Humoral response in early stages of infection of cod (*Gadus morhua* L.) with atypical furunculosis

Bergljot Magnadottir[1]
Berglind Gisladottir[2]
Sigridur Steinunn Audunsdottir[1]
Birkir Thor Bragason[1],
AND
Sigridur Gudmundsdottir[1]

[1]Institute for Experimental Pathology, University of Iceland, Keldur v. Vesturlandsveg, 112 Reykjavik, Iceland,
E-mail:bergmagn@hi.is Corresponding author
E-mail: ssa2@hi.is
E-mail: birkirbr@hi.is
E-mail: siggag@hi.is

[2]The Blood Bank, University Hospital, Snorrabraut 60, 105 Reykjavík, Iceland
E-mail: beg6@hi.is

ABSTRACT

Atlantic cod (*Gadus morhua*) was experimentally infected with the bacterium *Aeromonas salmonicida* subsp. achromgenes (Asa), which causes atypical furunculosis in cod and other fish species. Uninfected fish and fish infected with two different bacterial dosages were used and blood samples collected before infection and after 1 and 6 days. The effect of infection on the following humoral parameters was determined: the total protein serum level, the concentration of cortisol, two types of pentraxins and IgM, and on the specific and natural antibody and anti-trypsin activity. The results showed that infection by Asa caused a significant increase in the stress hormone cortisol while other parameters showed limited and in general suppressive response to infection. Neither CRP PI nor CRP PII, the two types of cod pentraxins, acted as typical acute phase proteins in the early stages of infection. The short duration of the experiment may have influenced the limited response observed but the results also reflect the relatively slow immune response of cod.

Keywords: Cod *Gadus morhua, Aeromonas salmonicida,* cortisol, pentraxin, natural antibodies

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Vessabundið viðbragð þorsks á fyrstu stigum sýkingar af völdum kýlaveikibróður

Þorskur (*Gadus morhua*) var sýktur með bakteríunni *Aeromonas salmonicida* undirteg. *achromogenes* (Asa), sem veldur sjúkdómnum kýlaveikibróður í þorski og fleiri fisktegundum. Ósýktur fiskur og fiskur sýktur með tveim mismunandi skömmtum af Asa voru notaðir og blóðsýni tekin fyrir sýkingu og eftir 1 og 6 daga. Áhrif sýkingar á eftirfarandi vessabundna þætti í sermi voru könnuð: heildar magn prótína, cortisols, tveggja gerða af pentraxínum og IgM, og á virkni sérvirkra og náttúrulegra mótefna og ensímtálma. Niðurstöður sýndu að sýking olli umtalsverðri aukningu á streituhormóninu cortisól en hafði takmörkuð og frekar bælandi áhrif á aðra þætti. Hvorki CRP PI né CRP PII, tvær gerðir af bráðaprótínum (pentraxínum) sem greinst hafa í þorski, sýndu dæmigerð bráðasvarsviðbrögð í sýkingu. Stuttur tilraunatími hefur sennilega haft áhrif á þau takmörkuðu viðbrögð sem greindust en gefa einnig til kynna tiltölulega seinvirkt ónæmisviðbragð þorsks við sýkingu.

INTRODUCTION

Since the establishment of the Marine Institute's Experimental Station at Stadur, Grindavík, in the late 1980's considerable progress has been made in hatchery production of cod (*Gadus morhua* (Linnaeus)) in Iceland (Steinarsson & Björnsson 1999, Björnsson & Steinarsson 2002) and it is expected that commercial cod farming will soon become a viable industry. Concurrently with the availability at Stadur of cod at different developmental stages, various research projects have been carried out at our Institute involving different aspects of cod's immune system and immune response (http://www.hi.is/gadus). This work, and work by other research groups, has shown that cod's immune response to infection or vaccination is unusual when compared to other fish species like, for example, the salmonids. Cod's specific antibody response seems to be deficient and its immune defence is characterised by non-specific cellular response like granuloma formation (Magnadottir et al. 2001, 2002, Pilstrom et al. 2005, Lund et al. 2006, 2008). High levels of non-specific, natural antibodies are also commonly seen in cod serum (Magnadottir et al 2009).

Various humoral parameters of cod have been characterized and their role in immune response or ontogeny studied (Magnadottir et al 2001, 2004, Lange et al 2004, 2005). Most recently the classical acute phase proteins, the pentraxins, have been isolated from cod serum and two types of CRP-like pentraxins characterized, referred to as CRP PI and CRP PII (Gisladottir et al. 2009). The role of these proteins in acute phase response and infection of cod is currently being investigated (Gisladottir 2008).

