Partial cross protection against *Ichthyophthirius multifiliis* in *Gyrodactylus derjavini* immunized rainbow trout

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Abstract

Partial cross protection against a skin-parasitic ciliate has been recorded in rainbow trout previously immunized with an ectoparasitic platyhelminth. The susceptibility to infection by *Ichthyophthirius multifiliis* differed significantly between naive and *Gyrodactylus derjavini* immunized rainbow trout. Fish partly immune to the ectoparasitic monogenean *G. derjavini* became less infected and experienced lower mortality than naive fish when exposed to *I. multifiliis* infections. *In vitro* studies on immobilization of theronts using decomplemented (heat-inactivated) serum from *G. derjavini* immune or non-immune hosts showed no immobilization. However, untreated serum from both immune and non-immune fish containing intact complement immobilized theronts (titre 128–256). In addition, non-specific priming of the host response with interleukin (IL-1), bacterial lipopolysaccharide (LPS), concanavalin A (Con A) or mannan did confer a partial resistance to *I. multifiliis* infection. This will suggest that non-specific factors including complement could be partly responsible for the host response against infections with this ciliate.

Introduction

Freshwater fish in most parts of the world are susceptible to the ubiquitous freshwater ciliate *Ichthyophthirius multifiliis*, which continues to represent a threat to both wild fish populations (Elser, 1955; Allison, 1963; Wurtsbaugh & Tapia, 1988; Jessop, 1995; Traxler *et al*., 1998) and cultured hosts (Fouquet, 1876; Buschkiel, 1910, 1937; Butcher, 1947; Bauer, 1958; Johnson, 1961; Wootten & Smith, 1980; Valtonen & Keränen, 1981; Mellergaard & Dalsgaard, 1987; Buchmann & Bresciani, 1997). Several studies have documented that a number of fish species are able to develop acquired resistance to challenge infections with this skin parasitic ciliate (Buschkiel, 1910; Bauer, 1958; Hines & Spira, 1974; Houghton & Matthews, 1993; McCallum, 1986; Clark *et al*., 1988; Cross & Matthews, 1992; Clark & Dickerson, 1997) which makes immunoprophylactic measures such as vaccination feasible (He *et al*., 1997). However, the responsible immune mechanisms are still subject to some dispute. Both cellular factors (Graves *et al*., 1985; Cross & Matthews, 1993; Cross, 1994; Sin *et al*., 1996) and humoral factors (Hines & Spira, 1974; Wahli & Meier, 1985; Clark & Dickerson, 1997) have been suggested to be involved in the host response but their relative importance are poorly defined. In addition, some studies have indicated that the protection is based on non-specific mechanisms whereby reactions to other ciliates offer protection against *I. multifiliis* (Goven *et al*., 1981; Wolf & Markiw, 1982; Dickerson *et al*., 1984; Ling *et al*., 1993). Rainbow trout do also develop acquired resistance against the ectoparasitic monogenean *G. derjavini* (Buchmann & Bresciani, 1998; Lindenstrøm & Buchmann, 1998) and the present study describes a partial cross protection against *I. multifiliis* in rainbow trout immunized against *G. derjavini.* In addition, a number of studies conducted *in vitro* and *in vivo* are described which indicate that

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non-specific factors are involved in this cross-protection against *I. multifiliis.*

Materials and methods

Fish

Fry (4–5 cm body length) of rainbow trout *Oncorhynchus mykiss* were purchased from a closed pathogen free system using recycled water (Fischer Fish, Zealand). In the laboratory they were kept in 200 l aquaria at 11.6°C (temperature controlled room) with aerated freshwater (pH 7.3, 390 mg l^{-1} $CaCO_3$), internal biofilters (Eheim) and partial water changes (75%) every week.These fish were used for infection experiments with *I. multifiliis* and *G. derjavini*. Larger fish (20 cm body length) which either had been exposed to *G. derjavini* for 12 months (immune fish) or had been kept uninfected (naive) were used for blood sampling.

Parasites

Laboratory stocks of *I. multifiliis* were established by transfer of trophonts on ten infected rainbow trout (obtained from a Danish trout farm supplied by natural stream water (Refsgaard 2) during an outbreak in the month of July) to ten specimens of naive rainbow trout fry in 200 l aquaria. By addition of a number of naive fish every 10 days continous cultures of the parasite stock were produced. A high infection pressure was created by leaving the aquarium without water exchange for 14 days. The low infection pressure was created by removing fish from the aquarium and changing the main part of the water volume.

