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Immune response of *Cryptobia*-resistant and *Cryptobia*-susceptible *Salvelinus fontinalis* to an *Aeromonas salmonicida* vaccine

B. F. Ardelli, P. T. K. Woo*

Department of Zoology, University of Guelph, Guelph, Ontario, Canada NIG 2W1

ABSTRACT: Brook charr *Salvelinus fontinalis*, both those susceptible and those resistant to the pathogenic haemoflagellate *Cryptobia salmositica*, responded well to a commercially available *Aeromonas salmonicida* vaccine. The proportion of susceptible and resistant charr which produced agglutinating antibody were similar, but resistant brook charr had higher agglutination titres than susceptible brook charr. Concanavalin A stimulated peripheral blood leucocytes of *Cryptobia*-resistant charr and the kinetic profiles suggest that maximal stimulation is dependent on mitogen concentration. *Cryptobia*-resistant brook charr displayed both cell-mediated and humoral responses suggesting that their immune functions are similar to those of *Cryptobia*-susceptible charr.

KEY WORDS: Humoral immunity · Cell-mediated immunity · Agglutinating antibody · Leucocytes · Concanavalin A

INTRODUCTION

Cryptobia salmositica (Katz) is a pathogenic haemoflagellate of *Oncorhynchus* spp. on the west coast of North America (Woo 1987). Forward et al. (1994) demonstrated that not all brook charr *Salvelinus fontinalis* were susceptible to *C. salmositica*. Susceptible brook charr with high parasitaemias did not suffer from cryptobiosis (e.g. they were not anaemic) and parasitaemias were significantly reduced when charr were immunized with an attenuated strain of the pathogen (Ardelli et al. 1994). Since infected *Cryptobia*-susceptible brook charr do not suffer from cryptobiosis, Ardelli et al. (1994) suggested that they may serve as reservoir host for the pathogen where their range overlaps with those of the susceptible *Oncorhynchus* spp.

Forward et al. (1993, 1994) showed that the *Cryptobia* resistance (innate immunity) in brook charr is controlled by a dominant allele and that this innate resistance to *C. salmositica* infection is inherited by progenies of resistant parent(s). Since it is now possible to breed *Cryptobia*-resistant fish, we suggest that they be considered in future stocking programmes especially in areas that are enzootic for cryptobiosis. However, critical studies should be conducted on the basic biology of *Cryptobia*-resistant brook charr before we embark on a selective breeding programme. Woo (1992) suggested that the studies should include careful examinations of their immune responses to available vaccines, their innate and acquired resistance to other diseases, and their growth rate and fecundity.

The main objective of the present study is to examine the acquisition of acquired immunological (humoral and cell-mediated) responses in *Cryptobia*-resistant brook charr to a commercially available *Aeromonas salmonicida* vaccine.

MATERIALS AND METHODS

Experimental design (*Cryptobia*-susceptible salmonids). Three groups of fishes (n = 20 group^{-1}) were used and each contained 2 sub-groups: 10 hatchery-raised adult rainbow trout *Oncorhynchus mykiss* (A1, B1, C1; 251.78 g ± 43.56) and 10 laboratory-raised

*Addressee for correspondence; E-mail: pwoo@uoguelph.ca

Cryptobia-susceptible brook charr *Salvelinus fontinalis* (A2, B2, C2; 225.18 g ± 66.37). Each fish in Group A and Group B was inoculated intraperitoneally with 250 000 *C. salmositica* in 0.2 ml of sterile cold-blooded vertebrate Ringer's solution (CBVR). The inoculum was from a single rainbow trout with a high parasitaemia (>1 000 000 *C. salmositica* ml^{-1} blood) and diluted with sterile CBVR. Each fish in Group C was inoculated intraperitoneally with 0.2 ml of sterile CBVR. At 4 wk postinfection each fish in Group A and Group C was inoculated intraperitoneally with 0.2 ml of a commercially available *Aeromonas salmonicida* vaccine (Furogen, Aquahealth Ltd, Charlottetown, Prince Edward Island, Canada) and each fish in Group B was inoculated intraperitoneally with 0.2 ml of sterile CBVR. At 7 wk postinoculation of *C. salmositica* (3 wk after inoculation of the *A. salmonicida* vaccine) each fish in Group A and Group C was inoculated intraperitoneally with an additional 0.2 ml of the *A. salmonicida* vaccine and each fish in Group B was inoculated intraperitoneally with 0.2 ml of sterile CBVR. Plasma samples from each group were collected weekly and stored at –20°C to determine agglutination titres against *A. salmonicida* (humoral response).

