. Fish

All fish were tagged with T-bar anchor tags inserted at the base of the dorsal fin and kept in 60-L glass aquaria that received aeration and flow-through seawater (120 L/h). All seawater was continuously pumped in from Kaneohe Bay and filtered with 5 μm filters and treated with UV before use in the fish aquaria. The water temperature was 27 ± 1 °C, and the salinity of the seawater was 34‰. Brood stocks of Mozambique tilapia (*Oreochromis mossambicus*) raised in fresh water were provided by Dr. Gordon Grau at the Hawaii Institute of Marine Biology, University of Hawaii. A brace of the brood stock was initially kept in fresh tap water in the SEA lab at HIMB for 2 days and acclimated to seawater by gradually increasing the salinity up to 34‰ throughout 2 weeks. All fish were fed approximately 2% of the body weight per day once daily with Silver Cup Trout Chow (Nelson and Sons, Murray, UT). All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

2.2. Parasite isolation and culture

Two different isolates of *C. irritans* were used in this study. The first isolate, designated W1, was obtained from an infected Mozambique tilapia kept for two weeks with coral sand collected from a display tank at the Waikiki Aquarium (Honolulu, Hawaii). The second isolate, designated K1, was obtained from an infected Mozambique tilapia kept for one week with bottom deposits collected from a flow-through circular fish tank at the Hawai'i Institute of Marine Biology, Coconut Island (Kaneohe, Hawaii). Both isolates were obtained in April, 2008 and passaged separately and continuously on naïve Mozambique tilapia in UV treated and filtered seawater at 27 ± 1 °C in 60-L fish aquaria. A naïve tilapia was added and replaced twice a month for propagation of tomonts in the aquarium. The health of the fish was observed daily and any dead fish or fish with lesions were removed from the tanks. Protomonts were collected from heavily infected fish with 1000 μl micropipettor under a stereo-microscope (American Optical, Model 41). One hundred protomonts were collected and washed four times and placed in sterile 120 ml specimen collection containers (Starplex Scientific, Ontario, Canada) with filtered (0.2 μm) seawater and supplemented with 100 I.U. ml^{-1} Penicillin G potassium and 100 μg/ml streptomycin sulphate [24]. After confirmation of encystment of the tomont, cultures were incubated for 2–3 weeks at 22–23 °C. Newly hatched theronts from the culture were used for DNA extraction, immobilization assay, and future propagation, and the remaining theronts were stored in sterile phosphate-buffered saline (PBS) at −80 °C until processing.

2.3. Experimental design

A total of 36 tilapia (mean weight 97.0 ± 2.5 g/length 16.7 ± 0.15 cm) were used for this study. They were randomly divided into two challenge groups. Each challenge of this study was run in triplicate (6 fish per tank). Each challenge group was exposed to either W1 or K1 strain for the primary exposure. Newly hatched theronts in concentrations of 20,000 theronts per fish were transferred to each aquarium. Two days after the initial exposure, the number of infected trophonts on the left pectoral fin was counted under an inverted microscope (Olympus IX; Olympus Optical, Tokyo) in order to measure the infection level for each fish. All fish were transferred into new, clean tanks every 5 days to avoid accidental reinfection by *C. irritans*. Fifteen days after primary exposure, each challenge group was subdivided into two subgroups. One subgroup from each challenge group was mixed together in one tank. These mixed groups were subsequently exposed to W1 or K1 strain (20,000 theronts per fish) for the secondary challenge study. As a result, depending on the order of exposures, 4 different challenge groups were formed (designated as fish exposed to W1W1, W1K1, K1W1, and K1K1), comprising 9 individuals each. Two days after the secondary exposure, the number of infected trophonts on the right pectoral fin was counted. And again, all fish were transferred into new clean tanks every 5 days to avoid accidental reinfection by *C. irritans*.

2.4. Sampling mucus and serum

Cutaneous mucus and blood were collected three times total throughout this study from all individual tilapia 2 days prior to initial and secondary theront exposures and 14 days after the secondary exposure. Fish were sampled at random from each challenge group and were anaesthetized by 0.2 ppm of tricaine methanesulfonate (MS 222: Finquel, Redmond, WA).Mucus was sampled from the anaesthetized fish by lightly swabbing one side of the fish 10 times from head to tail except fins with a sterile cotton swab. The cotton part of the swab was cut and placed in a 1.5 ml microcentrifuge tube containing 100 μl of PBS. The tube was vigorously vortexed for 2 min to elute the mucus, and excess liquid was removed from the swab by pressing against the side of the tube. The resulting liquid was centrifuged at 1000 g for 10 min, and the collected supernatant was distributed into aliquots of 20 μl in 200 μl microcentrifuge tubes and stored at −20 °C. Following mucus sampling, blood (200 μl) was withdrawn from caudal vein of the anaesthetized fish using a 1 ml syringe with a 23 gauge needle and transferred into a 1.5 μl microcentrifuge tube. Blood samples were allowed to clot overnight at 4 °C. Serum was obtained by centrifugation of blood at 1000 g for 10 min. Serum samples were distributed in aliquots of 20 μl in 200 μl microcentrifuge tubes and stored at −20 °C for later analysis. In this study, the serum and mucus collected from 4 different challenge groups (W1W1, W1K1, K1W1, and K1K1) following the secondary exposures were designated as W1W1-, W1K1-, K1W1-, and K1K1-antiserum or mucus antibody.

2.5. Immobilization assay

Blood serum and cutaneous mucus from individual fish were tested for immobilizing antibodies against both isolates W1 and K1.