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ORIGINAL PAPER

Towards a metalloprotease-DNA vaccine against piscine cryptobiosis caused by *Cryptobia salmositica*

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Abstract Cysteine protease is a metabolic enzyme, whereas metalloprotease is the virulent factor in cryptobiosis caused by *Cryptobia salmositica*. Recombinant DNA vaccines were produced with the insertion of either the metalloprotease or cysteine protease gene of *C. salmositica* into plasmid vectors (pEGFP-N). As expected, fishes (*Oncorhynchus mykiss* and *Salmo salar*) injected intramuscularly with the metalloprotease-DNA (MP-DNA) vaccine (50 μg/fish) were consistently more anemic (lower packed cell volume, PCV) than controls (injected only with the plasmid) at 3–5 weeks post-inoculation. Also, there were no difference in PCV between fish injected with the cysteine-DNA plasmids and the controls. In addition, agglutinating antibodies against *Cryptobia* were detected only in the blood of MP-DNA-vaccinated fish at 5–7 weeks post-vaccination and not in cysteine-DNA plasmids and the control groups. MP-DNA-vaccinated fish when challenged with the pathogen had consistently lower parasitemia, delayed peak parasitemia, and faster recovery compared with the controls. All fish vaccinated with attenuated strain were protected when challenged with the pathogen; this positive control group confirmed that the two vaccines operate through different mechanisms.

Introduction

Salmonid cryptobiosis is caused by the haemoflagellate *Cryptobia salmositica*. The parasite is normally transmitted by the freshwater leech, *Piscicola salmositica*, in streams and rivers on the west coast of North America. It has been recorded from all species of Pacific salmon, *Oncorhynchus* spp., and outbreaks of the disease with high mortalities have also occurred periodically in hatcheries and in sea cages in British Columbia, Canada, and in Oregon and Washington states, USA (see Woo 2003). The pathogen has been attenuated (Woo and Li 1990), and it has been used routinely as an experimental vaccine. It circulates in the blood and protects both juvenile (e.g., Sitja-Bobadilla and Woo 1994) and adult (e.g., Woo and Li 1990) salmonids from infection. Protection in vaccinated fish is via the production of complement-fixing antibodies (e.g., Li and Woo 1995; Feng and Woo 1998) and cell-mediated immunity (e.g., Li and Woo 1995; Ardelli and Woo 2002; Mehta and Woo 2002). A single dose of the attenuated *C. salmositica* vaccine protects rainbow trout, *O. mykiss*, for up to 24 months (Li and Woo 1997).

Anemia is one of the most consistent clinical signs of cryptobiosis (Woo 1979). The anemia is in part caused by an antibody-independent lytic factor and an immune-complex-forming component (Thomas and Woo 1988). *C. salmositica* has two proteases, a cysteine protease (49, 60, 66, and 97 kDa), which is a metabolic enzyme, and a 200-kDa metalloprotease (MP), which is the virulent factor (Zuo and Woo 1997a). A monoclonal antibody (mAb-001) produced against a 200-kDa surface membrane epitope (Feng and Woo 1996) inhibits the activities of the MP and part of the cysteine protease (Zuo et al. 2001). Under in vitro conditions, mAb-001 agglutinates live parasites, and it is therapeutic and prophylactic when injected into fish (Feng and Woo 1997). Live parasites exposed to mAb-001 do not multiply, and its oxygen consumption is inhibited. Also, incubation of the parasite with a cysteine protease inhibitor (E64) stops parasite multiplication, whereas ethylenediaminetetraacetic acid (EDTA; cysteine protease acti-

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vator) increases parasite multiplication (Hontzeas et al. 2001). Partial cysteine and MP genes from *C. salmositica* have been sequenced (Jesudhasan et al. 2007a, b) and transformed in *Escherichia coli* (Tan 2005).

Anderson et al. (1996) injected the first DNA vaccine (plasmid DNA encoding the glycoprotein G gene of infectious hematopoietic necrosis virus) into rainbow trout and demonstrated a protective immune response. Other studies that demonstrate the effectiveness of DNA vaccines against viral diseases in fishes have been carried out (Boudinot et al. 1998; LaPatra et al. 2001; Lorenzen et al. 1999, 2002; Mikalsen et al. 2004). Only one parasite (*Ichthyophthirius multifiliis*) has been used in a DNA vaccine study; however, no challenges were carried out, and consequently, its efficacy is not known (Lin et al. 2002).

The main objectives of the present study are to produce DNA vaccines (encoding the MP gene and the cysteine protease gene of *C. salmositica*) and to determine their efficacy in rainbow trout (*O. mykiss*) and Atlantic salmon (*Salmo salar*) against the pathogen.

Materials and methods

Cryptobia salmositica

Vaccine strain

Woo and Li (1990) attenuated the pathogenic *C. salmositica* and used it as an experimental vaccine to better understand the development of protective immunity in salmonids (Woo 2006). The strain was cultured at 10°C in 100-ml culture flasks with Eagle's minimal essential media (MEM, Sigma, St. Louise, MO, USA) supplemented with 25% fetal bovine serum (FBS, Gibco Life Technologies, Grand Island, NY, USA). The medium with FBS was adjusted to pH 7.4–7.5 with 3 M NaOH (Woo and Li 1990), and it was filter sterilized using Corning 500-ml-bottle top filters (Corning Incorporated, Corning, NY, USA). At the end of the log phase of growth (usually about 4 weeks), the parasites were dislodged from the surface of the flask by shaking and swirling of the flask. High numbers of parasites were collected by centrifugation of cultures at 19,400×*g* at 4°C for 25 min. The pellet was then resuspended in phosphate-buffered saline (PBS; pH 7.5) and centrifuged again. This was repeated three times, and the resuspended parasites were then diluted to the required numbers using PBS.

