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ORIGINAL PAPER

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An in vitro study on the mechanism of innate immunity in *Cryptobia*-resistant brook charr (*Salvelinus fontinalis*) against *Cryptobia salmositica*

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Abstract Fresh plasma from *Cryptobia*-resistant brook charr (*Salvelinus fontinalis*) lysed *Cryptobia salmositica* under in vitro conditions. However, the parasite was not lysed if the plasma was heat-inactivated at 37°C for 2 h or after the addition of ethylenediaminetetraacetic acid (EDTA) to chelate the free Mg^{2+} ions. Addition of Mg^{2+} ions to EDTA-chelated plasma restored parasite lysis. Treatment of *Cryptobia*-resistant plasma with either ethylenebis(oxyethylenenitrilo)tetraacetic acid, a Ca^{2+}-chelating agent, or cobra venom factor did not reduce their lytic titres. The alternative pathway of complement activation is the mechanism of innate immunity against *C. salmositica*. The present study also shows that there is functional heterogeneity amongst the complement components activated via the alternative pathway within a species (brook charr) and between species (brook charr and goldfish) of fishes.

Introduction

Cryptobia salmositica Katz 1951 (Sarcomastigophora: Kinetoplastida) is a haemoflagellate that infects all species of Pacific salmon, *Oncorhynchus* spp. It causes disease and mortality in some salmon stocks on the West coast of North America (Woo 1987, 1991, 1994). The clinical signs of salmonid cryptobiosis include exophthalmia, abdominal distension with ascites, general oedema, splenomegaly, anaemia (Woo 1979), positive antiglobulin reactions of red cells (Thomas and Woo 1988) and anorexia (Li and Woo 1991; Thomas and Woo 1992). The immune system in infected fish is depressed (Jones et al. 1986) and the anorexia contributes to the immunodepression (Thomas and Woo 1992). The amount of complement in the blood of infected rainbow trout is significantly reduced (Thomas and Woo 1989), and this may also contribute to depression of the immune system.

Very little is known about innate immunity and the mechanism of resistance in fish to parasitic infections (Woo 1992). In an earlier study, Forward et al. (1995) found that not all laboratory-raised brook charr, *Salvelinus fontinalis*, were susceptible to *C. salmositica* infection and that the resistance was inherited by the progeny of resistant fish. They showed that the plasma of laboratory-raised *Cryptobia*-resistant brook charr (resistant to experimental infection) lysed *C. salmositica*, whereas the plasma of *Cryptobia*-susceptible brook charr was not cryptobicidal under similar in vitro conditions. The main objective of the present study was to elucidate the mechanism of innate immunity in *Cryptobia*-resistant brook charr.

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Materials and methods

Materials

The plasma of brook charr used in the present study was obtained from the same laboratory-raised fish used in the earlier study (Forward et al. 1995). It was either freshly collected from fish via caudal vein puncture under MS-222 or collected earlier and stored at –100°C. Since the parasite was lysed when incubated in the plasma of *Cryptobia*-resistant brook charr but not when incubated in the plasma of susceptible brook charr (Forward et al. 1995), the in vitro plasma incubation test (Bower and Woo 1977) was used to study the mechanism of innate immunity in the resistant fish.

The T4 strain of *C. salmositica* (see Woo 1978, 1979) was cultured as previously described (Forward et. al. 1995). The parasite does not infect goldfish (*Carassius auratus*), the plasma of which lyses the parasite under in vitro conditions (Wehnert and Woo 1980). Hence, freshly collected goldfish plasma was used as an additional control in the study. In the in vitro plasma incubation test, all solutions and the microtitre plate were kept cold (4°C) at all times (Bower and Woo 1977), the fish plasma (treated or untreated – see below) was serially diluted with phosphate-buffered saline and about 500 cultured parasites (Woo and Li 1990) were incubated with the plasma at 4°C for 3 h. The titre was the highest dilution in which there were less than ten living parasites when the microtitre plate was examined using an inverted microscope (objective 10X, eyepiece 10X).