Experimental design (*Cryptobia*-resistant salmonids). Three groups (n = 5 $group^{-1}$; D, E, and F) of *Cryptobia*-resistant *Salvelinus fontinalis* (1373.2 g ± 205.76) were used. Preinoculation blood samples (1.0 ml^{-1} fish) were obtained on alternate weeks from each fish in Group D, Group E, and Group F. After 2 wk, each fish in Group D and Group E was inoculated intraperitoneally with 0.2 ml of a commercially available *Aeromonas salmonicida* vaccine (Furogen, Aquahealth) and each fish in Group F was inoculated intraperitoneally with 0.2 ml of sterile CBVR. At 4 wk postinoculation, each fish in Group D was inoculated intraperitoneally with 0.2 ml of sterile CBVR and each fish in Group E and Group F was inoculated intraperitoneally with 0.2 ml of the *A. salmonicida* vaccine. Plasma samples from each group were collected and stored at –20°C to determine agglutination titres against *A. salmonicida*. Peripheral blood leucocytes were isolated immediately and used to estimate lymphocyte proliferation (cell-mediated response).

Parasite count. Parasitaemias were determined using a haemocytometer; low parasitaemias were detected using the haematocrit centrifuge technique (Woo & Wehnert 1983). Blood was diluted in CBVR using a red (1:10) or white (1:200) cell micropipette. Diluted blood was dispensed into both chambers of the haemocytometer, allowed to settle, and parasites were counted under a microscope in either red cell chambers or white cell chambers.

Preparation of *Aeromonas salmonicida* antigen for agglutination test. *Aeromonas salmonicida* antigen (RS842) was grown on tryptic soy agar plates at 18°C for 24 h. Bacteria were harvested by washing cells from the plates with sterile phosphate-buffered saline (PBS), and washed by centrifugation (1350 rpm, 190 × *g*) in sterile PBS (pH 7.2). The washing was repeated and the cell pellet was suspended in sterile PBS (final optical density of 1.0 at 520 nm) for the agglutination test.

Detection of agglutinating antibody against *Aeromonas salmonicida*. Fish plasma was heat inactivated for 30 min at 40°C (Sakai 1981). 25 µl was dispensed into U-shaped wells in a microtitre plate (kept on ice) and serially diluted (2-fold dilution) in 25 µl of sterile PBS with safranin. The solution contained 1 ml of safranin stock solution (0.005% weight/volume) in 100 ml of sterile PBS. Suspended *Aeromonas salmonicida* (25 µl) was added to each well. Plates were incubated at 37°C for 2 h, removed, and placed at 4°C for 24 h to allow the bacteria to either settle or agglutinate. The end point was the reciprocal of the last dilution with a positive reaction (uniform layer of clumped bacteria across the bottom). In a negative reaction, the bacteria formed a small 'button' in the base of the well.

Isolation and processing of peripheral blood leucocytes (adapted from Denizot & Lang 1986). Fish were bled aseptically by swabbing the caudal peduncle with 70% alcohol, and, using a sterile syringe and needle, 1.0 ml of blood was withdrawn from the caudal vein and diluted 1:3 with heparin and RPMI (+ 2.0 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1% penicillin-streptomycin, and 25 mM Hepes, filter-sterilized, pH 7.3). Blood was transferred aseptically to 15 ml sterile disposable modified polystyrene centrifuge tubes with plug seal cap in a tissue culture cabinet. Blood was centrifuged at 50 × *g* for 10 min and the plasma was withdrawn. The cells were resuspended with 4 ml of RPMI (containing phenol red) and gently loaded onto 4 ml of sterile Ficoll-Paque using a 10 ml sterile serological pipette. The mixture was centrifuged at 2700 rpm (780 × *g*) for 30 min. Ficoll-Paque (containing leucocytes) was removed and washed twice in RPMI at 2700 rpm (780 × *g*) for 10 min. After washing, leucocytes were resuspended to 1.0 ml in PR-RPMI (– phenol red and 2-mercaptoethanol).