Acute phase response is a physiologically induced response to tissue injury, infection or trauma (Bayne & Gerwick 2001). This is a dynamic homeostatic process that aids healing and survival by removing and repairing damaged tissue and containing or destroying the infectious agent (Kushner 1982). The pentraxins, C-reactive protein (CRP) and serum amyloid P (SAP), are the typical acute phase proteins of mammals and both types have been demonstrated in the serum of some fish species, like plaice (*Pleuronectes platessa* L.) and rainbow trout (*Oncorhynchus mykiss* (Walbaum)) (White et al. 1981, Kodama et al. 1989, Jensen et al. 1995). Other species appear to have either CRP-like pentraxin, like cod, or SAP-like pentraxin, like Atlantic salmon (*Salmo salar* L.) and halibut (*Hippoglossus hippoglossus* L.) (Lund & Olafsen 1998).

The level of pentraxins in fish serum is relatively high compared to mammalian species but their role in the acute phase response may vary (Bayne & Gerwick 2001). Turpentine injection, which is commonly used to induce acute phase response, resulted in an increase in the serum CRP level of channel catfish (*Ictalurus punctatus* (Rafinesque)) within 2 – 4 days (Szalai et al., 1994) while a significant decrease was seen in a similar experiment on rainbow trout (Liu et al. 2004). Similarly, there are varied reports on the effects infection can have on the serum level or gene expression of pentraxins in fish. For instance, an apparent but statistically insignificant increase in the SAP serum level of Atlantic salmon was seen 2 days after infection with *A. salmonicida* subsp. *salmonicida* followed by a significant decrease on day 5 (Lund & Olafsen 1999), and in a recent study on zebra fish (*Danio rerio* (Hamilton)) immune response to *A. salmonicida* and *Staphylococcus aureus* showed an up-regulation of the CRP gene in response to the infections (Lin et al, 2007).

Several studies have indicated a close link between the neuro-endocrine and immune systems and stress is known to have a generally suppressive effect on the disease resistance of fish (Weyts et al. 1999, Yada & Nakanishi, 2002). This has also been demonstrated by the direct administration of cortisol to fish (Espelid et al 1996). However, there are also instances of cortisol having beneficial and possibly protective effects in the immune defence of fish (Weyts et al. 1998, Bilodeau et al. 2003).

The aim of the present study was to examine the effects of the initial stage of infection on a) the serum level of pentraxins and other immune

parameters and b) on the serum level of the stress hormone cortisol. The infective agent used was the bacterium *Aeromonas salmonicida* subsp. *achromogenes* (Asa), which causes atypical furunculosis in fish, including cod (Magnadottir et al, 2002). This is an endemic disease in Iceland and caused serious losses of farmed salmonids reared in brackish water before vaccination became a common practice (Gudmundsdottir 1998). No commercial vaccines are as yet available for cod and experimental vaccination has given variable and often poor protection (Gudmundsdottir & Bjornsdottir 2007).

MATERIALS AND METHODS

The fish

Atlantic cod (*Gadus morhua*) was obtained from the Marine Institute's Experimental Station, Staður, Grindavík, Iceland. The fish originated from wild gametes, which were fertilized and hatched at the experimental station and maintained at 7 – 8°C under normal cultural conditions (Steinarsson & Björnsson 1999, Björnsson & Steinarsson 2002). The mean weight (and standard deviation) of the fish used was 93.1 + 26.4 g. After transporting the fish to the Institute they were kept in 170 L tanks at 8°C in well aerated sea water, salinity 32 ‰, and allowed to acclimatize for a few days. Feeding was kept to a minimum during the experimental period.

Experimental infection

The bacterium

Aeromonas salmonicida subsp. *achromogenes* (Asa), strain F19/99 was used, originally isolated from wild cod in Iceland (Magnadottir et al. 2002, Gudmundsdottir et al 2003, Bjornsdottir et al 2005). The bacterium was passed three times through cod to ensure expression of virulence factors, and then re-isolated from kidney and cultivated on blood agar supplemented with 1.5% NaCl (BA-S) at 15°C for 72 h. The preparation of the bacterium for infection and determination of dosage (colony forming units (CFU)) was as described by Gudmundsdottir et al. (2003).

The infection of cod and sample collection

Fifty fish were used. Group 1 (n=19), the control fish, was injected intra-muscularly (i.m.) with 100 µl of phosphate buffered saline (PBS, Sigma, USA), group 2 (n=14) was injected i. m. with 100 µl containing $3x10^5$ CFU of Asa, and group 3 (n=17) was injected i.m. with 100 µl containing $3x10^6$ CFU of Asa. Before infection, six fish were killed by anaesthesia (with 50 mg ml^{-1} of tricaine methanesulfonate), and then 6 - 9 fish from each group were killed by anaesthesia 1 and 6 days later. The level of infection was determined by the re-isolation of Asa from the kidney and cultivation on BA-S. Blood was collected from the caudal aorta and serum collected after clotting overnight at 4°C and stored -80°C.