Table 1 Lytic titres determined in untreated and treated plasma from susceptible F1 brook charr (charr 1 and 2) and resistant goldfish, F1 brook charr (charr 3-10) and a human (NE # No end point – i.e. parasites were not lysed; – not done)

Source of plasma	Family origin	Plasma treatments[a]							
		Untreated	Heat (37°C)	+EDTA	+EDTA +Mg^{2+}	+EGTA	+EGTA +Ca^{2+}	+Co VF Incubation time	
								20 min	4 h
Goldfish	–	1:2	NE	NE	1:1	1:2	1:2	1:2	1.2
Human	–	1:1	–	–	–	–	–	1:2	NE
Charr 1	111	NE	NE	NE	NE	NE	NE	NE	NE
Charr 2	119	NE	NE	NE	NE	NE	NE	NE	NE
Charr 3	115	1:2	NE	NE	1:1	–	–	–	–
Charr 4	115	1:2	NE	NE	1:1	–	–	–	–
Charr 5	117	1:2	NE	NE	1:1	–	–	–	–
Charr 6	108	1:2	NE	NE	1:1	1:2	1:2	1:2	1:2
Charr 7	129	1:2	NE	NE	1:1	1:2	1:2	1:2	1:2
Charr 8	109	1:2	–	–	–	1:2	1:2	–	–
Charr 9	109	1:4	–	–	–	1:2	1:2	–	–
Charr 10	116	1:2	–	–	–	1:2	1:2	–	–

[a] Lytic titre

Table 2 Lytic titres determined for pre-inoculation plasma from 2-year-old F1 resistant (a–k) and susceptible (l–m) brook charr heated at 37° C for 2 h and supplemented with fresh plasma from susceptible charr donors (NE No end point – i.e. parasites were not lysed, – not done)

Plasma source	Family origin	Unheated[a]	Heated source plasma+fresh donor plasma[a]			
			Donor 1	Donor 2	Donor 3	Donor 4
Donor 1	140	NE	–	–	–	–
Donor 2	115	NE	–	–	–	–
Donor 3	111	NE	–	–	–	–
Donor 4	115	NE	–	–	–	–
Goldfish	–	1:2	NE	NE	NE	NE
Charr a	140	1:2	1:2	NE	NE	NE
Charr b	129	1:2	1:2	NE	NE	NE
Charr c	108	1:4	1:2	NE	NE	NE
Charr d	108	1:2	NE	NE	–	–
Charr e	119	1:2	NE	–	–	–
Charr f	111	1:2	NE	–	–	–
Charr g	115	1:2	NE	NE	–	–
Charr h	129	1:2	NE	NE	–	–
Charr i	115	1:2	–	NE	–	–
Charr j	117	1:2	–	NE	–	–
Charr k	108	1:2	–	NE	–	–
Charr l	111	NE	–	NE	–	–
Charr m	119	NE	–	NE	–	–

[a] Lytic titre

Plasma treatments

Individual samples of pre-inoculation plasma from goldfish, a human, and susceptible and resistant 2-year-old charr (F1; Forward et al. 1995) were subjected to the following treatments prior to incubation with living parasites using the in vitro plasma incubation test.

Heat inactivation of plasma

Plasma was heated at 37°C for 2 h and cooled to 4°C prior to use.

Chelation and subsequent supplementation with free Mg^{2+} ions

Ethylenediaminetetraacetic acid (EDTA) was added to fresh plasma to a final concentration of 0.01 *M*. A portion of the chelated plasma was re-supplemented with $MgCl_2$ to a final concentration of 0.08 *M* Mg^{2+}. EDTA was also added to some heat-inactivated plasma.

Chelation and subsequent supplementation with free Ca^{2+} ions

Ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) was added to fresh plasma to a final concentration of 0.01 *M*. Some chelated plasma was also re-supplemented with $CaCl_2$ to a final concentration of 0.024 *M* Ca^{2+}.