Leucocytes were diluted 1:20 with PR-RPMI and then diluted 1:1 in trypan blue (50 µl cells and 50 µl of 2% trypan blue). Living cells (not stained) were counted using a haemocytometer and cells were diluted to a final concentration of 1×10^7 in 50 µl using PR-RPMI.

Sterile plates (96-well cluster, flat bottom with lid, tissue-culture treated) were used. To each well was added 50 µl of cells, 25 µl of trout plasma (heat-inactivated, filter-sterilized) and 25 µl of Concanavalin A (in PR-RPMI) at concentrations of either 1.0 mg ml^{-1}, 0.1 mg ml^{-1}, 0.2 mg ml^{-1}, or 0.4 mg ml^{-1}. Each mitogen

treatment was performed in triplicate. Plates were incubated in air for 4 d at 18°C.

MTT assay to evaluate cell-mediated immunity (adapted from Denizot & Lang 1986). Culture media was removed by carefully inverting, flicking, and blotting the plate. To each well, 100 µl of a 1 mg ml^{-1} solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) in PR-RPMI was added. The plates were gently shaken and incubated at 18°C for 4 h. At the end of the incubation period, untransformed MTT was removed by carefully inverting, flicking, and blotting the plate. The formazan product in each well was dissolved in 150 µl $well^{-1}$ of dimethylsulfoxide (DMSO) for 15 min. The optical density was measured using a 560 nm test wavelength (measures MTT) and a 690 nm reference wavelength (measures background) in a microplate reader.

Statistical analysis. Data were analyzed using the Statistical Analysis System (SAS) (SAS Institute Inc 1985). An analysis of variance (ANOVA) was performed to determine significant differences in parasitaemias, agglutination titres, and optical density values between groups. Results were considered significant if $p \leq 0.05$ (Steel & Torrie 1980).

RESULTS

Parasitaemias

Significant differences were not detected in mean parasitaemias between rainbow trout in Groups A1 and B1 and between *Cryptobia*-susceptible brook charr in Groups A2 and B2. Parasitaemias in rainbow trout (Groups A1 and B1) fluctuated during the course of infection and peaked at 4 and 6 wk (Group A) and 4 and 8 wk (Group B) postinoculation. All rainbow trout (Groups A1 and B1) were still infected at 10 wk postinoculation (Fig. 1). Mean parasitaemias in brook charr (Group A2 and Group B2) peaked at 4 wk postinoculation, and rapidly declined. At 10 wk postinoculation, 2 of 10 brook charr still had low detectable parasitaemias (Fig. 1).

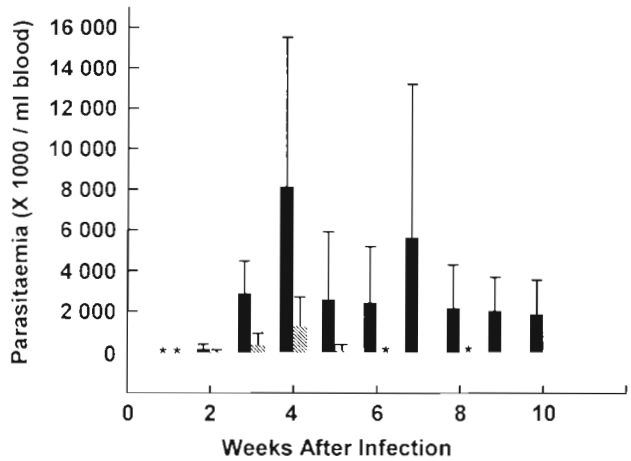

Fig. 1. *Oncorhynchus mykiss, Salvelinus fontinalis*. Mean parasitaemias (± standard deviation) in rainbow trout and brook charr infected with *Cryptobia salmositica*; black bars: parasitaemias in rainbow trout (Group A1); shaded bars: parasitaemias in brook charr (Group A2); (*) detectable parasitaemias