Pathogenic strain

The pathogenic *C. salmositica* was initially isolated from *Pisicola salmositica* feeding on *O. kisutch* on Vancouver Island, Canada. The parasite was cloned (T4 substrain), characterized (Woo 1978), cryopreserved, and stored at −85°C, and also was maintained by serial subpassage in rainbow trout. *C. salmositica* for in vitro cultures were obtained aseptically from an infected trout using a heparinized needle and syringe. Blood was withdrawn aseptically from an infected fish, and 0.5 ml was inoculated into a 50-ml culture flask with MEM and 20–25% FBS. The culture flask was incubated at 10°C at an angle so that the red blood cells settled at the bottom. The supernatant fluid containing parasites was withdrawn aseptically and inoculated into 100-ml culture flasks with medium. After 2–4 weeks, parasites were collected, washed with PBS (see above), and frozen at −20°C. For antigen preparation, frozen parasites were thawed and sonicated using a Vibracell ultra-sonicator (VC 50T, Sonics & Materials, Newtown, CT, USA) at 10 kHz. Parasites in PBS were kept on ice at all times, and the parasite suspension was sonicated with 10 to 12 bursts of 10 s each at intervals of 30 s to prevent overheating the lysate. Because Zuo and Woo (1997a) showed that no MP was detected in the pathogenic *C. salmositica* after 10 months of in vitro culture, all pathogenic parasites used to challenge vaccinated fish were cultured for not more than 2 months.

Parasitemia

Parasitemia was monitored using the hematocrit centrifuge technique (Woo 1970) and enumerated using a hemocytometer. High numbers of parasites were counted using a hemocytometer. Briefly, the blood was vortexed before dilution with PBS, and the diluted blood was transferred onto the hemocytometer. Parasites were counted under a light microscope (×10 ocular and objective ×16), and the number of parasites/ml fish blood was determined (Archer 1965).

Fish and experimental design

Fish

Full-sib families of Atlantic salmon (Chin et al. 2004) were obtained from the Atlantic Salmon Broodstock Development Program (ASBDP, St. Andrews, NB, Canada). Rainbow trout for vaccine studies were obtained from the Alma Research Station, University of Guelph. Fish were maintained in 125-l cylindrical tanks with aerated, UV-treated, recirculated well water at 10–12°C and allowed to acclimatize for 10–14 days in tanks before the start of an experiment. They were fed daily ad libitum with a 42% commercial protein diet (3PT-5PT, Martin's Feed Mills, Elmira, ON, Canada). Fish were anaesthetized with 2-phenoxyethanol (Fisher Scientific, Pittsburgh, PA, USA) before blood sampling and infection with parasites. All fish

were bled weekly except for the study using Atlantic salmon. Blood was withdrawn from each fish using a heparinized syringe and 25 $G^{5/8}$ latex-free needles (Becton Dickinson, Franklin Lakes, NJ, USA) from the caudal vein midway between the tail and the anal fin. The volume of blood collected depended on the size of the fish and ranged from 0.05 ml (from a ~50-g fish) to 0.2 ml (from a ~1-kg fish). After bleeding, the fish were allowed to recover in well-aerated water, and full recovery took about 10 min. Individual fish were identified using a PIT tag (AVID Canada, Calgary, Alta, Canada). All procedures with fish were done in accordance with the animal care policies of the University of Guelph.

DNA vaccine development

The methods used for genomic DNA and plasmid DNA extractions, polymerase chain reaction (PCR) amplification, cloning, transfection, and transformation are as described in Sambrook et al. (1989). Briefly, both the cysteine and MP gene were sequenced (Jesudhasan et al. 2007a, b), and inserted into plasmid vectors (Clonetech, pEGFP-N1; GenBank accession no. U55762) that had a neomycin/kanamycin resistance gene that allowed for kanamycin resistance in *E. coli* competent cells. The plasmid vector was kept on ice, and 2 μl of the vector (with the insert or 2 μl of the plasmid template without the insert) were added to sterile 1.5-ml centrifuge tubes containing 50 μl of Top10 cells (Invitrogen). The contents in the tubes were mixed gently by tapping, kept on ice for 30 min, transferred to a water bath at 42°C for 45 s, and returned to ice for 2 min. Sterile SOC medium (950 μl; lot # 102K8402; Sigma) was added to each tube, and tubes were incubated for 1 h at 37°C.

Transformants with the cysteine and MP genes were plated independently in 10-, 50-, and 100-μl quantities on kanamycin-treated (30 μg/ml) Luria–Bertani (LB) plates and incubated overnight at 37°C. Single colonies were selected, and the clones were confirmed by PCR (Mini-Cycler, MJ Research, Waltham, MA). The reaction mixture containing plasmid DNA with either cysteine or MP gene insert, 5 μl of 10× high fidelity PCR buffer, 0.2 mM of each deoxyribonucleotide triphosphate (dNTP), 1 μM of each primer, 5 U of Platinum® *Taq* DNA polymerase high fidelity (Invitrogen), and double-distilled water up to a final volume of 50 μl. DNA denaturation was carried out at 94°C for 3 min, and then a total of 35 PCR cycles were run under the following conditions: DNA denaturation at 94°C for 30 s, primer annealing at 65°C cysteine protease, 56°C MP for 40 s, and DNA polymerization at 72°C for 2 min. After the final cycle, reactions were terminated by a further run at 72°C for 5 min. PCR products were run on agarose gels (1%) to ascertain that the gene encoding the cysteine and MP gene were present. Selected colonies were then cultured overnight in kanamycin (30 μg/ml)-supplemented LB broth in a shaking incubator at 37°C, 180 rpm.

Plasmid DNA was extracted from *E. coli*-competent cells using both Plasmid Midi-kit (Cat # 12143, Qiagen, Mississauga, ON, Canada) and Gen-Elute Plasmid Maxi-Prep kits (Prod # NA0410-1KT, Sigma). After extraction, the plasmid DNA was resuspended in DNase/RNase-free water and quantified using spectrophotometric analysis with A260/A280 ratios (Ultrospec 3100, Amersham Biosciences, Buckinghamshire, UK). The plasmid vectors with or without the cysteine or MP insert were sequenced at the DNA sequencing facility, University of Guelph. The sequences were compared with previously uploaded *C salmositica* protease genes (Jesudhasan et al. 2007a, b) to ensure that the transformation had not muted the protease sequence.

