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NOTE

Temperature-dependent protection against *Ichthyophthirius multifiliis* following immunisation of rainbow trout using live theronts

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ABSTRACT: Rainbow trout *Oncorhynchus mykiss* Walbaum, 1792 fingerlings were vaccinated by intraperitoneal (i.p.) injection using live theronts of the skin parasitic ciliate *Ichthyophthirius multifiliis* Fouquet, 1876 at 2 temperatures (12 and 20°C), and protection against challenge infections was subsequently evaluated by bath exposure to live theronts. Vaccination conferred a relative protection (evaluated as the decrease in the number of established theronts) at 12°C and almost complete immunity at 20°C. Significantly increased immobilisation titers (using plasma immobilisation of live theronts) were found in immunised fish at Week 2 and 4 post-vaccination. Lysozyme activity of plasma from vaccinated fish increased from Week 1 to 4. Both immobilisation titers and lysozyme activity were significantly higher at 20°C. This study demonstrated that live theronts are good candidates for an antigen source for development of effective vaccines against white spot disease in this fish host, and further indicated that the protection of rainbow trout against *I. multifiliis* infection is highly temperature dependent and may be associated with both adaptive and innate response mechanisms.

KEY WORDS: Immune response · Vaccination · Fish · Parasites

INTRODUCTION

The skin parasitic ciliate *Ichthyophthirius multifiliis* Fouquet, 1876 is known to induce protective immunity in a range of freshwater fishes following sub-lethal natural infections (Buschkiel 1910, Bauer 1953, Hines & Spira 1974, Goven et al. 1980, Wahli & Meier 1985, McCallum 1986, Clark et al. 1988, Houghton & Matthews 1990, Cross & Matthews 1993, Gleeson et al. 2000, Sigh & Buchmann 2001, Matthews 2005). It is therefore appropriate to pursue the development of vaccines against this pathogen. Some previous studies indicated that injections with antigens from this parasite may induce immunity in various fish species (Clark et al. 1988, Ling et al. 1993, Wang et al. 2002). In these reports, the membrane-bound immobilisation antigen (i-A) was suggested to be crucial to the induction of immunity, and experimental vaccines based on recombinant i-A were shown to confer some protection to goldfish (He et al. 1997). Specific antibody production against this antigen in protected hosts was proposed to account for immunity (Clark et al. 1988, Xu et al. 2002). However, immunisation experiments with rainbow trout *Oncorhynchus mykiss* Walbaum, 1792 using various preparations of killed parasites were only able demonstrate limited protection (Dalgaard et al. 2002). Likewise, DNA-vaccination of trout using genes encoding i-A was shown to be non-protective, even though specific antibody production was induced against i-A in the host (Sigh 2004). Further, some studies point to the fact

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that innate factors such as lectins (Xu et al. 2001), complement (Sigh & Buchmann 2001) and leukocytes (Graves et al. 1985, Cross & Matthews 1993, Sin et al. 1996) could play a protective role. This is further substantiated by the finding that the susceptibility of naïve fish varies considerably (Gleeson et al. 2000).

We initiated studies in order to elucidate if other types of antigen preparations could confer immunity to rainbow trout against *Ichthyophthirius multifiliis*. Burkhart et al. (1990) previously demonstrated that injections of live theronts could induce immunity in channel catfish, and that this antigen preparation was superior to preparations of killed parasite stages. In the present study we report on protective immunity induced in rainbow trout by intraperitoneal (i.p.) injection of live theronts. Experiments were conducted at 2 temperatures (12 and 20°C) with the aim of elucidating temperature-dependent reactions against the parasite preparation. Immune parameters such as plasma immobilisation of theronts (indicative of the presence of specific antibodies; Sigh & Buchmann 2001) and lysozyme activity (indicative of innate immune reactions; Iwama & Nakanishi 1996) were determined in order to elucidate possible immune mechanisms activated by the treatment.

MATERIALS AND METHODS

Fish. Eyed rainbow trout *Oncorhynchus mykiss* eggs from the Danish fish farm Fousing (western Denmark, Jutland) were disinfected by iodophore (Actomar K30) bathing and brought to the Danish Center for Wild Salmon in Randers (Jutland), where they were hatched and reared to the age of 5 mo in a pathogen-free fish-culture recirculation system. Mean body weight of fish was 10.0 g (± 1.9 SD) (subsample of 20 fish). Fish were then transferred to the experimental recirculation system and acclimated for 30 d at 12 and 20°C before experimentation. Fish were fed pelleted dry feed (Biomar) (1% of the biomass daily).

Parasites. A laboratory stock of *Ichthyophthirius multifiliis* was isolated in a Danish trout farm (Refsgaard, Jutland) and maintained in culture at 12 to 13°C in fish tanks with susceptible and continuously replenished rainbow trout for 5 yr before experimentation.

Water. Municipal water from Frederiksberg County was used to fill fish tanks. Concentrations of ammonia, nitrite and nitrate were recorded by Aquaquant test kits (Merck) and were kept at a minimum by internal biofilters (Eheim) and water replenishment (50% twice a week).