Agglutinating antibody

Cryptobia-susceptible salmonids

Agglutinating antibody against *Aeromonas salmonicida* was not detected in fish in Groups A and C prior to injection with the microbial vaccine nor in fish in Group B (which were only infected with *C. salmositica*). At 1 wk postinoculation of the *A. salmonicida* vaccine, all brook charr (A2 and C2) had detectable antibody. In contrast, only 7 of 10 (A1) and 8 of 10 (C1) rainbow trout had detectable antibody at 1 wk postinoculation and antibody was detectable in all rainbow trout at 2 wk (A1) and 5 wk (C1) postinoculation (Table 1). Peak agglutination titres were reached in brook charr at 2 wk (A2) and 4 wk (C2) postinoculation of the vaccine and in rainbow trout at 5 wk (C1) and 6 wk (A1) postinoculation (Table 1).

Agglutination titres against *Aeromonas salmonicida* in *Cryptobia*-infected brook charr were significantly higher than those in uninfected brook charr at 2 wk ($p = 0.002$) and at 3 wk ($p = 0.014$) wk postinoculation of the *A. salmonicida* vaccine. In contrast, significant differences were not detected between the 2 groups of rainbow trout. After 4 wk, no significant differences were detected between responding fishes in Group A and Group C (Table 1).

Cryptobia-resistant salmonids

Agglutinating antibody against *Aeromonas salmonicida* was not detected in fish in Group C (naive controls) nor in preinoculation samples (Groups D, E and F). All fish in Groups D and E responded to the vaccine, with the exception of 2 wk (Group E) and 3 wk (Group D) postinoculation, in which only 3 of 5 fish responded. In addition, antibody was only detected in undiluted plasma in Group E at 2 wk postinoculation. Titres were similar for fish in Groups D and E at 4 and 5 wk postinoculation. Titres for fish in Group E increased at 6 wk (2 wk after second inoculation of the vaccine) postinoculation (Table 2).

Table 1 *Salvelinus fontinalis* and *Oncorhynchus mykiss*. Agglutinating antibody against *Aeromonas salmonicida* in fishes in Group A (inoculated with *Cryptobia salmositica* and *A. salmonicida*) and Group C (inoculated with *A. salmonicida*) (Expt 1). Data are number of fish responding/total number of fish (using undiluted plasma) and, in parentheses, mean antibody titre of responding fish ± standard deviation

Weeks post-inoculation of *Aeromonas* vaccine	Brook charr Group A2 (infected)	Brook charr Group C2 (uninfected)	Rainbow trout Group A1 (infected)	Rainbow trout Group C1 (uninfected)
1 [5][a]	10/10 (1.1 ± 0.32)	10/10 (1.0 ± 0)	7/10 (0)	8/10 (1.5 ± 0.71)
2 [6]	10/10 (2.2 ± 1.04)	10/10 (1.0 ± 0)	10/10 (1.2 ± 0.45)	6/10 (1.0 ± 0)
3 [7]	10/10 (1.56 ± 0.53)	10/10 (1.0 ± 0)	10/10 (1.0 ± 0)	9/10 (1.0 ± 0)
4 [8]	10/10 (1.71 ± 1.12)	10/10 (2.25 ± 1.49)	10/10 (1.17 ± 0.41)	9/10 (1.0 ± 0)
5 [9]	10/10 (1.57 ± 0.54)	10/10 (1.0 ± 0)	10/10 (1.0 ± 0)	10/10 (2.0 ± 1.42)
6 [10]	10/10 (1.2 ± 0.45)	10/10 (2.0 ± 1.73)	10/10 (1.84 ± 1.17)	10/10 (0)

[a][ ]: weeks postinoculation of *C. salmositica*

Mitogenic response of peripheral blood leucocytes (*Cryptobia*-resistant salmonids)

Although PBL were stimulated with each of the 4 mitogen concentrations tested, the pattern of stimulation obtained with these cells was optimal at a mitogen concentration of 1.0 mg ml^{-1} (Fig. 2). Significant differences were detected between Groups D and F at 1 wk ($p = 0.0036$) and 5 wk ($p = 0.0081$) postinoculation of *Aeromonas salmonicida*. Optical density values for Group E at 2 and 4 wk postinoculation were significantly higher than those values obtained for Group D at 1 wk ($p = 0.0001$) and 3 wk ($p = 0.0001$) postinoculation and for those values obtained for Group F at 1 wk ($p = 0.0001$) and 3 wk ($p = 0.0001$) postinoculation. Optical density values for Group F at 5 wk postinoculation were significantly higher than those obtained for Group D at 5 wk ($p = 0.0081$) and for those values obtained for Group E at 6 wk ($p = 0.0316$) wk postinoculation.