Serum analysis

Total protein concentration

Protein concentration was determined using a protein assay kit from Pierce (USA) based on a method by Bradford (1976) and following the producer's instructions. All samples were measured in duplicates. A standard curve was plotted using bovine serum albumin from the kit and the protein concentration of the serum samples extrapolated from this.

Cortisol concentration

A kit from Neogen Corp. (Lexington, KY, USA) was used following the manufacturer's instructions. All samples were measured in duplicates. A standard curve was plotted using a cortisol standard from the kit and the cortisol concentration of the samples calculated from this, as described by the manufacturer.

Serum IgM concentration

The ELISA method, described by Israelsson et al. (1991), was used with some modifications. Briefly, microwell trays (96 well, Maxisorp, Nunc) were coated with polyclonal rabbit anti-cod IgM antibody (a kind gift from the late professor L. Pilström, Department of Medical Immunology, Uppsala University, Sweden) 10 µg $well^{-1}$ in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6, Sigma), overnight

at 4°C. Residual sites were blocked with 1% bovine serum albumin in coating buffer for 1 h at room temperature and then serial dilutions of standard cod IgM (prepared at our Institute) and serum in PBS-T (PBS containing 0.05% tween 20) were incubated for 2 h at 37°C. This was followed by an incubation for 1 h at 37°C with mouse polyclonal anti-cod IgM antibody (prepared at our Institute) and then alkaline phosphatase conjugated rabbit anti-mouse Ig's (Dako) in PBS-T for 1 h at 37°C. Finally, nitro blue tetrazodium substrate (Sigma) was added and the reaction stopped after 30 min at room temperature with 1N NaOH and optical density (OD) read at 405 nm. A standard graph was plotted using the results from the standard IgM and the quantity of the IgM in serum was extrapolated from this. Extensive washing in PBS-T was carried out between each step.

Specific and natural antibody activity

Antibody activity was measured using an ELISA procedure previously described (Magnadottir et al 1999). MaxiSorp microwell trays were coated with 10 µg protein well^{-1} of a formalin killed and sonicated suspension of Asa diluted in coating buffer for measuring the specific antibody response or with 5 µg well^{-1} of trinitrophenyl conjugated bovine serum albumin (TNP-BSA) (prepared at our Institute) diluted in coating buffer for measuring the natural antibody activity (Magnadottir et al 2009). Following coating overnight at 4°C the trays were blocked with 0.1% semi-skimmed milk powder in coating buffer. Serum samples in duplicates, diluted 10^{-2} in PBS-T, were incubated overnight at 4°C, followed by incubation for 1 h at 37°C, first with mouse anti cod IgM antibody and then with alkaline phosphatase conjugated goat anti mouse Ig antibody (Dako), both diluted in PBS-T. Washing, developing and reading of the trays was as described above. Antibody activity was expressed as the OD value of serum diluted 10^{-2} after subtracting the blank values (PBS-T in place of serum).

Anti-protease activity

Anti-protease activity was measured using a modification by Magnadottir et al. (1999) of the method described by Bowden et al (1997). Twenty µl of serum were incubated with the same volume of standard trypsin solution (5 mg ml^{-1}, Sigma T-7409) in 1.5 ml microcentrifuge tubes for 10 min at room temperature. To this was added 200 µl PBS and 250 µl of 2% azocasein (Sigma), mixed well and incubated for 1 h at room temperature. Then 500 µl of 10% trichloroacetic acid was added, mixed well and incubated for a further 30 min at room temperature. The mixture was centrifuged at 6000 g for 10 min and 100 µl transferred to a 96 well, flat-bottomed, non-absorbent microtray (Nunc) containing 100 µl well^{-1} of 1N NaOH. Optical density was read at 450 nm. All samples were tested in duplicates. The blank contained PBS in place of serum and trypsin and the 100% reference wells contained PBS in place of serum. After subtracting the value of the blank the percentage inhibition of the trypsin compared to the reference sample was calculated for each sample. The anti-trypsin activity was expressed as the % inhibition.

Pentraxin concentration

CRP-PI

Capture ELISA was devised, following checkerboard titrations, to estimate the CRP PI concentration in cod serum. Microtrays were coated with polyclonal mouse anti-CRP PI purified immunoglobulins (prepared at our Institute), 10µg well^{-1} in coating buffer overnight at 4ºC. Blocking was with 0.1% skimmed milk powder in coating buffer for 1 hour at room temperature (22°C). Purified standard CRP PI protein in serial five-fold dilutions and serum samples diluted 1/500 and 1/2000, both diluted in PBS-T and in duplicates were incubated for 2 hours at room temperature. Biotin labelled anti-CRP PI mouse Ig´s (prepared at our Institute using an ECL protein biotinylation kit from Amersham Biosciences, UK) diluted in PBS-T was added and incubated for 1 hour at 37ºC. Next, alkaline phosphatase-linked

streptavidin (Dako, Dk.) diluted in PBS-T was added, and incubated for 1 hour at 37ºC. Washing, developing and reading of the trays was then as described above. A standard graph was plotted using the results for the purified standard CRP-PI and the quantity of the CRP PI in serum was extrapolated from this.