Treatment with lyophilized cobra venom factor

Fresh plasma from two resistant and two susceptible 2-year-old charr (F1) and a human were used for this study. The plasma (0.025 ml) was incubated for 20 min or for 4 h at 4°C with 0.025 ml cobra venom factor (CVF) derived from the Thialand cobra, *Naja naja kaouthia* (Sigma Chemical Co., St. Louis, Mo., USA). The stock solution contained 250 µg in 1.25 ml cold-blooded vertebrate Ringer's saline.

Complement supplementation of heat-treated plasma

Plasma from susceptible or resistant charr was heated at 37°C for 2 h to inactivate the complement. Following heating and cooling to 4°C, fresh plasma (0.025 ml) from four charr (donors 1–4, whose fresh plasma did not lyse *C. salmositica*; after the plasma had been removed for the supplementation study the susceptibility of the fish to the parasite was confirmed by i.p. inoculation; Forward et al. 1995) was added to the treated plasma.

Results

Plasma from goldfish and five *Cryptobia*-resistant charr (charr 3–7) heated at 37°C for 2 h and cooled to 4°C did not lyse the parasite (Table 1). This was confirmed using plasma from six other resistant charr. Fresh or stored (at –100°C) plasma from these resistant brook charr lysed the parasite. Similar results (non-lysis of the parasite) were obtained following heat treatment of plasma from these resistant charr (charr 3–7) and goldfish with EDTA (0.01 *M*). The experiment was repeated, and fresh plasma from resistant charr (charr 3–7) and goldfish treated with EDTA (0.01 *M*) did not lyse the parasite. However, plasma from goldfish and resistant charr (charr 3–7) treated with EDTA (0.01 *M*) and then supplemented with 0.08 *M* Mg^{2+} lysed the parasite; lytic titres were half of what they had been before EDTA treatment (Table 1).

Plasma from goldfish and *Cryptobia*-resistant charr (charr 6–10) treated with EGTA (0.01 *M*) also lysed the parasite. Lytic titres were unchanged except that the end point was halved in the plasma of one resistant fish (charr 9) after EGTA treatment; it was reduced from 1:4 to 1:2 (Table 1). Plasma from goldfish and resistant charr (charr 6–10) treated with EGTA (0.01 *M*) and supplemented with 0.024 Ca^{2+} lysed the parasite, and lytic titres were unchanged except for a reduction in titre in plasma from charr charr 9.

Human plasma incubated at 4°C for 20 min with CVF lysed the parasite. However, the same human plasma incubated at 4°C for 4 h with CVF did not lyse the parasite, whereas the same untreated plasma lysed the parasite. Plasma from goldfish and resistant charr incubated at 4°C for 4 h with CVF continued to lyse the parasite and lytic titres were unchanged.

Untreated plasma from susceptible charr (charr 1 and 2) or plasma treated with either heat, EDTA, EDTA plus Mg^{2+}, EGTA, EGTA plus Ca^{2+}, or CVF did not lyse the parasite (Table 1).

Fresh plasma from a susceptible charr (donor 1) added to heat inactivated plasma of eight resistant charr (Table 2, a–h) restored the lytic titres in only three charr (Table 2, a–c). The experiment was repeated with plasma from the same resistant charr (a–h) and the results were similar. Fresh plasma from another susceptible charr (donor 2) did not restore the lytic titres in heat inactivated plasma from 11 resistant charr (a–d and g–m) (Table 2). Similarly, fresh plasma from a third and fourth susceptible charr (donors 3 and 4) did not restore the lytic titres in heat-inactivated plasma of three resistant charr (a–c). Also, fresh plasma from donors 1–4 did not restore the lytic titre in heat-inactivated goldfish plasma.

Discussion

The present in vitro study suggests that the alternative pathway of complement activation is the mechanism of innate resistance in some brook charr to *Cryptobia salmositica*. The in vitro test indicates that the cryptobicidal property of plasma from goldfish and *Cryptobia*-resistant charr are lost after heat inactivation (Table 1). Goldfish are innately resistant to *C. salmositica* infection, and plasma of goldfish lyses the parasite via the alternative pathway of complement activation (Wehnert and Woo 1980). Consequently, goldfish plasma was used as one of the controls for all in vitro plasma incubation tests performed in the present study.