The DNA vaccines were lyophilized, resuspended in 1× PBS, and diluted to the desired concentration for inoculation into fish.

Experiment A—rainbow trout vaccinated with MP-DNA vaccine

Fifty-six rainbow trouts (average weight, 51.5±1.64 g) were used. They were randomly divided into five groups: (1) MP plasmid, (2) plasmid vector, (3) infected with pathogenic *C. salmositica*, (4) vaccinated with attenuated live *C. salmositica*, and (5) PBS control. Each group had 14 fish except for the infected and the PBS control groups in which there were only seven fish each.

Each fish in the MP group was injected with 50 μg/fish of recombinant MP-DNA (pEGFP–N1–MP) in 0.2-ml PBS (pH 7.5). Fish in the plasmid control (PC) group were injected with 50 μg/fish of non-recombinant plasmid vectors (pEGFP-N1) in 0.2-ml PBS. Plasmids in both groups were injected in the epaxial muscle just below the dorsal fin in four areas, two on the right and two on the left side of the fish (modified from Kanellos et al. 1999b). Fish in the infected (INF) group was each injected intraperitoneally (IP) with 20,000 pathogenic *C. salmositica* in 0.1-ml PBS, whereas those in the live vaccine (LV) group were each injected IP with 20,000 attenuated parasites in 0.1 ml PBS. Fish were bled weekly, and approximately 0.05 ml of blood was drawn from each fish from 0–7 weeks post-vaccination (wpv). Fish in the MP and PC groups were challenged with 20,000 parasites/fish at 7 wpv and bled weekly from 3–6 weeks post-challenge (10–13 wpv) and once more at 8 wpc (15 wpv).

The LV group was infected with live attenuated *C. salmositica* at 0 wpv. Each fish in the three vaccinated groups (LV, MP, and PC) was challenged with 20,000 pathogenic parasite at 7 wpv. The INF group was infected with 20,000 pathogenic *Cryptobia* 2 weeks after the other

groups were vaccinated or infected. This was a replacement group (initially called the PBS group), as all fish in the initial INF group died because of aeration problems. The INF group was also not challenged with the pathogen at 7 wpv as was done with the vaccinated groups. The LV and INF groups were killed and bled dry at 10 weeks post-infection (wpi).

Blood was centrifuged and the packed cell volume (PCV) was measured (Woo 1979). The plasma was collected and stored at −80°C for dot blots and agglutination tests. All initial parasitemias (1 wpi onward) were detected using the hematocrit centrifuge technique (Woo 1970), and if the tube had about 30 parasites above the buffy layer, the number of parasites in the blood sample was then determined using a hemocytometer (Archer 1965).

Experiment B—Atlantic salmon vaccinated with MP-DNA vaccine

Fifty-seven Atlantic salmon (average weight, 287.3± 8.37 g) were vaccinated, and they were assigned to the (1) MP group, (2) plasmid vector group, and (3) LV group. The MP group had three subgroups (n_1=10, n_2=10, and n_3=9), and the PC group had two subgroups (n_1=9; n_2=9). The LV group had only one group of ten fish. Fish were maintained as described earlier.

Recombinant MP, pEGFP–N1–MP (50 μg) in 0.2 ml of PBS was injected into the epaxial muscle of each fish as described above. The same was done for the PC group, except that the fish were only injected with the plasmid vector (pEGFP−N1). An additional ten fish (PBS control fish) were also injected in the epaxial muscle with filter-sterilized PBS (pH 7.5). The LV group was injected intraperitoneally (IP) with 100,000 live attenuated *C. salmositica*.

Fish were bled (0.1 ml) every 2 weeks from 0 until 18 wpv. Vaccinated fish were challenged with 30,000 pathogenic *C. salmositica* at 8 wpv. After challenge, parasitemia was monitored using the hematocrit centrifuge technique (Woo 1970) during low parasitemia and counted using the hemocytometer (Archer 1965) during high parasitemia. PCV was measured for each fish as previously described.

Experiment C—Atlantic salmon vaccinated with cysteine protease DNA vaccine

Thirty Atlantic salmon (average weight, 153.25±4.9 g) were used in this experiment. They were randomly divided into three groups, and each group (n=5 fish) has a replicate (n=5) in a second tank. Fish were injected with either (1) cysteine plasmid vaccine (pEGFP–N1–CP), (2) LV, or (3) PBS. Fish were maintained as described earlier.

Each fish in the CP group was vaccinated intramuscularly (IM) with 25 μg pEGFP–N1–CP in 0.2 ml PBS in the epiaxial muscle, and the PBS control group was injected IM with 0.2 ml PBS. Each fish in the LV group was vaccinated with 100,000 attenuated parasites intraperitoneally (IP) in 0.1 ml PBS. All fish were each challenged IP with 30,000 pathogenic *C. salmositica* at 6 wpv.

Fish were bled (0.1 ml per fish) every 2 weeks after vaccination and weekly after challenge with pathogenic parasites. Blood was centrifuged, and the PCV was measured. Hematocrit centrifuge tubes were examined for parasites after challenge, and if the tube had ~30 parasites above the buffy layer, the parasitemia was enumerated using a hemocytometer (Archer 1965).

Detection of free MP in MP-DNA-vaccinated fish

Seven rainbow trout antisera (collected at 0, 1, and 3 wpv) were diluted 1:2 with Tris-buffered saline (TBS) and 5 μl spotted onto a NC membrane. As a positive control, 5 μl of recombinant MP (0.195 μg/μl) was also dotted onto a nitrocellulose membrane. As negative controls, 5 μl of sera from two naive rainbow trout diluted 1:2 with TBS were dotted onto nitrocellulose membrane. The membranes were then blocked with (5% *w*/*v*) skim milk, incubated with mouse antibodies produced against the recombinant MP, conjugated secondary antibody, and substrate as previously described (Tan 2005).