CRP-PII

The same procedure as described above was used for measuring the CRP PII concentration in serum, mouse anti CRP PII purified Ig's, biotin labelled anti CRP PII Ig's and standard CRP PII protein replacing the CRP PI equivalents. The two antibodies, anti-CRP PI and anti-CRP PII did not cross-react (Gísladóttir et al. 2009).

Statistical analysis

StatView™ analysis system for Windows was used for statistical analysis. Unpaired t-test was used to examine the difference between two distinct groups and $P< 0.05$ was set as the critical value of significance.

RESULTS

Infection of cod by Asa

Asa was not isolated from the control fish at any time, and there was no difference between groups 2 and 3 with respect to the percentage bacterial isolation. Asa was isolated from about 13% of the infected fish on day 1 and from about 80% of the infected fish on day 6. No fish died from Asa infection during the experimental time.

Serum analysis

Total protein concentration

The mean serum protein level of the fish was 35.2 ± 6.5 mg ml^{-1}. Figure 1 shows the serum protein concentration of the three groups, before infection and 1 and 6 days after infection. Compared to the pre-challenge values a significantly reduced protein level was observed in the control fish (group 1) and group 3 ($P= 0.0013$ and 0.0041 respectively) at 1 d.p.i., while on day 6 the protein level of these groups had returned to a level comparable to the pre-challenge values. Group 2 showed no change in protein level on day 1, while on day 6 a significantly reduced protein level

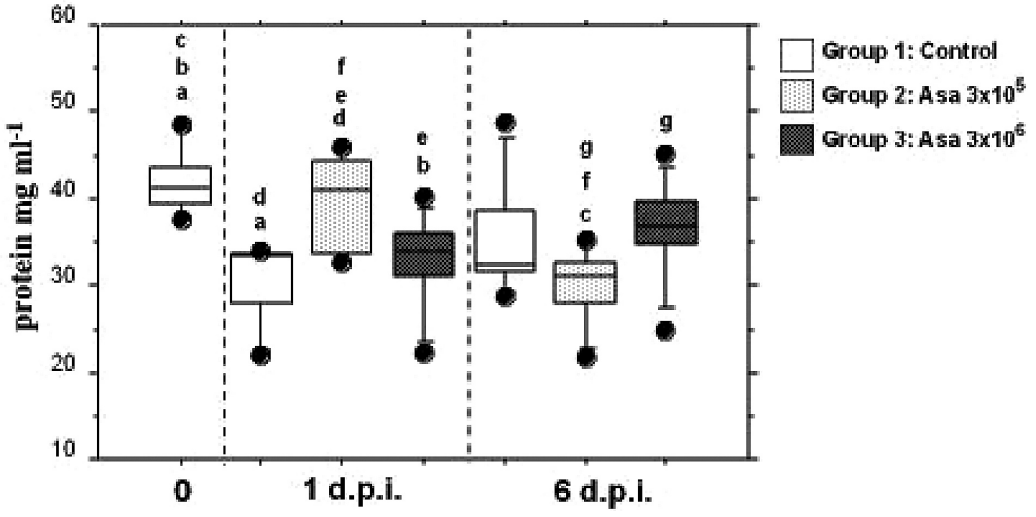

Figure 1. Box plots of serum protein concentration of non-infected (group 1) and infected (group 2 and group 3) cod before infection (0) and 1 and 6 days post infection (d.p.i.). The median is the line within each box, the boxes indicate 25 – 75 percentiles, the whiskers 10 – 90 percentiles, and the filled circles the extreme values. Corresponding markings indicate a statistically significant difference between the relevant groups ($P<0.05$).