The *Gyrodactylus derjavini* laboratory stock was kept in the laboratory for 2 years before use. It was originally established by transfer of infected rainbow trout from Paelebro trout farm (Jutland) to fish tanks with naive fish.

Experimental design

All experiments were conducted in a temperature controlled room (11.6°C). Rainbow trout were immunized against *Gyrodactylus derjavini* by exposing 30 fish to approximately 300 free parasites in small plastic aquaria (10 l) containing 3 l water (Buchmann & Bresciani, 1998). After 24 h infected fish were checked for infection and transferred to 200 l glass aquaria where they were kept for 8 weeks. During this period, the infection increased to more than 30 parasites per fish in week 5 followed by a decline to 0–2 parasites per fish in week 8, an infection pattern previously described by Buchmann & Bresciani (1998). Rainbow trout (naive or immunized against gyrodactylosis) were then exposed to *I. multifiliis* infection. In one experiment fish were subjected to a high *I. multifiliis* infection pressure and in another experiment to a low infection pressure by transfer of experimental fish to the infected aquaria mentioned above. *Gyrodactylus* immunized fish were tagged by cutting 10% of the dorsal part of the caudal fins, whereas the naive fish were tagged by cutting the corresponding ventral part. Fin cutting was performed on MS 222 (Sigma) (80 mg l^{-1}) anaesthetized fish. Mortality and the number of established trophonts were then counted with 3–8 days interval. In addition, 25 infected fish from the stock population (previously naive to both parasites but kept for 14 days in the high infection aquarium before initiation of experiments) were likewise examined for additional information.

Parasite counts

Fish were anaesthetized with MS 222 (Sigma) (80 mg l^{-1}) in small (200 ml) glass beakers. The established *I. multifiliis* trophonts on the fins were then counted with the aid of a subilluminated dissection microscope (magnification 6.6–50×). The density of parasites on various body parts was calculated based on the fin surface measurements provided by Buchmann & Bresciani (1998). A number of parasites was found on the gills and corpus. However, due to the difficulties in obtaining an accurate count on the body surface proper, only fins and corneae were included in this work.

Measurements of mucous cells and trophonts

Naive rainbow trout fry were subjected to infection as described above and removed after 48 h and 120 h. Fish were then fixed in neutral phosphate buffered 4% formaldehyde for 7 days whereafter fins were dissected, rinsed in PBS, stained in 1% Alcian Blue in 3% acetic acid, rinsed in distilled water and mounted in Aquamount. The length of mucous cells (200 cells per fish) and established trophonts were measured at 400–1000× magnification.

Serum

Blood samples were recovered by caudal vein puncture of MS 222 (80 mg l^{-1}) anaesthetized rainbow trout (body length 20 cm) immune or non-immune to *Gyrodactylus derjavini* (two fish in each group). Following centrifugation of blood (11,000 *g*, 5 min) serum was removed, pooled and used untreated or decomplemented (heat-inactivated 44°C, 20 min) for immobilization studies on theronts. For this purpose, serial two-fold dilutions with PBS (phosphate buffered saline) pH 7.2 were used.

Theronts

Trophonts were allowed to escape from heavily infected rainbow trout fry in 100 ml glass beakers containing 50 ml tap water (11.6°C). Following encystment and production of tomites in tomocysts, this stage was transfered to sterile glass beakers. When theronts were released the concentration of theronts was determined using a Sedgewick Rafter cell.

Immobilization assay

Theronts were added to a 96 well microtitre plate (Nunc) (five theronts in 125 μl water per well), thereafter serum dilutions were added in corresponding volumes. The activity of theronts were determined every 10 min for 2 h. The titre was determined as the reciprocal of the dilution where theronts first showed motility.

Non-specific priming of host response

A total of 72 rainbow trout were divided into five

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Table 1. Infection levels and mortality (%) in the stock population and in fish (naive and *Gyrodactylus derjavini* immune) exposed to a high infection pressure for 6 days. Only specific regions (fins and cornea) are included.