Agglutination tests

Agglutination tests were used to detect antibodies in sera/antisera of fish (vaccinated or unvaccinated) before the fish were challenged with pathogenic *C. salmositica*. Briefly, heat-inactivated sera were diluted (1:10, 1:50, and 1:100 times) with cold PBS and added to live *C. salmositica* at 10°C in 96-well microtiter plates (Feng and Woo 1996).

Experiment A

Rainbow trout antisera (1, 5, 6, and 7 wpv) from MP-injected group were diluted at 1:10, 1:50, and 1:100 with PBS and tested for agglutinating antibodies. The parasites from cultures were rinsed with PBS twice before being re-suspended in cold PBS. Approximately 10^5 parasites diluted in 25 μl PBS were added to each well in a 96-well microtiter plate. This was followed by the addition of diluted antisera (25 μl). Each antiserum had three replicate wells for each dilution. After 1-h incubation at 10°C, the wells were examined for agglutinated (clumped) parasites using an inverted microscope (ocular, ×10; objective, ×16). A positive was recorded if the clumps had more than five parasites per clump and if there were more than five clumps

per well. As controls, live *C. salmositica* were also incubated with sera from the plasmid-only group of fish, taken at 1, 5, and 7 wpv. Also, antisera from three other infected and recovered rainbow trout (positive controls), sera from three naive fish, and PBS (three wells) were used as controls.

Experiment B

As in experiment A, antisera from six Atlantic salmon (MP-vaccinated group) at 0 and 8 wpv were incubated (at dilutions of 1:10, 1:50, and 1:100) with three replicate wells for each antiserum dilution. Live *C. salmositica* (10^5 parasites in 25 μl PBS) were added into each well. Positive and negative controls were similar to those in experiment A.

Experiment C

The protocol was similar to that described in experiment A. Agglutination tests were conducted with antisera (0, 5, and 6 wpv) from the fish vaccinated with cysteine protease plasmid DNA group. Positive and negative controls were similar to those in experiment A.

Statistical analysis

The repeated measures analysis of variance (ANOVA) was used to detect significant differences in means between the treatments and between families of fish across time (wpv/infection/challenge) (Steel and Torrie 1980). If the sample failed the normality test, a non-parametric ANOVA on ranks was run with Kruskal–Wallis test for differences between groups. If there was a significant difference between the groups of fish, a Bonferonni's *t* test was run. A significant difference was recorded when $p<0.10$. A significance level of 0.10 was used to ensure that any significant differences would be detected, as the sample sizes were small, and to reduce type II error.

Results

Agglutinating antibodies

Experiment A

In the MP group, none of the 14 sera at 1 wpv had detectable agglutinating antibodies. At 5 wpv, four samples were positive and two samples were negative (four out of six with average titer of 100). By this time, 4 out of 14 of the MP-inoculated rainbow trout were killed because of tail rot. At 6 wpv, seven out of eight of the vaccinated antisera were positive for agglutinating antibodies (five out of eight with average titers of 50). At 7 wpv, six out of six antisera had agglutinating antibodies (average titers of 100). The number of samples tested were different from week to week because those with observable bacterial contamination were not tested, as fish sera were not collected aseptically.

No parasite agglutination was detected in the PC group at 5 and 7 wpv. Parasites in the sera were very active and multiplying rapidly. Also, naive sera (from three uninfected fish) and PBS (three samples) did not agglutinate the parasite; however, parasites in antisera from three infected and recovered fish were agglutinated.

Experiment B

Sera from all six of the MP-vaccinated fish agglutinated the parasite at 8 wpv, whereas their pre-immune sera (0 wpv) did not. Parasites incubated with PBS and naive sera were also negative, whereas sera from infected and recovered fish agglutinated the parasite.

Experiment C

No agglutinating antibodies were detected at any time in sera from fish injected with cysteine-DNA plasmids, whereas those from infected and recovered fish agglutinated the *Cryptobia.*

Detection of recombinant MP

Rainbow trout sera from 1 and 3 wpv (experiment A) were tested for free MP using mouse antibodies produced against the recombinant MP (Tan 2005) in dot blots. Results were inconclusive. Dot blots on naive rainbow trout sera with the addition of recombinant MP (positive controls) were positive, whereas naive rainbow trout sera without the addition of MP were negative (negative controls).

Packed cell volume

Experiment A

The MP and PC groups were vaccinated at 0 wpv and challenged with parasites at 7 wpv. The LV group was inoculated with the LV (at 0 wpv), and the INF group was inoculated with pathogenic parasites at 2 wpv of the LV group. Acute anemia was seen in the INF group at 5 wpi but not in the LV group. Fish in both the LV and INF groups were killed at 10 wpv (3 weeks post-challenge for the LV group and 8 weeks post-infection for the INF group), and the MP and PC fish were killed at 15 wpv (8 wpc). The PCV in the LV group returned to pre-vaccination level at 3 wpc because the fish were protected and were not bled after parasite challenge (Fig. 1).