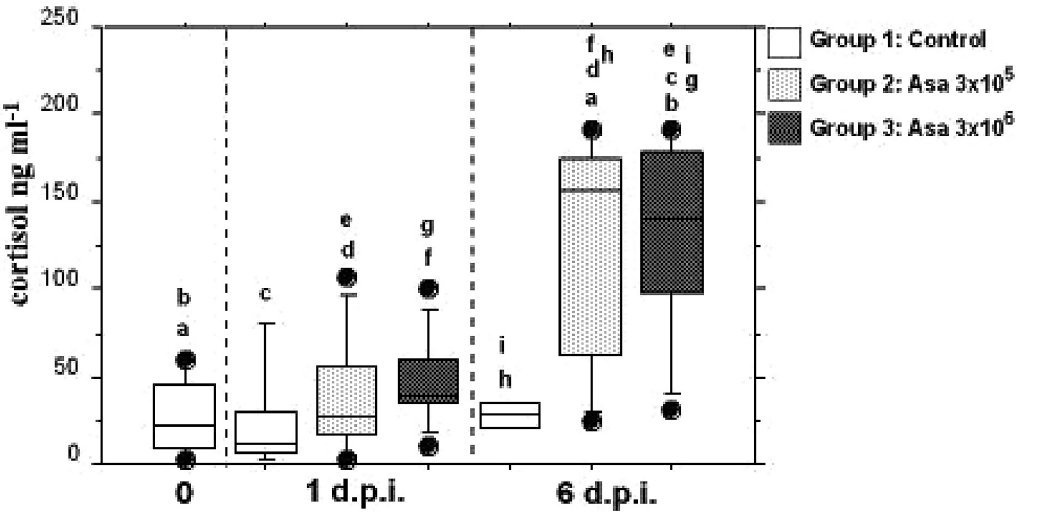

Figure 2. Box plots of serum cortisol concentration of non-infected (group 1) and infected (group 2 and group 3) cod before infection (0) and 1 and 6 days post infection (d.p.i.). The median is the line within each box, the boxes indicate 25 – 75 percentiles, the whiskers 10 – 90 percentiles and the filled circles the extreme values. Corresponding markings indicate a statistically significant difference between the relevant groups ($P<0.05$).

was observed compared to the pre-challenge level ($P=0.0003$) and compared to group 3 at this time point ($P= 0.0325$) but was not significantly different from the control fish at this time point.

Cortisol concentration

The mean cortisol level at pre-challenge was 26.7 ± 22.3 ng ml^{-1}. Figure 2 shows the serum cortisol level of the three groups, before infection and 1 and 6 days after infection. The cortisol level of the control group was not significantly changed at 1 and 6 d.p.i., while the cortisol level of both groups 2 and 3 showed slight increase at 1 d.p.i. and a significant, 4.5 – 4.7 fold increase (125 – 132 ng ml^{-1}, $P=0.0057$ and $P=0.0013$ respectively) at 6 d.p.i. compared to the control fish at the same time point. There was no statistical difference in the cortisol level of groups 2 and 3.

IgM concentration

The mean serum IgM concentration was 2.1 ± 0.5 mg ml^{-1} or about 5.7% of the serum protein content. Figure 3 shows the IgM concentration of the three groups, before challenge and after 1 and 6 days. Compared to the pre-challenge value there was no change in the IgM level of the control fish at 1 or 6 d.p.i. The IgM level of group 2 was unchanged at 1 d.p.i. but significantly reduced at 6 d.p.i. both compared to the pre-challenge value ($P=0.0234$) and to the control fish at this time point ($P=0.0387$). The concentration of group 3 was significantly reduced on day 1 ($P=0.0081$) compared to the pre-challenge value but not when compared to the other two groups (control and group 2) at this time point and the IgM level of group 3 had returned to the original level on day 6.

Specific and natural antibody activity

No specific activity against Asa was detected in any group at any time. The only statistical significant change in the natural antibody activity was seen in a reduced activity of group 2 compared to the pre-challenge value on day 6 ($P=0.0458$). However, the difference between the three groups was insignificant, both on day 1 and day 6.

Anti-trypsin activity

Figure 4 shows the anti-trypsin activity of the three groups, before infection and after 1 and 6 days. No significant change was seen in the

anti-trypsin activity of the control group at 1 or 6 d.p.i. compared to the pre-infection value. Group 2 showed no change in anti-trypsin activity at 1 d.p.i. but was significantly reduced on day 6, both compared to the pre-infection value and to the control group at this time point (P= 0.0017 and P=0.0106 respectively). Group 3 showed significantly reduced activity on day 1 (P=0.0225) compared to the pre-infection value but not compared to the control group at