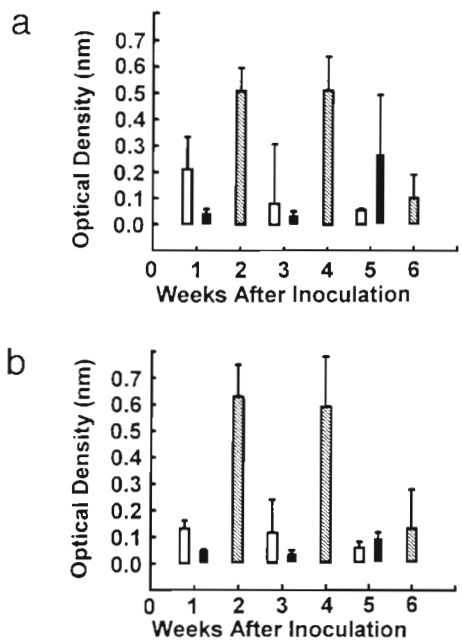

Fig. 2. Mitogenic responses of *Cryptobia*-resistant brook charr peripheral blood leucocytes. 10^7 leucocytes were incubated for 4 d with (a) 1.0 mg ml^{-1} and (b) 0.4 mg ml^{-1} of Concanavalin A. Bars represent mean optical density values (n = 5, r = 3) of stimulation indices from Group A (white bars), inoculated with *Aeromonas salmonicida* and CBVR; Group B (shaded bars), inoculated with *A. salmonicida* and given a booster 4 wk postinoculation; Group C (black bars), inoculated with CBVR and *A. salmonicida*

Table 2. Agglutinating antibody in *Cryptobia*-resistant brook charr inoculated with an *Aeromonas salmonicida* vaccine (Expt 2). Data are number of fish responding/total number of fish (with undiluted plasma) and, in parentheses, mean antibody titre of responding fish ± standard deviation

Weeks postinoculation of *Aeromonas* vaccine	Group D (*Aeromonas* vaccine)	Group E (*Aeromonas* vaccine)	Group F (naive controls)
0	0	0	0
1	5/5 (4.4 ± 2.19)		0/5 (0)
2		3/5 (0 ± 0)	
3	3/5 (3.33 ± 2.31)		0/5 (0)
4		5/5 (5.2 ± 1.09)	
5	5/5 (4.2 ± 2.49)		0/5 (0)
6		5/5 (5.6 ± 3.69)	

DISCUSSION

Both *Cryptobia*-susceptible (Expt 1) and *Cryptobia*-resistant (Expt 2) brook charr responded to the *Aeromonas salmonicida* vaccine. The proportion of brook charr (Group A and Group C) which produced

agglutinating antibody in Expt 1 was similar to those in Expt 2. However, brook charr in Expt 2 had higher agglutination titres than those in Expt 1. Since water temperature, water flow, oxygen, and total number of formalin-killed bacteria inoculated were similar in both experiments, differences in antibody production might reflect genetic differences because the fish came from different families of laboratory-raised fish.

Cryptobia-resistant brook charr (Group E, Expt 2) appeared to have a delayed response and did not show peak titres until 6 wk postinoculation of the microbial vaccine. Titres in *Cryptobia*-susceptible brook charr (Group A2, Expt 1) peaked at 2 wk postinoculation of the *Aeromonas salmonicida* vaccine and then slowly declined. The differences between *Cryptobia*-susceptible and *Cryptobia*-resistant brook charr might also be due to differences in the size of the fish. *Cryptobia*-resistant brook charr weighed from 981.6 to 1743.2 g while the weight of *Cryptobia*-susceptible brook charr ranged from 368.2 to 761.6 g. Dilution of the antibody and the vaccine, due to the larger volume of blood in the heavier charr, might have contributed to the delay in detectable levels of antibody in the larger resistant brook charr.