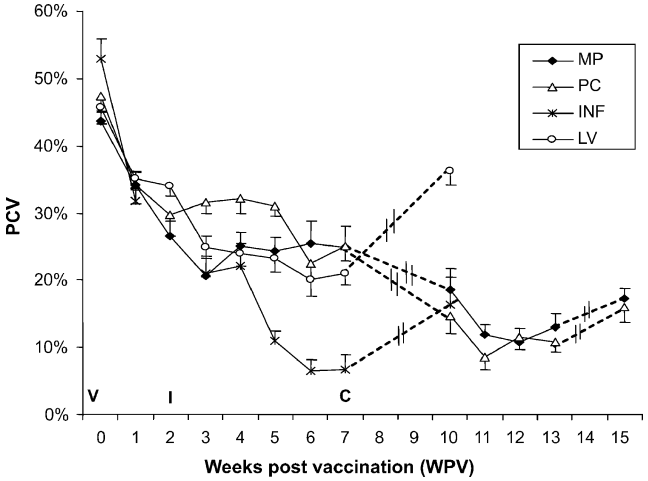

Fig. 1 Pack cell volume (*PCV*) in rainbow trout vaccinated with MP-DNA and controls. *MP* Metalloprotease-vaccinated fish (n=7), *PC* PC fish (n=10), *INF* fish infected with pathogenic *C. salmositica* (n=7), *LV* fish infected with live attenuated *C. salmotica* (n=14). *V* Vaccination with plasmid DNA or live vaccine, *I* infection with parasites (for INF group), and *C* challenge with pathogenic parasites at 7 wpv (MP and PC groups). *Bars* denote standard errors of treatment groups. *Broken lines* denote that no samples were taken during those periods

The PCV declined after inoculation of plasmids (MP and PC groups), and it was significantly lower (at $p<0.10$) in the MP group (23.6±0.029%) compared to the PC group (30.6±0.018%) at 3 wpv ($p<0.10$; Fig. 2). At 4 wpv, the PCV in the MP group rose to 25.0±0.021%, and it was not significantly different ($p=0.104$) from the PC group (30.8±0.024%). At 5 wpv, the PCV in the MP group (23.4±0.019%) was significantly lower ($p<0.10$) compared to the PC group (30.1±0.016%). After challenge (7 wpv), both the MP group and the PC group had the same trend in PCV and were not significantly different from each other. By 15 wpv, the MP group and PC group rose to 17.3±0.015% and 16.0±0.02%, respectively (Fig. 2).

In the MP group, some fish (e.g., 1/14 at 1 wpv, 3/13 at 2 wpv) started to exhibit signs of tail rot disease, and by 7 wpv (0 wpc), 7 out of 14 fish in the MP group were killed. In the PC group, 4 out of 14 fish were also killed because of tail rot disease. Data from tail-rot fish were not included when computing average PCV, and their sera were not tested for agglutinating antibodies. Data (fish without tail rot) from the MP and PC groups were replotted (Fig. 2) to show more clearly the trend in PCV and significant differences in these groups.

Experiment B

There were significant differences ($p<0.10$) in the PCV between the MP and PC group at 4 and 6 wpv (Fig. 3). The PCV for the MP group were 35.1±0.01% (4 wpv) and 35.6±0.01% (6 wpv), whereas the PCV for the PC group stayed at about 39% at 4 and 6 wpv. The MP and PC groups were not significantly different at any time after parasite challenge ($p>0.10$). There was no significant difference between the LV and PC group or between the LV and MP groups before challenge with the parasites. At 4 wpv, the variances of the MP and PC groups were not normally distributed, and so, a Kruskal–Wallis ANOVA on ranks was used.

Experiment C

There was no obvious anemia in all the groups except at 6 wpv in the LV group. There was a significant difference ($p<0.05$) between the CP and PBS groups when compared to the LV group after parasite challenge. The PCV trend for both the CP and PBS groups were very similar. After

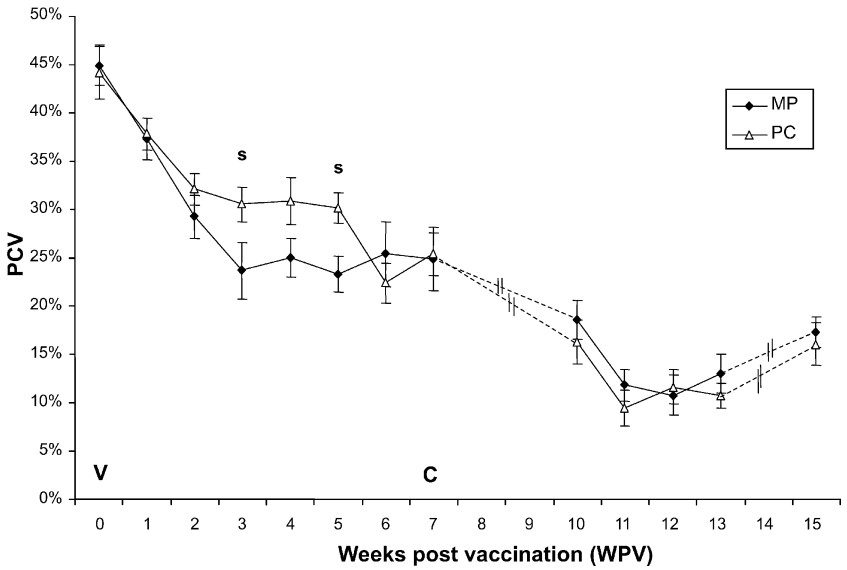

Fig. 2 Pack cell volume (*PCV*) in rainbow trout vaccinated with MP-DNA (n=7) and control plasmid DNA (n=10). *V* Time of vaccination, *C* time of challenge with parasites, and *s* significant difference between the MP and PC groups at p=0.10. *Bars* denote standard errors of treatment groups. *Broken lines* denote that no samples were taken during those periods

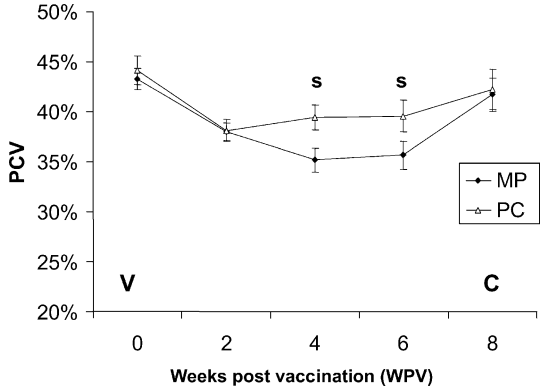

Fig. 3 Packed cell volume (*PCV*) in Atlantic salmon vaccinated with MP-DNA (*MP*, n=29) and PC (*PC*, n=18). *V* Time of vaccination, *C* time of challenge with parasites, and *s* significant differences. *Bars* denote standard errors of the treatment groups

challenge, there was a decline in PCV for the CP and the PBS groups. Again, the trend was similar for both, and there was no significant difference between them. However, after challenge, there was no decline in PCV for the LV group: They recovered to prevaccination levels (Fig. 4).