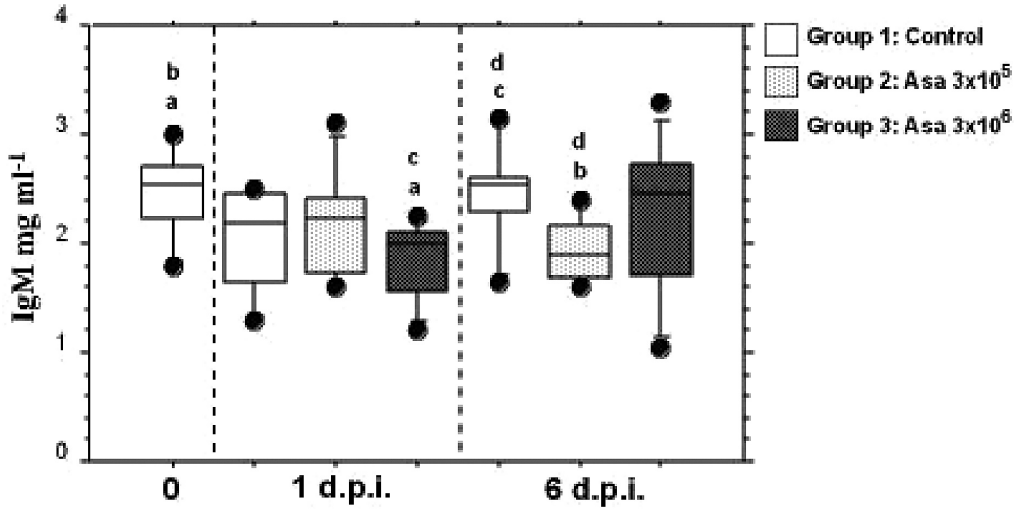

Figure 3. Box plots of the serum IgM concentration of non-infected (group 1) and infected (group 2 and group 3) cod before infection (0) and 1 and 6 days post infection (d.p.i.). The median is the line within each box, the boxes indicate 25 – 75 percentiles, the whiskers 10 – 90 percentiles, and the filled circles the extreme values. Corresponding markings indicate a statistically significant difference between the relevant groups (P<0.05).

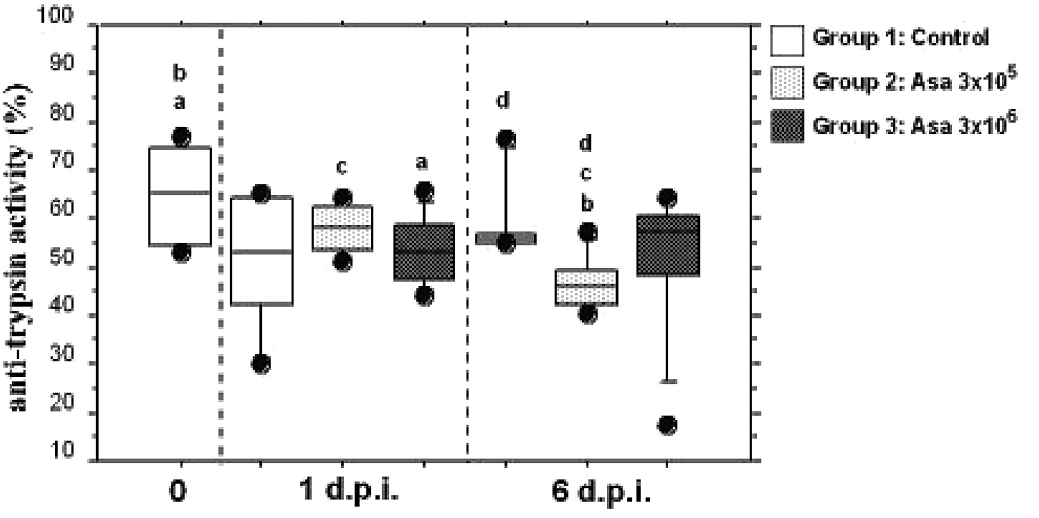

Figure 4. Box plots of anti-trypsin activity of non-infected (group 1) and infected (group 2 and group 3) cod before infection (0) and 1 and 6 days post infection (d.p.i.). The median is the line within each box, the boxes indicate 25 – 75 percentiles, the whiskers 10 – 90 percentiles, and the filled circles the extreme values. Corresponding markings indicate a statistically significant difference between the relevant groups (P<0.05).

this time point, and at 6 d.p.i. the anti-trypsin activity of group 3 was similar to the pre-infection values.

Pentraxin concentration

CRP PI

The mean CRP PI concentration and standard deviation of all three groups was 77.2 ± 29.2 μg ml^{-1}. Figure 5 shows the CRP PI serum concentration of the three groups, before injection and 1 and 6 days after injection. Compared to the pre-challenge values there was no change in CRP-PI level in any of the groups on day 1 while all 3 groups showed a significantly reduced level at 6 d.p.i (P=0.0275, 0.0045 and 0.0277 respectively for the control group, group 2 and group 3). However, there was no significant difference between the 3 groups at either time point, at 1 or 6 d.p.i.

CRP PII

The mean CRP PI concentration and standard deviation of all three groups was 58 ± 27 μg ml^{-1}. Compared to the pre-challenge value there was no change in the CRP PII level of the three groups at either 1 or 6 d.p.i. and the 3 groups showed similar CRP PII levels at both 1 and 6 d.p.i.

The results of these seven analyses are summarised in Table 2. In all instances considerable individual variation was observed with respect to all parameters.