Vaccine preparation. Theronts were isolated from tomocysts, produced as previously described by Buchmann et al. (1999). They were concentrated by centrifugation and adjusted to a concentration of 8000 theronts ml^{-1}. Each fish was injected intraperitoneally by 0.1 ml suspension (containing 800 theronts). Control fish were injected with sterile water containing BSA in a concentration corresponding to the protein content in 800 theronts (2 µg). No adjuvant was used.

Experimental design. Triplicate experiments were conducted in thermostat-controlled rooms at 12 and 20°C. Groups of 34 fish (3 groups at 12°C and 3 groups at 20°C, total of 204 fish) were anaesthetised with 80 mg l^{-1} MS222 (Sigma-Aldrich) and injected with either live theronts or BSA solution. Fish were then kept in triplicate fish tanks and blood was sampled at Day 0, 1, 7, 14 and 28. Four fish were included in each sample from each group.

Blood sampling. Blood of anaesthetised (80 mg l^{-1} MS222) fish was sampled by caudal vein puncture using heparinised syringes (1 ml). Blood was centrifuged (5000 × *g*) and the supernatant (plasma) recovered and frozen at –80°C.

Challenge infections. On Day 28 post-vaccination, fish were exposed to live theronts (2000 theronts per fish, isolated and concentrated as described above) at 12 and 20°C in 20 l aquaria for 12 h. Development of trophonts in fins and skin was then recorded on Day 4, 6, 7 and 10 post-challenge. The development time of trophonts in skin is longer at 12°C than at 20°C (Aihua & Buchmann 2001); therefore, visible skin trophonts were counted at Day 6 and 10 at 12°C and at Day 4 and 7 at 20°C. Fish were subjected to mild anaesthesia (30 to 50 mg l^{-1} MS222) and established trophonts in the fins and skin were counted under a dissecting microscope (6 to 50× magnification).

Immobilisation assays. Half of the plasma samples were heat-inactivated at 44°C for 20 min according to Sakai (1992) and used for immobilisation assays. These experiments were performed on glass plates with 2-fold dilutions (in phosphate-buffered saline [PBS]) of plasma samples. Volumes of 100 µl plasma and 30 theronts were used in each reaction. The number of moving, immobilised (non-moving cells with active cilia) and dead theronts (non-moving cells without active cilia) were then recorded every 30 min for 2 h. Immobilisation of 50% of the theronts was recorded as a positive reaction. The immobilisation titer was expressed as the reciprocal value of the highest dilution that produced immobilisation.

Lysozyme detection. Lysozyme activity was detected using the method of Lie et al. (1986). Briefly, 15 ml 1% agarose solution prepared with 0.1 M phosphate buffer (pH 6.6) containing *Micrococcus lysodeikticus* (50 µg l^{-1}) was poured onto a 10 × 10 cm glass plate and allowed to solidify. Wells (diameter 4 mm) were punched into the agarose layer and reference samples of chicken lysozyme (Sigma-Aldrich) with known

lysozyme activity (expressed in international units) was applied together with plasma-samples (native, diluted to a ratio of 1:10 in PBS) from rainbow trout. Samples on the agarose-covered glass plates were incubated for 20 h at 20°C, pressed under filter paper and air-dried. Subsequently, plates were stained with methyl-violet for 1 min, post-fixed in Lugol's solution for 15 s and finally destained with ethanol (96%) until clear zones were detectable. Diameters were measured and used for calculation of lysozyme activity in plasma samples, determined from a standard curve based on the activity of a dilution series of known concentrations of chicken lysozyme.

Data analysis. Infection levels were expressed as mean intensity (mean number of parasites per infected fish). Immobilisation titers were expressed as means. Mann-Whitney *U*-tests were used to detect differences between mean values (infection level, immobilisation titer, lysozyme activity) of experimental and control groups; differences were determined to be significant at $p < 0.05$.

RESULTS

Injection with live *Ichthyophthirius multifiliis* theronts conferred a high degree of protection to rainbow trout when evaluated by challenge with live theronts on Day 28 post-immunisation (p.i.). Protection was clearly superior at 20°C compared with 12°C: more than 30 trophonts per fish were detected in immunised fish at 12°C, whereas only 0 to 2 parasites could be found in immunised fish at 20°C (Table 1). Immobilisation titers increased significantly by Days 14 and 28 p.i. (Table 2). Lysozyme activity of plasma increased after only 1 wk, but was particularly high on Days 14 and 28 p.i. (Table 3). Highest immobilisation and lysozyme values were recorded in fish maintained at 20°C (Tables 2 & 3).