Parasitemia

Experiment A

The LV group was inoculated with the live attenuated vaccine at 0 wpi, and the INF group was infected with the pathogenic *C. salmositica* 2 weeks later. There was almost no detectable parasitemia in the LV group up until the fish were killed (10 wpi). The INF group had a peak parasitemia at 5 wpi, and then there was a slow recovery. These fish were not bled between 7 and 10 wpi and were killed at 10 wpi. At 10 wpi, there was still a high number of parasites in the INF group.

Fish in the MP and PC groups were vaccinated at (0 wpv) and challenged at 7 wpv. Parasitemias in the MP-vaccinated group were consistently lower than those in the PC group (Fig. 5). The PC group peaked at 3 wpc (with $5.6\pm0.78\times10^6$ parasites/ml of blood), whereas the MP vaccinated group had $3.56\pm0.8\times10^6$ parasites/ml of blood. Parasitemias in the MP group peaked at 4 wpc ($4.6\pm0.96\times10^6$ parasites/ml of blood). There was no significant difference in the peak parasitemias between the two groups. Recovery in the MP group was faster compared to the PC group ($p_{5\ \mathrm{wpc}}$=0.101). The variances for these two groups were not distributed normally, and thus, the Kruskal–Wallis ANOVA on ranks was used. By 5 wpc, the parasitemia in the MP group had dropped by 77.6% to $1.03\pm0.28\times10^6$ parasites/ml of blood. One week after peak parasitemia, the PC group had only dropped by 12.7% to $4.9\pm1.3\times10^6$ parasites/ml of blood (4 wpc). At 5 wpc, the plasmid DNA group had $2.56\pm0.69\times10^6$ parasites/ml of blood, and this increased slightly to $2.67\pm1.08\times10^6$ parasites/ml of blood at 6 wpc. At 6 wpc, the parasitemia in the MP group also increased ($1.35\pm0.39\times10^6$ parasites/ml of blood). The fish were terminally bled at 8 wpc, and the MP group had $8.27\pm2.16\times10^5$ parasites/ml of blood, and the PC group had $1.82\pm0.62\times10^6$ parasites/ml of blood.

Experiment B

All groups were challenged with 30,000 pathogenic *C. salmositica* at 8 wpv. There was no significant difference between groups after challenge. The MP group had a lower peak at 4 wpc ($11.96\pm1.46\times10^6$ parasites/ml of blood) compared to the PC group with a peak at 4 wpc of $14.6\pm1.87\times10^6$ parasites/ml of blood.

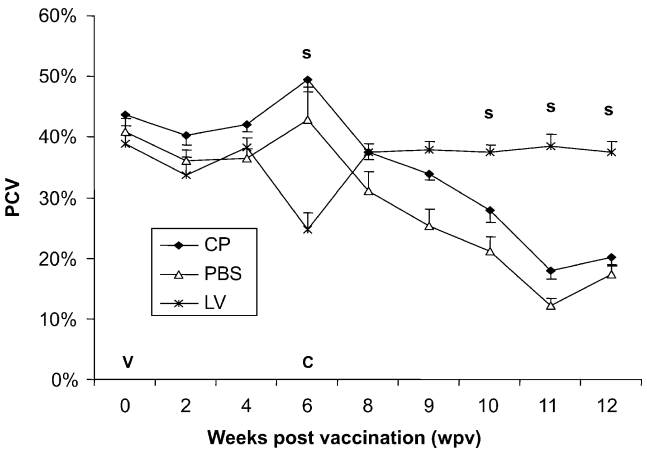

Fig. 4 Packed cell volume (*PCV*) in Atlantic salmon vaccinated with 47-kDa partial cysteine protease plasmid DNA (*CP*, n=10), live vaccine (*LV*, n=10), and PBS (n=10). *V* Time of vaccination, *C* time of challenge with parasites, and *s* significant difference at p=0.10 between the CP and PBS groups compared to the LV group. *Bars* denote standard errors of treatment groups

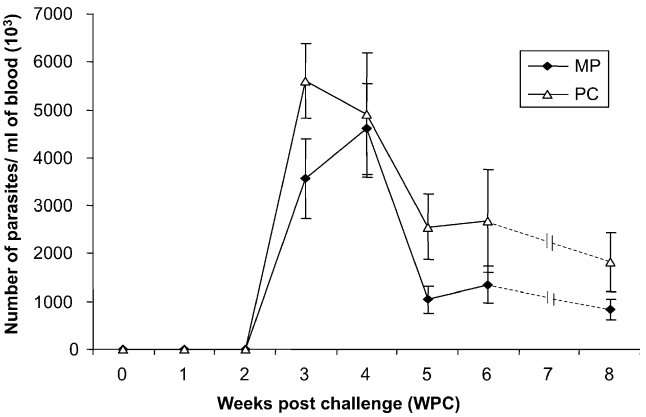

Fig. 5 Parasitemias in rainbow trout vaccinated with MP-DNA (*MP*, n=7) and control plasmid DNA (*PC*, n=10). These fish were challenge at 0 wpc (*week post challenge*), vaccinated at −7 wpc and killed at 8 wpc

In the PBS group, the trend in parasitemia was similar to the PC group, except that peak parasitemia at 4 wpc was $17.0\pm2.8\times10^6$ parasites/ml of blood, which is higher than the PC group (data not shown). There was no detectable parasites using the hemocytometer in the LV group after challenge.

Experiment C

There were no significant differences in parasitemias between the cysteine-plasmid-vaccinated and the PBS group.