Experiment	No. of fish	Mortality	Mean intensity (SD)
Stock population	25	#	159.6 (59.9)
Gyrodactylus immunized	17	18	79.7 (27.5)*
Naive	17	83	195.7 (51.3)

*Significantly different from naive fish, $P < 0.05$; #all stock fish died within the following 2 days.

groups. Groups were the untreated control or fish either immersed for 18 h in concanavalin A (10 mg l^{-1}) (BDH Biochemicals, UK, No. 44203) or mannan (50 mg l^{-1}) (Sigma M 7504) or injected intraperitoneally with interleukin 1 (IL-1, 50 ng per fish) (Sigma I 5396) or lipopolysaccharide (LPS, 50 μg per fish) (*E. coli* serotype O111, Sigma L-2630). After 18 h the fish were tagged by fin cuts and exposed in the same fish tank (70 l water) to a high *Ichthyophthirius multifiliis* infection pressure by cohabitation with ten heavily infected rainbow trout. The fish were examined for infection (number of trophonts on all fins) after 10 days and the survival determined after 26 days.

Statistics

The mean number of parasites per fish (mean intensity) and standard deviation (SD) was calculated for each group. Differences between groups were tested with the Mann-Whitney U-test (5% probability level). Spearman rank correlation coefficients (r) between density of trophonts in various parts (fins and corneae) and the density of mucous cells (Buchmann & Bresciani, 1998) in these structures were then calculated.

Results

High infection pressure

Rainbow trout immunized against *G. derjavini* experienced within 6 days a lower mortality and lower infection compared with naive fish exposed to a high infection pressure. The source of this infection was 25 fish infected with a mean of 159.6 trophonts per fish (table 1). These fish died within 2 days following examination. Only 17% of the naive fish survived at day 6 and all of these fish were dead within 7 days whereas 82% *G. derjavini* immune fish were still alive at day 6 and 7. However, the continued high infection pressure was lethal to the latter fish group within 11 days.

Low infection pressure

Gyrodactylus derjavini immunized fish experienced a significantly lower infection and mortality compared with naive fish (table 2). Mortality of naive fish reached 100% within 22 days whereas *G. derjavini* immunized fish merely showed a 30% cumulative mortality.

Site selection

The parasite distribution showed that particular fins were primarily chosen by *Ichthyophthirius multifiliis* (table 3). When the correlation coefficients between parasite density and mucous cell density were calculated, it was found that these parameters were positively associated. However, the correlations were only significant in naive fish (tables 4 and 5).

Mucous cell and parasite size

The Alcian Blue stained mucous cells in the pectoral fins varied in size from 16 to 28 μm. The smallest parasites were 28 μm long but some had increased in size to 93 μm 120 h post-exposure (table 6).

Immobilization assay

Theronts were not immobilized in heat-inactivated sera neither from host immune nor non-immune to gyrodactylosis. However, untreated sera (with complement) from both groups immobilized theronts (titres 128–256) (table 7).

Non-specific priming

Ten days after exposure to parasites the control group fish were infected with a mean of 13.9 parasites per host (table 8). However, the interleukin and LPS treated groups had mean intensities of 3.1 and 3.9, respectively. The mannan and Con A treated groups also showed a significantly lower infection (8.9 and 6.0, respectively) compared with the control. Despite this difference all groups experienced a mortality of 66% after 26 days continued exposure.

Table 2. Infection levels (fins and cornea) and mortality in naive and *Gyrodactylus derjavini* immune rainbow trout exposed to a low *Ichthyophthirius multifiliis* infection pressure.

	Number of fish		Mortality %		Intensity (SD)	
Days post-exposure	Immune	Naive	Immune	Naive	Immune	Naive
0	10	17	0	0	0	0
6	8	12	20	29	4.5 (3.2)*	21.7 (10.4)
9	8	12	20	29	10.9 (5.0)*	32.0 (13.0)
14	8	8	20	53	55.1 (23.3)*	176.9 (43.7)
22	7	0	30	100	16.1 (10.4)	–

*Significantly different from naive fish, $P < 0.05$.

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Table 3. Site selection of *Ichthyophthirius* trophonts on rainbow trout fins and cornea.