Table 1. *Oncorhynchus mykiss*. Mean (SD) no. of trophonts established in fins and skin following challenge exposure to live *Ichthyophthirius multifiliis* theronts at 12 and 20°C, recorded on Days 0, 4, 6, 7 and 10 post-challenge. Triplicate groups of 12 fish pooled. *Significantly different from control at same temperature, $p < 0.05$; **significantly different from fish immunised at 12°C (Days 6 and 10), $p < 0.05$

	No. of fish examined	Day 0	Day 4	Day 6	Day 7	Day 10
Immunised 12°C	36	0	–	4.53 (0.69)*	–	37.42 (6.22)*
Control 12°C	36	0	–	32.93 (40.06)	–	93.11 (16.39)
Immunised 20°C	36	0	1.02 (0.50)*,**	–	0.31 (0.13)*,**	–
Control 20°C	36	0	66.9 (23.97)	–	16.30 (5.92)	–

Table 2. *Oncorhynchus mykiss*. Mean (SD) immobilisation titres of plasma samples at 12 and 20°C on Days 0, 1, 7, 14 and 28 p.i.. Triplicate groups of 4 samples pooled. *Significantly different from control at same temperature, $p < 0.05$

	No. of fish examined	Day 0	Day 1	Day 7	Day 14	Day 28
Immunised 12°C	12	1.8 (1.3)	2.8 (2.7)	7.7 (4.6)	44.0 (43.6)*	34.3 (22.5)*
Control 12°C	12	2.5 (1.7)	2.2 (2.8)	5.1 (5.2)	3.8 (2.3)	3.3 (2.5)
Immunised 20°C	12	2.3 (1.4)	3.7 (2.6)	11.7 (10.5)	46.7 (42.2)*	46.0 (42.9)*
Control 20°C	12	3.2 (2.4)	3.8 (5.9)	8.5 (11.2)	4.2 (5.7)	4.3 (3.0)

Table 3. *Onycorhynchus mykiss*. Mean (SD) lysozyme activity (international units) at 12 and 20°C on Days 0, 1, 7, 14 and 28 p.i.. Triplicate groups of 4 samples pooled. *: Significantly different from control at same temperature, $p < 0.05$

	No. of fish examined	Day 0	Day 1	Day 7	Day 14	Day 28
Immunised 12°C	12	226.38 (57.78)	231.38 (71.37)	292.38 (56.53)*	305.58 (65.31)*	295.68 (63.93)*
Control 12°C	12	234.63 (32.83)	213.18 (40.34)	226.38 (54.61)	224.73 (67.61)	213.18 (49.11)
Immunised 20°C	12	242.88 (40.78)	257.78 (76.20)	320.43 (83.77)*	335.28 (56.21)*	318.78 (53.43)*
Control 20°C	12	195.03 (29.40)	272.58 (57.16)	242.88 (63.93)	241.23 (63.44)	190.08 (41.36)

DISCUSSION

Immunisation of rainbow trout achieved through i.p. injection of live theronts conferred protection against challenge infections of *Ichthyophthirius multifiliis* to rainbow trout *Oncorhynchus mykiss*. This was demonstrated both at 12 and 20°C; however, protection at 20°C was clearly superior when evaluated at Day 28 p.i.. The temperature-dependent immunity of fish is well known from studies with other pathogens and immunogens (Andersen & Buchmann 1998), but this study provides the first evidence of the phenomenon in the rainbow trout-*I. multifiliis* system. However, we cannot exclude the possibility that the activity (and antigen production) of the theronts also differed at the 2 temperatures, which should be investigated further.

The present study supports the results of Burkhart et al. (1990), who demonstrated that live theronts induced better protection in channel catfish than did other antigen preparations. Previous investigations showed only slight protection following immunisation of this host with homogenates of killed *Ichthyophthirius multifiliis* (Dalgaard et al. 2002, Sigh 2004). Live theronts seem to affect the host in a more efficient way than substances from dead parasites. However, it is not known if the induction of the response is based on mechanical actions of the theronts in the peritoneal cavity, or if tomites secrete immunogens at a higher or more immunogenic level. In the present study it was not determined if rainbow trout could have obtained the same protection at 12°C over a longer time frame: lower protection at 12°C could simply be due to delayed responses at this lower temperature (Andersen & Buchmann 1998).

The protective mechanisms of fish against *Ichthyophthirius multifilis* infections have not been fully elucidated. The role of specific antibodies has been emphasized as a result the fact that both ELISA and immobilisation titers increase following infection (Sigh & Buchmann 2001, Wang et al. 2002, Xu et al. 2002). This was also demonstrated in the present study. However, it should not be forgotten that a number of other factors could confer immunity against *I. multifiliis* infections. Sigh (2004) showed that DNA vaccination of rainbow trout using genes that encoded i-A elicited specific antibody production against i-A, but did not confer any protection against infection. Other immune components could therefore play a role, and it is noteworthy that lysozyme levels in plasma were elevated following immunisation. This may not suggest that lysozyme has an exclusive effect on the parasite, but could be indicative that other non-specific elements are activated by *I. multifiliis* infection (Iwama & Nakanishi 1996, Sigh et al. 2004). Therefore, factors (chemical or mechanical) associated with theronts that have the ability to induce protection should be further elucidated. The present study suggests that further investigations of anatomy, biochemistry, physiology, metabolism and behaviour of theronts (at various temperatures) should be pursued in order to describe the protective antigens and factors of theronts that are able to elicit protection in a host.

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