Discussion

The protective mechanisms of the two vaccines (live attenuated vaccine and MP-DNA vaccine) are distinctly different. As in previous studies (e.g., Woo and Li 1990; Sitja-Bobadilla and Woo 1994; Li and Woo 1995), the attenuated LV in the present study protected trout from infection when vaccinated trout were challenged with the pathogen. As expected, the MP-DNA-vaccinated fish were infected after challenge, but the vaccinated-challenged rainbow trout had lower parasitemias, and they recovered faster (see below).

The MP and the 47-kDa partial cysteine protease genes were sequenced (Jesudhasan et al. 2007a, b), were inserted into plasmid vectors to produce DNA vaccines (pEGFP–N1–MP and pEGFP–N1–CP, respectively), and used in the vaccination studies. Plasmid DNA controls (pEGFP–N1) that did not contain protease inserts (MP or cysteine) were also prepared and used. Two fish trials (rainbow trout and Atlantic salmon) were conducted using the recombinant MP-DNA, and the third trial (Atlantic salmon) was done with the 47-kDa partial cysteine protease plasmid DNA.

There were decreases in PCV within the first 2 weeks after rainbow trout and Atlantic salmon were inoculated with MP-DNA (pEGFP–N1–MP) or with plasmid vector only (pEGFP–N1). The decreases were partly caused by the bleeding. Subsequently, there were more significant decreases in PCV at 3 and 5 wpv in MP-vaccinated rainbow trout and at 4 and 6 wpv in MP-vaccinated Atlantic salmon after the secretion of MP from the DNA vaccine. At 4 wpv, the PCV difference between the MP-vaccinated rainbow trout and control groups were not significant ($p=0.104$). However, the anemia in both vaccinated Atlantic salmon and trout stabilized at about 5–6 wpv, as antibodies were produced against the MP, and the PCV started to rise again. We were unable to confirm the presence of recombinant MP in sera of vaccinated trout because of numerous factors. For example, some of the MP would be neutralized by antibodies, whereas others would also be depleted via other means (see below). Because we did not see similar decreases in PCV in the plasmid vector groups and also in the cysteine protease (pEGFP–N1–CP)-vaccinated group, we conclude that the anemia in the MP groups was specifically caused by the secreted MP and not by the plasmid nor to a metabolic protease (pEGFP–N1–CP).

An antibody produced in a mouse against the recombinant MP identified the purified recombinant MP when the MP was added to naive rainbow trout sera in a dot blot assay (Tan 2005). In the current study, the same dot blot assay was used to try to detect the secreted MP in vaccinated trout at 1 and 3 wpv. However, the results were inconclusive for at least three reasons other than being bound to antibodies. The 'unbound' MP would also be neutralized by the natural anti-protease a2-macroglobulin (a2-M) in fish (Zuo and Woo 1997b, c). Zuo and Woo (1997b) showed that, in rainbow trout, there was a significant decrease in a2-M at high parasitemia (acute cryptobiosis) and that the free MP in the blood was only detected during the recovery (chronic) phase of the disease. Also, MP would be used up to lyse fish erythrocytes (Zuo and Woo 1997d, 1998), cause lesions in other organs and tissue (Bahmanrokh and Woo 2001), and also be broken down by the immune system to generate specific agglutinating antibodies (see below) against the MP. Although we have no direct evidence that the injected MP-DNA plasmids were secreting MP, previous studies in fish (Hansen et al. 1991; Kanellos et al. 1999a, b; Dijkstra et al. 2001; Verri et al. 2003; Romoren et al. 2004) with reporter genes (chloramphenicol acetyltransferase [CAT] gene, β-galactosidase [*lacZ*] gene, and luciferase gene) have shown that the expression of plasmid DNA does occur after injection, and activity of some of these reporter genes can be detected 2 years later. Interestingly, Romoren et al. (2004) found that different formulations of plasmid DNA (lipopolysomes-formulated, chitosan-formulated, and naked DNA) with different injection routes (intraperitoneally, intramuscularly, and intravenously) caused differential expression of the luciferase gene, and only the muscle tissue at the site of injection was positive for DNA expression. Thus, we conclude that the MP-DNA plasmids injected into the rainbow trout and Atlantic salmon expressed the recombinant protein because of the reduced PCV and presence of specific *Cryptobia* agglutinating antibodies in the blood after vaccination.

In MP-vaccinated trout, agglutinating antibodies (against *C. salmositica*) with increasing titers were detected at 5, 6, and 7 wpv in trout but not at 1 wpv nor in the PC group. Also, agglutinating antibodies were detected in the MP-vaccinated Atlantic salmon and not in the PC. In another study (Tan 2005), agglutinating antibodies were detected at 5 wpv after whole *C. salmositica* lysate was injected in two full-sib families of Atlantic salmon. Also, the antibody production (determined using enzyme-linked immunosorbent assay, ELISA) trends in the two families were similar.

Pasnik and Smith (2005) showed low levels of antibodies against one of the major secreted fibronectin-binding proteins in hybrid-striped bass (*Morone saxatilis* × *M. chrysops*) at 2 wpv, and by 4 wpv, significant amounts of specific antibodies were detected, which were protective against *Mycobacterium marinum*. However, not all DNA vaccines are protective. Some, like the LACK antigen from *Leishmania*, were very immunogenic in mice, but double doses of complementary DNA (cDNA)-encoding LACK did not induce protection, and only a prime boost strategy with a recombinant vaccinia virus elicited a strong protection (Ramiro et al. 2003). Vermount et al. (2004) showed that injection of the recombinant fungal (*Microsporun canis)* keranolytic MP into guinea pigs resulted in strong antibody production at 2 wpv, and this was significantly increased by 4 wpv. However, the antibody did not prevent invasion by the fungus and development of dematophytic lesions. Xu and Liew (1994) found that vaccination of mice with DNA encoding the gp63 gene of *Leishmania infantum* significantly reduced the size of lesions, delayed onset of lesions, and lowered parasite load. Finally, Rafati et al. (2001) showed that vaccination with recombinant DNA-encoding cysteine proteases CPa and CPb from *Leishmania major* resulted in significant protection of mice against leishmaniasis. However, long-term protection was achieved only with boosters of either CP or cp genes (Rafati et al. 2001).