DISCUSSION

The present study has demonstrated that infection of cod induces some changes in humoral parameters during the initial stages of contagion. Apart from the increase observed in the stress hormone, cortisol, these changes were generally of a suppressive nature.

The difference in bacterial dosage of groups 2 and 3 was about 10-fold or $3x10^5$ and $3x10^6$ CFU respectively. This is a relatively high dosage resulting in about 80% infection of both groups within 6 days, but no deaths. In spite of the same degree of infection and similar cortisol levels the two infected groups, groups 2 and 3, showed subtle differences in the effects that infection appeared to have on some of the parameters measured. For example, the change in total protein and CRP PI concentration and in the anti-trypsin activity was similar in the

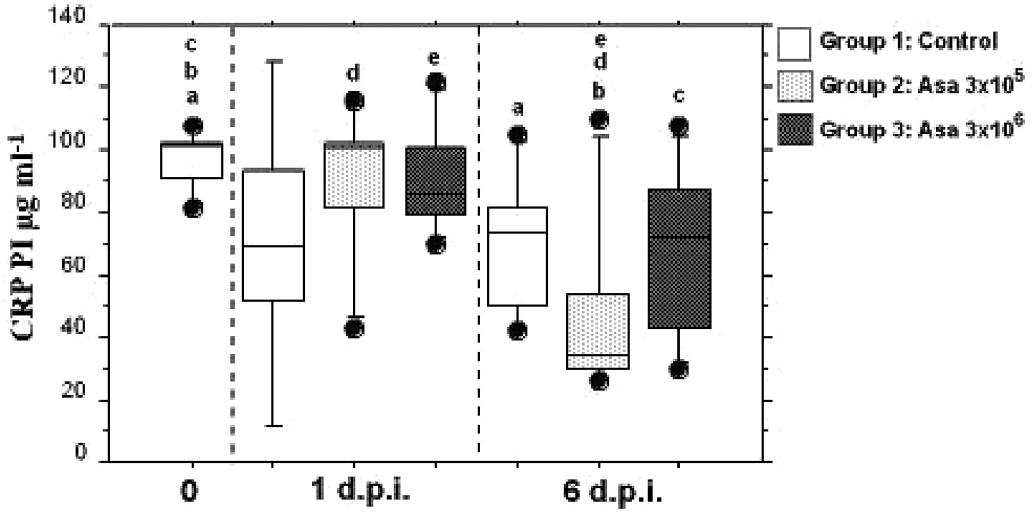

Figure 5. Box plots of pentraxin CRP PI concentration of non-infected (group 1) and infected (group 2 and group 3) cod before infection (0) and 1 and 6 days post infection (d.p.i.). The median is the line within each box, the boxes indicate 25 – 75 percentiles, the whiskers 10 – 90 percentiles and the filled circles the extreme values. Corresponding markings indicate a statistically significant difference between the relevant groups (P<0.05).

Table 1. A summary of the results compared to the initial status before infection, 0 indicates no change (statistical significance $P > 0.05$), + indicates an increase (statistical significance $P < 0.05$) and - indicates a decrease (statistical significance $P < 0.05$).

Parameters	Group 1: Control		Group 2: Asa 3x10^5 CFU		Group 3: Asa 3x10^6 CFU	
	1 d.p.i.	6 d.p.i.	1 d.p.i.	6 d.p.i.	1 d.p.i.	6 d.p.i.
Protein	-	0	0	-	-	0
Cortisol	0	0	0	+	0	+
CRP PI[1]	0	-	0	-	0	-
CRP PII	0	0	0	0	0	0
IgM[2]	0	0	0	-	-	0
Natural antibodies[1]	0	0	0	-	0	0
Anti-trypsin[2]	0	0	0	-	-	0

[1] The difference between the three groups was insignificant at 1 d.p.i. and at 6 d.p.i.
[2] the difference between the three groups was insignificant at 1 d.p.i.

control group and group 3 and different from group 2. The reasons for this difference between infected groups are not clear.

Infection appeared to reduce the serum protein level in all three groups. The control and group 3 showed a temporary reduced level at 1 d.p.i., returning to the pre-challenge level on day 6, while group 2 only showed a significant reduction at 6 d.p.i. This is comparable to the results obtained in a previous study and is discussed below (Magnadottir et al 2002).

Nutritional status and food usage are known to influence the serum protein concentration of fish (Love 1980). Similarly, stress and infection can reduce appetite of fish and affect the serum protein level (Moyer et al. 1993, Rehulka 1993). Feeding of the fish in the present study was restricted compared to the previous feeding conditions at the experimental station. This probably contributed to the reduced serum protein level observed, as well as loss of appetite due to the infection and stress.