Addition of EDTA to plasma from goldfish and resistant charr blocked the lytic activities. EDTA chelates both free Mg^{2+} and Ca^{2+} ions from the plasma and, therefore, one or both of these cations are required for lysis of the parasite. However, plasma from goldfish and resistant charr chelated with EDTA and supplemented with Mg^{2+} ions lysed the parasite, although the lytic titres were reduced. This shows that free Mg^{2+} ions are required for lysis of the parasite and that the concentration of Mg^{2+} ions in supplemented plasma (0.08 *M*) was not sufficient to restore the pre-chelation lytic titres in *Cryptobia*-resistant charr and goldfish. It is also possible that free Ca^{2+} ions are also required for lysis and that the EDTA concentration used (0.01 *M*) inhibited lysis by chelating all the Mg^{2+} ions but not all the Ca^{2+} ions.

The results show that free Ca^{2+} ions are not required in the lytic mechanism. EGTA chelates only Ca^{2+} ions. The lytic titres in EGTA-chelated plasma from a goldfish and four resistant charr (charr 6–8 and 10) supplemented with Ca^{2+} were unchanged from pre-treatment titres.

Complement is a part of the vertebrate immune system (Day et al. 1970) and is composed of a series of proteins in the plasma. Activation of the complement cascade is involved in lysis of some foreign cells, opsonisation, chemotaxis of macrophages and anaphylaxis (Leid 1988). The lytic activity of complement occurs in a cascade fashion and terminates with holes punched into the cell membrane of the activating organism (Sakai 1992). Complement can be activated through either the classic or alternative pathways.

The classic pathway is initiated by the formation of antigen-antibody complexes. The formation of these complexes requires specific antibodies against the activating organism. Consequently, the host must have previous exposure to the organism for complement to be activated through the classic pathway. All brook charr used in the present study were raised in the laboratory and had no previous exposure to *C. salmositica*. Also, the classic pathway requires free Ca^{2+} ions for activation of complement (Sakai 1983), and the lytic mechanism of goldfish

and resistant charr does not require free Ca^{2+} ions for activation.

Complement of fish contains heat-labile components (Sakai 1981), and the lytic mechanism of resistance in brook charr and goldfish plasma is dependent on heat-labile factors. Also, the alternative pathway requires free Mg^{2+} ions for the activation of complement (Sakai 1983), and the lytic mechanism in both goldfish and resistant charr requires free Mg^{2+} ions. The present study confirms that CVF has no anticomplementary effect on the plasma of bony fishes and suggests that the anticomplementary effect of CVF in human plasma is dependent upon the incubation time. CVF inactivates complement component C3 of humans (Muller-Eberhard and Fjellstrom 1971). Human plasma incubated in the present study at 4°C for 4 h with CVF did not lyse the parasite, whereas untreated plasma lysed *C. salmositica,* as did plasma treated for a short period. The apparent increase in cryptobicidal titre (in short-term-treated human plasma) is likely due to experimental variations. CVF, however, had no anticomplementary effect on the plasma of goldfish or resistant brook charr (present study).

Day et al. (1970) reported that CVF inactivated complement of mammals, birds, reptiles, amphibians and some cartilaginous fishes but had no anticomplementary effect on the plasma of two teleost, the common carp (*Cyprinus carpio*) and the paddlefish (*Polyodon spathula*).

The present study shows that there is functional heterogeneity among the complement components activated via the alternative pathway (innate immunity) within a species (brook charr) and between species (brook charr and goldfish). When added to heat-inactivated plasma from some *Cryptobia*-resistant brook charr, fresh plasma from *Cryptobia*-susceptible brook charr restored lytic titres against *C. salmositica* (Table 2). However, lytic titres of heat-inactivated plasma from some resistant brook charr and goldfish were not restored. Sakai (1981) has shown that there is no difference in the hemolytic activity of fish complement when antibodies from other fish species are used to lyse heterologous red blood cells and has concluded that teleost fish complements are appropriate for the complement-mediated immune reactions (acquired immunity) of teleosts.

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