Body structure	Surface area/fish (Buchmann & Bresciani, 1998)	Stock population (distribution of parasites)		*Gyrodactylus* immune (high infection, day 6)		Naive (high infection, day 6)		*Gyrodactylus* immune (low infection, day 14)		Naive (low infection, day 14)		Mean number of mucous cells mm^{-1} (Buchmann & Bresciani, 1998)
		%	No. cm^{-1}	%	No. cm^{-1}	%	No. cm^{-1}	%	No. cm^{-1}	%	No. cm^{-1}	
Caudal fin	1.14	18.1	17.3	18.7	13.1	15.3	26.3	30.4	14.7	15.3	23.6	116.7
Adipose fin	0.1	3.8	40.3	5.2	41.5	5.1	100.0	1.1	6.3	3.2	56.7	236.6
Dorsal fin	0.23	16.9	83.1	12.1	43.1	22.0	195.5	16.6	40.6	21.4	170.0	309.8
Pectoral fins	0.88	25.0	30.8	21.1	19.2	17.4	38.6	18.4	11.6	23.5	47.3	285.1
Pelvic fins	0.57	21.5	41.5	25.3	35.9	23.2	80.9	15.0	14.7	20.3	64.4	269.3
Anal fins	0.33	13.9	46.9	16.7	41.2	12.3	75.0	18.4	31.2	15.9	87.2	217.7
Corneae	0.30	0.8	2.9	0.9	2.1	4.7	31.1	0.1	0.4	0.4	3.3	49.9

Discussion

Rainbow trout responding to the ectoparasitic monogenean *Gyrodactylus derjavini* possess one or more mechanisms in the skin which provide some protection against the ciliate *Ichthyophthirius multifiliis*. These mechanisms are as yet poorly defined but the numerous investigations on the immune response in fish against this parasite provide an opportunity of studying of these reactions. It has been clearly shown (Clark & Dickerson, 1997) that specific monoclonal antibodies binding to ciliary antigens of *I. multifiliis* force the parasite to exit the skin of primed fish. Likewise, the specificity of the cellular reactions is indicated by inhibition of macrophage migration in the presence of *I. multifiliis* antigens (Sin *et al*., 1996). This, however, will not exclude a number of non-specific reactions counteracting a challenge infection with this parasite. The non-specific nature of some protective mechanisms against *I. multifiliis* was shown by Wolf & Markiw (1982), Dickerson *et al*. (1984), and Ling *et al*. (1993). They were able to induce protection against

Table 4. Spearman rank correlation coefficients between parasite density and mucous cell density at high infection pressure for 6 days.

Experiment	r
Stock population	0.679
Gyrodactylus immunized	0.667
Naive	0.714*

*$P < 0.05$.

Table 5. Spearman rank correlation coefficients between parasite density and mucous cell density at low infection pressure for 22 days.

Days post-exposure	Naive	Immune
0	–	–
6	0.57	0.43
9	0.82*	0.61
14	0.68*	0.49
22	–	0.45

*$P < 0.05$.

Table 6. Mucous cell size (pectoral fins) and size of established trophonts in the rainbow trout fins following different exposure times.

	Mucous cell size (μm)		Trophont size (μm)	
Exposure time	Min	Max	Min	Max
Control (no infection)	16	28	–	–
48 h	16	25	28	45
120 h	16	28	31	93

Table 7. Titres from immobilization assays (2 h incubation) using *Ichthyophthirius multifiliis* theronts and serial dilutions of pooled sera from either *Gyrodactylus derjavini* immune or naive fish.

Serum type	Complement	Immobilization titre
Immune	Present	128
Immune	Inactivated	1
Non-immune	Present	256
Non-immune	Inactivated	1

Table 8. Non-specific activation of host responses against *Ichthyophthirius multifiliis* (infection with trophonts 10 days after non-specific priming).

Group	N	Mean intensity	SD	P
Control	15	13.9	4.8	-
Mannan	14	8.9	3.3	< 0.006
Con A	15	6.0	3.1	< 0.001
LPS	14	3.9	2.1	< 0.001
IL-1	14	3.1	1.4	< 0.001

Mann-Whitney U-test.