In the present study, parasitemias in the MP-vaccinated rainbow trout and Atlantic salmon were lower than those in unvaccinated fish after parasite challenge. In rainbow trout, this was consistently lower that in the PC group. There was also a delay in achieving peak parasitemia in the MP-vaccinated group, and the recovery was also faster. However, because the sample size was small, the probability value (p=0.101) at 5 wpc was interpreted with some caution. Previous studies in mammals have shown that there are some protective effects when using DNA vaccines encoding virulent proteases. Ahmed et al. (2004) compared the immunogenicity of various antigens from *Leishmania major* in mice and found that cDNA-encoding gp63 (a MP) had a transitory protective effect that lasted 4 weeks. Vermount et al. (2004) showed that there was a delay in the onset of disease and smaller lesions in guinea pigs vaccinated with the recombinant fungal (*Microsporun canis*) keranolytic MP. Chlichlia et al. (2002), who vaccinated mice with a cysteine protease of *Schistosoma mansoni*, found no reduction in parasitemia, but there was a significant reduction in fecundity of female worms.

A similar course of infection was not observed in the MP-DNA-vaccinated Atlantic salmon after challenge because the fish were bled once every 2 weeks. The biweekly sampling was to address the concern that weekly bleedings (in the rainbow trout) might have contributed to the anemia. As the PCV trend in the Atlantic salmon was similar to that in the rainbow trout experiment and the LV group maintained a high PCV similar to the PC group, we also conclude that the anemia in the MP-DNA-vaccinated group was caused by the secreted MP. Unfortunately, the parasitemia in the vaccinated Atlantic salmon after challenge (bi-weekly bleeding) could not be monitored as closely as in the rainbow trout experiment, but the parasitemia in the vaccinated Atlantic salmon was still lower compared to the PC group.

The essential enzymatic action of the MP was present, as we detected anemia in the fish after vaccination. Also, antibodies generated by the vaccination agglutinated live *C. salmositica*, and this was similar to a monoclonal antibody (mAb-001) against a 200-kDa epitope on *C. salmositica* (Feng and Woo 1996). Although mAb-001 cross-reacted with the carbohydrate moieties of more than one protein in *C. salmositica* lysate (Feng and Woo 2001), it was generated against a 200-kDa epitope (Feng and Woo 1996). MAb-001 also inhibited respiration and multiplication of the parasite under in vitro conditions (Hontzeas et al. 2001). In the present study, the MP-DNA-vaccinated fish generated antibodies against the recombinant MP and would neutralize the lytic factor (MP) and would inhibit respiration and multiplication of the parasite. Neutralization of the lytic factor would have effectively 'disarmed' the pathogen and, thus, allowed the immune system of the fish to operate more efficiently so that infected fish recovered faster. This was the case in the MP-DNA-vaccinated rainbow trout after parasite challenge—the parasite load was lower, it took longer to achieve peak parasitemia, and the fish recovered faster. Targeting of the enzyme in the pathogen (*Trypanosoma congolense*) was also attempted in cattle. Acute anemia was observed after vaccination with recombinant cysteine protease, but vaccinated cows recovered faster and were healthier than the controls (Authie et al. 2001).

The 47-kDa partial cysteine protease (CP) plasmid DNA vaccine study was done to more specifically confirm its role against cryptobiosis. As mAb-001 does not inhibit the activity of the 49-kDa CP (Zuo et al. 2001), it is expected that the cysteine plasmids (CP) will not modulate the disease nor induce protective antibodies in vaccinated fish. There was no obvious drop in PCV after fish were vaccinated with the CP plasmid DNA as was found with MP-DNA. Also, no agglutinating antibodies were detected in the fish vaccinated with the 47-kDa cysteine protease plasmid DNA. These confirm the non-reactivity of mAb-001 with the smallest of the four cysteine protease bands in *C. salmositica* (Zuo et al. 2001). However, it is not known if the 49-kDa CP plays a part in protection when it is administered with the other three cysteine protease bands. Studies with leishmanial cysteine proteases CPa and CPb in mammals suggest that combinations of the cysteine proteases or CP plus MP (gp63) are required for better protection against

Trypanosoma and *Leishmania* (Authie et al. 2001; Rafati et al. 2001; Zadeh-Vakili et al. 2004; Mottram et al. 2004; Mahmoudzadeh-Niknam and McKerrow 2004). Although the cysteine protease that was tested in the present study was not the complete protease, previous work had shown that a partial cysteine protease might also elicit good immune responses (Law et al. 2003). In the cysteine protease vaccinated fish, the course of infection was opposite to the MP-vaccinated groups. The cysteine protease vaccinated group had higher parasite numbers than the PBS controls. Thus, it would seem that the 47-kDa partial cysteine protease might have antagonistic properties. Ahmed et al. (2004) also found that the previously protective p20 antigen did not induce protection but caused significantly larger lesions compared to saline PBS controls when mice were challenged with high numbers of parasites.

Although the LV does not cause disease in salmonids (e.g., Woo and Li 1990; Sitja-Babodilla and Woo 1994), it may cause a slight temporary anemia in fish (e.g., Atlantic salmon) where the strain multiplies (Ardelli and Woo 2002; Davy et al. 2007). This is because of considerable release of parasite antigens with the production of antibodies (e.g., complement-fixing antibodies; Li and Woo 1995) by the fish and the formation of immune complexes on the surface membrane of red blood cells (Thomas and Woo 1988). This would explain the temporary decrease in PCV (at about 6 wpv) in the vaccinated Atlantic salmon in the present study.

Our current study on trout and salmon indicates that vaccination with MP-DNA vaccine against cryptobiosis is a promising option. Dosages, boosters, frequency of boosting, and size of challenge dose are some of the questions that will be addressed in subsequent studies.

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