Paterson & Fryer (1974) found that antibody production in coho salmon *Oncorhynchus kisutch* was delayed in fish immunized with *Aeromonas salmonicida* in Freund's complete adjuvant compared with similar production in coho salmon injected with a soluble antigen (*A. salmonicida* endotoxin). They also suggested that soluble antigen would more rapidly neutralize and remove antibodies from the circulatory system than an antigen containing adjuvant. Vaccines with adjuvant are usually dissipated more slowly and antigenic stimulation is lower but prolonged, and it may take several wk before antibodies are detected. The *A. salmonicida* vaccine used in this study is in an aluminum phosphate adjuvant. Therefore, adjuvant would not explain the differences between *Cryptobia*-resistant and *Cryptobia*-susceptible brook charr because both fish families were inoculated with the same vaccine.

The *Cryptobia*-resistant brook charr responded well to the vaccine and had higher antibody titres than *Cryptobia*-susceptible fish. Salmonid populations may differ in their resistance to diseases, and this resistance has a genetic basis (Beacham & Evelyn 1992). Ehlinger (1977) showed that some strains of *Salvelinus fontinalis* were more resistant to furunculosis but more susceptible to gill disease. Similarly, a population of *Oncorhynchus mykiss* which was more susceptible to *Vibrio* spp. was less susceptible to *Renibacterium* spp. and *Aeromonas* spp. (Winter et al. 1980).

The peripheral blood leucocytes from *Cryptobia*-resistant brook charr proliferated in response to Concanavalin A. Mitogenic response has been reported in other fish species; for example, phytahaemagglutinin stimulated leucocytes from the paddlefish *Polyodon spathula* and the stingray *Dasyatis americana* (Olson 1967) and Concanavalin A and phytahaemagglutinin stimulated peripheral blood leucocytes from the nurse shark *Ginglymostoma irratum* (Lopez et al. 1974). Etlinger et al. (1976) demonstrated that patterns of mitogenic responses of leucocytes from the thymus, anterior kidney, spleen and blood of rainbow trout differed significantly with various lymphocyte mitogens. They suggested that there is lymphocyte heterogeneity in rainbow trout and that these lymphocytes have a unique tissue distribution.

Using MTT concentrations from 0.4 to 1.0 mg ml^{-1} (Fig. 2) with a constant number of cells per well, it was found that the amount of formazan produced was optimal at a concentration of 1.0 mg ml^{-1}. These results in the present study are consistent with the observations of Denizot & Lang (1986) as there was an increase in formazan product from 0.1 to 1.0 mg ml^{-1}.

The amount of formazan product is directly proportional to the number of viable cells present (Mosmann 1983). Also, activated cells produce more formazan than resting cells, which allows the measurement of activation even in the absence of proliferation. These properties are consistent with the cleavage of MTT only by active mitochondria (Mosmann 1983). In the present study, leucocytes from unimmunized resistant brook charr (Group C) had significantly lower mitogenic responses than brook charr immunized with the *Aeromonas salmonicida* vaccine (Fig. 2). The higher mitogenic response in vaccinated brook charr suggests that leucocytes were already activated (by *A. salmonicida*) prior to incubation. There were no detectable proliferations without the addition of mitogens. Thus, the higher optical density values in fish immunized with *A. salmonicida* is indicative of stimulation of a cell-mediated response in *Cryptobia*-resistant brook charr.

The results of the present study indicated that *Cryptobia*-resistant and *Cryptobia*-susceptible brook charr were able to produce antibodies against a microbial vaccine. In addition, the present study confirmed that *C. salmositica* does not cause clinical signs in *Cryptobia*-susceptible brook charr (Ardelli et al. 1994). *Cryptobia*-resistant brook charr displayed both cell-mediated and humoral responses suggesting that their immune functions were similar to those of *Cryptobia*-susceptible brook charr and rainbow trout. Thus, in situations where brook charr are being released into areas which are enzootic for cryptobiosis, it would be better to release fish with innate resistance to the parasite because this would not increase the number of reservoir hosts.

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