The increase in the cortisol of the infected fish, reaching a 4.8-fold value at 6 d.p.i., while the control fish remained at pre-infection level, was a clear serological indication of an association between stress and infection in cod and may be a significant factor in the immune defence of cod. Several studies have demonstrated that stress can have suppressive effects on the immune response of fish (Weyts et al. 1999). Within the short time span of the present experiment a correlation between a high cortisol level and suppressed immune parameters was not convincingly demonstrated, a possible exception being the reduced IgM concentration and lower anti-trypsin activity seen in group 2 at 6 d.p.i. but not seen in group 3. The pre-infection cortisol level of cod in the present experiment was comparable to the level reported by Perez-Casanova et al (2008) in resting cod (using the same cortisol kit). In their experiment a gradual temperature increase from 10 to 19°C over a 45 day period resulted in an increased cortisol level, reaching a maximum 2.9-fold value after 30 days when the temperature had reached 16°C and then returned to basal level. The present experiment did not last long enough to discover if the cortisol level would have continued to increase or returned to normal values at a later stage of infection, which seems to be the pattern commonly seen in other fish species following stress induction, including chronic stress (Fast et al 2008, Perez-Casanova et al 2008). In the case of infection there are some reports that the serum cortisol level may continue to increase with time in infected fish. For example, the cortisol level continued to increase for up to 40 days in Atlantic salmon infected with parasitic co-pepod, *Lepephtheirus salmonis* (Fast et al 2006).

The IgM concentration of cod serum is relatively high compared to many other fish species and the natural antibody activity, which is closely correlated to the IgM concentration, is also generally high (Magnadottir et al 2009). Both parameters were suppressed in the infected fish when compared to the pre-infection value and, as mentioned above, the IgM level was also significantly reduced in group 2 at 6 d.p.i. when compared to that of the control fish at the same time point. Various factors can

influence the natural antibody activity and the IgM serum concentration of cod, like temperature, age and infection (Magnadottir et al 1999, Bowden 2008). In a study by Saha et al (2004) *in vitro* administration of cortisol was shown to reduce the number of IgM-secreting cells and IgM secretion, which is in accordance with the present results. However, in a previous study discussed below, an apparent increase in natural antibody activity and IgM concentration was demonstrated four weeks after Asa infection (Magnadottir et al 2002).

Infection had suppressive effects on the anti-trypsin activity, which was significantly reduced in group 2 at 6 d.p.i., both compared to the pre-infection level and to the control group at this time point. Some reduction was also seen in group 3, but only at 1 d.p.i. and only when compared to the pre-infection level. Different serum components can contribute to the anti-protease activity of fish serum like α1-antitrypsin and α2-acroglobulin (Hjelmeland 1983, Bowden et al 1997). This is an important element in humoral defence and can inhibit or delay bacteria that have toxic proteases implicated in pathogenicity like Asa (Gudmundsdottir 1998). Previous studies have indicated that the anti-protease activity may be of special importance in cod at a low environmental temperature (< 7°C, Magnadottir et al 1999), while infection had limited effects (Magnadottir et al 2002).

In a previous Asa challenge experiment on cod, referred to above (Magnadottir et al 2002), serum samples were analysed for total protein and IgM concentration and natural antibody and anti-trypsin activity as in the present experiment. In the previous experiment lower bacterial dosage was used and samples for analysis were collected from the control and infected fish 4 weeks after infection. As in the present experiment it was found that the serum protein level of the infected fish was reduced compared to the control group. On the other hand, unlike in the present experiment, the IgM concentration and natural antibody activity had increased while the anti-trypsin activity was apparently not affected. Although not strictly comparable experiments, the results suggest that the initial response involves the drawing on, neutralizing or blocking of the relevant parameters while an enhancement or equilibrium is achieved at a later stage.

The role of the two CRP-type pentraxins in cod is still not clear. The normal level of pentraxins in cod serum (total mean value about 130 μg ml^{-1} or about 0.4% of the serum proteins) is high compared to the level seen in mammalian species but comparable to other fish species like carp (Cyprinus carpio (Linnaeus)) and rainbow trout (Cartwright et al 2004, Liu et al 2004). In the present experiment infection had no effect on the level of CRP PII but had slightly suppressive effects on the level of CRP PI when compared to the original pre-infection value. The conclusion is that neither protein acts as a typical acute phase protein during infection. Their role in acute phase response of cod induced by turpentine injection is presently being investigated at our Institute, while their possible function as pattern recognition proteins or complement activators remains to be examined (Bayne & Gerwick 2001).

In conclusion: Infection by Asa resulted in a significant increase in the stress hormone cortisol while other parameters showed a limited and generally suppressive response to infection. Cod pentraxins, CRP PI and CRP PII, did not act as typical acute phase proteins in the early stages of infection. The short duration of the experiment probably played a role in the limited response observed but also the results reflect the relatively slow immune response of cod.

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