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I. multifiliis by immunizing with *Tetrahymena* antigens. Presumably, antibodies towards ciliary antigens in the latter species are able to bind *I. multifiliis* antigens. Indeed, Goven *et al*. (1981) and Clark *et al*. (1988) showed a weak cross-reactivity of anti-*I. multifiliis* immunoglobulins to *Tetrahymena* ciliary antigens. However, controversial aspects of these vaccines were stressed by Dickerson & Clark (1994) and the immobilization assay presented here showed that the observed cross-protection was not associated with immobilizing antibodies. In contrast, complement from both immune and non-immune fish immobilized theronts which will indicate the role of non-specific factors. In addition, Graves *et al*. (1985) found that non-specific cytotoxic cells (NCC) offered catfish protection against a number of pathogens, including *I. multifiliis*. Recently (Evans *et al*., 1998) demonstrated that these cells recognize certain epitopes expressed by a range of cells and organisms (as *Tetrahymena*) which lead to the killing of the target cell. It cannot be excluded that such reactions are involved in the *G. derjavini* induced response observed in this work. Disturbance of the teleost epidermis will indeed initiate a number of strong non-specific reactions. It has been suggested that epithelial injury produced by monogeneans will elicit the production of interleukin 1 (IL-1) which subsequently activates leucocytes and induces mucous cells to secrete their contents (Buchmann & Bresciani, 1998). Besides mucopolysaccharides, the secretion of complement, lysozyme, immunoglobulins and C-reactive protein was likely to follow and mucous cells have been presumed to play a pivotal role against monogeneans (Buchmann & Bresciani, 1998; Sterud *et al*., 1998). A constitutive production of these antiparasitic molecules could explain that 50% of theronts invading the teleost epidermis are lost within 5 min even in naive fish (Ewing & Kocan, 1992). Whether such a non-specific reaction is responsible for the 79% rejection of parasites in immune fish (Cross & Matthews, 1992) is unknown, but it cannot be excluded that hyperactivation of this production following monogenean invasion is involved in partial protection against *I. multifiliis*.

The present study on the activation of non-specific reactions in the host by using treatments with IL-1, LPS, mannan and Con A supports this view. It is known that interleukin (IL-1) and LPS elicit hyperplasia of teleost epidermis, including mucous cells (Balm *et al*., 1995). Con A is known to activate lymphocytes and macrophages. In addition, IL-1 is an immunological key factor released by skin macrophages after infection with ectoparasites (Buchmann & Bresciani, 1998). Mannan should be considered immunologically relevant as mannose-rich regions in *G. derjavini* have been demonstrated to bind and activate complement factor C3 and thus play a role in immunity to this ectoparasite (Buchmann, 1998). Thus, the present study using these molecules indicates that non-specific responses offer some protection against *I. multifiliis*.

The mode of *I. multifiliis* infection in fish epidermis has not been fully elucidated. A direct penetration of epithelial cells has been suggested by Matthews (1994) who showed the perforatorium of invading theronts in close contact with the host nucleus. However, these parasites show site predilection. In the present work, the majority of parasites were found on the dorsal and pelvic fins and the pectoral fins often exhibit high parasite density. Other studies have shown a corresponding site selection (Buschkiel, 1910; Paperna, 1972; Hines & Spira, 1973; Kozel, 1986; Clayton & Price, 1988) and the present investigations indicate that this phenomenon is associated with differentiated mucous cell densities. Actually, the most heavily infected sites showed the highest mucous cell density and it is likely that parasites are attracted to these cells in naive fish. Parasites are not attracted over long distances to the host (Wahli *et al*., 1991) but respond chemotactically to serum components in mucus following random contact (Lom & Cerkosovova, 1974). Furthermore, mucous cell openings would be a suitable additional target for the invasion behaviour of theronts which included a directed rotation around the longitudinal axis (Nehresheimer, 1908; Buschkiel, 1910; Geisslinger, 1987). Mucous cells measure 16–28 μm which corresponds to the size of theronts. The smallest established trophonts found in the present study are 28 μm. The literature reports that theront sizes vary from 15 to 20 μm (at 25°C) to 60 μm (at 3–4°C) (Wagner, 1960). The smallest trophonts found by MacLennan (1942) in the fish epidermis were 31 μm at 4–20 h post-infection and Buschkiel (1910) found theront sizes to vary between 25 and 35 μm. In addition, the weaker association between mucous cell density and parasite density in immune fish can be caused by increased amounts of antiparasitic products released in mucus from immune fish. Correspondingly, a weak positive correlation between monogenean attachment and mucous cell densities on naive fish shifted to a negative correlation when the host responded (Buchmann & Bresciani, 1998).

The openings of mucous cells are located in between the epithelial cells and are easily accessible. If theronts also are able to penetrate these openings, this will explain the fast entry and lack of damage to the superficial epithelial cells seen in some studies. Actually, the theront invading the epithelium has been studied by SEM (Chapman, 1984; Kozel, 1986; Geisslinger, 1987) and by TEM (Ewing *et al*. 1985; Ewing & Kocan, 1992) who demonstrated that invasion took place between epithelial cells.

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